IMMUNE RESPONSES OF SHEEP TO RUMEN CILIATES AND THE SURVIVAL AND ACTIVITY OF ANTIBODIES IN THE RUMEN FLUID

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by

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Abstract

There has been an interest among ruminant nutritionists to develop new systems to manipulate rumen protozoa (ciliates) to maximise gains in productivity of ruminants. Some of the present techniques are of limited practical value for large scale application in the control of these organisms . An immunological approach was therefore chosen for investigation. The aim was to work towards a technique which would be acceptable to the livestock industry for the control of rumen ciliates.

The work in this thesis consists of a review of the highly specialized rumen ciliates, their implications in ruminant nutrition and a description of the research methods, the results and the conclusions drawn with regard to the prospects of establishing an immunological basis for the manipulation of rumen ciliates.

It was hypothesized that antibodies specific to rumen protozoa can be raised in saliva and milk of sheep and that these antibodies can survive and demonstrate activity in the rumen fluid which will be sufficient to modulate the activity of protozoa. Three areas were examined: (1) the ability of ciliate-antigens to elicit humoral and mucosal immune responses in sheep, (2) the structural intactness and activity of immunoglobulins in the rumen fluid (3) and the effect of antibody on the ciliates.

Preliminary studies on host immune responses were aimed at raising specific antibodies to ovalbumin in the saliva of two groups of wethers. This was done to select the most appropriate immunization protocol to optimize the induction of specific IgG and IgA antibody responses in the saliva of sheep. In these studies, two protocols were compared employing either intraperitoneal/intraduodenal or intraperitoneal/local routes of immunization respectively. In both groups, distinct IgG responses were detected in saliva and serum when antibodies were measured by enzyme linked immuno assays (ELISA).

Having determined the appropriateness of the strategy, the final stages of the study involved a group of lactating ewes, which were immunized intra-peritoneally with relevant antigens and challenged further by the same route. Specific IgG and IgA antibody responses were measured by ELISA in serum, saliva and milk respectively. All of these fluids had specific IgG antibodies to ciliate antigens. Milk also exhibited an enhanced IgA response. Surprisingly, no IgA response to ovalbumin was detected in the milk using the same technique.

The binding of these antibodies to surface antigens of rumen ciliates was investigated using indirect immunofluorescence by microscopy and specific binding to surface structures was clearly demonstrated.The activity of antibody in the rumen fluid was examined by incubating these antibodies for various periods of time in rumen fluid (in vitro) up to a total incubation period of 4 hours. Residual antibody activity was measured by surface binding to ciliates as measured by flow-cytometry. It was found that all of the samples of incubated antibody still bound to the surface structures of rumen ciliates, but that the amount bound varied between different species of ciliates.

The nature of the antibody molecules was examined after different periods of incubation in rumen fluid. This was done by proteolytic studies using purified sheep immunoglobulins IgG and IgA under simulated rumen conditions, employing colorimetric, radiometric and electrophoretic techniques. The studies revealed no degradation of immunoglobulin molecules (IgG and IgA) for the first 4 hours of incubation.

Studies were undertaken in vitro with all of the secretions to determine the effect of antibodies on live mixed rumen ciliate population. It was demonstrated that the serum antibodies had an immobilising effect on these organisms and that this resulted in significant reduction in the rate of predation by the ciliates on radiolabelled bacteria. Similar studies with salivary and milk antibodies did not demonstrate the same effects. An attempt has been made to explain the above observations and the need for further investigations has been suggested.

The data from the foregoing studies supported the hypothesis that protozoa-specific antibodies can be raised in ruminants and that these antibodies can survive and function biologically in the protease-rich rumen environment. The implications of the findings for future research have been highlighted. It is suggested that immunology can provide new directions in rumen ciliate research and possibly lead to practical measures for the control of these and other components of the rumen micro flora.

STATEMENT

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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DATE: 1st December 1993

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Section 1.0

General Introduction

Rumen ciliates are a group of single-celled protozoan organisms which inhabit the rumen of herbivorous animals like cattle , sheep , goats, buffaloes, deer and some other wild animals of the suborder-Ruminantia. These organisms gain entry following ingestion of saliva containing the ruminal bolus from neighbouring animals which harbour the organisms. It has been observed that if the recipient animals are young (< 3 weeks old), the protozoa may have difficulty in becoming established because of the high acidity of the young ruminant's stomach (Hungate, 1966). When young animals begin to consume forage, the pH rises in the rumen and conditions become favourable for the establishment of protozoa. In adult animals, the majority of organisms establish soon after ingestion, although certain species experience difficulties for a variety of reasons including protozoal antagonists (Eadie, 1962, 1967) and host specificity/resistance (Dogiel, 1947; Warner, 1962b; 1965).

The rumen is an essential part of the ruminant stomach, which harbours a myriad of microorganisms viz., bacteria, protozoa and fungi. It provides a special environment for microorganisms. It is warm (38-39^{o}c), has a low-redox potential and is rich in food materials such as plant fibres, starch, sugars and proteins. During the last three or four decades a great deal of work has been done to elucidate the biochemical and physiological potential of these organisms and the role they play in the digestive functions of the ruminant. These organisms ferment the ruminant feedstuffs- plant cell-wall constituents, starch and sugars to produce mainly volatile fatty acids, carbon-dioxide and water. The volatile fatty acids thus formed, constitute the major source of energy for the host animal. These micro-organisms also degrade the dietary protein to peptides and amino acids which are further fermented to ammonia. During the process of fermentaion, ATP is formed which is utilized by the microbial population to synthesize their own proteins from dietary nitrogen, thereby accomplishing their functions of growth and reproduction. In the process these microorganisms make up a considerable biomass which serves as a good source of protein for the host ruminant.

The relationship between the rumen microorganisms and the ruminant is central to ruminant nutrition. The metabolic and physiological attributes of the microorganisms are significant factors in

determining the production potential of the domestic ruminants. In order to harness this potential and improve ruminant productivity, more knowledge is required of the microorganisms and there is a need to develop new practical techniques to manipulate these populations. It is against this background that an immunological approach was considered to further these aims.

In the development of such an approach, the interactions of the rumen microbial flora and fauna were considered, viz., bacteria-bacteria, protozoa-bacteria and bacteria-protozoa-fungi. The bacteria-bacteria interactions are of a consortial nature and these interactions appear to be highly beneficial to the population. The protozoa-bacteria interactions reveal certain salient features which include (1) protozoa ingest and digest bacteria and reduce the bacterial biomass (Hungate, 1966; Coleman, 1975), (2) as a consequence there is increased intraruminal recycling of nitrogen through ammonia N to bacterial N to ammonia N (Nolan and Leng, 1972), (3) there is also a high level of recycling of protein nitrogen between the bacterial and protozoal pools, such that less than 50% of the gross incorporation of dietary nitrogen into bacteria and protozoa subsequently flows out of the rumen (Cottle et al., 1978), (4) only some ammonia is absorbed from the rumen and excreted by the sheep, accounting for the reduced availability in the outflow from the rumen, and (5) the biosynthetic processes involved in nitrogen recycling are energy consuming and reduce the net recovery of dietary energy by the ruminant. The interactions observed between protozoa and fungi suggest that protozoa either compete for nutrients or reduce fungal growth in other ways (Eadie and Gill, 1971; Orpin, 1975, 1984; Soetanto, 1986; Joblin, 1990; Newbold and Hillman, 1990).

Studies have been conducted to examine the effect of removal of protozoal organisms from the rumen. It has been reported that removal of protozoa is beneficial for the growth of cattle and for both growth and wool production in sheep, when the diet is deficient in rumen undegradable protein and rich in soluble sugars (Bird and Leng, 1978; Bird et al., 1979). These findings were subsequently clarified by a number of *in vitro, in sacco* and *in vivo* digestion trials (Demeyer and Van Nevel, 1979; Kayouli et al., 1984; Veira et al., 1983; Ushida et al., 1984, 1986, 1990; Ushida and Jouany, 1985 and Meyer et al., 1986). The findings of such trials clearly demonstrated that in animals without protozoa, (1) there was always an increase in efficiency of bacterial protein synthesis and (2) there was an increased flow of microbial protein to the small intestine. These observations suggested that the presence of protozoa in the rumen impaired the availability of dietary protein to the host animal.

Other studies on the kinetics of protozoa (Leng et al., 1981; Leng, 1982) demonstrated that a large proportion of rumen ciliates (62-85%) complete their life span in the rumen and are lysed within the rumen. It implies that protozoa stay long in the rumen and as a result have high maintenance energy requirements. Further they also predate heavily on bacteria and increase intra-ruminal recycling of nitrogen. These processes cause an undue wastage of ATP which in turn reduces the yield of total dry cells produced per mole of ATP available (Y_{ATP}) and thus the flow of protein to the small intestine. The net effect is a reduction in the protein-to-energy (P/E) ratio in the products of fermentation, [P/E ratio is an index which expresses the balance of protein to energy available to the animal for maintenance and production]. It is the higher P/E ratio in protozoa-free animals that has been cited as the main reason for their better performance compared with that of faunated animals. This has a direct bearing on the economic gains of livestock operations, which are invariably beset with high cost of protein feeds. It is with these considerations that the concept of defaunation (complete removal of protozoa from the rumen) has emerged as a possible nutritional strategy to maximise productivity in ruminants.

However there are no practical techniques available currently to defaunate large numbers of animals, either to maximise commercial gains in productivity or to gain better insight into hitherto unknown long-term effects of defaunation. There has been only limited research on techniques to achieve complete removal of rumen protozoa or to control individual populations. At present, none exist which have potential for controlling rumen protozoa under field conditions.

It was decided to explore a different approach to the manipulation of rumen protozoa (and the effect that it could produce on these organisms). Having considered the two living systems, unicellular protozoa and multicellular ruminant, which are intrinsically foreign to one another, immunology was chosen as a research tool. The rumen is not itself considered to have local secretory immunity but it is nevertheless in receipt of immunoglobulins. Sheep produce large volumes of saliva containing both IgA and IgG (Pahud and Mach, 1970). These immunoglobulins mix with the digesta during rumination. This thesis examines the hypothesis that salivary antibody has the potential to affect the balance of microflora in the rumen. It is hoped that the studies undertaken could lead to methods for manipulating not only the rumen protozoa but also other rumen microbial populations which occupy a similar niche in the rumen ecosystem.

The major objective of the present study was to determine whether any immunological interactions occur between protozoal antigens and the host animal and whether such interactions can be modified or manipulated to advantage. It is therefore pertinent at the outset, to review our current knowledge of rumen protozoa and the host ruminant.

Section 2.0

Review of Literature

2.1 Preamble

The review of literature focusses on the taxonomical and ecological background of rumen ciliates, their nutritional significance, the immediate advantages of controlling these organisms in terms of ruminant productivity, and the limitations of currently available practical techniques. Also it examines the prospects of applying immunological methods to develop new methods for controlling rumen protozoa by reviewing relevant immunological principles and concepts, the state of knowledge of ruminant immunology and the principal factors and strategies which could shape this project to work towards the desired goals.

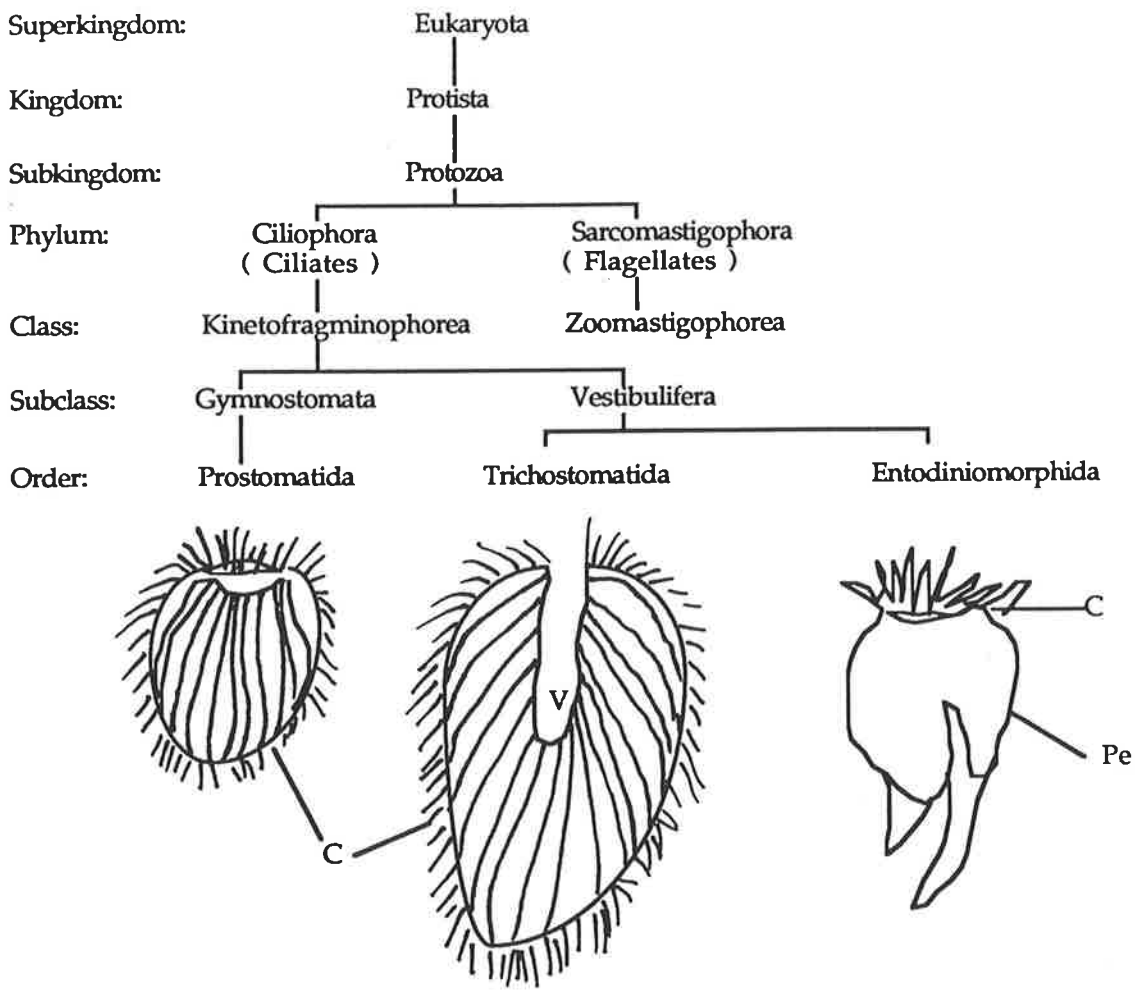
2.2 Taxonomy, metabolism and implications of protozoa in ruminant nutrition

2.2.1 *Classification of rumen protozoa*

The rumen protozoa are classified mainly on the basis of morphological features. They belong to the superkingdom Eukaryota. The arrangement of organisms into lesser taxa is not very consistent and different opinions have been advanced by various workers over many years. In the revised classification scheme (Levine et al., 1980), the taxon protozoa has been considered a subkingdom of the kingdom Protista (see Fig. 2.1). Organisms referred to as rumen protozoa belong to two major phyla, Ciliophora (ciliates) and Sarcomastigophora (includes flagellates). The rumen ciliates belong to the Class Kinetofragminophora (possessing kinetofragments in the oral area of body) and are divided into 2 Subclasses, Gymnostomata (no buccal cavity) and Vestibulifera (deep vestibulum). The ciliates are further divided into 3 orders. Those belonging to orders Prostomatida and Trichostomatida are characterised by uniform holotrichous ciliation (and are called holotrichs) whereas those belonging to the order Entodiniomorphida are recognised by the lack of complete ciliation and the presence of a firm pellicle. They also possess a tuft or tufts of cilia towards the anterior end of the body and are generally called entodiniomorphs (see Fig. 2. 1).

The order Prostomatida contains one family with a single genus, Buetschelia. The order Trichostomatida contains three genera, Isotricha, Oligoisotricha and Dasytricha belonging to the family Isotrichidae, and another genus Charonina belonging to family Blepharocorythidae (Fig. 2.2).

Fig.2.1 The main classification of rumen protozoa based on Levine et al. (1980). The drawings were adapted and redrawn from Ogimoto and Imai (1981).

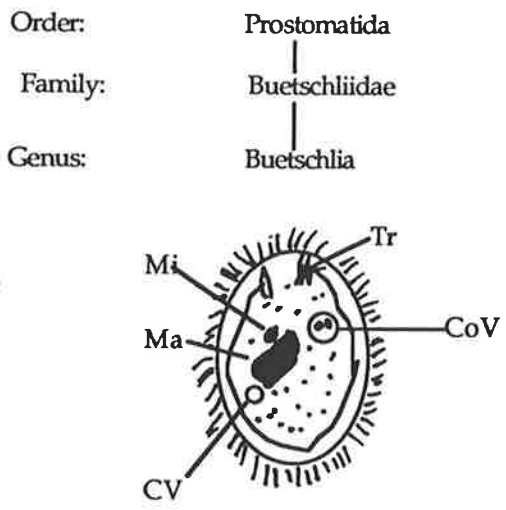


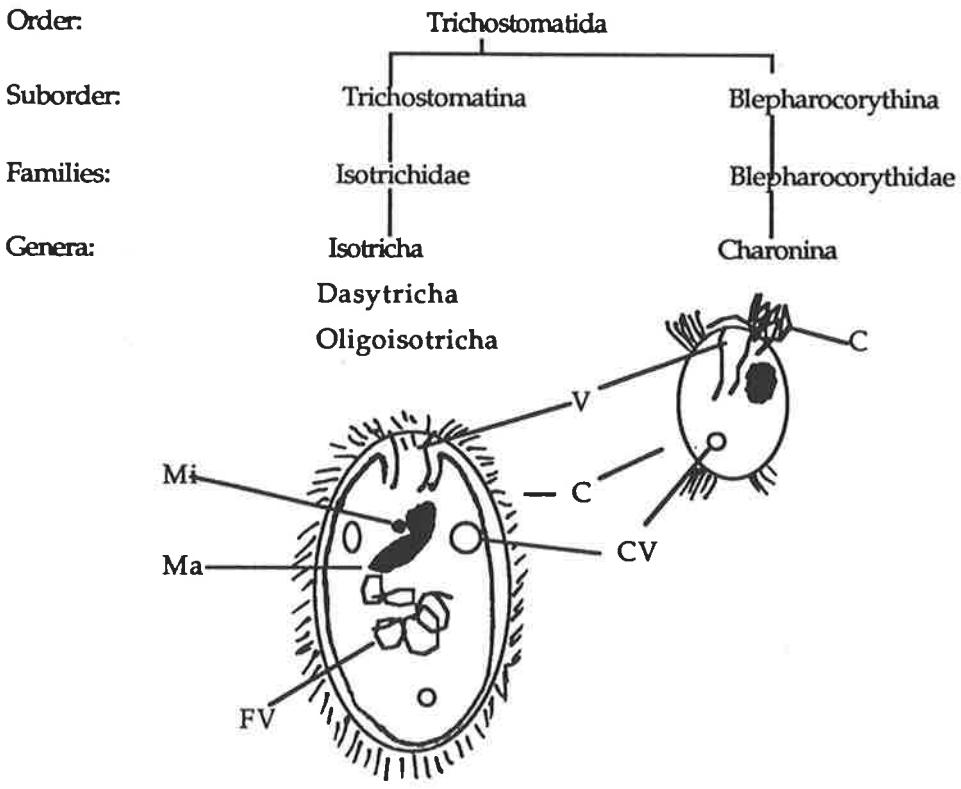
Holotrichs

Entodinium

C: cilia; Pe: pellicle; V: vestibulum.

Fig.2.2. The taxonomy of rumen holotrich ciliates (Levine et al. 1980).
The drawings were redrawn from Ogimoto and Imai (1981).

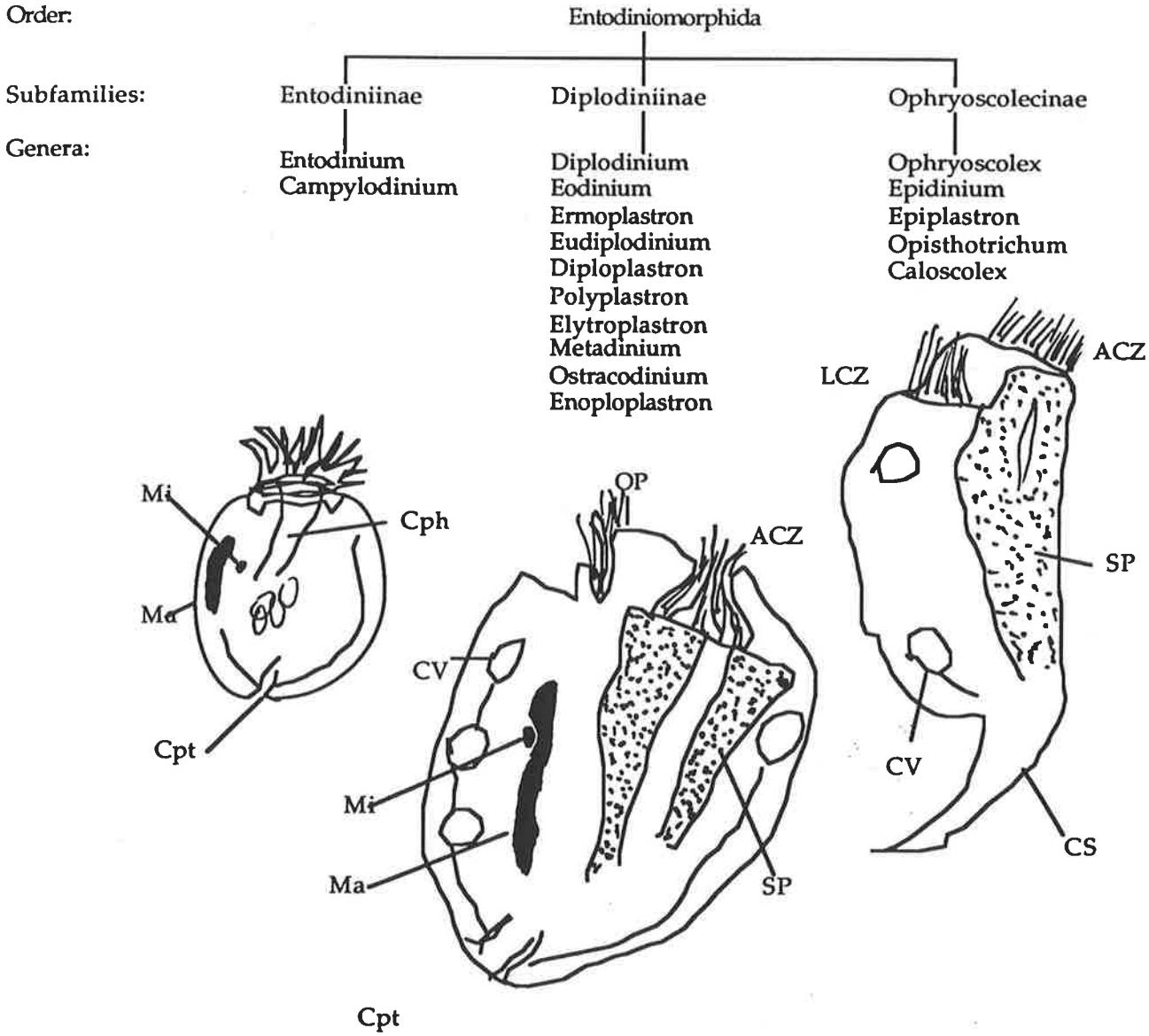




C: cilia; CoV: concretion vacuole; CV: contractile vacuole; FV: food vacuole;

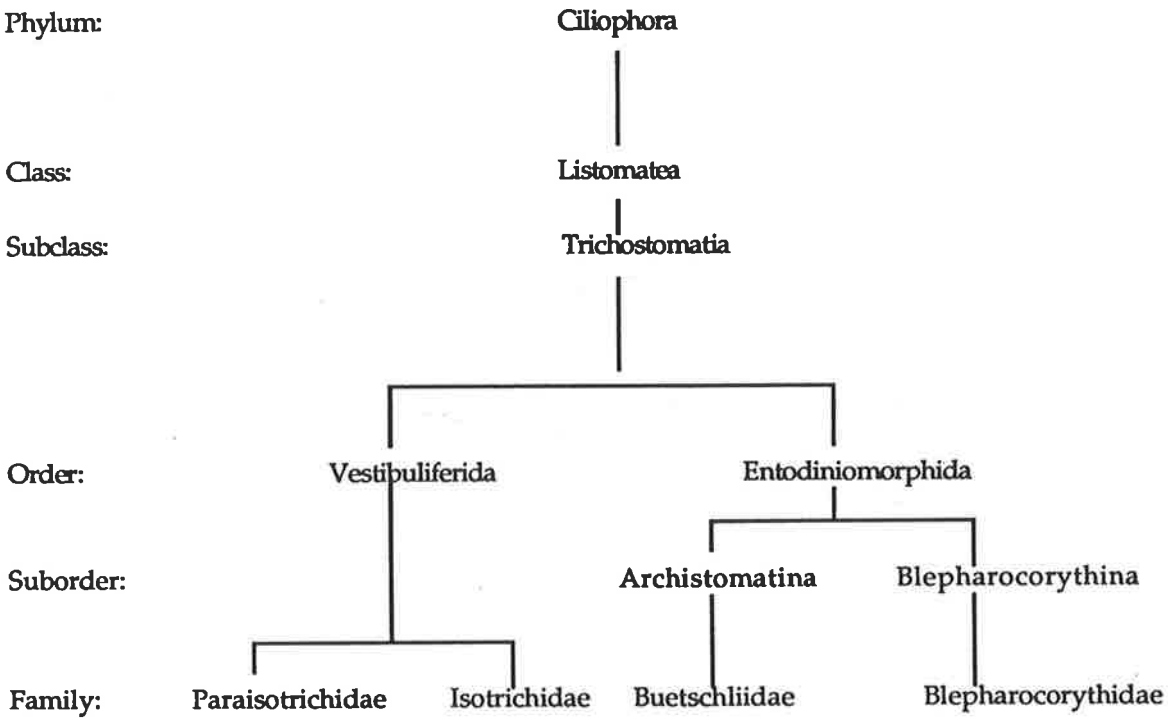
Ma: macronucleus; Mi: micronucleus; Tr: tichite; V: vestibulum.

Fig.2.3 The taxonomy of rumen entodinomorphid protozoa (Levine et al. 1980).
The drawings were redrawn from Ogimoto and Imai (1981)



ACZ: adoral ciliary zone; C: cilia; Cph: cytopharynx; Cpt: cytoproct; CS: caudal spine; CV: contractile vacuole; FV: food vacuole; LCZ: left ciliary zone; Ma: macronucleus; Mi: micronucleus; OP: operculum; SP: skeletal plate.

Fig.2.4 The revised classification scheme based on Lee et al. 1985.


Phylum:
Ciliophora
Class:
Listomatea
Subclass:
Trichostomatia
Order:
Vestibuliferida
Entodiniomorphida
Suborder:
Archistomatina
Blepharocorythina
Family:
Paraisotrichidae
Isotrichidae
Buetschliidae
Blepharocorythidae

The entodiniomorphs are represented by three distinct sub families, Entodiniinae, Diplodiniinae and Ophryoscolecinae. Each subfamily contains more than one genus and each genus contains several species (Fig. 2.3).

In a recent reappraisal (Lee et al., 1985) the Buetschliidae and Blepharocorythidae have been placed within the order Entodiniomorphida (see Fig. 2.4 for details).

Rumen protozoan flagellates belonging to the phylum Sarcomastigophora are divided into three orders, Retortamonadida (bacterivores with 2-6 flagella, Oxymonadida (2 pairs of flagella and 1 or more karyomastigonts) and Trichomonadida (4-6 flagella and an undulating membrane). Flagellates are reported to be less numerous than ciliates.

2.2.2 *Methods of identification*

Different techniques are used in the identification of rumen ciliates. These involve the morphological examination of fresh preparations of temporary or permanent mounts. Live ciliates may be examined by diluting rumen fluid in several volumes of normal saline at 39°C, recovering individual ciliates with a micropipette and mounting them on a glass slide for viewing under a light microscope at magnifications of 100 x and above. The detailed examination of rumen ciliates in fresh preparations is not always easy, so it is usually necessary to fix and stain them, preferably in a purified form.

In some early studies (Dogiel, 1927; Kofoid and Maclennan, 1930) unstained temporary mounts in glycerine were used to study the general proportions and structures of rumen ciliates. It was noted that all details except the finer fibrils can be seen in glycerine mounts. Whole mounts stained in iron- alum haemotoxylin were also found to be useful for examining morphological details. In later studies, Clarke (1964, 1968) fixed and stained organisms in acidified methyl-green and Ogimoto and Imai (1981) recommended the use of methyl-green-formalin-saline, pyronine-methyl-green or tincture of iodine for the demonstration of specific subcellular features (nuclei, vacuoles, skeletal plates, etc.).

Various silver impregnation techniques have also been developed for the specific staining of ciliate infraciliature. The popular methods include the ' dry ' silver nitrate method of Klein (1958), the ' wet ' silver nitrate method of Chatton and Lwoff (1930). All these methods have particular advantages or disadvantages for specific ciliate groups and that are not

always successful on individual ciliate species. The ultimate choice is based on trial and error. In addition to light microscopy, scanning and transmission electron microscopy has proven invaluable in the determination of ultrstructural features (Ogimoto and Imai, 1981).

2.2.3 *Enumeration of rumen ciliates and bacteria*

The usual practice in the enumeration of rumen ciliates is to estimate their number per unit volume of rumen fluid. Some earlier workers used blood-counting chambers of various kinds (Johnson et al., 1944; Moir, 1951 and Weller and Gray, 1954) but Oxford (1955) pointed out the disadvantages of using such chambers when the protozoa to be counted could exceed 200 microns in length and suggested using techniques similar those used by Adam (1951) for counting ciliate protozoa in the large intestines of horses. In keeping with these suggestions, (Boyne et al., 1957) and Purser and Moir (1959) developed methods for counting ciliates which are currently being employed by various workers.

These techniques rely on the fundamental assumption that protozoa are distributed uniformly throughout the rumen and that the protozoal concentration may be estimated from any aliquot of strained rumen fluid. The validity of such an assumption has been challenged (Warner, 1962a; Dehority, 1980) because it does not take into account the heterogeneous mixture of rumen contents (solids and liquid) nor the fact that several genera of protozoa have been found attached to plant material (Bauchop and Clarke, 1976; Orpin and Letcher, 1978; Bauchop, 1979) or the reticulo-ruminal wall of cattle (Abe et al., 1981). Warner (1962a) examined the efficacy of enumeration techniques and conclude that only a moderate error in the order of 20% occurred. Variations may occur due to differences in solute concentration in the rumen and also the local groupings of protozoa around particular foci, sometimes identifiable as feed partcles. Dehority (1984) improved subsampling procedures and reported that estimations of the numbers of protozoa per unit volume of rumen fluid and solid were not significantly different in samples taken before feeding. On the other hand, numbers estimated three hours after feeding demonstrated significant differences. It was also observed that the genera encountered in the solid and fluid fractions of rumen content differed markedly. However, until more accurate techniques are found, the current methods using counting chambers are valuable in the enumeration of these organisms.

In the enumeration of rumen bacteria Smith and Baker (1944) used an ordinary haemocytometer(0.1mm deep) and stained bacteria with iodine. Van der Wath (1948) used a Petroff-Hauser counting chamber (0.2mm

deep) and stained bacteria with Nile blue. Moir (1951) adopted a modification of Galls (1949) negative staining technique with nigrosin. None of these methods has been proven to be superior over the other, therefore the selection of a particular method appears to be empirical.

2.2.4 *Role of ciliates in the rumen*

The metabolic role of rumen microorganisms is central to ruminant nutrition. The role played by the organisms in metabolizing major ruminant feedstuffs determines both the survival and the production potential of domestic ruminants. Any study directed towards managing or modifying the population dynamics of rumen microorganisms must consider the importance of these organisms in metabolizing various forms of carbohydrate and protein in the feed of the ruminant.

2.2.4.1 *Action on cellulose*

Yoder et al., (1966) investigated the influence of mixed rumen protozoal and bacterial populations upon cellulose digestion *in vitro* and concluded that the addition of rumen protozoa to a suspension of rumen bacteria increased cellulose digestion substantially. Kayouli et al., (1984) examined the effect of defaunation on straw digestion *in vivo* and reported lowered digestibility (30%) in defaunated animals presumably due to the absence of protozoa. However, these earlier studies did not examine the relative importance of the different species of rumen bacteria and protozoa on the digestion of cellulose.

More recently, Coleman (1985, 1986) measured cellulase activity in protozoal and bacterial fractions prepared from animals with no protozoa, animals containing four individual species (namely, Epidinium ecaudatum caudatum, Eudiplodinium maggi, Ostracodinium obtusum bilobum, Entodinium caudatum) and two groups of animals containing natural Type A and Type B populations (Eadie, 1962, 1967).

The medium, which contained the combined natural B Type protozoal population plus the bacteria when compared for total rumen cellulase activity with that of the medium without any protozoa, an increase of 10-11 fold was noticed for the medium contained the protozoa. Even with other individual protozoal species examined, there was always a higher level of cellulase activity in all the ciliate fractions with the exception of Entodinium caudatum. In this case, the corresponding bacterial fraction showed an increase in cellulase activity in contrast to the fraction containing no protozoa thereby suggesting that Entodinium caudatum might have stimulated the growth of cellulolytic bacteria. These studies

demonstrate the importance of the types of protozoal species present in rumen fermentation. In contrast, the effects that holotrichs may have on cellulose digestion has not been determined although some studies have revealed poor activity on carboxy methyl cellulose (Williams and Harfoot, 1976; Van Hoven and Prins, 1977). Although most of the studies on digestibility have been concerned with cellulose, there is evidence that the protozoa are also important in the breakdown of pectin and hemicelluloses (Bailey and MacRae, 1970; Coleman et al., 1980; Williams and Coleman, 1985).

2.2.4.2 *Action on starch*

All entodiniomorphid ciliates, except possibly the smallest and those considered to be cellulolytic, engulf starch grains very rapidly (Hungate, 1975). Starch engulfed by protozoa is broken down slowly with liberation of acetic and butyric acids. In contrast, rumen bacteria degrade starch rapidly to lactic acid. If there is too much fermentation of starch by bacteria, the rumen pH falls and microbial activity declines with disastrous consequences on both rumen metabolism and the host ruminant. In this respect, the rumen ciliates play a more protective role for the host by way of their rapid engulfment of starch (Schwartz and Gilchrist, 1975; Mackie et al., 1978) and slower action on starch.The release of volatile fatty acids provides useful energy to the host animal. Apart from the engulfment of starch particles, the ciliates also metabolize exogenous lactic acid (Newbold et al., 1985) and engulf bacteria, thereby removing not only the starch but also the lactic acid-producing-bacteria.

2.2.4.3 *Action on sugars*

It has been shown that soluble sugars are the preferred substrate for rumen holotrichs and that they are attracted to them by chemotaxis (Orpin and Letcher, 1978; Murphy et al., 1985). The holotrichs are able to assimilate sugars as soon as they become available in the immediate environment . Entodiniomorphs also utilize sugars at different levels. Entodinium spp. take up maltose and glucose and incorporate them into protozoal polysaccharides (Coleman, 1969; 1979). Epidinium spp. take up glucose 100 times faster than Entodinial counterparts and they reserve this sugar as polysaccharide and also convert it into aminoacids and proteins (Coleman and Laurie, 1974, 1976). Polyplastron spp. incorporate simple sugars into polysaccharides (Coleman and Laurie, 1977).

2.2.4.4 End-products of carbohydrate metabolism

There have been many studies on the effects of faunation on the absolute and the relative proportions of acetic, propionic and butyric acids in the rumen, without any clear pattern emerging of the overall effect of protozoal populations on the production of these acids. When reporting on the production of individual acids by rumen ciliates, Abou Akkada and Howard (1960), Williams and Harfoot (1976) and Coleman (1978) found that the main products of entodiniomorphs were found to include butyrate and acetate whilst those of holotrichs were found to include lactic, acetic and butyric acids with traces of propionic acid. The reason for lowered production of propionic acid by rumen ciliates has been reported in another study by Whitelaw et al., (1984). These workers found that in faunated animals, there was competition for hydrogen for the production of methane, at the expense of propionate formation. This was due to the activity of methanogenic bacteria attached to the pellicles of most rumen protozoa (Vogels et al., 1980). In the same study it was also observed that there was a significant decrease in the digestibility of the ration in the faunated animals.

2.2.4.5 Ciliates and digestibility of fibrous feeds

It was mentioned in Section 1.0 that under some dietary conditions, defaunated animals have shown higher productivity than faunated animals. This has raised a number of questions because of the types of diets used in such studies and the influence that such diets would have had on the protozoal population. In the initial studies carried out in cattle by Bird and Leng (1978), diets were chosen to have large amounts of sugar, which promoted a dense population of protozoa in the rumen. In a subsequent study on ewes and lambs (Bird and Leng, 1985) where animals were fed either a basal diet of oaten chaff and sugar, or animals were grazed on native pastures, the productivity of unfaunated animals was again shown to be considerably higher than that of faunated sheep. In another trial (Demeyer et al., 1982), in which alkali-treated straw was the basal diet, defaunation increased growth rate by 37%.

It has been reported (Preston and Leng, 1987) that in animals fed straw-based diets, the population of protozoa was low and therefore it appeared that the causes of increased productivity due to defaunation in these instances were different to those sheep on high-energy-diets which generated a high population of protozoa. In the latter case, it is generally observed that the main effect of the absence of protozoa is the increase in

the P/E ratio in the end-products of fermentation. However, in animals on low-energy based diets of straw supplemented with urea and minerals, it was shown that defaunation resulted in large increases in the number of fungal spores in the rumen (Soetanto, 1986) and concomitant increases in both the rate of, and total, dry-matter digestibility of straw in nylon bags in the rumen. This increased rate of digestion might have increased the feed intake, thereby increasing productivity. Viera (1986) has recently reviewed the literature on the effects of the faunated state on apparent digestibility of dry matter in ruminants over a wide range of diets. He observed that digestibility is generally higher in the faunated compared with the defaunated state. However, the diets in most of those instances were of high digestibility (i.e. greater than 60%), whereas the low-energy diets referred to above came from straw-based-rations of 45-55% digestibility. Moreover, in sheep given a roughage diet (Orpin and Letcher, 1984) or wheat-straw (Soetanto et al., 1985; Romulo et al., 1986), it has been shown that after a reasonable period of readjustment of the rumen, digestibility was unchanged by defaunation. This may be because, when protozoa were eliminated from the rumen, (1) fungi proliferated and (2) the defaunation improved colonization of particles by cellulolytic bacteria and increased the comminution of larger particles. It has been reported in one of the recent studies that defaunation resulting in the increase of fungal population (Newbold and Hillman, 1990).

2.2.4.6 Ciliates in protein metabolism

Rumen ciliates metabolize both feed proteins and bacterial proteins. Only recently has there been some information presented on the relative importance of protozoa and bacteria in the degradation of feed proteins. Brock et al., (1982) found that the specific activity of bacterial proteases against azocasein was 4.4-6.5 times higher than that of protozoal enzymes. Ushida and Jouany (1985) then demonstrated that the presence or absence of protozoa had no effect on the rate of degradation of soluble proteins. However, the more insoluble the protein, the greater the decrease in its rate of degradation when the animal was defaunated. Similar results were obtained in experiments performed in vitro by Hino and Russel (1987), who concluded that protozoa play a significant role in the degradation of insoluble proteins. If a low protein ration contains mainly insoluble protein, it is then likely that ciliates will degrade a high proportion of this protein, reducing the quantity reaching the small intestine.

It has been shown that ciliates such as Epidinium ecaudatum caudatum, Polyplastron multivesiculatum and Isotricha spp. utilize free

amino acids for growth (Coleman and Laurie, 1974). Abou Akkada and Howard (1962) had earlier demonstrated that Entodinium caudatum exhibited peptidase activity against a range of dipeptides and glycyl glycyl glycine. Mixed rumen protozoal populations have also been shown to have high leucine amino peptidase activity (Forsberg et al., 1984). In another recent study, Wallace et al., (1987) observed that the ciliate protozoa, particularly small entodinia, were more active in some peptidase and deaminase activities than rumen bacteria.

It is not known to what extent the rumen protozoa assimilate ammonia for protein synthesis. There is evidence to suggest that 60-92% of dietary nitrogen is transformed into ammonia in the rumen and that 31-35% of protozoal nitrogen is derived from ruminal ammonia (Mathison and Milligan, 1971). However, it has been reported (Allison, 1970) that information concerning the relative rates of incorporation of ^{15}N from urea or ammonium salts suggest that ammonia is initially incorporated by the bacteria and these supply a major part of the nitrogen assimilated by the protozoa.

2.2.4.7 Engulfment of rumen bacteria

Coleman (1979) reported that all rumen ciliates engulf bacteria. Most of the protozoa, except for Entodinium caudatum, exhibited some degree of preference for one or more bacterial species. However, the rate of bacterial uptake and digestion differs for both bacterium and the protozoal species under investigation. Coleman (1975) used the known bacterial and protozoal population densities (10^9- 10^{10} and $2x10^6$ per ml rumen fluid respectively) in animals fed a restricted high grain ration and observed the rate at which Entodinium caudatum grown *in vitro* engulfed bacteria. He estimated that up to 10^8 bacteria per ml could be engulfed by the protozoa each minute. Such voraciousness may account for the observed action of rumen ciliates in reducing bacterial population densities (Kurihara et al., 1968). Tracer-dilution study by Cottle et al., (1978) who used ^{15}N to investigate the recycling of bacterial nitrogen, indicated that 6g of N_2 (i.e. approximately 36 g protein) was recycled between bacteria and protozoa each day in a sheep with 10^6 protozoa per ml and where 2.2g bacterial N and 1.3g protozoal N left the rumen each day. This recycling of bacterial carbon and nitrogen represents an energy and nitrogen loss as far as the host animal is concerned and it was considered to be of great importance under conditions where the protein content of the feed is low and of poor quality.

2.2.4.8 Dynamics of rumen ciliates

The studies of Weller and Pilgrim, (1974) and Bird et al., (1978), indicate that protozoa are retained selectively within the rumen, based on observations that the numbers of protozoa per ml of fluid in the omasum were only 20-50% of the numbers found in fluid taken from the rumen at the same time. Moreover, the estimates of protozoal pool size based on the product of numbers of protozoa per ml of rumen fluid and rumen fluid volume gave lower estimates of pool size than methods based on dilution of ^{14}C-labelled protozoa (Leng et al., 1981). Considerable differences were observed in the distribution of protozoa in different sections of the forestomach of the animal. These studies suggested that protozoa are not distributed homogeneously throughout rumen. This is supported by other studies (Akin and Amos, 1979; 1983; Grain and Senaud, 1984), showing entodiniomorphs attached to plant fragments and protozoa sequestrated to the reticulo-rumen wall as a thick protozoal mass on the reticulum (Abe et al., 1981). Microscopic observation indicated that the mass was primarily composed of holotrichs and counts revealed 53% Isotricha spp, 25% Dasytricha spp. and 22% entodiniomorphs. These authors speculated that this behaviour may be essential for the protozoa to maintain their population levels within the rumen. A series of studies (Leng, 1982; Leng et al., 1984; Ffoulkes and Leng, 1988) demonstrated that entodiniomorphs and holotrichs have a half-life in the rumen much longer than that of rumen fluid. During this time, there is heavy lysis of these organisms (60-70%). This presumably results in recycling leading to further loss of energy and nitrogen as NH_3. This means that a substantial amount of protozoal organic matter may not be available for the host and thus protozoa contribute only relatively small quantities of amino acids for intestinal digestion and absorption. There have been many attempts to measure the rates at which fluid, bacteria and protozoa leave the rumen. There is agreement among all of the authors that protozoa leave the rumen more slowly than free fluid or bacteria, though there is considerable disagreement on the extent of the reduction of the protozoal-flow. This is because the estimates of total and microbial N flows into the small intestine of ruminants *in vivo* are compromised by considerable technical difficulties associated with sampling of digesta, non-availability of ideal microbial markers and inaccuracies in laboratory analyses.

2.2.5 Productivity of ciliate-free ruminants (defaunated animals)

It has been mentioned previously that the activity of protozoa results in a decrease in the ratio of protein to energy in the end products of rumen fermentation. The beneficial response to defaunation is most likely to be due to an improvement in the protein economy of the animal. Coleman (1988) observed that within limits, at higher protein/concentrates levels in the ration, the presence of protozoa may had either no effect or a marginal stimulatory effect on the performance of ruminants. However, on low-protein rations, irrespective of the energy content, faunation has a depressive effect on the performance of the animals. This implies that the beneficial effect of defaunation may be best seen in situations where the dietary level of protein is insufficient or marginal to meet the nutrional requirements of the animals. While there is consensus on the above rationale, there are still criticisms levelled towards the concept of defaunation. These cite the negative response to defaunation, particularly lowered digestibility in defaunated animals. There are no consistent reasons forthcoming to explain this negative response. Different opinions have been expressed from time to time by various workers. Hungate (1975) observed that the amount of cellulose digested by the protozoa was relatively small in relation to the amount digested by the bacteria. Some other researchers (Jouany and Senaud, 1979; Jouany et al., 1981) indicated that only some species of protozoa are important in the digestion of cellulose, which is either acted on directly by these organisms or indirectly through the stimulation of the relevant bacterial populations. Coleman, (1988) stated that rumen ciliates are quantitatively important in the digestion of cellulose in the rumen.These observations are not in question.

It may be of relevance to mention that even the source of plant material appears to be important in the interpretation of digestive functions in the rumen. For instance, Amos and Akin, (1978) have shown that orchid grass (Dactylis glomerata) was more readily digested by protozoa than Bermuda grass (Cynodon dactylon). In these situations, all of the effects may not be ascribed to protozoa, because changes in the rumen environment such as rumen pH, ammonia concentrations and retention time of feed particles may all influence the extent of digestion

To confound the uncertainty surrounding the importance of protozoa in digestion, defaunation has not always depressed digestibility of feed materials in the rumen. In sheep, given high roughage-mainly Timothy grass (Phleum pratense), defaunation did not alter the digestibility (Orpin and Letcher, 1984). Following defaunation, the period of time required for the rumen microorganisms to recover appeared more critical in

determining the effect of treatment on rumen function. It was observed in that study that the number of viable organisms required at least 3-4 weeks to reach a plateau in the defaunated rumen. In the same study, the organic matter digestibility also showed a similar trend.

2.2.5.1 Live-weight gains in defaunated animals

The studies which measured the live-weight gains of faunated and defaunated animals may be divided into specific groups based on the type of diets used and the techniques used to defaunate the animals.The live-weight gains of lambs and steers given forage and sucrose diets were consistently higher in the defaunated animals (Bird and Leng, 1978; Bird et al., 1979; Burggraaf, 1980; De meyer et al., 1982; Bird and Leng, 1984a; 1984b). In these studies , the increased rumen volume may have accounted for some of the observed difference in the live-weight gains of defaunated and faunated animals, because it was apparent from some other studies that the removal of protozoa from mature wethers was associated with an increase in the volume of liquid in the rumen (Orpin and Letcher, 1984). This was attributed to the use of manoxol as defaunating agent.

In another study, (Burggraaf, and Leng 1980) when alkanate was used for defaunation, subsequent slaughter of animals confirmed a greater weight and volume of rumen contents in the defaunated lambs (1.3 kg heavier than in defaunated group). There was a 35% increase in the rate of live-weight gain in the defaunated animals, % of which was due to increased rumen weight. However, in a subsequent study conducted under similar conditions by Bird and Leng, (1984b), defaunation was associated with only a 9% increase in the rate of live-weight gain of lambs. In the latter study, the control animals were defaunated and then refaunated using the same type of defaunating agent. This exercise may have caused an increase in rumen volume of control animals as well thus reducing the influence of the rumen weight on the differences between the defaunated and the faunated groups.

After suggestions that defaunation may only be beneficial in situations where animals receive energy-rich diets, a series of studies was undertaken using low-energy diets (low-quality forage-based diets) (Bird and Leng, 1984a; Bird and Leng, 1985; Habib et al., 1989). These studies clearly indicated that the rate of live-weight gain of animals given either high- or low-quality forage diets was higher for those that were defaunated. An interesting observation in one of these studies was that in a grazing situation, defaunation increased the live-weight gains of lambs born to defaunated dams. It was mentioned that in this instance, the method of

defaunation (obtaining lambs from defaunated dams) apparently did not have any effect on the volumes of either the rumen or the caecum (Bird and Leng, 1984a; Bird and Leng, 1985; Bird, 1989).

Protozoa may have an important nutritional role in animals given diets high in cereal starch. When animals have received diets containing 47% maize (Christiansen, 1965) or straw and tapioca (De meyer et al., 1982) the defaunated groups have registered a depression in live-weight gain. In contrast, in some other studies, it has been reported (Becker and Everett, 1930; Habib et al.,1989) that defaunation is not always associated with a depression in live-weight gain of animals receiving diets containing grain. Such differences in results suggested that the level of grain in the diet and the type of cereal starch may determine the outcome of defaunation in such situations..

2.2.5.2 *Wool-growth in defaunated animals*

Studies on wool-growth have been conducted with classes of sheep; lambs (Bird and Leng, 1984b); sheep < 1 year old (Bird and Leng, 1984a, Cottle, 1986a; 1986b) and ewes (Bird and Leng, 1985).The results of these studies showed that, with the exception of animals fed on high-grain based diets (Cottle, 1986a, 1986b) wool production is higher in defaunated animals. The increased wool growth in defaunated animals is another strong indication that more protein is reaching the small intestine of these animals, because it has been clearly established that wool growth is always protein-limited (Reis, 1969). Most of these studies were conducted under Australian conditions and it was shown clearly that the extra wool production was not achieved as a consequence of extra food intake. This emphasizes the fact that removal of protozoa may increase the availability of nutrients and subsequently the efficiency of utilization of the absorbed nutrients. More recent studies have reaffirmed the earlier findings (Cottle, 1988a; 1988b; Forster and Leng, 1989a; 1989b, Habib et al., 1989) and also demonstrated that the improved wool-growth after defaunation occurred with both low- and high-protein diets. It is of interest to note that in defaunated adult ewes, a lower wool-growth response was observed compared with any other sex-groups. This may have been due to an extra-drain of protein for foetal development and lactation in these ewes.

2.2.6 Economic merits

Defaunation has been emphasized as a means of improving ruminant profitability (Bird, 1989), based on the fact that it increases the availability of protein to the host ruminant. Such an effort could offer substantial

financial benefits to extensive livestock production systems in natural low quality pastures as in inland Australia as well as to intensive husbandry systems, may it be for meat or wool production. It has been reported that the defaunated sheep tended to have more meat and less fat though the proportions of meat and bone were the same in carcasses of faunated and defaunated animals (Demeyer et al., 1982; Van Nevel et al., 1985).

The merits of faunation versus defaunation have not been properly evaluated in cattle reared for milk production. The production of high milk yields do necessitate the use of expensive protein supplements. Defaunation, in these instances, may reduce the need of these supplements by improving the availability of protein in the lower digestive tract of dairy animals. In the developing countries, the use of supplements is less an option and it may become possible to boost financial gains in a number of production areas by manipulation of rumen protozoa. Such a concept may even find application with exotic pseudo-ruminants such as alpaca, llama, etc. because of the potential value for their fleece.

2.2.7 *Practical limitations of techniques for manipulation of rumen ciliates*

Various techniques have been adopted by different researchers to study the effects of removal of protozoa on rumen function and the performance of ruminants. These techniques have included (1) the use of chemicals, (2) the isolation of animals at birth, (3) dietary manipulations (4) and the use of breeding from ciliate-free dams.

2.2.7.1 *Use of chemicals*

Coleman (1988) noted that when copper sulphate, (Becker and Everett, 1930), dioctyl sodium sulphosuccinate (manoxol) (Abou Akkada et al., 1968), nonyl phenol ethoxylate (Terric GN9) (Bird and Leng, 1978) or sodium lauryl diethoxy sulphate (alkanate 3SL3) (Burggraf and Leng, 1980) were used to reduce protozoal populations, occasional fatalities occurred and the detergents severely affected the consistency of the rumen fluid. Treated animals also often refused food for several days. In using chemicals there are also other problems: (1) these chemicals are not only toxic to protozoa but probably also kill other microorganisms and may also affect host rumen tissues, (2) the method offers two unattractive options- either drenching all of the animals and reinoculating the control animals or drenching only the group of animals randomly assigned for the treatment of defaunation. With the first option, there is no guarantee that the reinoculated animals are representative of the untreated group because of

the nonspecific effects of treatment and the spectrum of protozoa in the inoculum used for refaunation. The second option may also be criticized on the grounds that the differences between chemically-treated animals and the control animals cannot be attributed to the removal of protozoa alone. The chemical may have altered the composition of the remaining microbial population and also affected the rumen itself.

2.2.7.2 *Isolation of new-born animals at birth*

This technique involves the removal of new-born animals from their dams at birth and rearing them in isolation (Pounden and Hibbs, 1950; Abou Akkada and El Shazly, 1964; Eadie and Gill, 1971). Besides the practical difficulties encountered in keeping animals in isolation, there are other criticisms of this technique. For example, when new-born animals are inoculated with fresh rumen fluid containing protozoa (i.e. faunated), the inoculated animal receives not only protozoa but many other microorganims (bacteria and fungi of a different consortia) from the donor animals. Therefore differences observed between control and ciliate-free animals cannot be attributed to the addition of protozoa alone. Doubts also have been raised as to whether ciliate-free animals separated from their dam at birth develop a normal microbial flora in the rumen. Even if they do so, it is likely to be at a much slower rate than in the young which remain with their dams.

2.2.7.3 *Dietary manipulation*

In this approach, either mixed concentrates (Christiansen et al., 1964) or barley cubes (Whitelaw et al., 1972) have been fed to animals continuously for at least 4-5 weeks. This was claimed to have resulted in the decline of protozoal population. However, the efficacy of this method has not been well documented and it is unlikely to produce complete defaunation. Furthermore, changes in other components of the rumen flora may also be expected.

2.2.7.4 *Breeding from defaunated dams*

This is a technique where the dams are defaunated in the early stages of pregnancy, thereby giving sufficient time for the microbial population to recover and approach stability prior to parturition. The progeny from these dams have an equal opportunity of acquiring rumen-adapted microbes. Faunation of the control group is achieved through inoculating with protozoa obtained from in vitro cultures.

2.3 Practical problem and prospects of immunology

The improved productivity in defaunated ruminants has been highlighted by these methods of defaunation, although defaunation has not always resulted in consistent changes in ruminant performance. The inconsistency may be partly explained by the limitations of the techniques employed under experimental conditions. It appears that these techniques are of restricted value in wide-scale application under field conditions.

It must be emphasized that there are still a number of areas which merit continued research efforts in respect of manipulation of rumen protozoa. A deeper understanding is required of (a) the long-term effects of defaunation on the establishment of other microbial populations, (b) the effect of the size of the protozoan population or of individual species of ciliates on both the bacterial populations and on the overall changes in rumen fermentation, and (c) the results of such manipulations on the health-status of the host ruminant. These efforts require development of new systems which can target specific organisms and establish conditions both to increase the knowledge of these organisms and to observe changes that could occur in the rumen environment. It is in pursuit of such diversed research interests that the present immunological study was initiated.

There are few references in the literature relating to the application of immunological procedures to the study of the rumen microbial populations (Hobson et al., 1962; Sharpe et al., 1975; Conway de Maccario et al., 1982 and Sato et al., 1990a; 1990b) and none in respect of rumen protozoa. However various principles of immunology have been applied widely to research on true parasitic protozoan organisms. Parasite antigens have been characterized; host humoral and cellular immune responses have been assessed and, in some cases, natural or recombinant vaccines have been produced. Similar attempts on the whole subject of immune reponses to rumen ciliates and on their reciprocal responses to antibodies produced in the host ruminant may demonstrate basic physiological control mechanisms that regulate the rumen microflora, as well as methods that can be used experimentally or in the field to achieve better rumen function.

Once an immunological basis is established, it may open up possibilities to examine hitherto unknown aspects of rumen protozoa. For instance, more could be learned about the cell-surface antigenic characteristics, e.g., cilia, cell-walls, cytoplasmic membranes, etc., which may help to provide taxonomic characters useful in the identification of different genera and species of rumen ciliates. Based on such findings, it

may be possible to manipulate individual species or populations of rumen ciliates and thus the effects of such organisms on the establishment of other microbial populations and on the net fermentation patterns in the rumen. The detection of species-specific antigen may allow the production of specific antibodies that could be used to purify individual species. All of the foregoing observations stimulated the present research. A review of the immune system in general and its features in the ruminant animal is therefore necessary.

2.4 Immune system and its features in ruminants

The ruminants possess a highly-developed immune system. It shows many similarities to that in some other better-studied species such as rodents and humans, while retaining certain distinctive features. The immune system, in general, is composed of a diverse array of cell types found either organised into primary and secondary lymphoid tissues or circulating in blood and lymph. The major cellular components are lymphocytes and reticulo-endothelial cells (macrophages and related phagocytic cells), which in tissues such as bonemarrow, lymph nodes, spleen, Peyer's patches and especially the thymus, may be associated with stromal and functional epithelial elements.

Experiments on bursal resection in birds (Warner, 1965) and thymectomy in birds (Cooper et al., 1966) and mammals (Miller, Marshall and White, 1962; Aronson, Janovic and Walisman, 1962) and observations on children with congenital immunodeficiencies (reviewed Good, 1991) revealed the division of lymphocytes into the B (for bursal, or in mammals, bone-marrow derived) cells and T (for thymus-derived) cells. T cells were found to be critical for cell-mediated immunity while B cells were essential to the development of humoral immunity. This functional split was, however, found to be imprecise, as it was soon shown, that although B cells actually make antibodies, T cells are also required for optimal antibody production in response to most protein antigens (Claman et al., 1966 ; Davies et al., 1964; Miller and Mitchell, 1968). Subsequent research revealed a functional heterogeneity of lymphocytes(Cantor and Weissman, 1976). This introduced the concept of functional compartmentalization of lymphocytes into regulatory and effector categories.The overall pattern of immune responses is a net result of interaction between and among a number of lymphocyte subsets. Included in this array are regulatory T cells (helper, suppressor and contra-suppressor), effector T cells (cytolytic and delayed-type-hypersensitivity-inducing cells), B cells with different triggering requirements, as well as

subsets of macrophages and dendritic cells with different accessory functions.

The macrophages and dendritic cells are elements of the reticuloendothelial system, which interact actively with the lymphocytes and enable them to carry out their functions of recognition and elimination of foreign molecules(antigens). The reticuloendothelial system *per se* is a mononuclear phagocytic system located primarily in the venous sinuses of the bone marrow, spleen and liver and the lymphatic sinuses in other lymphoid tissues. The cells that participate in immune inductive events include primarily macrophages, Langerhans dendritic cells, scattered dendritic cells in most tissues, veiled cells in lymph and interdigitating cells in organized lymphoid tissues. These cells are commonly designated as antigen-presenting cells (APCs).

Macrophages are found in most tissues, sometimes surrounding blood vessels, or close to epithelial cells. They sometimes line venous and lymphatic sinuses or lymphatic endothelial cells. The Langerhans-Dendritic cells (L-DCs) are a family of related cells distinct from the macrophages. They are found in large numbers in epithelia and are probably precursors of interdigitating cells in the thymus-dependent areas of the lymphoid tissues. L-DCs comprise the Langerhans cells of the skin, the "veiled" cells of afferent lymphatics, the dendritic cells of the spleen and some of the " interdigitating" cells of the lymphoid organs (Silberg- Sinakin et al., 1980). Besides macrophages and dendritic cells, B lymphocytes also act as APCs. All of these cells, along with T lymphocytes, are involved in a series of reactions, following engagement with any molecule which is recognized as non-self (foreign) by antigen-specific T and B lymphocytes. The reaction of these different sets of immune cells to a foreign antigen is commonly referred to as immune response.

2.4.1 Immune response-an overview

The immune response is directed specifically towards certain determinants (epitopes) of the foreign molecules (antigens) which are recognized by structurally-related receptor molecules on the membranes of T or B lymphocytes which have complimentary paratopes. In the case of T cell recognition, antigen is taken up by the antigen-presenting cells, processed and expressed on the surface in association with class II histocompatibility molecules if it is processed in the endocytic compartment, or with class I histocompatibility molecules if it is associated with a replicating organism such as a virus if it is processed in the cytosol. The histocompatibility molecules were originally identified in experiments

involving grafting of tissues between related and unrelated animals and for this reason they were described as transplantation or histocompatibility antigens and their function in antigen presentation has been recognized only recently. They are in fact gene products which play a vital role in the shaping of the T cell repertoire and the ability of individuals to respond to particular antigens. The major histocompatibility antigens are coded by genes which are grouped together on one particular chromosome (chromosome 6 in man and chromosome 17 in mice) forming the major histocompatibility gene complex (MHC). In man the MHC is known as the HLA (human leucocyte antigen) complex; in mice it is known as H-2 (histocompatibility locus 2) complex. In ruminants, it is designated as the BoLA (bovine lymphocyte antigen) complex in cattle, the OLA (ovine lymphocyte antigen) complex in sheep, and the CLA (caprine lyphocyte antigen) complex in goats. Other farm animals also have a similar complex, SLA of swine, ELA of horses and B- complex of chicken. The genes of the MHC are mainly of two classes. The class I code for class I antigens, which are expressed on virtually all cell surfaces. The class II genes code for class II antigens which are expressed mainly on certain lymphoreticular cells such as macrophages, dendritic cells and B lymphocytes. The MHC molecules are peptide receptors which transport processed antigens to the cell surface where they can be recognized by T lymphocytes. The associative recognition of processed peptide and self MHC molecule is a phenomenon termed MHC restriction. Processed antigen associated with class I histocompatibility molecules is recognized by CD8 positive T cells, while processed antigen presented in association with class II histocompatibility antigens is recognized by CD4 positive T cells. There are also class III genes in the MHC which encode a group of serum proteins including some components of complements, some blood group antigens and some cytokines such as Tumour Necrosis FactorTNF.

2.4.1.1 Antigen-presentation

Antigens are presented to the immune system through two different pathways, namely an exogenous pathway and an endogenous pathway. In the exogenous pathway, the antigens are taken up by APCs by endocytosis, processed in the endocytic pathway and presented to the surface in association with class II molecules. In the endogenous pathway, the antigens are synthesized endogenously (e.g. viral antigens), processed and presented on the surface in association with class I molecules. Elucidation of these pathways has been one of the outstanding recent achievements of immunology and cell biology (Townsend et al., 1985; Morrisons et al., 1986

). Germain (1986, 1991) first articulated these pathways and they have been the subjects of recent reviews (Braciale and Braciale, 1991; Monaco, 1992; Neefjes and Ploegh, 1992).

2.4.1.2 Cell-mediated immune responses and cellular interactions

In cell-mediated immune responses, the resting T lymphocytes are triggered by antigenic peptides associated with MHC molecules, become activated and display specific effector functions. The key activation element during T cell responses to antigens is a complex receptor. It consists of two components. One consists of polymorphic subunits, namely, the T cell antigen receptor (TCR), whose function is to recognize and specifically bind antigenic peptides presented by MHC molecules. The second, a complex termed " CD3 " is non-polymorphic believed to function as the signal-transducing unit of the receptor complex. The TCR and CD3 associate non-covalently to form the functional receptor. T cells also have certain other surface glycoproteins which function as adhesive molecules, some of which also function in T cell activation. Of these, CD4 and CD8 are expressed on mutually exclusive populations which are popularly known as Helper T (Th) cells and cytotoxic or cytolytic T cells (CTLs) respectively. The CD4 molecule is believed to bind to nonpolymorphic regions of the class II molecules and strengthen the adhesion of the T cell and the APC (Marrack et al., 1983; Bierer et al., 1989). This is probably associated with binding of the TCR to the same MHC class II molecules on the APC, and the binding of the CD4 and TCR to the same ligand generates a more potent activating signal, allowing fewer ligands to activate a T cell (Janeway, 1988a; 1988b). Interactions between CD8 and class I MHC molecules have a similar function in the generation and effector functions of cytolytic T cells.

During the close interaction between APC and Th cells, these two groups of cells mediate their functions by secreting cytokines. Cytokines are a group of protein cell-regulators, variously called lymphokines, monokines, and interleukins (depending on their origins). Some are produced by a wide variety of cells in the body besides the immune cells. The helper T-cell-derived lymphokines (as they are popularly known) act either directly on the antigen or induce phagocytosis of the same by the mononuclear-phagocytic system, thereby establish protection against foreign molecules. However these mechanisms also exert great influence on the generation of other forms of immunity, in particular, humoral and cytotoxic T cell immunity.

2.4.1.2.1 T cell help for B cell antibody responses (Humoral Immunity)

Humoral immunity refers to mechanisms of resistance that depend on the development of antigen-specific antibodies in various body fluids. Some antigens can activate B lymphocytes directly (so-called T independent or T1 antigens). Bacterial lipopolysaccharides (LPS) can activate B cells to divide and secrete antibody without an obligatory requirement for help from other cells. These are referred to as type 1 T-independent antigens (T1-1). Other bacterial polysaccharides (type 2 or T1-2) arerelatively T-independent but activation falls short of maturation to antibody-secreting cells unless factors are available from macrophages and / or T lymphocytes. Development of humoral immunity against most protein antigens has an obligatory requirement for antigen-specific T cell help, usually provided by $CD4^+$ T cells. Antibody production against these T-dependent antigens therefore requires recognition of native antigen by B cells and processed antigen by the T_h cells. Specialized antigen presenting cells are required to process antigen, present it in association with MHC class II molecules and provide co-stimulatory signals- either as soluble mediators (IL-1 or IL-6) or cell-surface ligands (B7 / BB1 molecule, Linsley et al., 1991). Activated T cells in turnrelease a train of other lymphokines. Among the currently recognized T-cell-derived lymphokines are interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 5 (IL-5), interferon-gamma (IFN-g), granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukin 3 (IL-3). In addition T cells also secrete interleukin 6 (IL-6). Of all of these lymphokines, particularly IL-2, IL-4, IL-5 and IL-6 appear to play a vital role in the activation and differentiation of B cells which are committed to the production of antibodies against T-dependent antigens. T and B cell collaboration in antibody responses had been clearly demonstrated by various workers using a hapten-carrier system (Ovary and Benacerraf, 1963; Mitchison, 1971; Katz et al., 1973). Whilst direct interaction between MHC-matched T and B cells has been shown to be required for the maximum activation of resting B cells, the latter can also be partially activated through a direct receptor cross-linkage of Surface Immunoglobulins. Resting B cells, with their membrane immunoglobulin (Ig) molecules acting as receptors, bind antigens on which there is multiple representation of particular antigenic determinants. Such antigens can cross-link independent membrane Ig molecules, creating a biochemical signal in the cell. This signal, together with IL-4, activates the B cell. With additional mediators such as IL-5, IL-2 and IL-6, activated B cells can differentiate into antibody-secreting cells.

However B cell responses to most T dependent antigens, in which each antigenic epitope is unique and therefore cannot cross-link surface immunoglobulin molecules, requires direct or " Cognate " interaction with the antigen-specific T_h cell. The mechanism of cognate help has been demonstrated recently by Lanzavecchia (1985) and reviewed by Noelle and Snow (1990). In cognate interaction, antigen is bound by B cells with specific Surface Immunoglobulin receptors, endocytosed and, after processing, presented as peptide fragments on the cell surface bound to class II MHC molecules. T cells specific for the antigen MHC- complex bind to the B cell and in the context of this cell pair, T cells cause B cell activation through the mediation of relevant lymphokines and by direct receptor-ligand interactions..

2.4.1.2.2 Influence of interleukins on isotype expression

The differentiated antibody-secreting cell derived from the activated B cell is the plasma cell. These cells secrete large amount of antibody molecules which possess the same idiotype and antigen-specificity as that of membrane-expressed IgM and IgD found on the progenitor B cells. Some progeny of activated virgin B cells undergo class-switch and give rise to memory cells and plasma cells that express antibody molecules of isotypes other than IgM. The " class- switch " or "isotype-switch" involves further rearrangement of the VDJ region of the heavy chain genes and expression of C_H genes. The studies in murine species clearly demonstrated that T cells or their products influenced isotype expression, either by stimulating B cells that have already undergone the class switch or by actually inducing the class switch themselves (Isakson et al., 1982; Kawanishi et al., 1983; Kiyona et al., 1984). Recent reviews on isotype regulation by lymphokines (Coffman et al., 1988; Snapper et al., 1988) revealed that IL-4 induces not only IgG_1, but also IgE production in lipo-polysaccharide (LPS) stimulated murine spleen B cells, but it also suppresses IgM, IgG_3, IgG_{2a}, and IgG_{2b}. On the other hand, IFN-g was found to induce IgG_{2a} production but suppressed the expression of IgG_1, IgG_3 , and IgG_{2b} and IgE in murine B cells. It may be noted that IL-4 and IFN-g act as reciprocal regulatory factors in the expression of relevant isotypes. IL-5 acted on LPS-stimulated murine splenic cells to induce a three to six fold in increase in IgA production. Whilst IL-4 has been shown to be involved mainly for the early activation of resting B- cells and IL-5 in the growth of activated B cells, the final differentiation stages of B cells are mainly influenced by IL-6. This results in the induction of Ig production in the presence of other factors (Hirano et al., 1984). Furthermore, IL-6 was found to act on B lymphoblastoid cells at the

mRNA level and to increase the biosynthesis of IgA by cells committed to this isotype (Kikutani et al., 1985). Despite the considerable advances in the understanding of the control of immunoglobulin production, still there are certain questions remaining unanswered, in particular, as to why the preponderance of IgA is limited to mucosal tissues.

2.4.1.2.3 Suppressor T cell activity

Whilst T_h cells stimulate humoral immune responses, T cells also have the capacity to suppress immune responses. In different experimental systems, a variety of cell types, including T cells, B cells and macrophages has been cited as mediators of immune suppression (Katz et al., 1974; Zubler et al., 1977; Baird and Kaplan, 1977). However, in the majority of the situations, T cell suppressor populations have been identified (Boyse et al., 1968; Cantor et al., 1975; 1976). T cell subset carrying surface molecule CD8 is strongly correlated with suppressor T cell activity. However, the nature of I-J restriction of these suppressor cells is not clear and the functional role that might be played by the CD8 molecules in these cells is not known. While the phenomenon of suppression is undoubted, the mechanisms involved remain unclear. In particular, the existence of a subset of T cells whose function is devoted to suppression has been questioned in recent years (Moller, 1988). It is not relevant to pursue mechanisms of suppression here, but it is important to note that it may involve a number of T cell functions, including cytotoxicity, the action of suppressive cytokines, the activity of " veto " cells and the effects of anti-idiotypic interactions.

2.4.2 *Humoral immunity in ruminants*

Humoral immunity refers to the state of immune defence prevailing in extracellular fluids like plasma, lymph, saliva, milk, bronchial fluid, tears, nasal and uro-genital secretions. It is mediated through the production of antigen-specific antibodies, which are effector molecules produced by B lymphocytes in response to an antigen. The antibodies are immunoglobulin molecules and the major classes of imunoglobulins that are found in ruminants include IgM, IgG, IgA and IgE. The IgG class is subdivided into IgG_1 and IgG_2. Babel and Lang (1976) reported on the presence of a third subclass IgG_3. Allotypic variants of IgG_2 have been characterized(Heyermann et al., 1992).

Like in many other species, immunoglobulin G (IgG) predominates over other classes of immunoglobulins in serum and accounts for approximately 90% of the total serum immunoglobulins (Duncan et al., 1972; Williams and Spooner, 1975). In the external secretions like milk and

colostrum, unlike in most other mammals, again IgG is the major class of immunoglobulin in ruminant animals. However, the saliva, tears and nasal secretions of ruminants contain immunoglobulin A (IgA) predominantly as in other mammalian counterparts. The intestinal secretions of these animals show a relative prominence of IgA in comparison to IgG, and milk and serum have relatively low concentrations of IgA in a descending a fashion (see Table 2.1).

2.4.2.1 The mucosal immune system

The mucosal immune system is associated with the mucosal surfaces of the gut, respiratory tract, urogenital tracts, pharynx, tonsils, the ducts of the salivary glands, etc. These mucosal surfaces represent the largest organ system in vertebrates and constitute a large area exposed to exogenous agents including microorganisms. Hence these mucosal tissues are defended by a local immune system with properties and functions that in many respects are separate from systemic immune system. The wall of the gut functions as the largest immunological organ in the body. It contains aggregates of lymphoid follicles referred to as Peyer's patches (PP) which appear as nodules along the luminal wall of the intestine. These PP are situated beneath a specialized layer of epithelium [follicle associated epithelium (FAE)] within which are found specialized membranous cells called the M cells. Between adjacent follicles are tufts of villi, the epithelium of which is columnar. The FAE consists of epithelial cells with microvilli, while the M cells have microfolds which facilitate the approach of particles and microorganisms to the M cell surface. Within the PP, the germinal centre comprises primarily B cells, while the regions between the follicles are occupied primarily by T cells. Much work concerning the function of the PP has been done in rodents (reviews: Mayrhofer, 1984; Carlson and Owen, 1987; Neutra and Kraehenbuhl, 1992). The results obtained in rats and mice have been interpreted to suggest that the PP responded to intraluminal antigen and produced precursors of IgA-secreting plasma cells, which eventually home to intestinal mucosa. It has been confirmed that M cells are in close association with lymphoid cells and macrophages which invaginate into their cytoplasm (Owen and Jones, 1974; Bye et al., 1984) and they have been characterized by their ability to internalize particulate material by endocytosis.

A number of studies in mice revealed that M cells are involved in the uptake and transport of antigens from the intestinal lumen into the underlying lymphoid tissue (Owen, 1977; Bockman and Cooper, 1983; Wolf and Bye, 1984).Transcytosis of macromolecules has also been demonstrated

for M cells in calves (Landsverk, 1987). While both soluble and particulate uptake by the FAE has been reported (Bockman and Cooper, 1983), several other studies in mice have shown the ability of the epithelium of the absorptive villous mucosa (a) to absorb both low and high molecular antigens (Warshaw et al., 1971), (b) to process the absorbed proteins to constituent peptides (Stern and Walker, 1984) , (c) and to constitutively express class II (Ia) antigens of the MHC (Mayrhofer et al., 1983). Also this epithelium has been shown to contain large number of intraepithelial lymphocytes (IEL) and they play a role in the induction of suppressor T cells mediating systemic tolerance to dietary antigens (Bland and Warren, 1986).

The gut mucosa of domestic ruminants exhibits a number of features in common with other species but there are some interesting differences. In neonatal calves and sheep there are many small PP throughout the jejunum and upper ileum but in the lower ileum there is a large continuous patch (Pabst and Reynolds, 1986). Moreover, in sheep, it was reported that the large continuous patch of the lower ileum regressed with age and it was completely involuted by 15 months (Reynolds and Morris, 1983) while the jejunal PP persisted in adult animals. There are also marked differences between the surface markers on follicle lymphocytes of the ileal and jejunal PP, suggesting that there are more immature cells in the ileal PP (Miyasaka et al., 1984; Hein et al., 1989). It was observed that ileal PP of lambs contained virtually only B lymphocytes, whereas, the jejunal PP had a mixture of both B and T cells. Furthermore, the T cells comprised both $CD4^+$ and $CD8^+$ subpopulations, whereas the ileal PP follicles were almost devoid of T lymphocytes in their follicles. Another difference is that the FAE of the jejunal PP has M cells, whilst the ileal PP has no typical M cells. In terms of functional importance, it has been suggested that the large continuous patch of ileum may play a role as a source of B cells, a role normally filled by bone marrow in other mammalian species and by the bursa of fabricius in birds. This has been confirmed by the finding that lymphopoiesis occurs in this PP without the requirement of antigen (Reynolds and Morris, 1984; Reynolds, 1987), that the removal of the ileal PP in sheep fetuses near term or shortly after birth impairs the post-natal expansion of circulating B lymphocytes (Gerber et al., 1986). The evidence for the presence of lymphoid tissues at other mucosal sites in sheep has been cited through a number of studies, which shall be discussed in subsequent sections.

2.4.2.1.1 Regulation of mucosal immune responses

The regulation of the mucosal immune responses has not been studied in ruminant animals to the same extent as in laboratory rodents and humans . It is relevant to summarize mechanisms that have been studied in rodents in order to develop an insight into the predominance of IgA in mucosal immunity. At a molecular level, it involves genomic modifications and rearrangements of DNA segments coding for the immunoglobulin molecule (Honjo et al., 1982; Strober and Jacobs, 1985), controlled by the influence of T cells on B cell development (Kawanishi et al., 1982; Kawanishi and Strober, 1983a, b). The latter workers suggested that special T-cell subsets from murine PP caused B cells to switch from IgM class to IgA by-passing expression of IgG. In contrast, T cells from the spleen failed to stimulate expansion of surface-IgA bearing cells in a B cell population derived from PP. The Peyer's patch environment rather than the source of uncommitted B cells appears to determine committment to IgA synthesis. In an adoptive transfer experiment, where syngenic lymphoid cells were transferred adoptively to recipient rats whose lymphoid systems have been destroyed by γ-irradiation, it has been shown that lymphocytes from any source, e.g. peripheral lymph nodes or spleen were able to produce IgA provided they were placed in the GALT environment (Denham et al., 1984). It has been surmised by Hall (1986) that specialized dendritic macrophages which are abundant in the GALT (Mayrhofer et al., 1983) of rats may play a role in providing the unique microenvironment that favours gut mucosal IgA responses. A similar conclusion was reached by Spalding and Griffin (1986) from studies of immunoglobulin isotypes synthesized *in vitro* in the presence of dendritic cells derived from various sources.

2.4.2.1.2 Migration of lymphocytes and cellular basis of humoral immunity in the intestine

The most extensive recirculation of lymphocytes occurs through the gut. The recirculation of lymphocytes appears similar between different species of animals. The generation of large lymphoid immunoblasts in the GALT and the homing properties of these cells is also similar in a variety of species. Organ-specific patterns of lymphocyte recirculation are particularly prominent in sheep (Cahill et al., 1977; Scolley et al., 1976; Hall et al., 1977) in comparison with rodents (De Freitas et al., 1977). The recirculatory and homing behaviour of lymphocytes has generally been interpreted in terms of immune surveillance, as a mechanism for recruiting, selecting and

stimulating specific immunocompetent cells by exposing them to antigens and for distributing immune effector cells to sites of antigen deposition and focuses of infection. The immunoblasts in the intestinal lymph arise, like immunoblasts elsewhere, by the transformation of small lymphocytes in response to antigenic stimulation (Gowans et al., 1962). In rats, it was shown that many of the large lymphoid immunoblasts in the thoracic duct lymph (which is principally of intestinal lymph from the mesenteric nodes) home to the lamina propria of the gut (Gowans and Knight, 1964). This was subsequently confirmed by other workers (Griscelli et al., 1969: Hall et al., 1972). Griscelli et al., (1969) showed that cells from mesenteric lymph nodes homed preferentially to the intestine, whereas cells of peripheral lymph nodes migrated to peripheral nodes. It was postulated that the progenitor cells resident in PP germinal centres migrated via mesenteric lymph node, thoracic duct lymph and circulation prior to extravasating in the intestinal lamina propria (Husband and Gowans, 1978). A similar migrating pathway of antibody containing cells responding to intestinal antigen has been demonstrated in ruminants (Husband et al., 1979). Most of the migrating immunoblasts in mice and rats were shown to be IgA-committed cells (Mandel and Asofsky, 1968; Williams and Gowans, 1975). Certain other studies also revealed that the majority of terminally-differentiated B lymphocytes (plasma cells) in the rodent and human lamina propria contained IgA (Crabbe et al., 1965; Tomasi and Bienenstock, 1968; Crabbe et al., 1970) and similar results have been reported in pigs (Bennel and Husband, 1981a) and in ruminants, although there are relatively more IgG containing cells found in the thoracic duct lymph (Lee and Lascelles, 1970; Husband et al., 1979). In some studies, it has been observed neither the isotype nor the presence of antigenic material seem to be involved in mediating the extravasation of immunoblasts to the lamina propria of the gut. This has been clearly demonstrated in experiments conducted both in rats and sheep respectively. Immunoblasts from the thoracic duct lymph of rats home just as readily to the antigen-free gut of unsuckled new-born rats, (Halstead and Hall, 1972) or to subcutaneous implants of sterile foetal gut (Moore and Hall, 1972) as they do to the antigen-laden gut of adults. Similar results have been obtained in the foetuses of sheep (Hall, Hopkins and Orlans, 1977). In the latter study it was also shown that immunoblasts that homed to the gut were committed to make IgG or IgM . Furthermore, T immunoblasts from intestinal lymph, which neither display nor contain immunoglobulin, are also capable of homing to the gut (Sprent, 1976; Rose, Parrot and Bruce, 1976), though they may be more likely to enter the Peyer's patches than the lamina propria. It

may be mentioned, in passing, that recirculation studies in lambs have shown that the lymphocytes of ileal PP traverse a path which is to a large extent outside the path which is normally observed of immunoblasts of jejunal PP of sheep and PPs that of rodents and humans (Reynolds et al., 1985).

Whilst the route of migration of lymphocytes from lymphoid tissue in the intestine to the intestinal lamina propria has been established, the mechanisms enabling cells of mucosal origin to extravasate preferentially from the circulation into the mucosal lamina propria have only recently been elucidated (Streeter et al., 1988; Nakacha et al., 1989; Briskin et al., 1993).

2.4.2.1.3 The common mucosal immune system

It has been demonstrated in a series of studies that after antigen-induced proliferation and partial differentiation, both B and T cells cells enter the regional mesenteric lymph nodes, and after further differentiation they are transported through the thoracic duct into the circulation (Gowans and Knight, 1964; Craig and Cebra, 1971; Butcher, 1986). Streeter et al., (1988) demonstrated that these cells have surface determinants, called adressins, on endothelial cells in mucosal and glandular tissues. They appear to be involved in the recognition of mucosal tissues by immunoblasts originating in the GALT. It has also been observed that whilst most of the activated B and T cells migrate from the circulation back to the lamina propria, a substantial proportion, perhaps 10-20%, end up in mucosal tissues outside the intestine (Rudzic et al., 1975; Roux et al., 1977; McDermott and Bienenstock, 1979; Weiß-Carrington et al., 1979). Based on the results of these studies, Bienenstock and Befus (1980) advanced the idea of a common mucosal immune system, which refers to the interconnected network of migration of activated lymphocytes between the distant mucosal sites within the body. It emphasized the point that the mucosae of different sites are provided with a common pool of recirculating lymphocytes and that precursors of plasma cells from one tissue can seed the mucosa at other sites, resulting in the production of secretory immunoglobulins (usually IgA) in the alimentary, respiratory, genito-urinary and naso-lacrimal ducts, salivary glands and mammary tissues.

The concept of the common mucosal immune system has raised enormous interest to explore possible ways of immunizing animals and humans against various forms of infections. The best known entity providing specific immune protection in mucosal surfaces is the secretory

IgA (SIgA). In many mammals, including man, the bulk of immunoglobulins in secretions of the mucosae of alimentary, respiratory, uro-genital tracts; the naso-lacrimal ducts; and exocrine glands such salivary glands, lacrimal glands and mammary glands are of the IgA class. IgA in external secretions occurs exclusively as SIgA with a sedimentation rate of 11S and a molecular mass of 385,000 to 450,000 daltons. The IgA in rodents and ruminants is secreted locally by plasma cells, for instance in the intestinal lamina propria (Vaerman et al., 1973; Beh et al., 1974; Quin et al., 1975) as a dimeric molecule. Dimeric IgA comprises two IgA molecules joined by another polypeptide (J chain) both of which are synthesized by the plasma cells. Dimeric IgA bind to the polymeric immunoglobulin receptor (secretory component), which is expressed by epithelia at mucosal sites. The immunoglobulin is internalized in vesicles by receptor mediated endocytosis and transported to the luminal surface where it is secreted as the IgA-J-chain-SC complex (hence the term SIgA). Secretory IgA may also originate from the serum, as observed for biliary IgA in rats (Jackson et al., 1978) and sheep (Sheldrake et al., 1984) and the same apply to a varying extent in other secretions (Sheldrake et al., 1980).

The distribution of various classes of immunoglobulins in cattle and sheep and their physicochemical properties have been reviewed extensively (Butler 1969; 1981; 1983; Lascelles et al., 1986) and Table 2.1 shows the concentration of the various isotypes of bovine and ovine immunoglobulins in serum and other secretions. It may be observed that IgG_1 and IgG_2 are present in nearly equal amounts in serum and represent 30% of the total serum protein. The next most abundant isotype is IgM, while IgA is at low concentration as found in most mammals other than man (Heremans, 1974). However, unlike in rodents and man, the levels of immunoglobulin isotypes show distinct variations in certain mucosal and glandular secretions. In cattle, the normal intestinal secretion contains nearly equal concentrations of both IgA and IgG_1, with very low IgG_2. This seems to be consistent with the findings of Curtain et al (1971) and of Newby and Bourne (1976), who reported more IgG than IgA cells in the small intestine of calves and even more IgG_1 in intestinal secretions. However this data was not reflected in the organ cultures of Butler et al., (1972) where it was reported on the prominence of IgA synthesis in many local tissues including the intestinal mucosa. This was further confirmed in another study (Allen and Porter, 1975) where all regions of the calf gut showed marked predominance of IgA plasma cells. Plasma cells secreting IgA also predominate in the sheep intestine (Lee and Lascelles, 1970; Beh and Lascelles,1974). Yet even in sheep, Curtain and Anderson (1971)

demonstrated nearly equal numbers of IgG_1 and IgA cells in the gastro-intestinal tract of these animals, again supporting the strong representation of IgG_1 along with IgA in the " Thiry-Vella " loop secretions of the ovine species as shown in Table 2.1.

In the saliva of both cattle and sheep, IgA comprised nearly 80-90% of the total immunoglobulins. The studies in sheep indicated that part of the sIgA content of saliva originated locally from plasma cells located near the glandular epithelium of the non-parotid glands whereas the remainder was transported selectively from the serum (Lee and Lascelles, 1970; Watson and Lascelles, 1971; 1973). This was further supported in a recent study where it was demonstrated that iodine-labelled both IgA and IgM were transported selectively into saliva from the serum (Scicchitano et al., 1986).

In contrast, in the colostrum of ruminants, unlike in rodents and humans, IgA constitutes less than 10% of the total immunoglobulin (Table 2.1). The major isotype in colostrum is IgG_1. The overwhelming presence of IgG_1 in cow's colostrum has been ascribed to the selective transfer from the blood serum, commencing 2-3 weeks before parturition (Brandon, Watson and Lascelles, 1971). In bovine milk, the total immunoglobulin concentration is only about 1% of that in colostrum, the predominant isotype remains IgG_1, but the proportion of IgA is increased. Similar studies in sheep have shown that the selective transfer of IgG from serum continues into lactation in much less intense fashion (Watson and Lascelles, 1973).

Reaginic antibody similar to human IgE has been described in cattle (Wells and eyre, 1971; Hammer et al., 1971). Recently, IgE-containing cells has been quantitated in bovine lymphoid tissues (Gershwin and Olsen, 1985), with the greatest numbers present in bronchial and mesenteric lymph nodes. The data showed a similar trend in IgE distribution to those reported for other species.

2.4.2.2 *Selective transport of IgG_1*

Butler (1983) stated (quoting the personal communication of Leary and Larson) that in the transport of IgG_1, a vesicular mechanism operates within the cytosol rather than lateral transport through tight junctions. Specific binding of IgG_1 to mammary epithelial cells had been demonstrated in cattle (Hammer et al., 1969), with binding affinities as high as 10^{11}l / m (Sasaki et al., 1977). The binding receptors have been shown to be Fc-specific both in cattle (Kemler et al., 1975) and in goats (Micusan and Borduas, 1977) and receptors for IgG_2 may be present (Sasaki et al., 1977).

Table 2.1: Concentrations of immunoglobulins (mg/ml)in blood serum and various secretions of sheep and cattle. Values represent average with ranges within brackets

Source	Species	IgG_1	IgG_2	IgM	IgA
Serum	Cattle	11.2 (6.0-15.1)	9.2 (5.0-13.5)	3.05 (10.6-4.3)	0.37 (0.06-1.0)
Serum	Sheep	17.9 (22.6-12.1)	6.3 (4.1-8.8)	2.40 (1.70-3.6)	0.16 (0.05-0.32)
Colostral whey	Cattle	48.2 (39.0-61.1)	3.98 (2.1-4.3)	7.10 (5.0-9.9)	4.70 (3.8-6.3)
Milk whey	Cattle	0.40 (0.28-0.51)	0.06 (0.03-0.08)	0.15 (0.10-0.19)	0.11 (0.05-0.32)
Parotid saliva	Sheep	0.0017	0.0003	0.0001	0.0012
Submaxillary saliva	Sheep	0.0572	0.0100	0.0049	0.5050
Thiry Vella loop fluid	Adult sheep	2.97	1.48	0.30	4.87
Normal intestinal fluid	Cattle	0.25	0.06	trace	0.24
Bile	Cattle	0.10	0.09	0.05	0.08
Tears	Cattle	0.32	0.01	0.18	2.72
Nasal secretion	Cattle	1.56		0.40 (0.23-0.56)	2.81 (1.8-3.9)

Adapted from Lascelles et al., (1986).

To clarify the possibility that colostral and milk IgG_1 originates from production within the glandular tissue as well as from the serum, Newby and Bourne (1976) used radio-labelled IgG_1 given intravenously to calculate changes in specific activity and establish that nearly 100% of the colostral IgG_1 was of serum origin in bovine milk. Similar transfer of IgG_1 from blood into colostrum and milk occurs in ovine species (Brandon et al., 1972; Sasaki et al., 1976). However, Local synthesis of IgG_1 has been demonstrated in organ cultures (Butler et al., 1972), IgG_1 plasmacytes have been observed in milk (Yurchak et al., 1971) and IgG_1 plaque-forming cells have been demonstrated in milk (Chang et al., 1980). It appears therefore, that some IgG_1 is produced locally and this local population of IgG_1-producing cells might arise from a distal site antigenic stimulation in the gut mucosa.

In sheep and cattle, the higher ratio of IgG_1/ IgG_2 in saliva when compared to serum (Mach and Pahud, 1971; Watson and Lascelles, 1973) indicated a combination of selective IgG_1 transport (Curtain et al., 1971; Watson and Lascelles, 1973) and local synthesis (Morgan et al., 1981).

In the intestinal secretion of bovines, Newby and Bourne (1976) reported that IgG_1 was selectively transported from the plasma. In sheep, IgG_1 does not appear to be transported selectively from the serum (Cripps et al., 1974), although there is disagreement upon this point (Curtain and Anderson, 1971).

In the respiratory mucosa and lung, the higher percentage of IgG_1 than IgG_2 secreting plasma cells suggested that some IgG_1 may originate from local production in respiratory secretions (Allen et al., 1979: Morgan et al., 1981).

With regard to the existence of transport mechanisms for IgG_2, it has been generally reported that IgG_2 is not selectively transported into ruminant intestinal secretions, bile or saliva (Sheldrake et al., 1984; Scicchitano et al., 1986; Sheldrake et al., 1985c; Watson and Lascelles, 1973). However in certain studies, it has been observed that inflammation of mammary gland resulted in serum transudation and elevation of albumin and IgG_2 levels in milk (Butler et al., 1972; Mackenzie and Lascelles, 1968; Guidry et al., 1980). This increase is coincident with elevation of polymorphonuclear neutrophils (PMN) in the gland (Anderson and Andrews, 1977) and these cells are known to specifically bind IgG_2 to their membranes. IgG_2 has been reported to be cytophilic for the cell membrane of polymorphonuclear neutrophils (Watson, 1975). In the normal gland, levels of IgG_2 are generally low, but local synthesis has been reported (

Butler et al., 1972). Newby and Bourne (1976) estimated that 98% of the IgG_2 was produced locally.

2.4.2.3 *Transport of other immunoglobulins.*

It has been reported that the secretory component (SC)- dependent transport mechanism does exist for both dimeric IgA and for pentameric IgM in ruminants (Butler, 1983). In cattle, Newby and Bourne (1976) observed that more than 50% of the IgA in milk was of serum origin. Butler (1981) demonstrated that injection of anti-SC into cattle resulted in a selective appearance of this antibody in both bile and saliva, suggesting that an SC-dependent pathway for IgA transport from serum is present in cattle. The phenomenon is also consistent with the prevalence of SC in mucosal epithelial cells of cattle (Yurchak et al., 1971; Butler, 1973). IgA transport in all mammals involves a complexing of dimeric IgA with SC on epithelial cells, followed by a vesicular transfer (Kuhn and Kraehenbuhl, 1979; Renston et al., 1980). Such a transport mechanism is reported to be operative for dimeric IgA synthesized by local plasma cells as well as for serum dimeric IgA. Cripps and Lascelles (1976) in their studies on the origins of immunoglobulins in salivary secretions of sheep observed that an inverse correlation exists between concentrations of IgG_1 and IgA in secretions This suggests that there may be a competition between the two isotypes for the vesicular mechanism, although the nature of such competition is not known.

Levels of IgM in colostrum are often higher than levels of IgA (Table 2.1). IgM is present in low concentration in most mucosal secretions (Butler, 1974: Lascelles and McDowell, 1974; Saif and Bohl, 1979). In respiratory and intestinal secretions IgM appears to be derived predominantly from serum (Cripps et al., 1974; Scicchitano et al., 1984c), although considerable number of IgM-containing cells have been detected in the bovine (Allan and Porter, 1975) and the ovine intestinal lamina propria (Lee and Lascelles, 1970). Newby and Bourne (1977) reported that less than half of the IgM in cows milk was of serum origin. The serum versus local origin of IgM remains unresolved. In ovine mammary secretions, IgM appears to be predominantly of local origin and derived from plasma cells resident within the mammary gland (Brandon et al., 1971; Watson and Lascelles, 1973). It is more than likely that IgM may be transported selectively into milk, saliva and other secretions by the SC mechanism (Scicchitano et al., 1986). Polymeric IgM has been shown to bind successfully to SC with an affinity constant similar to that of polymeric IgA (Weicker and Underdown, 1975; Socken and Underdown, 1978).

2.4.2.4 *Structural features of immunoglobulins / antibodies.*

Immunoglobulins belong to the class of proteins called globulins and they have several structural features essential for their immune functions. The two most important of these features are specificity and biologic activity. When specific immunoglobulins are produced in response to antigenic stimulation by the immune system, they are designated as antibodies. Each immunoglobulin molecule is made up of two larger (heavy or H) and two smaller (light or L) polypeptide chains. These chains are held together by a number of disulphide bonds. Immunoglobulin molecules are classified on the basis of the structural variations in H chains encoded by the respective C_H genes and they also vary in carbohydrate content. The H chains of different classes (isotypes) of immunoglobulins, are designated with Greek letters, as follows:

Immunoglobulin class (isotype)	Heavy chain
IgM	μ
IgG	γ
IgA	α
IgD	δ
IgE	ε

It is the nature of the H chains that confers on the molecule its unique biologic properties, such as its half life in the circulation, its ability to bind to certain receptors, or its ability to activate compliment upon combination with antigen.

While there are five different major classes of immunoglobulins, there are two major classes of L chains, called k and l. Any individual of a species produces both types of L chain, but the ratio of k- chains to l- chains varies with the species (mouse: 95% k ; human: 60% k). However, in any one immunoglobulin molecule, the L chains are always either both k or both l. Unlike mice and humans, ruminant L chains were reported to be nearly all of the l- type (Hood et al., 1967; Beal and Squires, 1970).

The disulphide bonds occur both between(inter-chain) and within (intra-chains) the respective chains. The intra-chain disulphide bonds cause the formation of loops in the H and L chains. Each loop in the polypeptide chains forms the compactly folded domain (globular region with a tertiary structure). In fact, H chains have four (IgG and IgA) or five (IgM and IgD) domains, separated by short unfolded stretches and L chains have two

domains each. The first domain on L and H chains is highly variable, in terms of amino acid sequence, from one antibody to another, and it is designated V_L and V_H accordingly. The second and subsequent domains on both chains are much more constant in amino acid sequence and are designated C_L or C_H1, C_H2, and C_H3.

While reviewing on bovine immunoglobulins, Butler (1983) observed that bovine immunoglobulins and those of other ruminants are homologous to their counterparts in other mammals. This has been illustrated by gel diffusion tests in which heterologous antisera against bovine immunoglobulins cross-react with the respective isotypes of various other species, especially those from other ruminants. Interspecies homology was shown to be largely restricted to the Fab portions of the ruminant IgG. The comparative studies on vertebrate immunoglobulins suggested that μ and L chain determinants are more evolutionarily conserved than gamma and alpha chains (Mehta et al., 1972; Curtain and Friedenberg, 1973). Data from studies on bovine humoral immune responses suggested the typical pattern of B- cell differentiation in the production of immunoglobulins was like that in other species, although IgD was not identified in cattle and has therefore not been available as a marker for the differentiation process (Gottlieb, 1982; Huang et al., 1981). Recently, Knight and Becker (1987) isolated genes encoding bovine mu, gamma, alpha and epsilon chains employing recombinant DNA technology.

2.4.2.5 *Effector activities of immunoglobulins*

Functionally, the immunoglobulin molecule consists of two fragments. One fragment, termed the Fab, (fragment antigen binding) is concerned with the specific binding of antibody to its antigen (Fab-mediated functions). The specific antigen binding site is created by regions of both heavy and light chains (V_L, C_L domains) which show extreme variability in their amino acid sequences. The other fragment of the antibody molecule, the Fc (fragment crystallizable) is responsible for the biological activity of antibody molecule and in particular, the binding of various isotypes to Fc receptors and the polymeric immunoglobulin receptor. Furthermore, after antigen has been bound to the Fab part of the intact molecule, a conformational change in the Fc portion of the antibody molecule exposes a binding site for the C1 component of complement on the CH2 domains of IgG and the CH3 domains of IgM.Thus a given clone of B cells can potentially produce antibodies of various isotypes, each of which

may combine with the same epitope but each confers on the antibody different biological functions.

Many important biological properties are attributed to antibodies. These include neutralization of toxins; immobilization of microorganisms; neutralization of viral activity; antibody dependent cellular cytotoxicity (ADCC); activation of serum complements to facilitate the lysis of microorganisms and opsonization to promote phagocytosis and destruction of microorganisms by phagocytic cells. Another important biological activity of IgG antibodies is their ability to cross the placenta from the mother to the fetus, although this does not occur in ruminant species. The ruminant immunoglobulins have many structural similarities to rodents and humans.

In ruminants, IgG_1 and IgG_2 predominantly occur as monomeric molecules in all body fluids in which they have been studied (Mach and Pahud, 1969; Duncan et al., 1972; Tewari and Mukkur, 1975). Higher polymers of IgG are observed in both serum and secretions in cattle (Sullivan et al., 1969; Hammer et al., 1970). Currently there is no evidence if these higher polymers of IgG contain J chain.

The effector activities of ruminant immunoglobulins in bacterial, viral and protozoal immunity have been well documented in the literature. IgG is the major immunoglobulin in the serum of all the mammals. Its relatively long half-life compared to IgA and IgM, makes it an important molecule in the immune defence mechanisms. When bivalent IgG antibodies combine with microorganisms, cross-linked complexes are formed by virtue of the multi-determinant nature of the antigens leading to the phenomenon of agglutination (clumping together). The clumped antigen-antibody complexes are phagocytized and destroyed by phagocytic cells. Although IgG can agglutinate antigen, IgM molecules are more efficient agglutinating antibodies. In comparison to the IgG molecule, which has a four-chain structure, the IgM molecule has five such units, each of which consists of two L and two H chains. Each pentameric molecule contains a single J-chain polypeptide. It has been reported that J-chains obtained from bovine IgM were found to be antigenically indistinguishable from those obtained from SIgA of bovine origin (Komar et al., 1975).

In ruminants, IgG plays an essential role in the colostrum (Watson, 1980), where it provides humoral immunity to the neonate. It has been demonstrated that maternally derived IgG_1 in the colostrum acts locally in the lumen of the gut of lambs and calves, when they were challenged with

rotaviruses (Snodgrass and Wells, 1978; Snodgrass et al., 1980; Fahey et al., 1981). Recent data indicated that IgG anti K99 pilus antibody in colostrum is more efficient on a molar basis than IgM derived from the same source.(Altman and Mukkur, 1983). IgG_1 is the major immunoglobulin in colostrum and milk. In calf-rearing, the feeding of colostrum is of paramount importance to prevent diarrhea and to produce a profitable herd replacement. A new-born calf should receive minimally 2 litres of the first colostrum within the first four hours of life (Fred Trout, 1991). It is very much emphasized that the most important determinant in feeding colostrum to the calf is the colostrum immunoglobulin mass (concentration x volume). The colostrum should contain at least 30mg/ml of IgG_1 (Ig content > 60 mg/ml). Roy (1990) observed that to ensure complete protection from enteropathogens, the neonatal calf should receive at least 7 kg of colostrum, containing approximately 400g Ig over the first 24 to 36 hours of life.

It has been well established in sheep (Watson, 1975), cattle (McGuire, Musoke and Kurtti, 1979; Howard, Taylor and Brownlie, 1980) and goats (Micusan and Borduas 1977) that IgG_2 is the only immunoglobulin isotype which is cytophilic to neutrophils.. This property of IgG_2 is of crucial importance in resistance to those microbial infections in which phagocytosis is an important effector mechanism, e.g. staphylococcal mastitis (Watson, 1976) and theileriosis (Musoke et al., 1982).

The role of secretory-IgA (SIgA) in milk to ruminants has not been studied in detail. Studies in other animals and humans have revealed that colostrum from cows hyperimmunized by multiple injections of Cryptosporidium parvum into the mammary gland, provided efficacious passive prophylaxis and therapy against cryptosporidiosis (Fayer et al., 1989a; 1989b; 1990; Tzipori et al., 1987; Ungar et al., 1990;). In all these studies, both IgG_1 and IgA of ruminant milk has been to shown to have contributed in the reduction of parasite number. Such studies indicate that the IgG and S-IgA molecules may play a similar protective role in the intestinal tract of neonate ruminants. All of these observations stress the strategic importance of immunologically manipulating mammary tissue for specific purposes.

Furthermore, it has been described that IgA antibodies directed against the iron-binding molecules produced by bacteria exert a powerful bacteriostatic effect in synergy with lactoferrin (Funakoshi et al., 1982), and IgA antibodies also exert antiparasitic effects providing protection against Taenia taeniaformi, although the mechanism is not clear (Lloyd and Soulsby, 1978).

The multivalent oligomeric nature of sIgA allows it to cross-link target microorganisms efficiently in the mucus-rich environment in the digestive, respiratory and genital tracts (McGhee and Mestecky, 1990). For a considerable period of time, the effects of IgA in the mucosal secretions were thought to be mainly of a passive nature, but over the last decade or so, a number of studies made it clear that its relative resistance to proteolysis in the gut its structural configuration, hydrophilicity, and charge (confered by the Fc part of the S-IgA) give this immunoglobulin special mucosal defence properties (Underdown and Schiff, 1986).

In the intestinal secretions, the role of secretory IgA as an efficient agglutinating antibody has been cited in a number of studies. In these studies, IgA has been shown to reduce contact between the antigen and the systemic immune system by reducing absorption of antigens by the intestine and the lung(Walker et al., 1972; Andre et al., 1974; Stokes et al., 1975). In these situations, it has been presumed that binding of soluble antigens, including antigens and toxins released from microorganisms, to S-IgA antibodies forms highly agglutinated macromolecular complexes that are readily incorporated into the mucus layer and eliminated from the gastrointestinal and respiratory tracts. These observations have been supported by similar findings in pigs, where it has been demonstrated that IgA against Vibrio cholera will prevent disease by interfering with the attachment of the bacteria to the intestinal wall (Fubara and Freter, 1973).

The role of IgG in the intestinal secretions has not been defined clearly. It has been speculated that in view of its recognized resistance to proteolysis by chymotrypsin (Broch et al., 1977a, 1977b) it may play a role in prevention of bacterial colonization or in toxin neutralization.

In recent years, the roles of IgG and SIgA have received considerable attention in caries research in laboratory animals and humans. The oral cavities of these animals are continually exposed to various microbial organisms and both saliva and the gingival fluid have been shown to mediate immune functions through the major classes of antibodies. One of the preliminary reports indicated that patients with hypogammaglobulinaemia had low levels of anti Streptococcus mutans antibodies, harboured increased numbers of Strep.mutans and showed more caries than comparable controls (Cole et al., 1977). In a series of studies, it has been shown that the adherence of homologous and immunologically related cells of Streptococcus mutans to smooth enamel-like surfaces can be markedly inhibited by antisera against the whole cells (Olson et al., 1972; Mukasa and Slade, 1974; Hamada and Slade, 1976; Kilian et al., 1981). One of these studies (Olson et al., 1972) reported that either

IgG or IgM antibodies inhibited adherence of S.mutans and the authors hypothesized that this inhibition is brought about by antibodies directed against an enzyme, dextran sucrase, which is essential for these organisms to synthesize the extracellular polysaccharides needed by these bacteria to adhere to smooth enamel surfaces. In another study, McGhee and Michalek, (1986) demonstrated that the firm attachment of S.mutans can be inhibited by salivary S-IgA antibodies directed at the S.mutans glycosyltransferases which catalyse the synthesis of extracellular polysaccharides required for the accumulation of the bacterium. Kilian et al. (1981), while pointing to the role S-IgA, provided additional evidence for the complex interdependence of adherence and agglutination. They showed that S-IgA is but one of several salivary agglutinating factors, that are involved in bacterial agglutination. Coating of bacteria with antibody molecules is known to occur in vivo (Brandtzaeg et al., 1968), and it may conceivably block specific receptors on the bacterial surface, or reduces the hydrophobicity and negative charge of the bacteria (Magnusson et al., 1979), with ensuing inhibition of adherence.

The effector roles of antibodies in protozoal immunity in ruminants have been examined mainly in parasites that inhabit either the blood cells or the gut environment. In blood-born infections, both antibody-mediated and cell-mediated immune responses are induced in the control of the disease. In African trypanosomiasis of cattle, an increased IgG and IgM antibody activity against the variable surface glycoproteins (VSG) of the infecting trypanosomes has been demonstrated (Nantulya et al., 1979; Masake, Musoke and Nantulya, 1983), by the ability of IgM and IgG antibodies to neutralize the infectivity of homologous trypanosomes. Ngaira et al., (1983) investigated the effect of bovine anti-trypanosome antibodies on the interaction between T. brucei organisms and bovine peripheral blood monocytes and suggested that the bovine mononuclear phagocytic system, acting in concert with VSG-specific antibodies, could be a major mechanism of parasite clearance in the infected host. Similar evidences have been presented in theilerial infections in cattle (Musoke and colleaques, 1982; 1984). In bovine babesiosis, it was concluded that opsonization was probably the basis of protection by the immune system (Mahoney, 1986).

In helminth infections of the ruminant gastrointestinal tract, the emphasisis has been generally on the involvement of local hypersensitivity reactions and their role in the rejection or expulsion of the worms. Early reports suggested that IgG1 was responsible for anaphylactic activity in sheep (Curtain, 1969; Hogarth-Scott, 1969). Of late, IgE has been implicated (Emery et al., 1993).

The role of IgA in immunity to helminth parasites has yet to be resolved. A substantial IgA antibody-containing cell response has been associated with reduced numbers of worms, following a challenge with antigens from Ostertagia in sheep (Smith et al., 1983). There is some evidence that subpopulations of neutrophils may bear IgA membrane receptors (Shen and Fanger, 1981). However, the direct role of different classes of antibodies has yet to be completely resolved.

2.4.3 Cell-mediated immunity

The term ' cell-mediated immunity ' is generally restricted to immune protection that is afforded by T cells which are involved in either the direct (cytotoxic T cells) killing of infected cells, tumour cells and cells in both allogenic and xenogenic transplants or in mediation of macrophage activation and delayed-type hyper-sensitivity. The majority of cytotoxic T cells (CTLs) are derived from the $CD8^+$ subpopulation of T lymphocytes and they recognize antigen in the context of class I MHC restriction. A minority of CTLs are $CD4^+$ and recognize antigen in association with class II MHC molecules. Both types of CTLs have their origin from a pool of non-lytic precursor cells which need the co-operation of Th cells before they finally mature into functional CTLs in response to their specific antigens. The importance of Th cells appears to be primarily for the provision of IL-2, without which most CTLs fail to mature and proliferate. There is also another subpopulation of mononuclear cells refered to as large granular lymphocytes which included Natural Killer (NK) cells and Killer (K) cells. Non-specific killing of certain tumour target cells is attributed to NK cells, and killing of antibody-coated target cells is due to K cells activity. Very little is known about the target cell recognition by NK cells. Cytolysis by CTLs require intimate contact between a viable effector cell and its target and this is normally mediated through T cell receptor interaction with specific ligands on the target cell. Following the contact with a specific target cell, cytolytic cells secrete perforins, cytolysins and serine proteases which cause the death of the target cells.

Cell-mediated immune reactions, including MHC-restricted cellular toxicity, natural killer-cell activity and antibody-dependent cell-mediated cytotoxicity (ADCC) have also been considered to play a role in intestinal immunity against enteric infections in a number of different species. Although there are no precise data which indicate that cell-mediated immunity cannot act in the intestinal lumen, or at the interface between the lumen and the epithelial surface, it is generally assumed that cellular immunity is largely expressed within the intestinal tissue and as such

would be more effective against organisms which penetrate the mucosa (such as Salmonella and Shigella). Little is known about CMI in the lumen of the gut. In one particular study, local T-cell dependent cell-mediated immune reactions in the gut have been demonstrated to allografts, and to infections with Giardia muris or Nippostrongylus brasiliensis (Ferguson and McDonald, 1976). There is limited information about such reactions at the rumen mucosa.

2.4.4 *Immunological memory*

One of the characteristics of the immune system is memory following the primary immune response to an antigen. Any subsequent encounter with the same antigen usually results in a quicker and larger response, even years after the first encounter. The process is ascribed to the presence of clones of antigen specific B and T memory cells, selectively expanded by earlier contact with antigen. The phenomenon is referred to as anamnesis and the responses as anamnestic. While the existence of an immunological memory in systemic immunity has been known for a long time, its existence has not been clear at mucosal surfaces. Many earlier studies have failed to demonstrate anamnesis or secondary responses at the mucosal level which are either much greater or more rapid than the primary responses (Ogra and Karzon, 1969; Bazin, Levi and Doria, 1970; Porter et al., 1974; Taubman and Smith, 1974). The intestinal SIgA response to antigens like cholera toxin were found to be of relatively short duration, lasting for few weeks in mice and rats (Pierce and Cray, 1982; Lycke and Holmgren, 1989). However, repeated oral exposure with cholera B subunit vaccines resulted in long-lasting immunity in both animals and humans, which strongly suggested the existence of a potent mucosal immunologic memory in these species (Svennerholm et al., 1982; Quiding et al., 1991). It was found to be consistent with the isolation and characterization of specific memory B cells in the rat's gut mucosa (Lycke and Holmgren, 1989).

It has been shown in another study that non-replicating antigen must persist if B-cell memory is to be maintained (Gray and Skarvall, 1988) . Recently, in an adoptive transfer experiment, Gray and Matzinger (1991) have proposed (as one of the three mechanisms that may account for immunologic memory) that the original antigen persists in specialized reservoirs. The only known repository for long term retention of antigen is the surface of follicular dendritic cells, which are found only in B- cell follicles. In this paper, they showed that both helper and cytotoxic T cells keep their ability to generate secondary responses only in the presence of the original priming antigen. The results indicated that, in contrast to the

traditional view, the maintenance of T cell memory required the presence of antigen, suggesting that memory, like tolerance, is an antigen-dependent process rather than an antigen-independent state. The state of systemic unresponsiveness to orally administered haptens, proteins, and particulate antigens has been termed oral tolerance (Tomasi, 1980). Some authors have shown that certain strains of mice could be rendered tolerant to ovalbumin (OVA) by prior oral feeding (Saklayen et al., 1983; Mowat et al., 1986). On the other hand, oral tolerance to SRBC cannot be induced in C3H/HeJ mice. These mice, when given SRBC by IP injection, after prolonged gastric intubation with SRBC, elicited good secondary responses of the IgM, IgG, and mainly IgA isotypes (Kiyona et al., 1982). Interestingly, oral administration of antigen induces simultaneous responses, i.e., oral tolerance in systemic tissues and IgA responses at mucosal sites. (Challacombe and Tomasi, 1980). These findings are of great interest in analysing the natural responses to rumen microflora in the gut. Furthermore, some of these findings reinforce the present concept of that continuous stimulation of the gut mucosa is necessary for greater success in the development of mucosal vaccines. Perhaps the importance of the local route of immunization to produce secretory antibody responses is as much to load follicular dendritic cells in mucosal lymphoid tissue with antigen as it is to prime local B cells in an environment which favours committment to IgA synthesis.

2.4.5 *Characteristics of the sites for immunological interactions*

While rumen ciliates inhabit the rumen they also get swept into the upper reaches of the gut as far as the buccal cavity during the act of rumination (Dulphy et al., 1980; Orpin, 1984; Ulyatt et al., 1986). Thus, the live organisms have their access limits confined to the section of the alimentary tract from the lips to the reticulo-rumen. It is in these regions that these organisms may be subject to an immunological interaction in the event of successful immune responses in the host animal. It is appropriate to consider some of the relevant physiological characteristics of the ruminant animal and the potential that immunization against rumen protozoa may offer in the control of these organisms.

2.4.5.1 *Rumination and secretion of saliva*

The word ' rumination ' refers to the act of regurgitating ingested feed materials from the reticulo-rumen into the buccal cavity for further chewing of the cud. This is a physiological characteristic of ruminants. The value of rumination for the animal is to break down the coarse fibrous

plant materials by remastication accompanied by re-insalivation and reswallowing of the bolus for an efficient microbial action in the rumen. The process of rumination is brought about by orderly and synchronized movements of the reticulum and rumen. Such movements aid in mixing the newly ingested food with that already in the rumen and mixed food is moved forth and back between the oral cavity and the reticulo-rumen. It has been reported in the literature that large number of rumen ciliates are attached to the feed material and are therefore potentially will be swept into the buccal cavity along with the regurgitated food. Although the number of organisms reaching the oral cavity has never been estimated, numbers must be great, because sheep and cattle commonly spend about third of their time in rumination depending upon the nature of the diet. Studies with sheep (Weston and Hogan, 1967; Welch and Smith, 1968; 1969a; 1969b) indicate that sheep spend about 8-9 hours per day in rumination.

When ruminants are eating food, saliva is secreted by the parotid, the submaxillary and the sublingual salivary glands (Bailey and Balch, 1961b; 1961c). Besides these three well defined glands, other glands also contribute to saliva production. These include the dorsal buccal glands in the hard and soft palate; medial buccal glands and the ventral buccal glands in the cheek; labial and pharyngeal glands; and some additional glands in the lateral margins and root of the tongue (Kay, 1960). Saliva from the various glands drains into the oral cavity via ducts. The parotid and ventral buccal glands produce serous saliva which is thin and watery, mainly containing only protein but no mucin. The dorsal and medial buccal and pharyngeal glands produce mucous type of saliva, while the submaxillary (or mandibular), sublingual and labial glands produce mixed secretions. It has been reported that the submaxillary glands secrete only during eating and volumes as great as 480ml per hour are produced in cows, whereas the parotid glands secrete continuously, although their activity is greatly enhanced during the mastication of food (Bailey, 1961). Various factors are said to govern the flow of saliva, including the nature of the diet, the act of eating and rumination (Bailey and Balch, 1961). These authors reported that saliva secreted by cattle receiving various diets during a 24 hour period was always greater in volume during periods of rest and rumination than during the periods spent eating. The mean secretion rates in sheep during rest and rumination have been reported to be higher than during eating (Wilson, 1963).

With respect to immunoglobulins, the saliva of ruminants contains predominantly S-IgA (Table 2.1). In the studies of Pahud and Mach (1970), it was reported that mixed saliva in cattle had a mean IgA concentration of 56mg/100ml, while sheep and goats the concentration was approximately 20mg/100ml. IgA represented 60-95% of all immunoglobulins, with remaining 5-40% represented by IgG. Considering the total daily volume of saliva secreted by a sheep, the output of IgA in the secretions could exceed 1 gram/day. There are few studies concerning the specific roles of any of these immunoglobulins in the oral and rumen environment of ruminants, apart from surmising a possible role of maintaining an ecological balance of flora in the buccal cavity and the rumen. It is not known whether any specific antibodies are produced by the host animal which may have an effect on the rumen ciliate population. Rumen ciliates may be recognized as foreign material by the host during the course of their sequestration to the reticulo-rumen wall or when their metabolic or degradation products are absorbed by the host during the course of normal digestion. In this event, they could induce either tolerance or specific humoral response. Even in the event of such recognition, it may still be possible to boost the antibody levels in saliva by an anamnestic response to appropriate immunization with relevant antigens.

The pH of the saliva ranges between 8.0 to 8.6 The most important function of ruminant saliva is thought to be as a buffer to keep rumen pH within the physiological ranges to which the tissues and the microorganisms are adapted.

2.4.5.2 *Rumen environment*

The rumen may be considered as the most appropriate site for potential immunological interactions with protozoa, because it forms the natural habitat of the rumen ciliates. The rumen has a high water content and more than 70 % of it is derived from saliva (Church, 1988). Thus, saliva helps to maintain a desirable physio-chemical environment for microbial fermentation, in addition to acting as a buffer. The pH of the rumen can range from 5.5 to 7.2 and within this range the activity of the major components of the microflora remain considerably unaffected. The redox potential in the rumen is usually between -250 and -450mV, reflecting the absence of oxygen and excess of reducing power (Van Soest, 1982). Rumen temperature is generally maintained at a constant range of 38-40^{o}c.

There are very limited studies on the presence of antibodies either in the rumen wall or rumen contents (Mach and Pahud, 1971; Sharp et al., 1975). In all of these studies, there is little evidence for the presence of

antibodies in these locations. It has also been reported that the rumen wall constitutes a poor site for immune responses, because the epithelium is non-glandular and highly keratinized (Dobson et al., 1956). It may be of interest to mention that in a recent study, when calves less than a week old were inoculated orally and boosted through the same route with ruminal bacterial species, it was reported that lymph nodes associated with the forestomach (rumen, reticulum, omasum) contained a preponderance of IgG producing cells relative to the mesenteric lymph nodes (Sato et al., 1990). From the foregoing discussions, it may be noted that saliva could be the major source of immunoglobulins which will end up ultimately in the rumen. However, the suitability of the ruminal conditions for antigen-antibody interactions has not been studied in detail, apart from limited studies on the proteolysis of bovine γ globulins as part of a larger study on proteolysis in the rumen fluid (Wallace, 1983).

2.4.5.3 Rumen microbial proteases

As already discussed, there are prospects for immunological interactions to occur between rumen ciliates and specific antibodies in the oral and rumen environments. However, the nature and proteolytic properties of rumen fluid and microbial population must be given due consideration. Proteolytic bacteria have been found to comprise between 12 and 38% of the total bacterial population of the rumen (Bryant and Burkey, 1953; Fulghum and Moore, 1963). Proteolytic enzymes of ruminal bacteria appear to be predominantly cell-associated, as little proteolytic activity can be detected in cell-free rumen fluid (Blackburn and Hobson, 1960; Brock et al., 1982). The data of Kopecny and Wallace (1984) suggested that the majority of proteases produced by ruminal bacteria are periplasmic or associated with either extracellular capsular or coat materials. The pH optima for mixed bacterial proteases are generally reported in the range of pH 6.0 to 7.0 (Blackburn and Hobson, 1960: Kopecny and Wallace, 1982). On the basis of the effects of various protease inhibitors, ruminal bacteria appear to produce serine proteases, thiol proteases, and metalloproteases, but little carboxyl-like protease activity has been observed (Brock et al., 1982; Kopecny and Wallace, 1982). When the ability to hydrolyse specific synthetic substrates has been examined , rumen bacteria have been found to possess enzymes with trypsin-like, chymotrypsin-like, carboxypeptidase-like, and aminopeptidase-like activities (Brock et al., 1982; Wallace and Kopecny, 1983). Similar types of studies have been conducted with rumen protozoa (Coleman, 1983; Forsberg et al., 1984). These investigations found little activity of protozoal enzymes against casein at neutral pH, whereas

others (Abou Akkada and Howard, 1962; Brock et al., 1982) measured some activity under these conditions. Coleman (1983) found an optima of pH 3.2 as compared with 5.5 (Shinchi and Kandatsu, 1981) and 6.0-9.0 (Forsberg et al., 1984) for the activity of protozoal enzymes against casein. Coleman et al., (1983) reported that the proteolytic enzymes produced by rumen entodiniomorphid protozoa are of ' thiol ' and ' carboxyl ' types. Forsberg et al (1984), who worked with mixed rumen ciliate populations, revealed that these organisms exhibited proteolytic activity associated with cysteine-proteinases, aspartic proteinases and aminopeptidases. It may be emphasized that rumen microorganisms, particularly bacteria and protozoa, are capable of producing a mixture of proteolytic enzymes. The potency of the rumen microbial proteases on the ruminant's own immunoglobulins has not been well evaluated in terms of survial and biological activity.

Moreover, it appears that there has been no characterization of proteolytic enzymes relating to bacteria inhabiting the oral cavity of ruminants. This may be due to the relatively low incidence of oral infections encountered in these animals, although periodontitis has been reported in some parts of the world as a serious problem in sheep. In the inflammations of the gingiva, bacterial species such as Fusobacterium necrophorum and certain spirocheates have been implicated in sheep. Apart from these clinical reports, there has been hardly any research pertaining to the production of proteolytic enzymes by the oral microflora in ruminants. However, in humans, studies of the proteases of oral bacterial pathogens and on their influence on various classes of immunoglobulins has been an active area of dental research. Representative strains of Bacteroides and Capnocytophaga species have been reported to cause both cleaving of IgA and IgG into intact Fab and Fc fragments, as well as complete degradation (Kilian, 1981). It has been found that oral pathogens such as Bacteroides gingivalis in humans produce several proteases which cleave IgG and IgA non-specifically (Grenier et al., 1989).

This project is aimed at establishing an antigen-antibody interaction in the foregut section of the alimentary tract (i.e., in the oral cavity and in the reticulo-rumen). In view of the dynamic situation that prevails between the oral and rumen anatomical sites during rumination, it is appropriate to investigate the effect of proteases on immunoglobulins and their interaction with the ruminal microbial-flora.

2.4.5.4 *Proteolysis of ruminant immunoglobulins*

A series of proteolytic studies have been carried out by a number of researchers on bovine immunoglobulins (IgG_1 and IgG_2). These studies were undertaken either to examine the biological activity of these molecules in proteolytic environments or to characterize the chemical structure of the molecules. In all of these studies, individual purified enzymes were used. It has been reported that bovine IgG_2 is more resistant to degradation with pepsin than is IgG_1 (de Rham and Isliker, 1977; Kanamura et al., 1977; Butler and Kennedy, 1978). The pFc' fragment generated from IgG_2 appeared to be larger than the IgG_1-pFc' (Butler and Kennedy, 1978). In this particular study, it is of interest to note the fate of bovine IgGs when it is subjected to pepsin digestion. A 30 hour digest of an equal mixtures of IgG_1 and IgG_2 resulted in total degradation of IgG_1 to $F(ab)_2$ and pFc', leaving most of the IgG_2 intact.

IgG_1 was shown to be more resistant than IgG_2 to trypsin (de Rham and Isliker, 1977). It was also observed that IgG_1 remained unaffected by chymotrypsin (Brock et al., 1977a; 1977b). Micusan and Borduas (1977) in their studies with goat IgG subclasses, reported that IgG_1 was more resistant to papain than IgG_2 either in the presence or absence of cystine.

There appear to be no specific data available currently on the enzymatic degradation of ruminant IgA or IgM by serine proteases, pepsin or bacterial proteases. There are limited studies on the proteolysis of secretory component (SC). It has been reported that the bovine SC is susceptible to proteolysis (Labib et al., 1976). This is paradoxical in light of similar studies of SC in other species which showed a greater resistance to enzymatic degradation as SIgA compared with dimeric IgA (Tomasi and Czerwinski, 1968; Brown et al., 1970; Shuster, 1971; Lindh et al., 1977). The resistance of the secretory IgA of various species has been reported to be due to the secretory component blocking access to critical enzyme-sensitive sites on the IgA dimer.

2.4.5.5 *Fate of immunoglobulins in the neonate*

In the neonates of ruminants, the immunoglobulins of the colostrum are exposed to enzymatic processes in the oral cavity and gastrointestinal tract. It has been observed that the combination of low gastric acidity, the buffering effect of colostrum and the rapid passage of milk into the duodenum make peptic digestion very unlikely (Mylrea, 1966). In the upper part of the small intestine, immunoglobulins are exposed to proteolytic action by two major pancreatic proteases trypsin and

chymotrypsin respectively. Colostral secretions have been shown to contain a trypsin inhibitor which has been postulated to account for the survival of IgG (but IgM not associated with SC) during the first day or two after birth. The relative resistance of ruminant immunoglobulins (IgG) to trypsin and chymotrypsin have been mentioned in the preceding section (2.4.5.4). In addition, the colostrum and milk also contain SIgA. The relatively higher degree of resistance of SIgA in the milieu of the gastrointestinal tract has been clearly demonstrated in other species. Thus, if both IgG and IgA molecules are of the correct specificity, they should be able to protect against pathogenic organisms and also affect the behaviour of rumen microorganisms. If antibody levels in the rumen can be manipulated by immunization, it is possible that rumen fermentation patterns can be manipulated for specific production requirements.

2.4.6 Factors in the induction of immunity

There are some external as well as internal factors which determine the level of immune response that is manifested by any animal. Alhough the internal factors are an inherent property of an animal, the immune response is very much dependent on the experimental conditions of the system. These include the nature of antigens, choice of adjuvants, types of immunization procedures, size and duration of the dose and the age and preimmune status of the animal.

2.4.6.1 Nature of antigens

The nature of antigens includes the molecular size, complexity, structural stability and degradability of the molecules. In general, large molecules are better antigens than small molecules but it does not follow that all large molecules are antigenic. Molecular rigidity is important, and non-rigid molecules such as gelatin are poor antigens, while lipids are non-antigenic. It should be complex as well. Lipo-polysaccharides, polysaccharides and proteins are well known antigens. The immunogenicity of a molecule also depends to a great extent on its degree of foreigness. The greater the difference between the foreign antigen and the animal's own antigens, the greater will be the immune response.

2.4.6.2 Choice of adjuvants

The immune response to an antigen is enhanced by the incorporation of substances called adjuvants. Many adjuvants act by slowing the release of antigen into the body. They may be either insoluble salts (aluminium hydroxide, aluminium potassium sulphate, aluminium phosphate) or

water-in-oil emulsions (Freund's complete and incomplete adjuvants). Others are or surface active agents (lanolin, saponin and phospholipid liposomes) and polyanions like dextran-sulphate, sodium alginate or suramin. The salts and oil emulsions induce granulomata in the tissues and the antigens within the granuloma slowly leach into the body and so provide prolonged antigenic stimulus. In the Freund's Complete Adjuvant (FCA), killed tubercle bacilli (Mycobacterium tuberculosis) are incorporated into the water-in-oil emulsion. FCA not only forms a depot, but the tubercle bacilli contain a compound called muramyl dipeptide (MDP, n-acetyl muramyl-L-alanyl-D-isoglutamine) which acts on macrophages to produce interleukin-1, interleukin-6 and TNF, which stimulate the cells of the immune system and so enhances immunity.The surface active agents act on cell membranes to enhance immune reactivity and polyanions too are believed to operate in a similar fashion, perhaps enhancing phagocytosis and uptake of antigen. Adjuvants are also important in recruiting antigen presenting cells to sites of antigen deposition.

2.4.6.3 Age of the animal

The age of the animal has an important influence on the relative degree of immune responsiveness. In a number of studies, it has been demonstrated that ruminant animals are more susceptible to infectious diseases during their first six months of life. This phenomenon is well observed in nematodes infections (Manton et al., 1962; Benitez-Usher et al., 1977; Dineen et al., 1978). Recently it has been demonstrated clearly for bacterial infections (Weiss et al., 1986). This lower resistance of young animals to various diseases has been attributed to immunological hyporesponsiveness. However, it must also be remembered that aquisition of immunity to new infections is progressive, especially in the case of parasitic infections. It has been observed that the ovine neonate has a fully developed immune system at birth (Al Salami et al., 1984). Whilst discussing the onset of immune responsiveness in the sheep foetus, these authors referred to the ability of the foetus to respond to certain antigens and not to others. It is not clear why the immature ruminants have lower immune responsiveness than the adults. It has been suggested that the colostral antibody in neonates might suppress the development of active immune responses (Husband and Lascelles, 1975). It has been further speculated that some of the non-specific humoral and suppressor factors in the colostrum might add to the suppression of the immune responses (Drew et al., 1983). Recently, Hooper et al., (1986) observed that there was

an increased activity of suppressor T lymphocytes and Levin and Gershon (1988) have reported that there was impaired antigen-processing and presentation by antigen-presenting cells in neonates. These findings indicate that the immune response mechanisms may be functioning under specific restraints in young ruminants .

2.4.6.4 Genetic influence

The ability to respond to an antigen varies with the genetic make-up of the animal. It is known that pure polysaccharides are immunogenic when injected into mice and humans but not when injected into guinea pigs. Much additional information on the genetic control of immune responses has accrued from the use of inbred strains of animals . According to Biozzi (1984), there are probably a number of distinct but independent genes, including those of major histocompatibility complex (MHC), involved in regulating immune responses. The extreme polymorphism of MHC class II molecules has been directly related to variation in T-cell dependent immune responses (Guillet et al., 1987). Even in outbred species, it has proved possible to link immune responsiveness with MHC class II alleles , using a T cell proliferation assay to a multi-epitope antigen (Hiryama et al., 1987). Current understanding of the genes regulating immune responsiveness to complex proteins in outbred populations as cattle, sheep, goats etc., remains unclear. Alleles of MHC class II products have not yet been clearly defined in ruminant populations although considerable polymorphism has been demonstrated at the DNA level in cattle (Anderson et al., 1986). Recently, there were a number of studies which analysed the T cell recognition of bovine MHC class II antigens in relation to antigen presentation and immune response gene effects (Glass and Spooner, 1990; Glass et al., 1991a, 1991b). These studies also detected distinct class II haplotypes with functional significance in antigen presentation. The foregoing studies represent a major step forward in studies on the effects of genetic make up of ruminant species in determining patterns of resistance and susceptibility of individuals to disease and their responses to vaccines.

2.4.7 New approaches in the development of vaccines

Advances in biotechnology have brought in a new field of vaccine production. Several approaches are being studied in attempts to make vaccines more effective, safer and cheaper. An ideal vaccine elicits protective immunity and memory, so that subsequent exposure to the organism will result in elimination of the organism and in boosted immunity. The isolation of the protective portions of the antigen has

facilitated the development of new vaccines that use only subunits of the pathogen or peptides representing protective epitopes. The latter may be in the form of recombinant proteins, recombinant organisms expressing the epitopes, chemically synthesized peptides conjugated to immunogenic carriers or anti-idiotypic antibodies. Several problems have limited the development of vaccines using recombinant antigens. Purified proteins have generally been observed to be less immunogenic. They tend not to induce the major-histocompatibility complex class-I restricted T cells that are important for protecting against a wide range of viral and other infectious dieseases. They may also induce systemic tolerance when given to naive animals, particularly through the oral route (Mowat, 1987). Recently it has been suggested that lipophilic immune-stimulating complexes (ISCOMS) may provide an oral immunization vector for the induction of a wide range of immune responses to protein antigens (Mowatt and Donachie, 1991). It would be extremely useful if such vaccines could be given by the oral route, because they will induce not only systemic immunity but also the mucosal immunity.

The difficulty of choosing a suitable adjuvant for purified proteins has been overcome by the use of genetically-modified microorganisms that express recombinant protein antigens. Alhough these organisms do not require the use of any adjuvant, the development of these systems are still in their infancy.

Use of synthetic peptides is another preferred approach because of its safety over gene-splicing techniques but it does require the use of adjuvants, it is expensive and it does not usually lead to the priming of T_h cells against the natural antigen. Immune responses to these vaccines are not therefore boosted by infection.

The anti-idiotype vaccines can be made by taking a monoclonal antibody against the epitope in question and using it to immunize an animal. The anti-idiotypes produced can be used in turn to vaccinate an animal. The anti-anti-idiotypes formed may be protective, directed not only against the anti-idiotypes but also against the original antigen. The anti-idiotype vaccines are an attractive proposition in animal production research as an active immunization technique to increase growth in cattle, sheep and pigs (see review: Holder et al., 1991). However, as with synthetic peptides, they do not prime T_h cells and the immune response is not boosted by natural infection.

While most of the new approaches remain theoretically attractive, many important aspects need to be addressed, especially the safety and acceptability of the proposed vectors, the development of appropriate

formulations and also possibly the net effect on the immune effector mechanisms.

2.4.8 Immunogenicity of rumen ciliate antigens

There are no publications dealing with rumen protozoal antigens. These organisms are highly specialized to live in the ruminal environment, and because they are not considered to be invasive, they may not even be recognized by the host immune system. However, this does not preclude the potential of these organisms to behave as immunogens/ antigens. An immunogen is any agent capable of inducing an immune response. There are three characteristics that a substance must possess to be immunogenic: (1) foreigness, (2) high molecular weight, and (3) chemical complexity. The rumen ciliates have an evolutionary history of development in the foregut of ruminant animals. Their transmission from host to host occur after birth through ingestion of materials/saliva either from the dam or from neighbouring animals. Unlike rodents, the ruminant neonate possesses a fully-developed immune system at the time of birth and it can respond to non-self molecules. The outer coat or pellicle of rumen holotrichs is monolayered, while it is a five-layered structure in entodiniomorphod ciliates. The descriptions of ultra-structural details suggest that the pellicle may be a protein-carbohydrate complex. There are no studies in the literature that characterize the biochemical composition of the pellicle-membrane of rumen ciliates. In holotrichs, the pellicle also has a complete somatic ciliature. The entodiniomorphid ciliates bear closely-packed groups of cilia called " syncilia " or" synciliary tufts " on their outer coat. It has been shown that the adoral ciliary zone could be divided into two parts (Imai et al., 1983). The outer part is composed of membranelle-like structures rather than tufts, while the inner part is composed of aggregates of cilia. It may be said that rumen ciliates, in general, do have surface components which have the potential to act as immunogens.

Very interesting studies have been done with certain ciliated free-living protozoa (namely, Glaucoma-Colpidium, Paramecium and Tetrahymena species) using antisera specific for their surface antigens. In a study with ciliates belonging to the Glaucoma- Colpidium group, Robertson (1939) demonstrated agglutination and death of the organisms at higher serum concentrations. In further serological studies on Paramecia, emphasis has been placed on elucidating the nature of antigen-antibody reactions and the antigenic relationships between morphologically similar group of organisms (Bernheimer and Harrison, 1940). Certain interesting features have been observed in the agglutination reactions of Paramecia in

specific antiserum stating though these reactions are very infrequently observed, usually takes the form of rosette development (Harrison and Fowler, 1946). It has been further shown that Paramecia have distinctive surface changes which are not observed in antibody reactions with bacteria. These surface changes include an early development of a gelatinous and sticky antigen-antibody product in the form of small balls at or near the ends of the cilia, followed by a collection of a similar gelatinous product between the cilia. When extensive amounts of precipitate are formed, there often occurs a shrinkage and crenation of the cell. The interior components are not frequently afflicted by antibody; if this occurs, the most significant changes are an acute dilation and paralysis of the contractile vacuoles. It may be inferred from the foregoing that although the investigations with non-parasitic protozoa are not as extensive as those with parasitic organisms, it appears likely that similar antigen-antibody reactions may be expected with rumen ciliates.

A thorough exploitation of the antigen system of Paramecium has been used for the purpose of gaining knowledge about cellular heredity and variation using specified mating systems of these organisms (see reviews: Sonneborn, 1947; 1950; 1951; Beale, 1954). More recently, there are also a number of biochemical studies, reporting on the identification and characterization of surface structures (both of cilia and membranes) with the objective of understanding the structure and functions of these organelles in Paramecium and Tetrahymena respectively (Adoutte et al., 1980; Dentler et al., 1980; Dentler 1988; 1992; Merkle et al., 1982). The foregoing studies may not have a direct relationship to rumen ciliates, but they provide support and direction to explore the immunogenic potential of these organisms.

The heterogeneity of the rumen ciliate population is an important factor which may dilute the potency of their antigens, especially when there are still practical difficulties encountered in the isolation and preparation of pure clonal populations. Furthermore, antigenic diversity among various morphological species of rumen ciliates is to be expected, although there may also be phylogenetically related species. However, because the relative importance of individual species to the nutrition of the sheep is not known and because of the difficulty in obtaining pure preparations of single species, mixed protozoa were used as antigens in this project.

2.4.9 *Immunization strategies for the induction of immune responses at mucosal sites*

Any attempt to establish immunological control on rumen ciliates must be mediated via the mucosal secretions. Saliva is the obvious choice for such intervention. Colostrum and milk are another medium through which the establishment of rumen ciliates might be interrupted in neonates. This could be achieved through hyperimmunization of ewes against ciliates prior to parturition. Another possibility may be direct immunization of neonates at birth in order to induce gut immune responses.

Induction of antigen-specific responses in the mucosae depends on route, dose and the type of antigens used in the study. Vaccines containing antigen with high survival and multiplication rates in the mucosal environment induce better immunity than vaccines containing either killed cells or non-replicating antigen (Fuhrmann and Cebra, 1981; Lycke and Holmgren, 1986). An exception is cholera toxin, a non-replicating antigen that induces a significant mucosal immune response (Eson and Ealding, 1984; Kostert and Pierce, 1983; Pierce, 1984). Although ' live ' antigen-containing vaccines are more immunogenic at mucosal sites than non-replicating antigens-containing vaccines, only a few safe mucosal vaccines have been developed. Therefore, inactive non-replicating antigen/vaccines are used to study immunization regimes aimed at inducing mucosal immune responses. When these antigens are administered orally to the experimental animals, the induced responses were observed to be of relatively short duration (Pierce and Gray, 1982; Lycke and Holmgren, 1989) and there is a need for repeated immunization schedules. In order to overcome this difficulty, certain investigators have adopted parenteral routes (intra-peritoneal- I/P) in rodents and lambs respectively (Pierce and Gowan, 1975; Husband, 1978). The procedure stimulated a vigorous IgA-antibody-containing cell (ACC) responses from GALT following a subsequent oral challenge. Intraperitoneal immunization also induces a concomitant systemic antibody response.

In keeping with the idea of common mucosal immune net-work, the foregoing strategies, have been the subject of intensive investigation to explore the prospects of establishing suitable immunization regimes against enteric, respiratory and mammary infections.

2.4.9.1 *Intestine*

In sheep, it was shown that with a single I/P immunization followed by intraduodenal challenge, it was possible to generate a population of ACC in

the jejunal lamina propria (Husband et al., 1979; and Beh et al., 1979). A large percentage of the ACC was found to be of IgA isotype, but there were also significant numbers of IgG ACC. Some of the later studies confirmed these findings (Sheldrake et al., 1985a; 1985b; Scicchitano et al., 1984). In another study with lambs, Husband (1980) demonstrated that a single I/P imunization alone afforded protection to these animals, when they were challenged subsequently with Salmonella typhimurium. Based on these observations, it was pointed out that there would not be any need for subsequent oral boosting. In these studies, the antigens were prepared in oil based Freunds- adjuvant for I/P immunization. This may have ensured a constant stimulation of the Peyers patches (PP) through the serosal wall. Similar strategies have been found to be equally successful in containing Colibacillosis infections in porcine animals (Husband and Seaman, 1979). In principle, I/P immunization constitutes an effective way of inducing gut immune responses in these species.

2.4.9.2 Mammary glands

Mammary gland is not a mucosal surface, but it contains an epithelium and in many species it forms part of the common mucosal immune system. In a number of species, gut-stimulation leads to the relocation of IgA specific ACC to the mammary tissue and increased levels of S-IgA in the milk (Rudzik et al., 1975; Roux et al., 1977; McDermott and Binenstock, 1979; Weiz-Carrington et al., 1979; Jackson et al., 1981). The contribution of GALT to the ovine mammary sites has been evaluated in a series of studies (Sheldrake et al., 1985a;1985b;1985c) and it was observed that IgA producing plasma cells originating from the GALT do not appear to migrate to ruminant mammary glands in large numbers. However, there is a considerable IgG_1 immune response in the gland following I/P immunization. Although the origin of these cells is not known, there are other studies which demonstrate clearly that both IgG_1 and IgA are transported readily from serum into mammary secretions (Watson and Lascelles, 1974; Sheldrake et al., 1984; Sheldrake et al., 1985). Thus I/P immunization via systemic antibody, may generate a large proportion of the antigen-specific antibodies transferred into the mammary secretions.

2.4.9.3 Salivary glands

A limited number studies in ruminants suggest that both local production and transfer of IgA from the serum are responsible for antibodies in salivary secretions (Cripps and Lascelles, 1976; Scicchitano et al., 1986). It has also been reported that there is a selective transfer of IgG_1

from the blood into saliva (Cripps and Lascelles, 1976; Watson and Lascelles, 1973). Studies in man and animals have demonstrated various ways of inducing the appearance of S-IgA antibodies in the oral secretions. Injection of whole or broken cells of Streptococcus mutans via subcutaneous (S/C) or oral sub-mucosal routes gave significant protection against caries in monkeys (Bowen et al., 1975; Cohen et al., 1979). In some other studies, using similar immunization schedules, it was shown that the caries reduction was associated with high serum titres of IgG, IgM and IgA, but only a slight increase in salivary IgA levels was obtained (Lehner et al., 1977; Caldwell et al., 1977). Immunization of monkeys with killed cells of S. mutans by subcutaneous deposition in the vicinity of salivary glands resulted in the production of specific antibody in saliva and serum and also reduced caries caused by these organisms (Emmings et al., 1975; Evans et al., 1975). Similar results were obtained in rats using the same routes (vicinity of salivary glands) (Taubman and Smith, 1974). A specific salivary IgA response was demonstrated in another study conducted in rats (McGhee et al., 1974)

When the oral submucosal route of immunization was attempted in monkeys with either live or dead S. mutans, it failed to elicit a significant S-IgA response(Walker et al., 1981). In contrast, in rats, oral immunization with killed S mutans cells was found to stimulate specific S-IgA in saliva and milk, but not in serum of rats. Of particular interest here is the observation that the ingestion by human volunteers of capsules containing killed S. mutans also resulted in an increased specific S-IgA response in saliva although there was no detectable increase in serum antibody levels. However, salivary titres fell rapidly within two months (Mestecky et al., 1978).

Attempts to enhance secretory IgA response have included altering the form of the antigen, the route and schedule of administration, and the adjuvants used (Pierce, 1978; Husband, 1993; Keren et al, 1988). These workers employed combinations of parenteral injections of antigen and per enteric routes (either I/P- oral or I/M-oral or I/P-I/D) and these have stimulated secretory IgA responses to levels previously attainable only after multiple oral administrations of antigens. Although the foregoing attempts were addressed mainly to induce gut-immune responses, direct application of those routes or modification of same has not been investigated in ruminants for the induction of antigen-specific antibodies in the salivary secretions.

This review has examined a range of data to identify a strategy for immunization of sheep against rumen ciliates to induce mucosal

antibodies in external secretions, particularly saliva and milk, and design experiments to investigate the structural and functional aspects of these molecules in rumen fluid and the possible effect on live organisms under similar conditions.

2.5 Formulation of a hypothesis

The main questions addressed in the project were (1) whether rumen protozoa, as inhabitants of the rumen, would be immunogenic to the host ruminant? (2) If so, what would be the pattern of distribution of protozoa-specfic antibodies in the general body fluids, serum, saliva and milk? (3) What would be the effects of those antibodies on the rumen population?

A series of experiments were designed to find answers to these questions. The outcome of those experiments is discussed in the following chapters. They test the hypothesis (1) rumen ciliates can elicit immune responses in sheep and that the ciliate-specific antibodies can survive and maintain their activity in protease-rich environment where they can inhibit the activity of the resident ciliate population.

Section 3.0

Immunization Protocols for the Induction of Immune Responses against Ovalbumin in the Saliva of Sheep

3.1 Introduction

The saliva of ruminants contains major classes of immunoglobulins, particularly IgA and IgG_1 (see Table 2.1). As mentioned earlier in Section 2.4.9, any attempt to establish immunological control on rumen ciliates may have to be mediated through salivary antibodies. There have been few investigations into methods for selectively inducing immune responses in the saliva of ruminants. In contrast, as discussed in detail under Sub-section 2.4.9.3, there have been many reports of full induction of SIgA responses in rats, monkeys and humans by combination of different immunization protocols. Some of these protocols have included local injections into the oral mucosa or feeding of specific antigens in both rats (Taubman and Smith, 1977; Smith et al., 1977) and rhesus monkeys (Bahn et al., 1977). These procedures have resulted in the elevation of both IgA and IgG antibodies in saliva and decline in dental caries. Other methods have included local percutaneous injections in the vicinity of the salivary glands in either rhesus monkeys (Emmings et al., 1975; Evans et al., 1975) or rats (Taubman and Smith, 1974; McGhee et al., 1974). Oral immunization with killed Streptococcus mutans cells was found to stimulate specific SIgA in saliva and milk of rats but not in serum (Michalek et al., 1976; 1978). Ingestion of killed S.mutans cells by human volunteers has been shown to result in SIgA response in saliva, but no noticeable increase in the serum (Mestecky et al., 1978).

When killed or inactive non-replicating antigens/vaccines were administered to experimental animals, the induced mucosal secretory antibody responses were found to be of relatively shorter duration (Pierce and Cray, 1982; Lycke and Holmgren, 1989) and there was always a need for repeated immunization schedules. The contribution of the gut-associated lymphoid tissue (GALT) in generating IgA precursor cells has been documented (Craig and Cebra, 1971; Rudzik et al., 1975; Husband and Gowans, 1978 ; reviewed, Bienenstock and Befus 1980) Memory IgA responses have been induced in rodents by adopting intraperitoneal (I/P) routes of immunization (Pierce and Gowans, 1975). In sheep, it has been shown that it is possible to generate a substantial specific antibody-containing cell response of the IgA isotype in the gut mucosa by I/P priming and that this response can be boosted by

intraduodenal (I/D) route (Husband et al., 1979). Priming of the GALT with a specific antigen has been shown also to influence the population of IgG producing cells in the gut lamina propria (Beh et al., 1979; Bennel and Husband, 1981). Plasmablasts generated in the GALT extravasate from the blood to the lamina propria of mucosal surfaces, probably involving a mechanism similar to that outlined by Streeter et al., (1988). Proliferation of these relocated cells appears to occur only at sites where the antigen is subsequently presented (Husband and Gowans, 1978). Those studies have not extended the observations to either salivary mucosal sites or to the salivary secretions of ruminants. The present experiment was designed to test the efficacy of some of these immunization routes in the induction of specific antibody responses in the saliva of sheep with a view to developing a protocol suitable for subsequent studies concerning the immune responses of sheep to rumen ciliates. The main aims were to determine whether a secretory antibody response could be induced in the saliva of ruminants and to examine the possibility of boosting such responses subsequently by presenting antigens at or near the salivary glands of these animals.

3.2 Materials and Methods

3.2.1 Experimental animals

Short-wool merino wethers, 8 months old were maintained on the standard drenching programme at the university farm. The animals were housed in individual pens and fed once daily on a sheep-shed ration containing 500g oaten straw and 1000g pellets (9.6MJ/Kg DM and 7.8% CP). Water was made freely available to all of the animals.

3.2.2 Immunization

The animals were allocated randomly to two groups for separate experiments, 3A and 3B respectively. The immunization protocol and sampling schedule are shown in Table 3.1. In experiment 3A, five animals were immunized (I/P) with ovalbumin (OVA) (Sigma Chemicals) using a dose of 40mg in 5ml phosphate-buffered saline (PBS, pH 7.2), emulsified with 5ml of complete Freund's adjuvant (CFA). Booster injections were repeated two weeks later intraduodenally (I/D), each animal receiving 100mg OVA mixed with 2.5g of DEAE-dextran (Pharmacia Pty. Ltd.) in 50 ml PBS (ref. Beh, 1979). Intraduodenal injections were administered after the duodenum had been exposed by laparotomy.

In experiment 3B, six wethers of similar description were primed by the I/P route at a dose rate of 30mg OVA in 4ml of PBS plus an equal volume of CFA. 24 days later all of the animals were percutaneously boosted (10mg OVA in 5ml PBS / animal) in the vicinity of the salivary glands.

Table 3.1 Immunization protocol and sampling schedules[a]

Experiment (days)	No of Animals	Primary immunization Route	Schedule(days)[b] of sample collections	Secondary immunization[c] Route	Schedule of sample collections
3A	5	I/P	0, 9, 11,	I/D	13,20, 25, 28
3B	6	I/P	0, 14,	Local	24,36, 43, 50

[a] Serum and saliva samples were obtained from all animals 1 day before primary immunization and the postimmune sampling followed the schedule as shown in the above Table.

[b] Days from commencement of immunization

[c] Secondary immunization done on day 13 in Experiment 3A , whereas in Experiment 3B, it was done on day 24

3.2.3 Collection of samples

Approximately 20ml of saliva was collected from each animal into ice-chilled tubes after pilocarpine stimulation (0.1mg/Kg BW -intramuscular injections). The collected samples were clarified at 10, 000 x g for 10 min to sediment the debris and the supernatant was aliquoted into 1 ml Eppendorf tubes which were stored at -80^{o}C until needed. Blood collected at the same time from the jugular vein was allowed to clot and the serum was stored as 1ml portions at -80^{o}C until further use.

3.2.4 *ELISA reagents*

Rabbit anti-sheep IgG, (heavy and light chain specific) was obtained from Mr.R Wilkinson (Central Veterinary Laboratory, Institute of Medical and Veterinary Science, Frome Road, Adelaide, South Australia). Murine IgG_1 monoclonal antibody (MAbs-F3-4B4) specific for ovine IgA was purchased from Dr.J.C.Chin (Central Veterinary Laboratory, Department of Agriculture, Sydney 2167). Monospecific rabbit anti-sheep IgA was donated by Dr. A.J. Husband, (University of Sydney, N.S.W.). Mouse anti-sheep whole immunoglobulin, alkaline phosphatase-conjugated goat-anti rabbit and rabbit anti-mouse whole immunoglobulins were donated by Dr.P. Ey (Department of Microbiology and Immunology, University of Adelaide, S.A).

3.2.5 *Checkerboard titrations to optimize the ELISA protocol*

Briefly, ovalbumin (antigen), was used at a range of concentrations to coat ELISA trays. Positive and negative reference sera and saliva, rabbit-anti-sheep IgG (rendered monospecific), mouse monoclonal antibody specific for ovine IgA (MAbs-F3-4B4), and the two conjugates were all tested in checkerboard titrations. Reference samples were added to wells coated with various concentrations of antigen, while the concentrations of second antibodies and the conjugates were kept constant. Then dilutions of the second antibodies (anti-ovine) were tested against various dilutions of the corresponding conjugates with the concentration of coating antigen and primary anti-sera held constant. Thereby the optimal concentration of each reagent was determined to give an absorbance value of ≥ 1.0 with the positive reference control and < 0.1 with the negative control.

3.2.6 *ELISA protocol*

Anti-ovalbumin (anti-OVA) antibodies of various classes in serum and salivary samples were detected as follows. Flexible 96-well polyvinyl microtitre ELISA plates (Costar, catalog number 2595; Data Packaging Corp., Cambridge, Massachusets) were coated with the OVA at 10μg/ml (diluted in a buffer containing 0.5M Tris-HCL, pH 7.5; 0.15M NaCl; 0.008M NaN_3; TSA) by overnight incubation at 4°C. The trays were then blocked with 0.05% Tween 20-saline containing 0.01% bovine serum albumin (TS/BSA). Serum diluted 1/20 and saliva diluted 1/2 in TS/BSA were added to duplicate wells for the detection of IgA. Similarly serum dilutions of 1/200 and saliva 1/2 in TS/BSA were used for the detection of IgG. Subsequently, the serum and saliva samples were diluted two-fold sequentially in TS/BSA down the plate, giving eight dilutions. The plates were incubated at 37°C for 1h and then followed by another 1h incubation at 4°C. At the end of primary incubation,

the plates which carried the saliva samples were washed 4x with TS/BSA containing 10mM EDTA and 5mM L-cysteine, while the plates which had serum samples were washed 4x with TS/BSA. After the wash, either mouse monoclonal antibody F3-4B4 specific for IgA (1/5000 dilution) or monospecific rabbit-anti-sheep IgG (1/2500 dilution) in TS/BSA, was added. The plates were incubated as before, first 1h at 37°C and then 1h at 4°C. At the end of second incubation, the trays were washed 4x in TS/BSA and incubated with either alkaline-phosphatase conjugated rabbit anti-mouse Ig (RAMG) or goat anti-rabbit Ig (GARG) diluted 1/1000 with an enzyme diluent for 1hour at 37°C and another 1h at 4°C, depending on the second antibody used. Finally at the end of the 3rd incubation, plates were washed (4x) with 0.05% Tween-saline containing Mg^{++} and Zn^{++} (1mM $MgCl_2$ and 1μM $ZnCl_2$) and p-nitrophenyl phosphate (phosphatase substrate 104-105; Sigma) was added to all wells at a concentration of 1mg/ml in 10% (vol/vol) diethanolamine buffer. After the final incubation at 37°C for 4 h, the colour development was measured with an ELISA reader (Titertek Multiskan -Flow Laboratories) at 405nm.

Each assay included an antigen control (TS/BSA substituted for antisera), positive and negative serum/ saliva controls, (serum and saliva obtained from an animal with high antibody titres to OVA served as a positive control, similarly samples obtained from an unexposed animal served as negative controls , respectively), conjugate control and substrate control. The TSA control was included for identification of non-specific colour development, while negative controls were for measurement of background. The enzyme activity of the monoclonal control, conjugate control and substrate control was always found to be < 0.05 OD units.

3.2.7 Determination of ELISA end-point.

The ELISA end point (titre) was defined as the highest dilution which provided an absorbance value ≥ 0.200 absorbance for serum samples and≥ 0.100 for saliva, which was twice the OD value of the reference negative controls at their highest concentrations.

3.2.8 Quantitation of total immunoglobulin isotypes in saliva by ELISA

3.2.8.1 Preliminary determination of specificity of class-specific reagents and optimal conjugate concentration

Purified preparations of sheep IgG (donated by Dr. D. Auclair, Department of Animal Sciences, University of Adelaide, South Australia) and IgA (prepared as described in Section 6.0) were used to coat duplicate rows of wells at 5μg/ml and 10μg/ml respectively. The plates were incubated

overnight at 4°C. Following overnight incubation, plates were washed and blocked with TS/BSA, and were further incubated with the relevant class-specific antibodies [monospecific rabbit anti-sheep IgG (1/2500) and mouse monoclonal F3-4B4 (1/5000)] 1 hour at 37°C and another hour at 4°C. At the end of incubation, the plates were washed 4x in Tween-saline (0.05%) containing Mg^{++} and Zn^{++} and titrated with specific conjugates (GARG and RAMG) to determine the dilution that could give an OD value in the range of 1.2 -1.5 at 405nm. Specificity of the class-specific antibodies was verified by cross-over tests.

3.2.8.2 Quantitative assay of immunoglobulin isotypes in saliva

Assays were performed in 96-well microtiter ELISA plates (Costar, catalog number 2595; Data Packaging Corp., Cambridge, Massachusets). All reactants were added in 0.1ml/well and all washes were done three times using TS/BSA. For the assay of total IgG, wells were coated with 5µg/ml monospecific rabbit anti-sheep IgG in TSA, pH 9.6 and incubated at 4°C overnight. After washing, serial two fold dilutions ranging from 500-1 ng/ml of purified sheep lung IgG (used as standard) and dilutions of saliva (1:100-1:12800) were added to coated duplicate wells and to an uncoated well, which served as a control for non-specific binding. Plates were incubated overnight at 4°C and washed. The bound complex was incubated with mouse anti-sheep whole immunoglobulin (1/1000 dilution) for 1 h at 37°C and another 1 h at 4°C. At the end of second incubation, the plates were washed 4x in TS/BSA and incubated with alkaline-phosphatase conjugated rabbit anti-mouse Ig (RAMG) diluted to predetermined level (1:1000) for 2 hours as before. Following the third incubation, the plates were washed with 0.05% Tween-saline containing Mg^{++} and Zn^{++} , and p-nitrophenylphosphate (Sigma) was added After the final incubation at 37°C for 4 h, plates were read at OD_{405} in an ELISA reader (Titertek Multiskan-Flow Laboratories) when the OD of the highest concentration of the standard was 1.1-1.2. A standard curve was constructed to determine the IgG content of pooled saliva samples (Fig. 3.3).

Similar procedures were followed to determine total IgA. The heavy chain specific rabbit anti-sheep IgA was used to coat the wells at 1:2000 dilution. Purified sheep lung IgA (see Sect. 6.0) was used as a standard to provide concentrations in the range 500-1 ng/ml by two-fold serial dilution. The dilutions of saliva for incubation ranged from 1:800-1:51200. The bound complex was detected with mouse monoclonal antibody against ovine IgA diluted to predetermined level (1: 5000), followed by RAMG conjugate. IgA

contents of the pooled samples of saliva were quantitated from the standard curve (Fig. 3.3).

In order to standardize the ELISA for the quantitation of total isotypes, a sample of saliva known to contain high titres of antibodies to OVA was used. Two dilutions were selected that gave OD values just less than the maximum OD performance in the upper segment of the linear parts of the standard curves for IgG and IgA respectively. These dilutions were then used as reference samples to standardize for interassay variations.

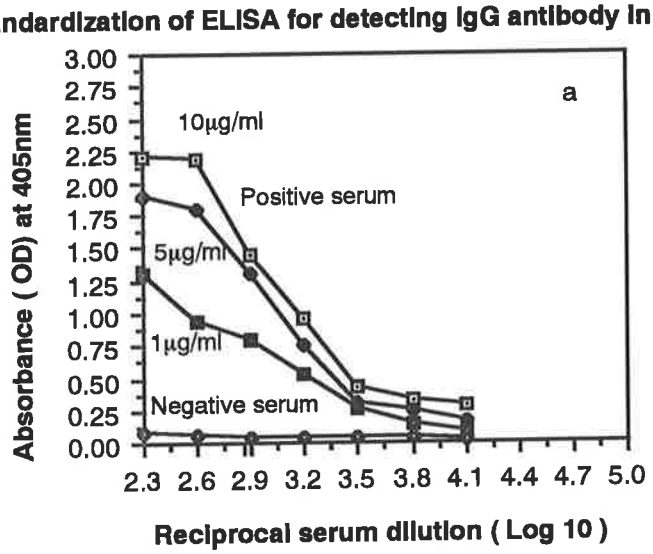
3.2.9 Statistical analysis

The assays on samples of saliva and serum collected from each animal on the respective days of sampling were normalized by means of the reference samples and the titres were log-transformed. Analysis of variance on log-transformed data was done based on a completely randomized design (Steel and Toorie, 1960). The significance between group geometric means of different day's samples was determined by LSD test.

3.3 Results

3.3.1 Optimization of the ELISA procedure

Reference positive and negative antisera were titrated on ELISA plates coated with a range of concentrations of OVA. When optimal concentrations of class-specific antibodies (Mab specific against sheep IgA, monospecific rabbit anti-sheep IgG) and corresponding conjugates were used, the optimal densities developed with positive reference samples, were significantly greater than the optical densities of the negative samples at all antigen dilutions. No specific antibodies of any class were detected at any dilution of the negative reference sera, even when reacted with the highest antigen concentration (see Fig. 3.1a). The optimal antigen-coating concentration was determined, having taken into consideration the following factors, viz., (a) the negative control samples give only low background reading on antigen-coated wells at dilutions where the positive controls provide an OD of at least 1.0, (b) the slope of the linear part of the titration-curve: highest optical densities were obtained in wells coated with OVA at 10μg/ml and comparison with results obtained at a coating concentration of 5μg/ml suggest that 10μg/ml is near-saturating (Fig. 3.1a). From series of assays, the following conditions were adopted. Microtitre plates were coated with 10μg/ml of OVA, class-specific antisera were used at dilutions of 1/5000 (MAbs-anti IgA) and 1/2500 (rabbit anti-sheep IgG), while respective conjugates were used at 1/1000. The same conditions were found to be satisfactory for assay of IgG and IgA antibodies in saliva (Fig. 3.2).



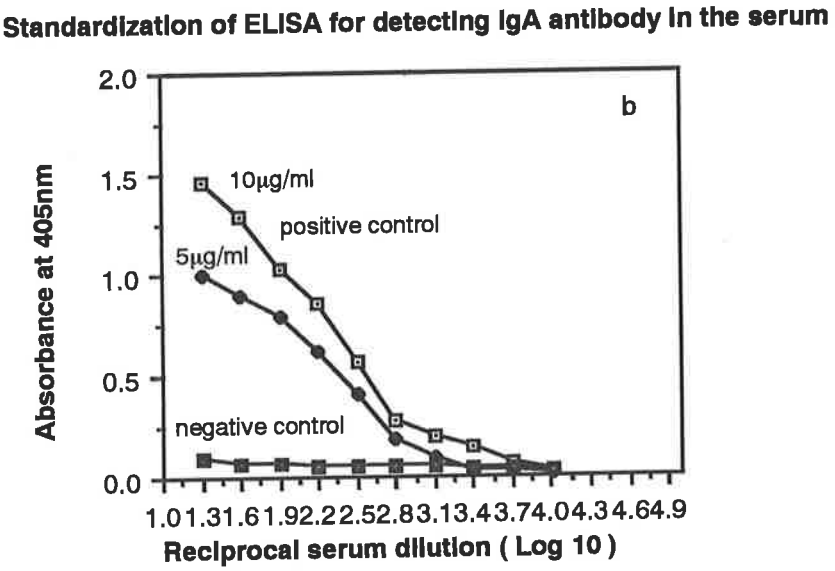


Fig.3.1 Standardization of ELISA for detection of specific antibodies in the serum.(a)- Detection of IgG anti-OVA antibodies in reference positive and negative antisera by titration on plates coated with 1µg/ml, 5µg/ml or 10µg/ml of OVA. Results are shown for the negative antiserum titrated on plate coated with 10µg/ml. The dilution of class-specific antibody (monospecific rabbit anti-sheep IgG) and conjugate (goat anti-rabbit Ig,) were constant at 1/2500 and 1/1000 respectively. (b)- Detection of IgA anti-OVA antibodies in reference positive and negative antisera by titration on plates coated with 5µg/ml or 10µg/ml of OVA. Results are shown for the negative serum titrated on plates coated with 10µg/ml. The dilution of class-specific antibody (mouse monoclonal anti-sheep IgA) and conjugate (rabbit anti-mouse Ig) were constant at 1/5000 and 1/1000 respectively.

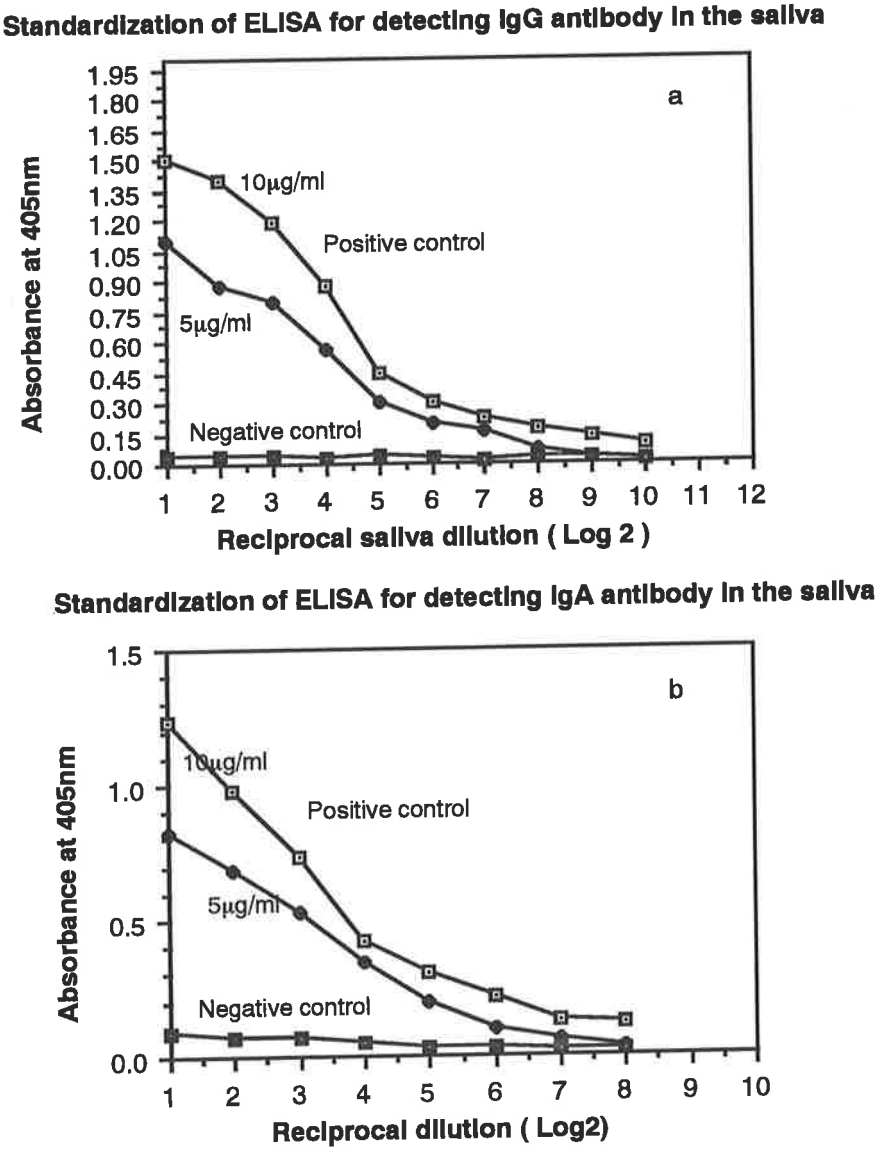


Fig.3.2 Standardization of ELISA for detection of specific antibodies in the saliva. (a)- Detection of IgG anti-OVA antibodies in reference positive and negative antisera by titration on plates coated with 1µg/ml, 5µg/ml or 10µg/ml of OVA. Results are shown for the negative antiserum titrated on plate coated with 10µg/ml. The dilution of class-specific antibody (monospecific rabbit anti-sheep IgG) and conjugate (goat anti-rabbit Ig,) were constant at 1/2500 and 1/1000 respectively. (b)- Detection of IgA anti-OVA antibodies in reference positive and negative antisera by titration on plates coated with 5µg/ml or 10µg/ml of OVA. Results are shown for the negative serum titrated on plates coated with 10µg/ml. The dilution of class-specific antibody (mouse monoclonal anti-sheep IgA) and conjugate (rabbit anti-mouse Ig) were constant at 1/5000 and 1/1000 respectively.

3.3.2 *Total IgG and IgA*

The concentrations of total IgG and IgA in the pooled samples of saliva obtained on different days of sampling for the experiments 3A and 3B are shown in Table 3.2. The standard curves for the estimation of total IgG and IgA in saliva are shown in Fig. 3.3. The concentration of each immunoglobulin isotype (IgG and IgA) was calculated from the mean of the estimates made at two dilutions of each sample, using the regression equation for the linear part of the respective curves. The concentration of each isotype (IgG and IgA) estimated at each of the dilutions did not differ by more than ± 10% from the mean. When total IgG levels were compared between preimmune and postimmune days' samples, no remarkable variations were observed for those samples from experiment 3A. The picture was slightly different in experiment 3B, where the total IgG concentration on day 36, after boosting was significantly different to the concentrations at any other sampling time ($P<0.01$). Total IgA concentrations were only measured for those samples in which specific IgA antibodies were detected and for preimmune saliva. Only one of the pooled immune samples (day 13) from experiment 3A showed a significant difference in the total IgA content when compared to that of the preimmune saliva ($P< 0.05$). Since the concentrations of specific antibodies in the saliva might have been influenced by variations in the rate of saliva flow following pilocarpine stimulation and by physiological variation in the rate of secretion and selective transfer of immunoglobulins from blood into saliva from day to day sampling, the specific antibody titres of IgG and IgA in the saliva were normalized by dividing the absolute values by the concentration of total immunoglobulin isotypes. The results are therefore expressed as units of antibody per mg of immunoglobulin (See Table 3.3).

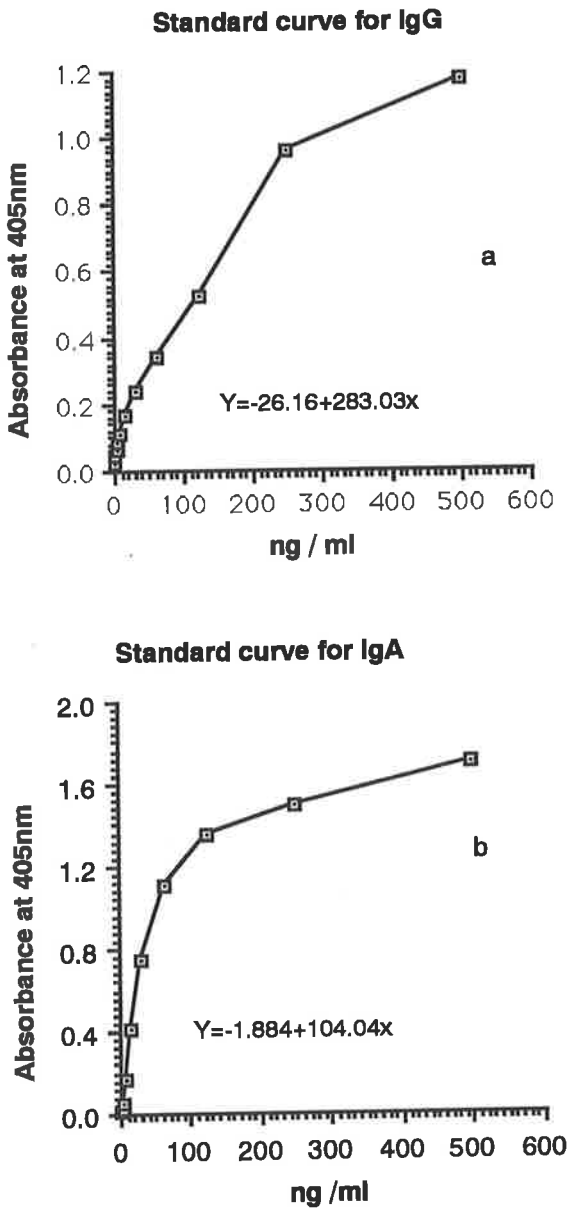


Fig. 3.3: Standard curves for the estimation of total IgG and IgA in the saliva- Regression equations represent the linear part of the respective curves.

Table. 3.2 Concentrations of total immunoglobulin isotypes in the saliva by ELISA (values in mg/ml)

Isotype	Day of sampling						
	0	9	11	13	20	25	28
Expt 3A	Mean± SD[a]						
IgG	0.03±0.003	0.03±0.002	0.04±0.004	0.04±0.001	0.04±0.003	0.03±0.005	0.004±0.002
IgA	0.25±0.007	0.27±0.002	0.24±0.004	0.20±0.004[b]	ND[c]	ND	ND
	Days of sampling						
Expt 3B	0	14	24	36	43	50	
IgG	0.05±0.004	0.04±0.003	0.05±0.001	0.07±0.008[d]	0.05±0.005	0.04±0.001	
IgA	0.31±0.008	0.32±0.001	0.30±0.002	ND	ND	ND	

[a]SD- Standard deviation
[b]Significantly different at P<0.05
[c]ND-not done
[d]Significantly different at P<0.01

Table 3.3 Specific IgG and IgA antibody titres against OVA in serum and saliva of immunized sheep

Days[a]	Titre[b]							
	Serum				Saliva			
Expt 3A	IgG		IgA		IgG		IgA	
0	000		000		000	NV[c]	000	NV
9	2253	(7.72± 0..45)	28	(3.34± 0.20)	64	(4.16±0..28)	2133	8 (2.08±0.40) 30
11	3197	(8.07± 0..49)	80	(4.38± 0.38)	54	(3.99±0.17)	1350	11 (2.43±0.20) 46
13	5825	(8.67± 0.49)	159	(5.07± 0.75)	54	(3.98± 0.38)	1350	23 (3.12±0.20) 115
20	2670	(7.89± 0.59)	000		108	(4.68± 0.44)	2700	000
25	3828	(8.25± 0.52)	000		76	(4.33± 0.33)	2533	000
28	4536	(8.42± 0.45)	000		76	(4.33± 0.33)	1900	000
L.S.D P<0.01	1.27		1.52		1.09		0.75	
Expt 3B								
0	000		000		000		000	
14	3605	(8.19± 0.33)	255	(5.54 ± 0.21)	72	(4.27±0.21)	1800	8 (2.08±0.57) 25
24	2080	(7.64 ± 0.52)	179	(5.19± 0.38)	64	(4.16±0.31)	1300	4 (1.39±0.44) 13
36	2951	(7.99± 0.39)	69	(4.23±0.88)	228	(5.43± 0.66)	3257	000
43	2080	(7.64± 0.27)	33	(3.49±1.13)	161	(5.08±0.58)	2300	000
50	1920	(7.58 ± 0.30)	000		114	(4.74±0.52)	1900	000
L.S.D P<0.01	1.04		2.23		1.36		1.64	

[a]Days from the commencement of immunization

[b]Titre values have been expressed as geometric means (with the corresponding natural log values and standard errors indicated within brackets) from four sheep per group in experiment 3A and six sheep per group in experiment 3B respectively.

[c]NV- normalized titres of antibodies in saliva (geometric mean), expressed as units/mg immunoglobulin. Serum titre values have not been normalized on the assumption that they were not subject to any undue variations during recovery unlike saliva.

3.3.3 Time-course of isotype-specific antibody responses

Table 3.3 depicts the titres of IgG and IgA antibodies in the serum and saliva of the sheep used in experiments 3A and 3B respectively. In experiment 3A, the titres are geometric means from four sheep, whilst those from experiment 3B are from six sheep. In absolute terms, titres of specific IgG antibody in the serum were 42 to 54 times higher in the serum than in the saliva (Expt. 3A). Such comparisons in experiment 3b showed a similar trend. When the titres of class-specific antibodies in the saliva were normalized against the total immunoglobulin isotypes, the IgG, IgA titres in the saliva reached comparable levels to the titres registered in the serum in respect of the class-specific IgG and IgA antibodies. It must be mentioned that the titres of IgG and IgA antibodies were not normalized in the serum on the assumption that the concentration of antibodies in the original samples of serum would not have been influenced for similar reasons as those mentioned for saliva. It is evident from Table. 3.3 and Fig. 3.4 that neither the preimmune serum nor preimmune saliva showed any detectable OVA-specific antibody of either class. When the sheep were subject to a primary I/P followed by a secondary I/D, specific-IgG antibodies appeared by 9 days after priming and peak titres were recorded on day 13 in the serum and thereafter remained around that level throughout the remainder of the total of 28 days (Fig.3.4a). All of the values were significantly greater than the preimmune levels.

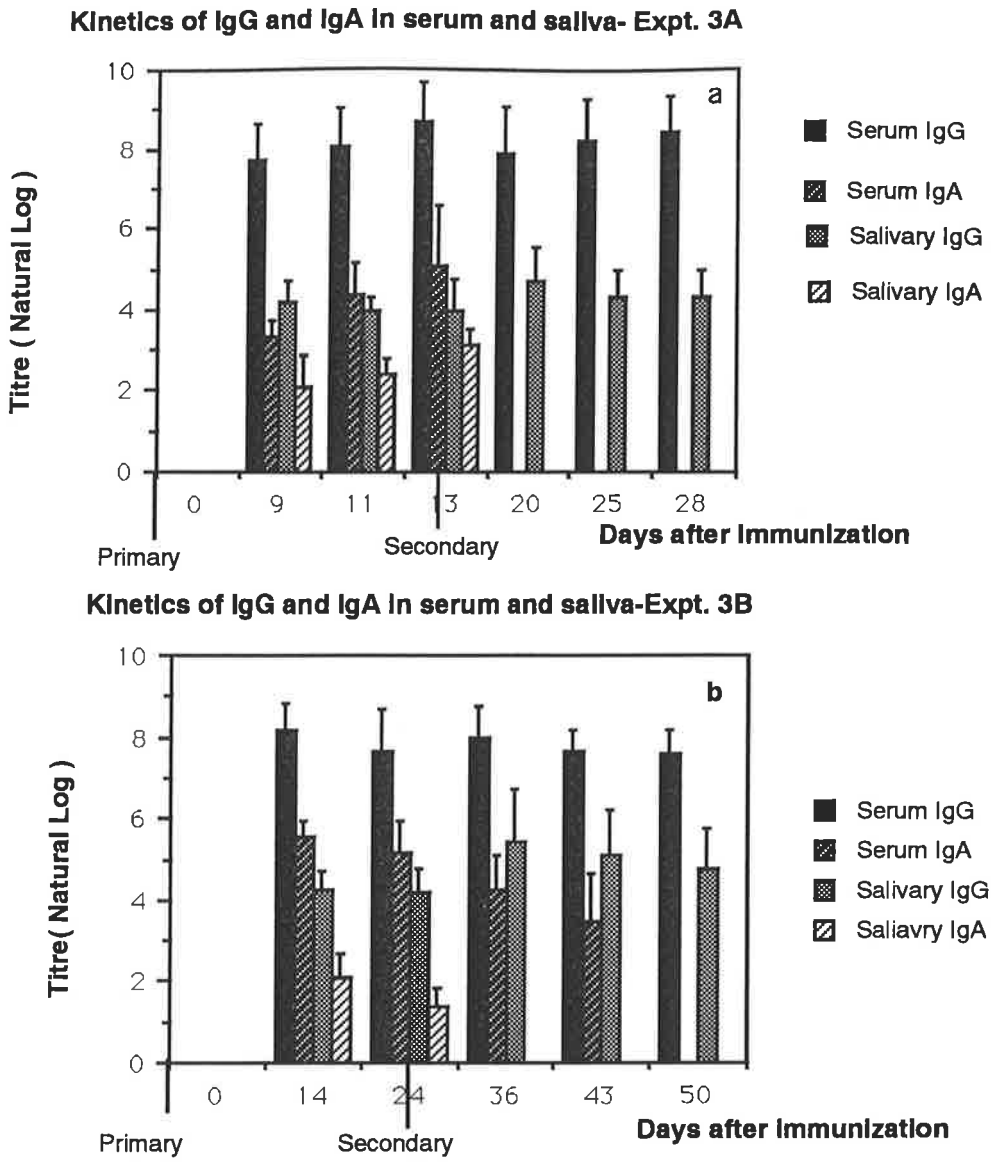


Fig.3.4 Serum and salivary IgG and IgA responses to ovalbumin. (a) Values represent geometric means of four sheep in Expt. 3A and (b) that of six sheep in Expt. 3B respectively. All values were clearly greater than the preimmune levels in respect of IgG throughout the experimental period. Levels of IgA were also elevated after primary immunization. After both secondary immunizations, IgA levels dropped below the levels detected by the ELISA assay.

A similar trend was observed in experiment 3B for both serum IgG and salivary IgG, regardless of the differences in routes adopted during secondary immunization. No significant differences were observed between the various postimmune samples, indicating that local challenge did not boost either serum or salivary antibody levels. In respect of specific IgA antibodies, it is evident from Table 3.3 that intraperitoneal immunization with OVA gave rise to a

primary serum and salivary anti-OVA response ($P<0.01$) in serum and saliva in both experiments. No secondary IgA antibody response was observed in serum or saliva in either immunization protocol.

3.4 Discussion

The studies reported here have investigated the effect of selected immunization protocols in eliciting specific IgG and IgA responses in the serum and saliva of sheep. Intraperitoneal (I/P) priming induced specific anti-OVA IgG and IgA antibodies in the serum. This is not surprising because I/P injection is a commonly used route of parenteral immunization. However, it has also been used to prime for mucosal IgA responses (Pierce and Gowans, 1975; Husband and Gowans, 1978; Husband, 1978) and this probably accounts for the serum IgA response that was also observed (Husband, 1978). A single I/P immunization in sheep can generate a large population of specific antibody-containing cells (ACC) in the jejunal lamina propria (Husband et al., 1979; Beh et al., 1979). Of these, a large percentage was found to contain IgA, although there were also detectable numbers of IgG-containing ACC as well. It appears that I/P immunization, when the antigens are prepared in oil-based Freund's adjuvant, may stimulate the Peyer's patches directly through the serosal wall and sustain appreciable production of IgA (and to a lesser extent IgG) producing ACC in ruminants.

Following the primary response, IgG antibody levels in serum and saliva remained elevated for the remainder of the observation period (up to 28 days in experiment 3A and 50 days in experiment 3B). Whether secondary immunization by the intraduodenal (I/D) route (expt. 3A) or percutaneous (P/C) route in the vicinity of salivary glands (expt. 3B) contributed to this sustained IgG antibody response is not known, because a control group receiving primary immunization alone was not included. However, it seems likely that the depot nature of the primary immunization (in CFA) was a major contributing factor. The reason for the decline of specific IgA antibodies after the booster injections is not very clear. Neither a single I/D booster dose nor a P/C dose of OVA in the vicinity of salivary glands enhanced the systemic IgA responses. IgA antibodies in serum were not detectable beyond day 13 after priming in experiment 3A or day 43 in experiment 3B. On the basis of the evidence from these two experiments, it is not possible to decide whether I/D boosting had an inhibitory effect on IgA antibody production or whether local immunization around the salivary glands prolonged the serum IgA titre. However, the difference between the longevity of the serum IgA antibody responses between the two experiments may be simply fortuitous,

because local boosting had little effect on salivary IgA titres in experiment 3B. It is noteworthy that protocol of I/P priming and I/D boosting did not result in a secondary intestinal IgA response in sheep (Beh et al., 1979). The failure of I/D challenge to induce a secondary IgA response could be due to immune exclusion of antigen by local secretory antibodies (Elson, 1985; Mowatt, 1987).

Intraperitoneal priming resulted in significant titres of IgA and IgG antibodies in stimulated saliva. Salivary antibody levels appear to parallel levels in serum and were not affected by either I/D or local salivary gland challenges with antigen. Salivary IgA antibodies also paralled levels in serum, although in experiment 3B the serum response was more sustained than the salivary response. Neither route of challenge was effective in boosting salivary IgA antibodies. The lack of secondary IgA antibody response following I/D challenge is similar to the result obtained by Beh et al. (1979), who observed no secondary IgA antibody response after a similar immunization procedure (without DEAE-Dextran) primed 2 weeks previously by I/P immunization. DEAE-dextran was included in the booster injections for the current study based on observations that DEAE-dextran enhanced antibody-containing cell response when ovalbumin was infused simultaneously with DEAE-dextran (Beh, 1979). It was not possible to compare the results because of differences in immunization protocols between the two studies . However, it appears that inclusion of DEAE-dextran did not produce a sustained response.

Furthermore, the findings of the present study were in contrast with the success of I/P priming and I/D challenge in producing local secretory antibody responses in rats (Pierce and Gowans, 1975; Husband and Gowans, 1978) and in sheep (Husband et al., 1979). Husband and Gowans (1978) observed the importance of local antigen in expanding mucosal antibody responses in rats. However, no boost in salivary IgA antibodies was observed after attempted local stimulation of the salivary glands (Expt. 3B), despite evidence of ongoing IgA response in serum and saliva at the time of challenge.

The failure to induce a secondary IgA antibody response in saliva appears to reflect the rather varied experience of others in inducing local immunity in this region as well as the intestinal immune response in general. While there are several reports of salivary IgA responses after experimental immunization (see reviews: McGhee and Michelek, 1981; McGhee and Mestecky, 1990), titres of antibody have usually been low and in some cases have been either absent or variable (Walker et al., 1981; Lehner et al., 1977; Caldwell et al., 1977). It was not clear whether such observations were influenced by variable intestinal immune

responses as reported by some researchers (La Brooy et al., 1980). There is a clear need to improve understanding of how IgA antibody responses in saliva are controlled physiologically and for better ways of achieving immunization in the oral cavity. It is by no means clear at present whether such IgA antibody as is observed in saliva is synthesized locally or delivered by the blood from other areas such as the gut. However, the studies of Scicchitano et al. (1986) indicate that at least some salivary IgA has its origin from blood-borne IgA in sheep.

The origin of the specific antibody isotypes detected in the serum and saliva in experiment 3A and 3B has not been ascertained. It can only be surmised that I/P priming would have stimulated both the gut-mucosal and collateral systemic immune responses, explaining the presence of post-primary IgG and IgA titres in the serum and saliva respectively. In particular, the greater part of IgA antibody and a certain percentage of IgG were most likely to be of gut-mucosal origin. After I/P priming, some of the isotypes produced in the lamina propria of the gut would have reached the blood circulation, and along with the IgG produced by extra-intestinal lymphoid tissues would have found its way into the saliva via the circulation. It has been demonstrated that greater than 80% of IgG_1 in the sheep's saliva is of serum origin (Cripps and Lascelles, 1976).

In another study, the transfer of IgA into saliva from sheep plasma has been reported (Sccichitano et al., 1986). It has been observed that most of the IgA in serum is derived from the small intestine and that all of it is dimeric IgA in ruminant animals (Beh, Watson and Lascelles, 1974). It is commonly known that dimeric IgA, derived from the blood stream and appearing in the capillary filtrate of an external secretory organ possessing transmembrane secretory component (SC) receptors, would be actively transported into external secretions (Binenstock and Befus, 1983). The findings of IgA in parotid saliva of sheep is of interest in this instance (Watson and Lascelles, 1973b; Cripps and Lascelles, 1976). It has been reported that parotid salivary tissue is virtually devoid of plasma cells, yet the parotid saliva contained IgA, all of which is expected to be derived from blood. The observed kinetics of IgA in the serum and saliva seem to be consistent with the foregoing statement. The relative contribution of serum derived and locally produced antibodies by the localized plasma cells of the submaxillary or the submandibular salivary gland could not be confirmed in the present study, because the concentrations of serum immunoglobulins IgG and IgA were not determined on the assumption that serum titres were not subject to undue variations during recovery unlike saliva. Therefore the specific activity ratios between saliva and serum [titres per mg Ig in saliva / titres per mg Ig serum]

could not be calculated. In the absence of these data, it is not proper to draw conclusions with regard to local production of antibodies. In addition, the re-location of the specific antibody producing cells from the gut mucosae to salivary mucosal sites and their contribution to the overall levels of specific isotypes in the saliva cannot be easily ruled out. These areas warrant further investigation.

In summary, after an intraperitoneal immunization with desired antigens, a good primary immune response may be obtained both in respect of IgG and IgA in the saliva. While the IgA response could not be boosted by the methods of challenge employed here, the sustained serum IgG response was accompanied by sustained IgG antibodies in saliva. If IgG antibodies are functional in saliva and in the rumen, then immunization methods that produce long-term serum IgG antibody responses may be all that is necessary to achieve rumen immunization.

Section 4.0

Purification of Rumen Ciliates for Immunization

4.1 Introduction

Immune responses arise as a result of exposure to substances that are generally referred to as antigens or, more correctly as immunogens. There are certain characteristics that a substance must possess to be immunogenic: a) foreignness, b) high molecular weight, and c) chemical complexity. One of the major aims in the current study was to examine the degree of foreignness of the rumen ciliates to the ruminant animal. The foreignness of any invading organism is determined by the structural aspects of its components and in the case of proteins, by the phylogenetic distance between that organism and the host. On these grounds, its rumen protozoa would be expected to be potential immunogens for the ruminant.

The rumen ciliates are a conglomeration of two distinct orders, Vestibuliferida and Entodiniomorphida (see Fig. 2.4). Each order in turn contains several genera and species that invariably occur simultaneously in the rumen of sheep and cattle at any one time. Apparently there are no studies reported in the literature investigating into the antigenicity of these organisms to ruminant animal. It may be logical to utilize either distinct classes of these organisms or individual species in order to reduce the complexity of the immune responses. However, the axenic culture of individual species of ciliates are not available. Furthermore, the present immunological study is one of first of its kind and the impact of the ciliates on the host ruminant is being considered as that of the whole complex rather than the effect of individual genera or species. It was therefore decided to use the crude mixture of these ciliates as antigens. The source of ciliates is rumen fluid, which teems with myriads of bacteria and fungi as well as protozoa all of which are involved actively in the fermentation of plant cellulose, hemicellulose and other components of animal feeds. In producing even a crude ciliate preparation from such an environment, there is need to establish procedures to ensure a high rate of recovery of ciliates in a very much purified form.

The current available techniques (simple filtration and centrifugation) are of limited use in obtaining these organisms in large numbers to prepare antigens for purposes of immunization. Therefore, a simple procedure has been developed to obtain a high yield of purified ciliates for the said purpose.

4.2 Materials and Methods

4.2.1 Harvesting of rumen ciliates

Rumen ciliates were always harvested from two castrate male sheep, which were fitted with a permanent rumen canula. These animals were maintained on a shed-ration containing 9.6M.J/Kg dry matter and 7.8% crude protein. Each animal was given 800g shed-ration (as fed) and 1Kg oaten straw once daily in the morning. Rumen contents were collected 3-4 hours after feeding with a suction device and transferred directly into an air tight bottle. Within 5 minutes of collection, the contents were taken to the laboratory and filtered through a layer of nylon gauze (pore-size 300μ) to remove all the large plant materials and any other large particles. The container was then bubbled with CO_2 for 10-15minutes and incubated in a water bath at 39°C for 30 minutes.

4.2.2 Purification step

At the end of 30 minutes incubation, a 40 ml sample of strained rumen fluid (SRF) was applied to a 50ml syringe with a compressed layer of cotton wool which was packed on top with teased absorbent cotton wool (approximately 0.5g). The syringe was filled up to 50ml mark with 10ml of warm PBS that has been equilibrated with CO_2. A clamp was then released to allow SRF to drip into screw-capped containers (24mm internal diameter, 11mm high, approximate volume 50ml) that were prewarmed and maintained at 39°C. The columns were eluted twice with warm 50ml of warm CO_2- equilibrated PBS and the effluent was collected into additional containers, so that each container had approximately 40ml of effluent. This procedure was then repeated with further 40ml aliquots of SRF, with the soiled cotton wool replaced with freshly-teased cotton wool between each aliquot. The filtered contents were allowed to settle for 45minutes at 39°C. The resultant supernatants were aspirated to remove the flocculant debris at the surface and the ciliates settled at the bottom of the containers were resupended in 40ml warm PBS and centrifuged at 250g for 3 minutes. The supernatants were aspirated and the pellets were resuspended again in 40ml of warm CO_2- equilibrated PBS and centrifuged for 3 minutes. The procedure was repeated three times and the purified pellets were finally resuspended in 4 ml PBS. A cocktail of protease-inhibitors was then added at final concentrations of 200μM phenylmethylsulfonyl fluoride (PMSF), 100μM L-[1-tosylamido-2-phenyl] ethylchloromethylketone (TPCK), 100μMN-∞-tosyl-L-lysine chloromethylketone (TLCK) and 100μM p-nitrophenyl-p'-quanidino-benzoate-HCL (NPG-B) and the purified

suspension of ciliates were stored at -20°C until further use. Photomicrographs of purified preparations are shown in Plates 4.1 and 4.2 repectively.

4.2.3 *Estimation of the recovery of purified ciliates*

Initially the counting of rumen ciliates was done by a technique that was described by Boyne et al., (1957). The method was modified slightly for convenience. Briefly, 5.0 ml of SRF was diluted with an equal volume of formol saline [10% (V/V) formaldehyde solution in 0.85% (W/V) sodium chloride] One millilitre aliquots of these samples were transferred into culture tubes and to these were added 9 ml of 30% glycerol solution, resulting in a 1/20 dilution of the original rumen contents. To the mixture, two drops of methyl green dye were added and the contents were mixed and allowed to stand for at least 4h for better staining of the organisms. This made identification rapid and facilitated differential counts of rumen ciliates.The diluted sample was pipetted into a MacMaster type of counting chamber (depth 1mm, total volume $10mm^3$ made in our laboratory) and the cells were counted at a magnification of x100. If the diluted rumen contents still carried high cell densities, further dilutions were made with 30% glycerol. In the counting of ciliates, an eyepiece was used with a graticule that projected an area of exactly $1mm^2$ on to the lower slide ($1mm^2$ x 1mm depth= $1mm^3$). A total magnification of x100 contained the 1mm square completely within the field of vision. For each sample, the counting chamber was filled 5 times, and each time , organisms were counted in ten separate fields that were chosen at random. The purified samples were also counted in the same way and the recovery of cells after cotton wool filtration was estimated accordingly.

4.2.4 *Identification of species in the suspension of mixed rumen ciliates*

The ciliate protozoa were identified to the level of genera. No attempt was made to identify the species of every cell examined because of the constraints and the difficulty of identifying internal structures in protozoa engorged with food particles (e.g. starch grains). The identification of genera (and species) of rumen ciliates utilized the scheme as described by Ogimoto and Imai (1983). In addition, the descriptions given by Dogiel (1927) and Kofoid and Maclennan (1932) were referred to whenever required. An attempt was made to stain the ciliates for detailed morphological investigations using iron-haemotoxylin. Some of the purified and identified species are shown in Plates 4.1, 4.2 and 4.3 respectively.

4.3 Results

In order to test the capability of cotton wool filtration to assist in the purification of rumen protozoa, two different conditions were examined: (a) the diluted strained rumen fluid (DSRF) was chilled on ice for 10 minutes and was then applied to the prescribed cotton wool columns equilibrated to 4°C and eluted with cold PBS. All procedures, including sedimentation and centrifugations, were done at 4°C, and (b) the DSRF was maintained at 39°c at the time of applying to the cotton wool column; prewarmed PBS (39°C) was used for elution and the sedimentation of the cotton-wool filtrates was done by incubating the filtrates in the water bath at 39°C. Only the centrifugation procedures were carried out at room temperature.

It was found that there was a very poor recovery of rumen ciliates (<10 percent- data not shown) when the process was carried out at 4°C. Furthermore, most of the organisms were immotile and appeared dead. In contrast, when the procedures were carried out at 39°C there was a good recovery of purified ciliates and they had excellent motility. Hence, all the subsequent purification procedures were carried out under conditions stipulated in (b). Table 4.1 presents data on the recovery of rumen ciliates after cotton wool filtration. The observations were made on a batch of 10 replicate runs, repeated on different days. The samples of strained rumen fluid (SRF) before dilution and filtration, contained entodiniomorphs at a maximum concentration of $3.8 \times 10^{5}/ml$ and a minimum concentration of $1.0 \times 10^{5}/ml$, while the concentration of holotrichs ranged from 8×10^{4} to $0.6 \times 10^{4}/ml$. After extraction and filtration through the cotton wool column, the mean percentage of recovery of entodiniomorphid ciliates was found to be 51.2±8.8% The recovery of holotrichs was 48.8±9.9%. In the experiment illustrated in Table 4.1, 10 aliquots of rumen fluid (40ml per aliquot) were purified and the recovery measured in each case. The concentration of ciliates in the final pool (10 x 4ml) of purified organisms was $1x10^{6}/ml$ entodiniomorphs and $9.9x10^{4}/ml$ holotrichs respectively.

Table 4.1 Recovery of rumen ciliates after cotton wool filtration. Results of observations on 10 aliquots, each of 40 ml, from a single rumen fluid sample. Cells recovered from filtrations of each aliquot were resuspended in 4ml of PBS.

Rep	No of cells before filtration				No of cells after filtration			
	Entodiniomorphs		Holotrichs		Entodiniomorphs		Holotrichs	
	Conc[a]	Total in 40ml SRF	Conc[b]	Total in 40ml SRF	Total in 4ml PBS[c]	% recovery	Total in 4ml PBS[d]	% recovery
1	1.0	40	1.3	52	16.0	40	15.6	30
2	1.6	64	1.4	56	35.2	55	25.2	45
3	1.1	44	0.6	24	16.7	38	7.9	33
4	2.3	92	0.9	36	46.0	50	18.4	51
5	1.4	56	1.1	44	34.7	62	26.4	60
6	1.6	64	2.0	80	30.7	48	50.4	63
7	3.8	152	8.0	320	88.2	58	169.6	53
8	2.6	104	1.6	64	66.6	64	31.4	49
9	1.9	76	1.5	60	34.2	45	32.4	54
10	2.0	80	1.0	40	41.6	52	20.0	50
Grand Total		772		776	409.9		397.0	
Conc[e]					1.02×10^6/ml		9.9×10^4/ml	
Mean						51.2		48.8
SD						± 8.8		± 9.9

[a]Concentration of entodiniomorphid ciliates $\times 10^5$ /ml

[b]Concentration of holotrichs $\times 10^4$ /ml

[c]Total number of entodiniomorphid ciliates $\times 10^5$

[d]Total number of holotrichs $\times 10^4$

[e]Concentration in 40ml (10x4) of pooled filtrates

4.4 Discussion

It was necessary to prepare a large numbers of rumen ciliates in a purified form for the intended immunization study. Some of the earlier techniques described for the isolation of rumen ciliates from rumen contents are based on simple filtration and centrifugation procedures. They were found particularly useful in the preparation of protozoal suspensions

for metabolic studies. Oxford (1951) isolated holotrichs by first straining rumen liquor through one or more layers of cheese cloth or similar material to remove the large particulate plant debris. The diluted rumen liquor was then transferred to a separating funnel where it was allowed to stand for 30-60 minutes at 39°C for sedimentation. Sedimentation was achieved under gravity or centrifugation (200g, 30sec). The surpernatants were removed by suction. The ciliates from the sedimented fractions were subject to washing procedures consisted of successive resuspension and sedimentation in buffer. The procedures also have been described to isolate individual genera or specific protozoal groups either by differential centrifugation (Coleman and Sandford, 1979), density gradient centrifugation (Mangan and West, 1977) or more specifically by filtration (Newbold et al. 1987). A method of separation involving the principle of electromigration has been attempted (Masson et al 1952). Although these methods are useful in the isolation procedures, they have not been intended to enrich ciliates for purposes of immunization. In order to meet the latter objective, cotton-wool extraction procedure was attempted.

The basis for this procedure was a consideration of a number of methods that have been described in the literature for the isolation and enrichment of T lymphocyte sub-populations. These fall into two categories. The first category employs the specific immunological features of the lymphocyte population removing selectively the unwanted population by passage through immuno-affinity columns made of plastic beads or nylon wool (Julius and Herzenberg, 1973; Kokkinopoulos et al., 1992). The second category of methods consists of empirically-derived procedures. For unknown reasons, B lymphocytes tend to stick preferentially to materials like glass beads (Rosenthall et al., 1972) or nylon wool (Eisen et al., 1972). Two other groups of workers (Hogg and Greaves, 1972; Mayrhofer and Whately, 1983) have used cotton wool in the isolation of lymphocytes. It has been stressed that a single column will produce 100% viable suspensions from samples containing up to 80% dead cells (Hogg and Greaves, 1972). According to these authors, the preferential adherence of stimulated lymphocytes to cotton wool was ascribed to higher fragility and lower viability of these cells under column conditions. At the same time, the non-stimulated cells remained highly viable and they passed freely through the column. A similar parallel may be envisioned when the diluted rumen fluid is allowed to pass through the cotton wool column. All the dead plant materials and any other damaged cells adhere to the cotton wool and the live ciliates pass through the column. The percentage recovery of both major groups (entodiniomorphs and holotrichs, 51.2 and 48.8% respectively

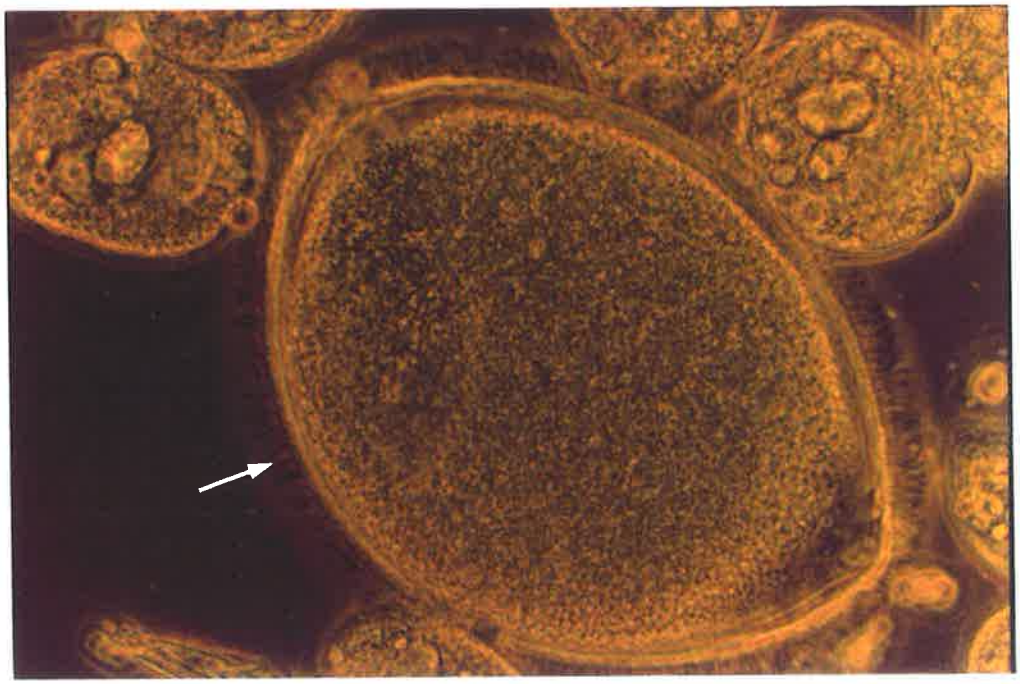
) appear to be quite consistent with the figure of 60% as reported for the recovery of purified intra-epithelial lymphocytes after cotton wool extraction by Mayrhofer and Whately, (1983). The key to the separation is slow flow-rate, allowing flocculation of colloids on the cotton wool fibres and prevention of mat formation which clogs the filter.

The effluents from the cotton wool columns were subject to repeated sedimentation and resuspension procedures, similar to what has been adopted in some other earlier studies (Coleman and Sandford, 1979) to ensure removal of the majority of fungal zoospores, flagellate protozoa and bacteria. The composition of the buffer solutions used in the washing procedures by various workers has varied, but the common criteria of pH (7.2), temperature (39°C) and anaerobiosis were adhered to strictly while using CO_2 equilibrated PBS as the sole buffer solution in the purification procedures. The rumen ciliate preparations obtained in this manner were not only highly motile at the completion of each filtration but they were also almost free of any contamination. The purity of the preparations is illustrated in plate 4.1 and 4.2 respectively. Although contamination by free bacteria was minimized by the cotton wool filtration and sedimentation technique, adherent and ingested bacteria associated with the ciliates are unavoidable in the ciliate suspension. In the final preparation of the ciliates for immunization purposes, steps were taken to further reduce bacterial contamination by incubating the thawed suspensions of frozen rumen ciliates with antibiotics (ref Section 5.0). An important charateristic of the separation procedure was that the ratio of entodiniomorphs to holotrichs was similar in SRF and in purified preparations (Table 4.1). There was therefore no selective loss of any of the major subpopulation of ciliates

In conclusion, it may be said that a simple and rapid (90 minutes) method has been developed to harvest and enrich rumen ciliates. Repeated replicate runs (10 per batch) provided, when pooled, highly-purified ciliates in sufficient number to make up a minimum of three to four doses of vaccine, each containing 10^7 entodiniomorphs and 10^5 holotrichs. The results presented in Table 4.1 are derived from a single preparation of SRF but similar results were obtained in other experiments. They provided the immunogen for immunizing and boosting the immunity of a number of sheep.

Plate 4.1: High-power photomicrograph of ciliate protozoa isolated and purified from the rumen contents of a sheep: Panel 1, A holotrich with cilia covering the outer surface (thin arrow) x425; Panel 2, An entodiniomorphid population with a large Polyplastron in the centre (large arrow head) and a group of smaller entodinia on the right (small arrow head) x 400.

1



2

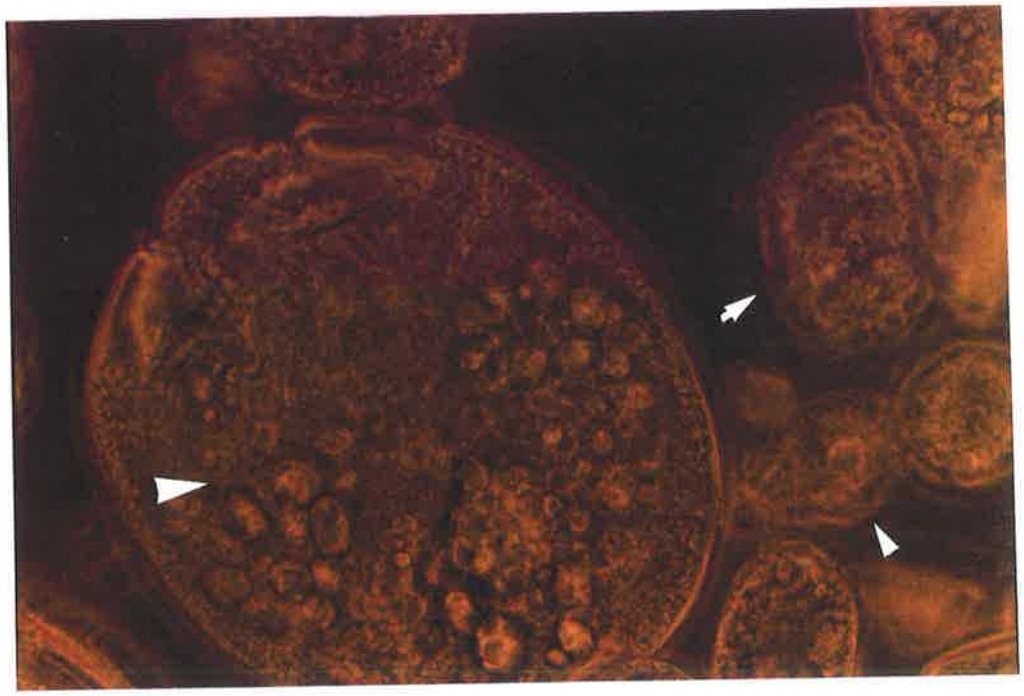
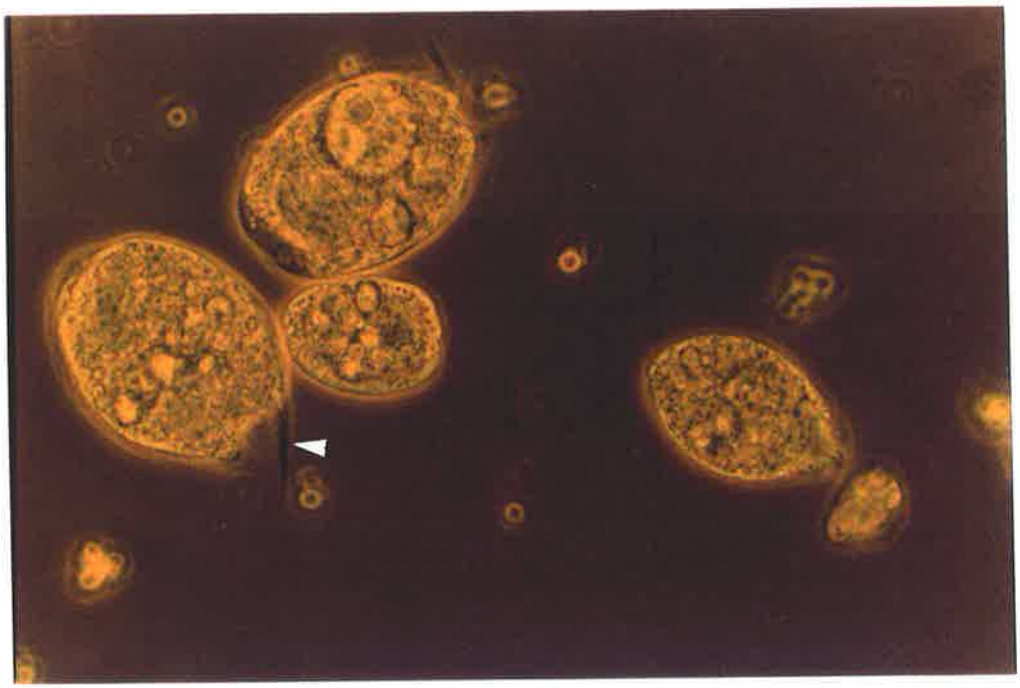


Plate 4.2: Photomicrograph of Entodinium caudatum with an intermediate sized caudal spines (see arrow head) and another group of entodiniomorphid ciliates x 320.

1



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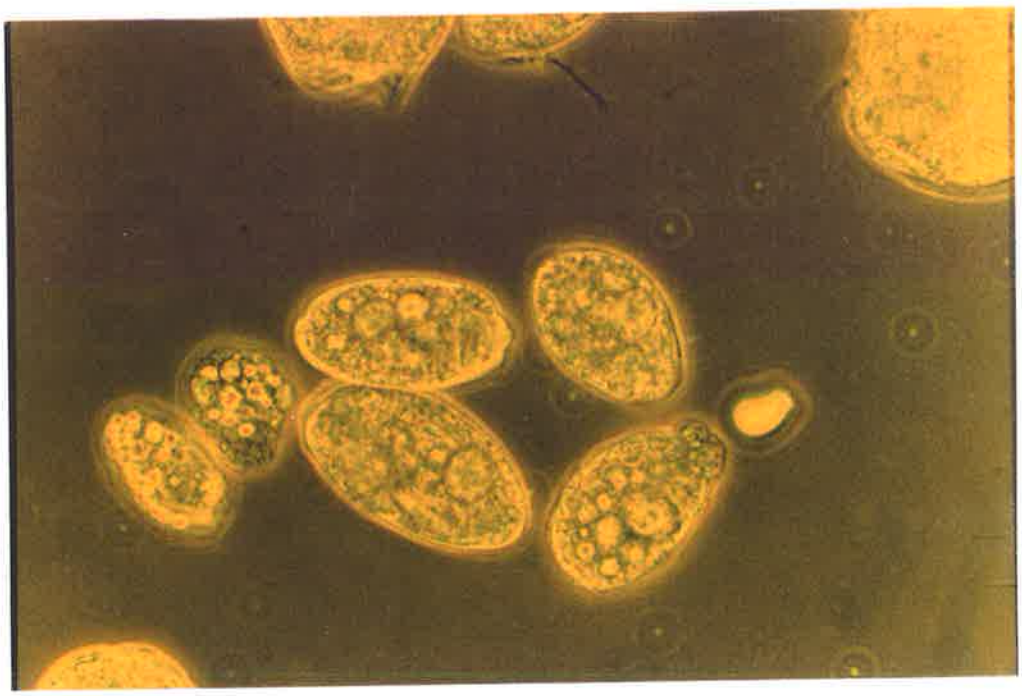


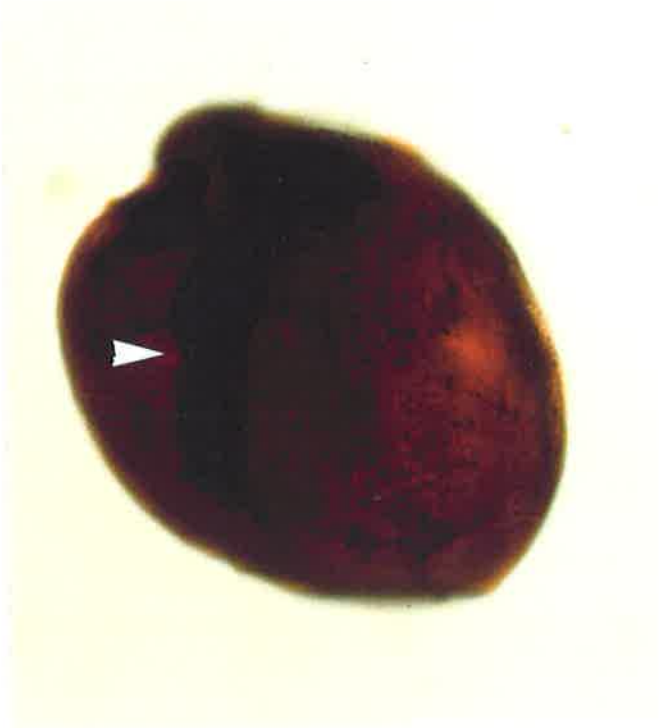
Plate 4.3: Photomicrograph of an entodiniomorphid population stained with iron-haemotoxylin.

Panel 1: A Polyplastron with an ovoid body. The macronucleus is rod-shaped with an indentation on the left side (white arrow head) x 400.

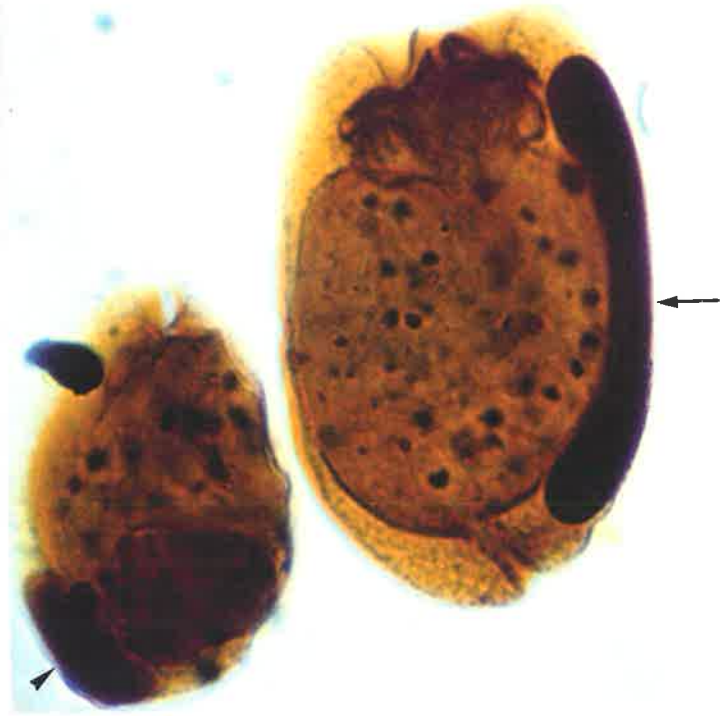
Panel 2: An Entodinium longinucleatum with a remarkable long macronucleus (thin arrow). An smaller Entodinium sp. with a short and thick macronucleus (black arrow head) x 400.

Panel 3: Unidentified smaller entodinia x 200.

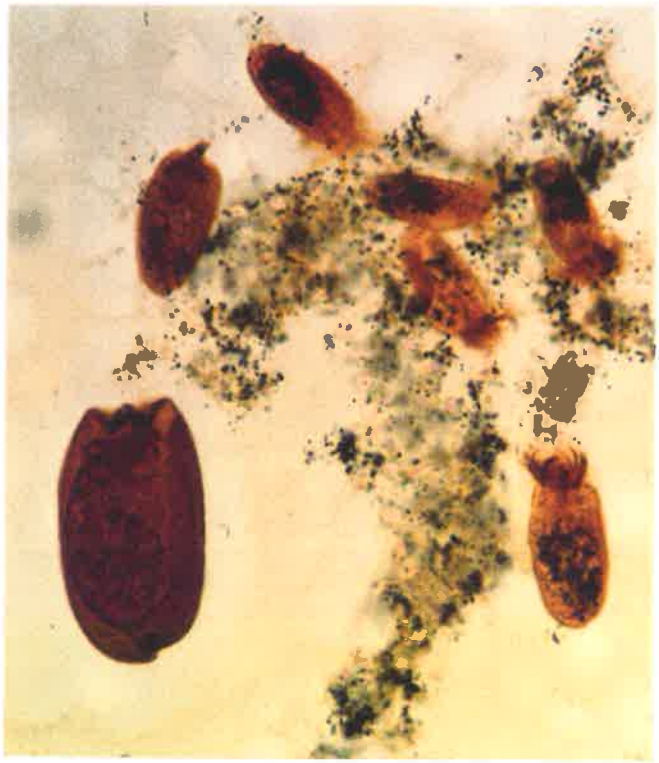
1



2



3



Section 5.0

Induction of Immune Responses to Rumen Ciliates in Sheep

5.1 Introduction

Rumen ciliates inhabit the rumen which forms an essential part of the digestive tract of ruminant animals. There have been suggestions in the past that conditions are favourable for the formation of natural antibodies against the rumen microflora and that the large load of these organisms present in the rumen might induce high titres of specific antibodies (Reiter and Madsen, 1963; Reiter and Oram,1967). However, this depends on whether there is a capacity for local antigen sampling and processing in the rumen wall or on how much undegraded microbial antigen survives digestion in the true stomach and is delivered to the small intestine. The epithelium of the gut in general provides a barrier against foreign materials and microorganisms present in the lumen, but pathogens nevertheless enter the body through this cellular layer. Protection of the vast mucosal surface of the gut depends in part on cells of the localized gut-associated lymphoid tissues (GALT). Antigens of the gut microbial flora stimulate the GALT and generate immune responses including antigen specific T lymphocytes and B lymphocytes that produce polymeric immunoglobulin A (IgA) antibodies. A feature of the mucosal immune system in ruminants, which distinquishes it from that of other species , is the prominence of IgG1 relative to IgA. The data in Table 2.1 showed that in the intestine of ruminants, although there is a prominent IgA system, IgG1 is also represented strongly. It has been established that the concentration of IgA is higher in the intestinal lymph than in the blood and virtually all is dimeric in ruminant animals (Beh, Watson and Lascelles, 1974). These authors reported that most of the IgA in serum is derived from the small intestine.

Another striking feature of the mucosal immune system of ruminants is exhibited in the saliva and milk of these animals. The submaxillary saliva of sheep has a predominant level of IgA, and it has been reported that the submaxillary gland contains numerous IgA-producing plasmacytes, whereas the parotid gland is virtually free of plasma cells (Watson and Lascelles, 1973b). However the content of IgA in parotid saliva of sheep indicated that IgA is selectively transferred into saliva from the blood-stream, presumably by virtue of the secretory component (SC) receptors on the glandular epithelium (Watson and Lascelles, 1973b; Cripps and Lascelles, 1976). Transport of IgG1 from blood

into both parotid and submaxillary saliva has been demonstrated clearly (Cripps and Lascelles, 1976). These authors showed that the transport of IgG_1 from blood into both parotid and submaxillary saliva was relatively selective for IgG_2 (Cripps and Lascelles, 1976) and the magnitude of selective transfer has been observed to be similar to that for the lactating mammary gland (Watson and Lascelles, 1973a).

In the mammary secretions, IgG_1 is predominant overwhelmingly in colostrum on account of its selective transfer from blood shortly before parturition (Brandon et al., 1971). Selective transfer continues into lactation though at a slower rate (Watson and Lascelles, 1973a). The relative contribution of serum-derived and locally produced IgA has been estimated in sheep mammary secretion (Sheldrake et al., 1984). During early and mid-lactation, serum IgA was selectively transported to mammary secretion on a large scale. Estimates of local production indicated that the bulk of IgA was locally produced during involution of the mammary gland.

The foregoing description of the distribution and the kinetics of immunoglobulin classes, in particular IgG_1 and IgA, reflect the possible contribution by the common mucosal immune system as propounded by Bienenstock et al (1979). In this system, the gut stimulation leads to the release of antibody precursor cells from the Peyer's patches, which migrate to a variety of mucosal sites. There have been continued attempts to selectively induce salivary and mammary antibody responses in mice and primates through oral and local immunization strategies (Goldblum et al., 1975; Mestecky et al., 1978; Challacombe and Lehner, 1980; Kiyono et al., 1982; Morisaki et al., 1983). In ruminants, investigations into specific salivary immune responses against particular antigens have not been carried out, apart from the detection and quantitation of non-specific immunoglobulin classes in the saliva of these animals. However, the effect of intraperitoneal and intramammary immunization, and local infusion into udders have been studied by different group of workers using killed bacterial antigens (Lascelles et al., 1966), monomeric and polymeric forms of flagellin (McDowell and Lascelles, 1969) or ovalbumin (OVA) (Sheldrake et al., 1985a; Sheldrake et al., 1985b). These studies demonstrated clearly that immunization either a few weeks before parturition or two to three weeks after parturition produced a substantial level of IgA and IgG_1 antibodies in the milk in the ensuing lactation.

The salivary antibody responses to intraperitoneal immunization of sheep to OVA in the saliva and serum of sheep has been described in Section 3.0 of this project. In the present Section, the effect of intraperitoneal immunization of ewes with rumen ciliates has been

evaluated to test the hypothesis that antibodies against rumen protozoa can be raised in the saliva and milk of sheep.

5.2 Materials and Methods

5.2.1 Experimental animals

Eight merino ewes were obtained two weeks after lambing from the university farm. Two animals were allotted to Group I (control group) and six animals to Group II (test group). Animals were kept in individual pens and given 500g/day lucerne hay (65% dry matter digestibility and 21 g nitrogen/kg dry matter) plus 1000g/day sheep pellets (70% dry matter digestibility and 7.8 g nitrogen/kg dry matter. These diets were offered until the end of the trial period.

5.2.2 Preparation of immunogens

Ovalbumin (Grade III, Sigma Chemicals) was prepared as a 40mg dose in 5ml phosphate-buffered saline (PBS, pH 7.2) and it was emulsified in an equal volume of complete Freund's adjuvant for primary immunization. For booster injections, a reduced dose was emulsified in incomplete Freund's adjuvant.

In the preparation of protozoal antigens, the following procedure was adopted. Samples of frozen purified suspensions (5ml) containing 2x 10^7 cells (with protease inhibitors-see sub-section 4.2.2) were thawed and ampicillin was added at the rate of 0.1mg/ml to each sample. This was then incubated at 39^{o}c for 24 hours to reduce any contaminating bacteria. At the middle of the incubation period, the same amount of antibiotic was added again and the incubation continued. At the end of the incubation period, each sample was emulsified in equal volume of complete Freund's adjuvant for the primary immunization. Vaccines for the subsequent immunizations were all emulsified in incomplete Freund's adjuvant and contained 1.7x 10^7 protozoa/ dose.

5.2.3 Immunization

Ewes were immunized according to the protocol in Table 5.1. During primary immunization, two ewes in group I were immunized intraperitoneally (I/P) against OVA with a 10 ml dose of 40mg / animal prepared as described above. Each ewe in group II was immunized (I/P) against rumen ciliates (2x 10^7 per dose) in complete Freund's adjuvant. At the end of 24 days, while group I animals received a further dose of OVA (20 mg/ewe), group II animals were boosted with rumen ciliates (1.7x 10^7 / animal) in 5ml of PBS emulsified in 5 ml incomplete Freund's adjuvant.

Following the secondary immunization, both groups received a dose of protozoal antigens (1x 10^7 cells/dose, in incomplete Freund's adjuvant) one month after the primary injection.

Table 5.1 Immunization protocol and sampling schedule

Group	Antigen	Primary Immunization[a]		Secondary Immunization[b]	
		Route	Sampling Schedule[c] (Days)	Route	Sampling Schedule[c] (Days)
Group I (2)[d]	OVA	I/P(0)[e]	0,7,14	I/P(24,31)[e]	24,31,38,45
Group II (6)[d]	Ciliates	I/P(0)[e]	0,7,14	I/P(24, 31)[e]	24,31,38.45

[a] Primary immunization for Group I consisted of 40mg of ovalbumin dissolved in 5ml sterile PBS and emulsified with 5ml FCA, delivered via intraperitoneal injection.

Primary immunization for Group II consisted of a dose of 2.0x10^7 purified ciliates mixed with Freund's Complete Adjuvant (FCA).

[b] For the secondary immunization GroupI animals received 20mg ovalbumin in 4ml PBS+4ml of incomplete Freund's adjuvant (IFA), whereas Group II received rumen ciliates (1.7x10^7cells/animal) in IFA. In both cases, immunization was (I/P). In the final immunization, both groups received ciliates antigens at the same dose level (1x 10^7) in IFA on day 31.

[c] Days from commencement of immunization

[d] Number of animals used in each group

[e] Days on which animals were immunized

5.2.4 *Collection of samples*

Similar procedures were adopted in the collection of sera and saliva as described in Section 3.0. The samples were collected prior to priming and at various times following primary and challenge inoculation. Milk samples for the study were collected by hand milking and stripping approximately 25 ml from both teats from each ewe. The collected samples were centrifuged at 10,000g for 15 minutes, and the clarified whey fraction was aliquoted into 5 ml vials and stored at -80°C.

5.2.5 *Detection of anti-OVA IgA antibodies by ELISA*

Anti-OVA IgA levels in milk were measured using an enzyme-linked immunosorbent assay (ELISA). The procedure was essentially similar to that described under Sub-section 3.2.6. A saline solution

containing 0.05% Tween-20 and 0.01% bovine serum albumin (TS/BSA) was used as diluent. Samples of milk-whey from the two ewes in Group 1 were diluted 1:5 and thence by doubling dilutions across the 8 wells of the ELISA plate (Costar, Catalog No 2595, Data Package Corp., Cambridge, Massachusets), coated with OVA (see 3.2.6). At the end of primary incubation, the plates were washed 4x with TS/BSA and incubated with the second antibody (mouse monoclonal antibody against ovine IgA). Prior to the addition of alkaline phosphatase-conjugated rabbit anti-mouse Ig (whole immunoglobulins, RAMG), the plates were washed again with TS/BSA. Finally, after incubation with the conjugate, the plates were washed with 0.05% Tween-saline containing Mg^{++} and Zn^{+++} and the substrate p-nitrophenyl-phosphate (104-105, Sigma Chemicals) was added. The colour development was measured at 405nm after a further incubation at 37°C for 4h.

5.2.6 Procedures for detection of antibodies against rumen ciliates

5.2.6.1 ELISA

For coating of the ELISA plates, rumen ciliate antigens were prepared by thawing purified frozen ciliates (with protease inhibitors) and mixing them 1:1 with a buffer containing 10mM Tris buffer (pH 7.6), 5mM EDTA, left at 4°C for 2h, and then sonicating at a power of 400 watts for 5-6 min in short spells on ice (Labsonic 1510). More than 75% of the ciliates were disrupted by this method, probably releasing both cell-wall and cytoplasmic antigens. The cell lysates were centrifuged at 13,000g for 3 min and the supernates were aliquoted into sterile Eppendorf tubes (250µl/tube) and stored at -20°C until further use.

Prior to coating of the plates, frozen cell-lysates were thawed, diluted 1:10 in a buffer containing 0.5M Tris-HCL, pH 7.5; 0.132M NaCL; 0.0008M NaN_3 (TSA) and the concentration of protein estimated from the absorbance at 280nm and 260nm respectively.

The protozoal antigens were diluted in TSA and used at 10µg/ml to coat the ELISA plates (Costar catalog number 2595; Data Packaging Corp., Cambridge, Mass.). Briefly, the ELISA plates were coated with 80µl of ciliate antigens per well and incubated overnight at 4°C. Antigen-coated ELISA plates were then blocked with TS/BSA. Samples of saliva were diluted initially 1:2 for detection of all classes of antibodies. Milk was diluted initially 1:5 for IgA and IgM antibodies, and 1:100 for IgG antibodies. In the case of serum, samples were diluted initially 1:25 for IgA and IgM antibodies, and 1:500 for the detection of IgG antibodies. TS/BSA was used as the diluent. The samples were then diluted two-fold, giving eight serial

dilutions. Plates were incubated for 1h at 37°C followed by another 1h incubation at 4°C. The second antibodies [mouse monoclonal specific for IgA, monospecific rabbit anti-sheep IgG, rabbit anti-sheep IgM (the latter was donated by Dr A.J. Husband, University of Sydney, N.S.W)] and the conjugates were added at optimal concentrations (as determined by checkerboard titrations) and incubated further for 1h at 37°C and then further 1 hour at 4°C. Between each incubation, ELISA plates were washed 4x with TS/BSA. After the final incubation with the relevant alkaline phosphatase conjugate, the plates were washed 4x with 0.05% Tween-saline containing Mg^{++} and Zn^{+++}. p-Nitrophenylphosphate (phosphatase substrate 104-105, Sigma Chemicals) was added as a 1mg/ml solution in a 10% (vol/vol) diethanolamine buffer to all wells. After incubation at 37°C for 4 hours, optical densities were read on a Titertek Multiskan Mc (Flow Laboratories, Irvine, Ayrshire) at 405nm. Samples from individual animals which gave the highest optical density in trial runs were used as the positive control in each test run. Similarly, samples known to have low antibody titre against rumen ciliates were included as negative controls.

The mean and the standard deviation (SD) of the maximum absorbance values (OD) obtained for the negative (preimmune) control samples were calculated and values 2 SD greater than the mean were regarded as the threshold for positivity in test samples. The end point titre of a sample was taken as the last dilution giving an OD either equal to or greater than this threshold value. Furthermore, in case of anti-ciliate IgA and IgG antibody in saliva and milk, the reciprocal of the end-point dilutions of preimmune and postimmune samples were related to changes in the total concentrations of the respective immunoglobulin classes. The result was then expressed as units of specific antibody per milligram of total IgA or IgG respectively.

5.2.7 Testing for the presence of antibodies against mixed rumen bacteria

The rumen fluid was collected from the same wethers which were used to obtain rumen ciliates for immunization. After straining through a 300μ defined aperture nylon gauze, the bacterial fractions from the strained rumen fluid (SRF) were recovered by a sequential centrifugation procedure, (initially at 2000g for 10 min to ensure complete removal of protozoal fractions, and then at 13,000g for 5 minutes to recover the bacterial pellets). The pellets of bacteria were washed three times in phosphate buffered saline (PBS, pH 7.2), resuspended in 1ml of PBS containing protease-inhibitor cocktail containing final concentrations of 200μM PMSF, 100μM

saline (PBS, pH 7.2), resuspended in 1ml of PBS containing protease-inhibitor cocktail containing final concentrations of 200μM PMSF, 100μM each of TLCK, TPCK and NPG-B respectively (see 4.2.2) and stored at -20°C. Before use, the frozen bacterial suspensions were thawed and sonicated to break up the clumps of cells. ELISA plates were coated with whole cells diluted to 8x 10^7 cells/ml (TSA). Following overnight incubation at 4°C, the plates were blocked with TS/BSA for 1h. Immune serum (diluted 1:100 in TS/BSA) and milk (1:10 in TS/BSA) obtained from individual animals were added to duplicate wells on the plates and doubling-dilutions were made across the plate, giving eight serial dilutions. The plates were incubated for 1h at 37°C and then 1h at 4°C. After washing, the plates were further incubated with second antibody (monospecific rabbit-anti sheep IgG) for 1h at 37°C and 1 h at 4°C followed by a third incubation with alkaline phosphatase-conjugated goat-anti rabbit IgG (GARG). After the final wash in 0.05% Tween-saline, the substrate was added to all of the wells, and after 4h incubation at 37°C, the optical densities were read at 405nm with the ELISA reader . A sample of serum known previously to have high concentrations of natural antibodies against mixed rumen bacteria was used as a positive control and a lamb serum with minimal binding activity to rumen bacterial antigens was used as a negative control. One row of each plate had controls for substrate and conjugate respectively.

5.2.7.1 Absorption tests for any sign of cross-reactivity of antibodies

Samples of preimmune sera, immune sera (1:100) and milk (1:5) from sheep immunized against rumen ciliates were absorbed with an equal volume of mixed rumen bacterial cells (10^7 /ml), prepared as described under sub section 5.2.7. The mixtures were kept at 37°C for 2h and then centrifuged at 13000g for 5 min. The supernatants were then tested by ELISA for any residual reactivity towards rumen bacterial and protozoal antigens as before. Similar absorption of samples of preimmune sera, immune sera (1:100) and milk (1:5) was also carried out with an equal volume of mixed rumen cilates (10^6 cells/ml). After absorption, rumen ciliates were spun out and the supernatants were tested for reactivity by ELISA against protozoal and bacterial antigens.

5.2.8 Determination of concentration of total IgG and IgA

Concentrations of total IgG and IgA in pooled saliva and milk collected on different sampling days were measured by ELISA. Procedures similar to those described in sub-section 3.2.8 were adopted for the quantitation of individual isotypes . The ELISA was standardized for the

quantitation of total isotypes in milk and saliva. Purified ovine IgA (0.5mg/ml) and ovine IgG (1mg/ml) were used to construct the standard curves. The ELISA plates used in the assays were coated with monospecific rabbit anti-sheep IgG for the quantitation of IgG and rabbit anti-sheep IgA (1:2000 dilution) for quantitation of IgA. Similar dilutions of saliva as mentioned in sub-section 3.2.8, were added to the ELISA plates. The dilutions of milk for incubation ranged from 1: 400 to 1: 102400 for IgG and 1:200 to 1:25600 for IgA.

5.2.9 Analysis of antibody binding to surface antigens of rumen ciliates

5.2.9.1. Monoclonal antibodies

The panel of murine monoclonal antibodies (MoAbs) specific for sheep IgG_1 and IgG_2 were purchased from McMaster Laboratory, Commonwealth Scientific and Industrial Research Organization, Glebe, New South Wales, Australia 3052. Irrelevant monoclonals were obtained from the Department of Microbiology and Immunology, University of Adelaide). They are summarized in Table 5.2:

Table 5.2 Monoclonal antibodies used to detect surface bound sheep antibodies on rumen ciliates and irrelevant monoclonals used as controls. The antibodies were used as culture supernatants.

Monoclonal	Specificity	Mouse isotype
McM_1	Sheep IgG_1	IgG_{2a}
McM_3	Sheep IgG_2	IgG_3
F_3-$4B_4$	Sheep IgA	IgG_1
6c6b	Human IgA	IgG_1
7b6c	Human IgG_1	IgG_{2a}

5.2.9.2 Indirect Fluorescent-Antibody (I FA)Test

Rumen ciliates were harvested and purified according to the procedures described under Section 4.0. Purified cells were resuspended in phosphate buffered saline (PBS) and maintained at 37°C at a concentration of $1x10^7$ cells /ml. Aliquots, (100µl), of this suspension were transferred to 1.5 ml Eppendorf tubes. Each was made up to 0.4ml with warm PBS (37°C) and the cells were centrifuged at 150g for 3minutes. The pellets were then resuspended in either preimmune (control) or immune samples (0.2ml) of serum, milk or saliva at dilutions in PBS as shown in Table 5.3.

Table 5.3 Dilutions of preimmune and immune samples for the Indirect Fluorescent Antibody (IFA) assay

Samples	Dilutions
Serum	1:10, 1:20, 1:40, 1:100, 1:200, 1:400, 1:800, 1:1600
Milk	1:5, 1:10, 1:20, 1:40, 1:80, 1:160
Saliva	1:2, 1:4, 1:8, 1:16, 1:32, 1:64

After incubation at 37°C for 1hour, the cells were centrifuged as before, and washed twice in 0.5ml aliquots of warm PBS (37°C) and resuspended in relevant mouse monoclonal antibodies against the respective classes of sheep immunoglobulins. After a further incubation at 37°C for 1 hour, the cells were washed twice again in 0.5ml aliquots of warm PBS, resuspended in 50 µl of fluorescein conjugated goat anti-mouse IgG (1:20 dilution in PBS) and incubated for 1h at 37°C. The optimal dilution of the conjugate was obtained by titrating against a 1:4 dilution of a positive serum and selecting the greatest dilution giving the maximum specific fluorescence. After washing twice in warm PBS, the cells were fixed in 1% paraformaldehyde and stored at 4°C. The samples analysed were pooled sera or secretions (milk and saliva) collected on day 14 after primary immunization and each titration was performed in duplicate. The controls in the study included known positive sera or milk, preimmune samples and irrelevant monoclonals and secondary antibodies. Autofluorescence was also assessed on unincubated samples of ciliates, and binding by conjugate alone was also tested. The fluorescein-stained rumen ciliate cells were examined with 10x, 20x and 40x objectives using an Olympus fluorescence microscope. The fields were scanned rapidly under low power to ensure uniformity of staining and from typical areas four or five high power fields were selected for reading. The replicate samples from the same tubes were used in flow cytometry analysis.

5.2.9.3 Measurement of intensity of fluorescence by flow cytometry

The fluorescence of mixed rumen ciliates was measured on a FACScan interfaced to a computer containing Consort 30 Software (Becton-Dickinson) for data storage and analysis. The excitation wavelength was 488nm; 10,000 cells were analysed in each sample. Rectilinear forward-angle light scatter and side light scatter were used to define the viable cell fraction. The threshold for positivity was set for each experiment by evaluating the fluorescence intensity of the controls (untreated cells only,

untreated cells and conjugate, untreated cells/irrelevant monoclonals/fluorescein-conjugate, untreated cells/relevant monoclonals/fluorescein-conjugate. The fluorescence of cells incubated in an irrelevant mouse monoclonal was used as the background in all experiments. The software Lysis version I (Becton-Dickinson) was employed to calculate the fluorescence intensity and the related statistics of different dilutions of the respective samples.

5.2.10 Statistical analysis

Due to variation in the antibody responses between animals, the mean titre for the six ewes in Group II was calculated for each sampling day and the means were used to obtain the fold rise in antibody titres. To fulfill parametric assumptions, reciprocals of end-point dilutions were log-transformed (natural logarithms) before applying statistical tests. A one way analysis of variance was then done, based on a completely randomized design (Steel and Toorie, 1960). The significance between means was analysed by the LSD test. Similarly the values of relative fluorescence intensity calculated from data measured by flow-cytometry were also subject to analysis of variance.

5.3 Results

5.3.1 Purpose of group 1 animals

Two lactating ewes were included in the above group to serve as controls for the animals immunized with ciliates in Group II. It was important to determine whether any specific IgA antibodies detected in milk were due to the immunization with ciliate antigens and not due to hormonally regulated recruitment of antigen-specific immunoblasts (originating in the gut mucosa - Weisz-Carrington et al., 1978). When the two ewes were immunized with the control antigen OVA, as shown in the immunization schedule (Table 5.1), OVA specific IgA antibodies appeared in the milk from the first week and remained elevated for the duration of the experiment. The booster injection (day 24) did not produce any further increase in the antibody titre (see Figure. 5.1).

When the ewes were immunized against rumen ciliates on day 31, IgA antibodies specific for ciliate antigens were observed in samples of milk from day 38 (7 days after immunization against ciliates) and the levels had increased further by day 45 (14 days after imunization). Neither the samples collected on day 31 nor any samples collected prior to day 31 showed any IgA specific to rumen ciliates (see Table 5.4).

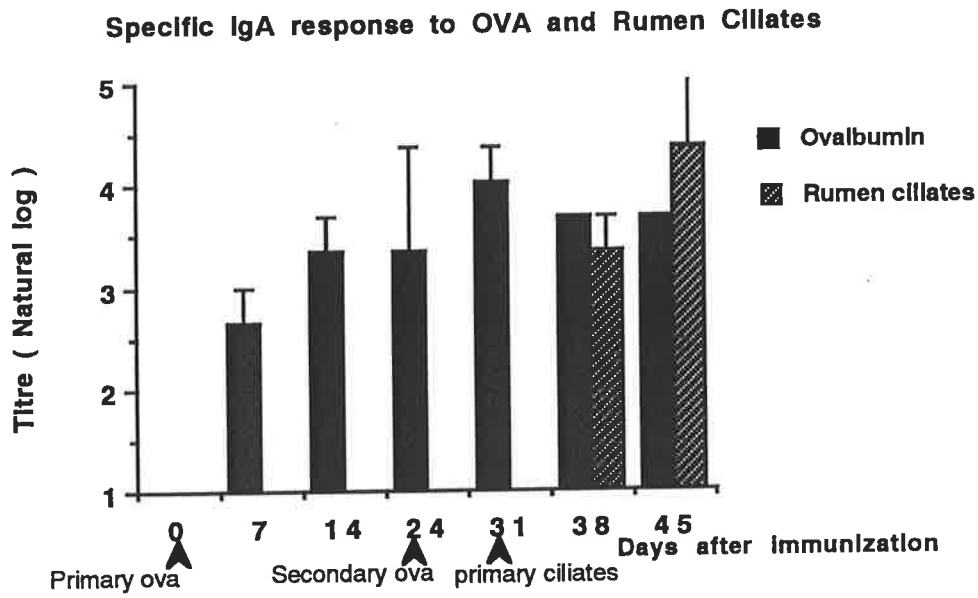


Fig. 5.1 Specific IgA antibodies to ovalbumin and rumen ciliates in the milk after immunization with the respective antigens. Values are average titres of two ewes (Group I ± SD).

Table 5.4 Specific IgA antibody responses to ovalbumin and rumen ciliate antigens in the milk of two ewes (Group I)

Days[a]	IgA[b]	
	Ovalbumin	Rumen ciliates
0	000	000
7	14 (2.65±0.35)	000
14	28.2 (3.34±0.35)	000
24	28.2 (3.34±1.04)	000
31	56.8 (4.04±0.35)	000
38	40.0 (3.69±0.00)	28.2 (3.34±0.35)
45	40.0 (3.69±0.00)	79.8 (4.38±0.69)

[a]Days from the commencement of immunization

[b]Titre values have been expressed as geometric means (n=2) Numbers in parenthesis are corresponding natural log values and standard errors

5.3.2 *Specificity of the ELISA*

In ovalbumin-coated wells, preimmune samples of milk did not show any background colour development. In the case of wells coated with rumen ciliate lysates, preimmune samples (saliva, serum and milk) were found to contain some background antibody. The specificity of this was examined by absorption studies.

5.3.3 *Antibodies against rumen bacteria and absorption studies*

The reactivity of the preimmune and immune sheep sera and milk with rumen bacterial antigens was examined by ELISA using plates coated with mixed rumen bacteria. Intense colour developed when both preimmune and immune samples were tested against bacterial antigens. The mean optical density of paired wells at each dilution of both preimmune and immune sera were matched against the threshold value (mean + two SD of the negative control, i.e., a sample of lamb serum with minimal binding activity to rumen bacterial antigens) and absorbance values similar to the figures shown in Table 5.5 was observed. Absorption with mixed rumen bacterial antigens reduced the absorbance values to the background level of the negative control for both preimmune and immune sera and milk (data not shown) when the samples were tested against the bacterial antigens. However, when the same samples were tested against the ciliate lysates, colour development was similar to that observed prior to absorption. Likewise, when the samples absorbed with mixed rumen protozoal antigens were tested against bacterial antigens, intense colour developed for both preimmune and immune sera and milk (see Table 5.5).

5.3.4 *Specific antibodies against rumen ciliates*

The kinetics of the IgG and IgA antibody responses were followed after the primary and subsequent secondary immunization with mixed rumen ciliate antigens.

5.3.4.1 *Specific antibodies in saliva*

The primary intraperitoneal immunization was followed by a rise in salivary IgG antibodies against rumen ciliate antigens (see Table 5.6 and Fig. 5.2). When the group mean titres were compared with the preimmune value, statistically no significant difference was observed between day 0 and day 7, but by day 14, there had been a five fold increase in titre ($P<0.01$). The subsequent booster dose further increased the level of IgG antibodies on day 31 to seven times the preimmune level. However, this latter

increase was not significant when the absolute titres were normalized against the total IgG to compensate for dilution of the stimulated saliva samples. The levels of antibody in the saliva increased from 153 units/ mg to 1017 units/ mg of immunoglobulin (Ig) after boosting (see Table 5.6).

Table 5.5 Mean ELISA readings (absorbance at $405nmx10^3$) for preimmune and immune samples at different dilutions against rumen mixed bacterial antigens before and after absorption.

Type of sample	Absorbance at different dilutions (serially diluted two-fold)	
	Before absorption with bacterial antigens	After absorption with ciliate antigens
Serum	Starting dilution 1:100	
Preimmune	569,459,349,222,145,73,46,20	521,428,327,216,133,67,30,11
Immune	589,452,350,215,150,70,40,20	530,439,324,214,111,61,20,5
Milk	Starting dilution 1:10	
Preimmune	398,271,168,75,36,14,5,6	349,246,113,57,35,15,9,10
Immune	411,309,171,83,39,17,10,11	358,250,115,57,33,11,6,4

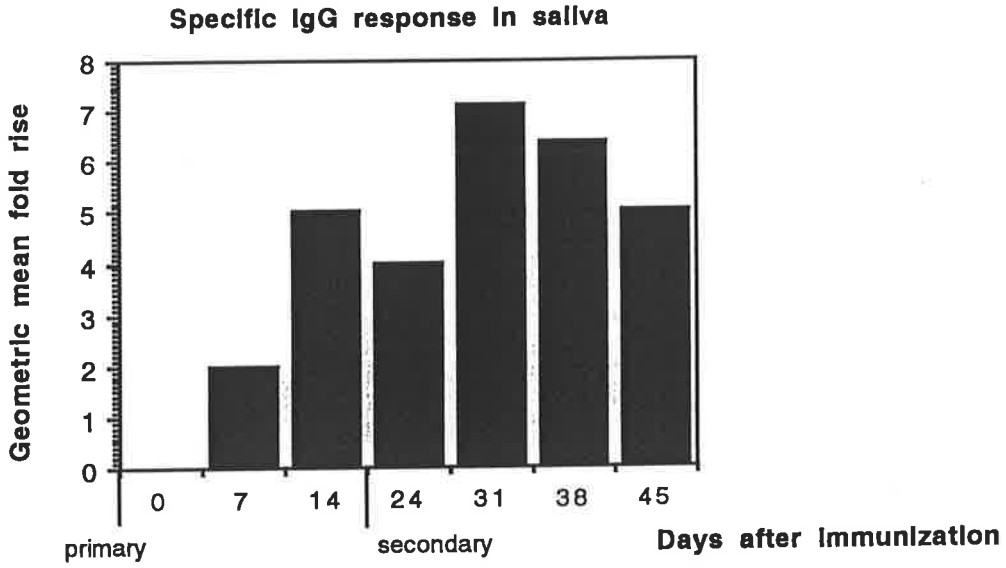


Fig. 5.2. Effects of intraperitoneal immunization on specific IgG responses to rumen ciliates in saliva. Antibody responses are given as the geometric mean fold rise in antibody titre over preimmune titre. The results were similar when plotted using antibody units normalized for IgG content.

An IgA antibody response in saliva was detected in only three of the ewes. The level of antibody in one of the responding ewes was above preimmune levels up to 24 days after primary immunization. In the other two ewes, the response was above preimmune levels for only the first two weeks (day 14). In the remaining three ewes in Group II, no IgA antibody response was detected in the saliva at any stage after primary or secondary immunization. There was no significant change in the total IgA concentration in pooled saliva before and after immunization and the IgA contents of all samples were similar. Therefore, the IgA contents of individual samples were not measured and the titres of IgA antibodies in the responding ewes were not normalized against total immunoglobulin concentrations (see Table 5.6).

Table 5.6 IgG and IgA antibody titres against rumen ciliate antigens in the preimmune and immune saliva of sheep (Group II)

Days[a]	Titre[b]					
	IgG		Total IgG[c]	NV[d]	IgA[e]	
0	5.7	(1.74±0.78)	0.036±0.008	153	000	
7	11.4	(2.43 ±0.66)	0.044±0.008	250	10.1	(2.31± 0.33)
14	28.5	(3.35±0.93)	0.045±0.010	664	33.1	(3.47± 0.57)
24	22.2	(3.10±0.96)	0.045±0.008	493	20.0	(3.00± 0.87)
31	40.4	(3.69±0.96)	0.047±0.006	860	Not done	
38	36.6	(3.58±1.02)	0.036±0.005	1017	Not done	
45	28.5	(3.35±1.16)	0.050±0.008	598	Not done	
L.S.D[f]. (P<0.01)		0.823	0.012			

[a]Days from the commencement of immunization; Day 0 values for IgG and IgA antibody titres were for preimmune samples relative to negative control (background).

[b]Titre values have been expressed as geometric means of the reciprocal of the end-point dilution (n=6). Numbers in parenthesis are corresponding natural log values and standard errors

[c]Total IgG- concentration of immunoglobulins (mg /ml) are mean of two duplicates ± standard deviation.

[d]NV- normalized values of salivary antibody titres expressed as units/mg immunoglobulin. Serum titre values have not been normalized on the assumption that they are not subject to undue variations during recovery.

[e]IgA antibody titres are means of the three responding ewes. The other three animals did not show any detectable IgA antibodies relative to the preimmune sample.

[f]L.S.D- Least significant difference used for statistical comparison of natural log titre values.

5.3.4.2. Specific antibodies in serum

Intraperitoneal immunization (I/P) with rumen ciliates resulted in a serum IgG response of six ewes in Group II. IgA antibodies were undetectable in the preimmune sera of the animals relative to the background of the negative control and no change was found after immunization with ciliate antigens.. After primary I/P immunization, IgG antibody titres were elevated 3 fold by day 7 in all of the ewes and five and a half fold by day 14 (see Fig.5.3).

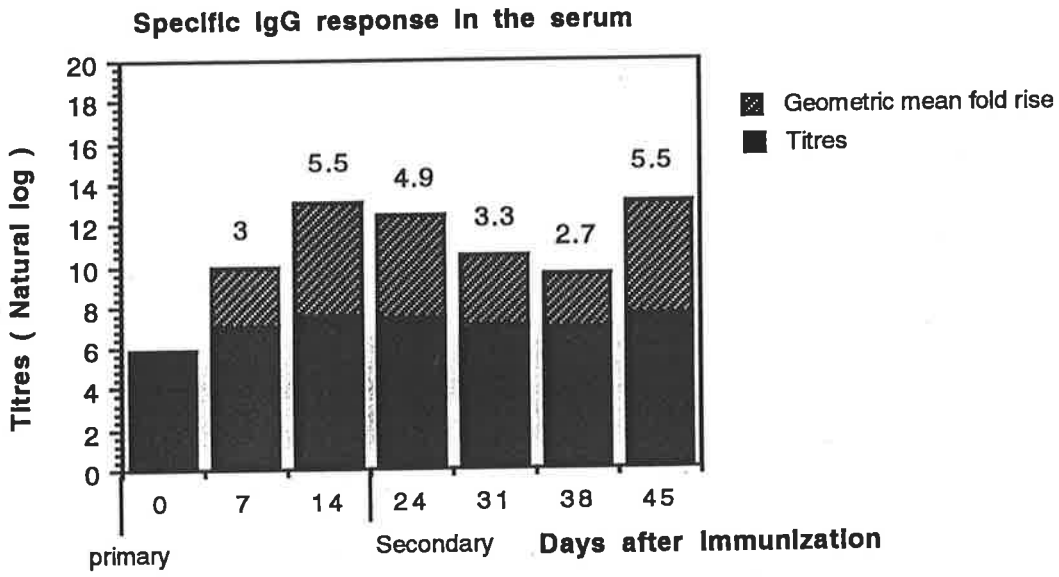


Fig. 5.3. Specific IgG antibody responses in serum to rumen ciliates. Values represent the natural log transformed mean of each day's sample of six ewes and the geometric mean fold rise in specific IgG antibody titre over the preimmunization titre.

It is evident from Fig. 5.3 that the booster dose at day 24 did not alter the level of specific IgG antibody response up to the end of the trial period and such differences as were observed were not significant. However, the mean fold rise in specific IgG antibody titre over the preimmune level (days 14-45) did represent a significant increase ($P<0.01$, see Table 5.7).

5.3.4.3 Specific IgG antibody response in milk

A specific IgG antibody response was detected in all six ewes. Responses were compared to preimmune levels. A rise above the geometric mean level of antibody in the preimmune milk was detected from the first week after primary immunization and the titre approached a plateau by two weeks (Table 5.7). The levels rose further after the booster immunization. Analysis of variance revealed a significant difference between preimmune and postimmune titres ($P=0.001$). Differences

between mean titres from day 14 were not significant throughout the experimental period (see Table 5.7). Titres of IgG antibodies in milk were also normalized to compensate for variations in the total IgG concentrations in individual samples, but this had little effect on the relative titres.

5.3.4.4. Specific IgA antibody response in milk

The data in Table 5.8 show specific IgA antibody levels in samples of milk collected on different days during the four to eight week period of the experiment. The data show that there was a significant increase ($P<0.01$) in IgA antibody levels in milk following intraperitoneal immunization. Total IgA concentration was also determined throughout the experimental period and mean values are presented for pooled samples of each stage. The Table 5.8 also shows specific IgA anti-protozoa antibody levels expressed as units per mg of total IgA in the milk. The striking feature of these data is the relatively high titres of IgA antibody found in the mammary secretions of ewes even after primary immunization.

5.3.5 Detection of antibodies binding the surface antigens of ciliates

5.3.5.1 Reading of the IFA test

After staining with low dilutions of immune samples (serum and milk) holotrichs showed a bright green fluorescence with a distinct halo around the periphery, whilst entodiniomorphid ciliates exhibited specific surface fluorescence, (see Plates 5.1 and 5.2). As dilution increased, the fluorescence decreased until at the end point it either disappeared compleletely or reached background levels similar to those observed with cells stained in preimmune samples (see Plate 5.3). The titre was considered to be the highest dilution at which most organisms exhibited continuous peripheral fluorescence. Some of the ciliate species exposed to lower dilutions of preimmune samples exhibited localized areas of brilliant green fluorescence at either the posterior or the anterior end (see Plate 5.3). However, the overall degree of brightness of the cells treated with preimmune samples was found to be less, while the autofluorescence of negative controls was yellow-orange and easily distinguished from that of specific fluorescence. (Plate 5.4). Based on the foregoing description, the end points of titrations were reached when greater than 50% of the cells displayed a level of fluorescence sufficient to give the appearance of a completely outlined cell wall. Such a criterion reproduced essentially the same titres of IgG_1 antibodies for samples when the assays were repeated on ten separate occasions. Titres are expressed as the reciprocal of the highest

dilution fulfilling the above criterion for positivity and the results are shown in Table 5.9.

Table 5.7 IgG antibody titres against rumen ciliates in milk and serum

Days[a]	Titre[b]			
	Milk			Serum[e]
	IgG	Total IgG[c]	NV[d]	IgG
0	25.3 (3.23±0.31)	0.36±0.003	70	361 (5.89±0.15)
7	35.5 (3.57 ±0.38)	0.34±0.004	104	1097 (7.0±0.28)
14	361 (5.89±0.30)	0.34±0.030	1062	1998 (7.6±0.25)
24	403 (6.00±0.27)	0.31±0.007	1300	1808 (7.5±0.21)
31	639 (6.46±0.23)	0.41±0.010	1558	1212 (7.1±0.23)
38	639 (6.46±0..23)	0.36±0.009	1775	992 (6.9±0.25)
45	508 (6.23±0.27)	0.36±0.003	1411	1998 (7.6±0.25)
L.S.D[f]. (P<0.01)	0.767	0.053		0.722

[a]Days from the commencement of immunization

[b]Titres have been expressed as geometric means (n=6). Numbers in parenthesis are the corresponding natural log values ± standard errors.

[c]Total IgG- concentration of immunoglobulins (mg /ml) are the means of two duplicates ± standard deviation.

[d]NV-Normalized titre of milk expressed as units of antibody/mg of IgG. Serum titres have not been normalized on the assumption that they are not subject to undue variations in the total concentration of IgG.

[e]Serum IgG titres represent geometric means (average of six ewes)

[f]L.S.D- Least significant difference used for statistical comparison of natural log titre values.

Table 5.8 IgA antibody titres against rumen ciliate antigens in the milk

Days[a]	Titre[b]			
	IgA		Total IgA[c]	Normalized titre value[d]
0	6.6	(1.88±0.63)	0.029±0.001	228
7	35.5	(3.57 ±0.33)	0.028±0.000	1268
14	70.8	(4.26±0.21)	0.031±0.001	2284
24	50.4	(3.92±0.29)	0.028±0.001	1800
31	79.8	(4.38±0.40)	0.033±0.004	2418
38	71.5	(4.27±0.33)	0.042±0.003	1702
45	79.8	(4.38±0.25)	0.033±0.003	2418
L.S.D. (P<0.01)		1.25	0.012	

[a]Days from the commencement of immunization

[b]Titre values have been expressed as geometric means (n=6). Numbers in parenthesis are the corresponding natural log values ± standard errors.

[c]Total IgA- concentration of immunoglobulins (mg /ml) are mean of two duplicates ± standard deviation.

[d]Normalized titre of milk expressed as units/mg immunoglobulin.

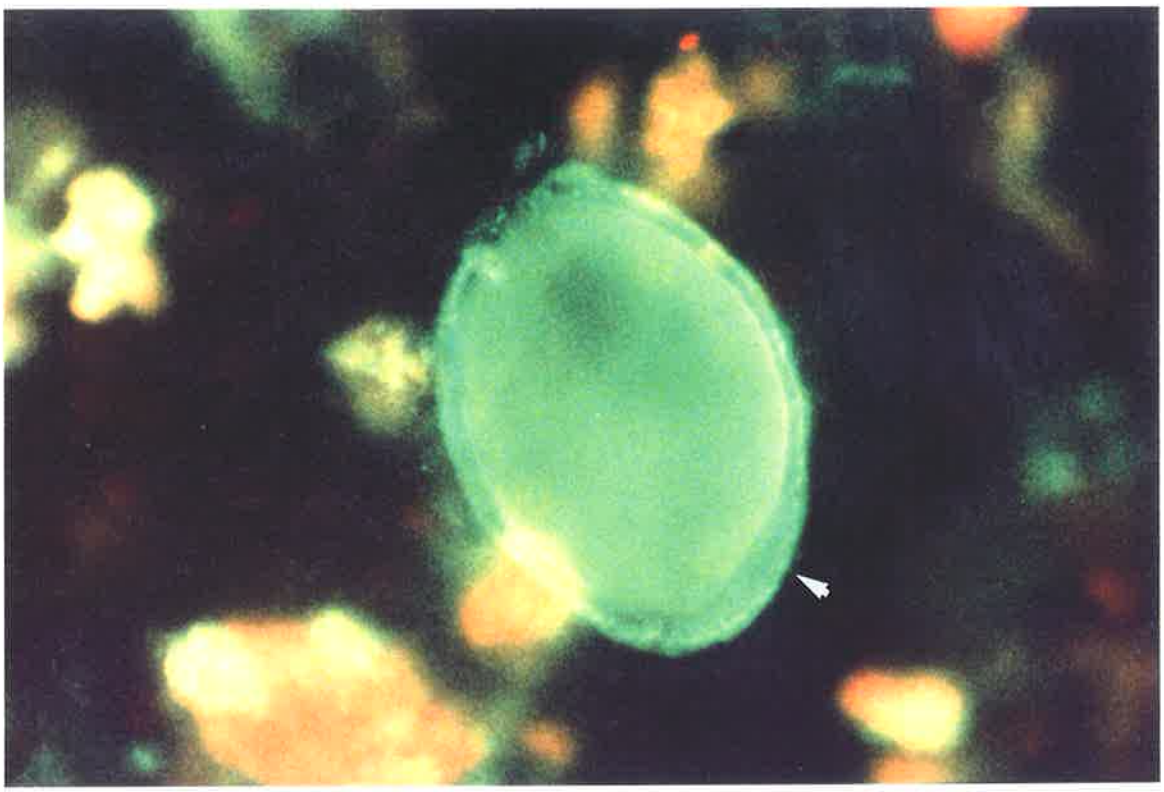
Table 5.9 Results of indirect-fluorescent antibody test

Samples	Titres of IgG_1	
	Preimmune	Immune
Serum	1:10	1:200
Milk	1:5	1:40

Plate 5.1: Positive immunofluorescence staining of rumen ciliates (holotrich) by pooled immune samples.

Panel 1: A holotrich showing bright green fluorescence with a distinct halo (white arrow head);
Panel 2: Phase photomicrograph of the same specimen.

1



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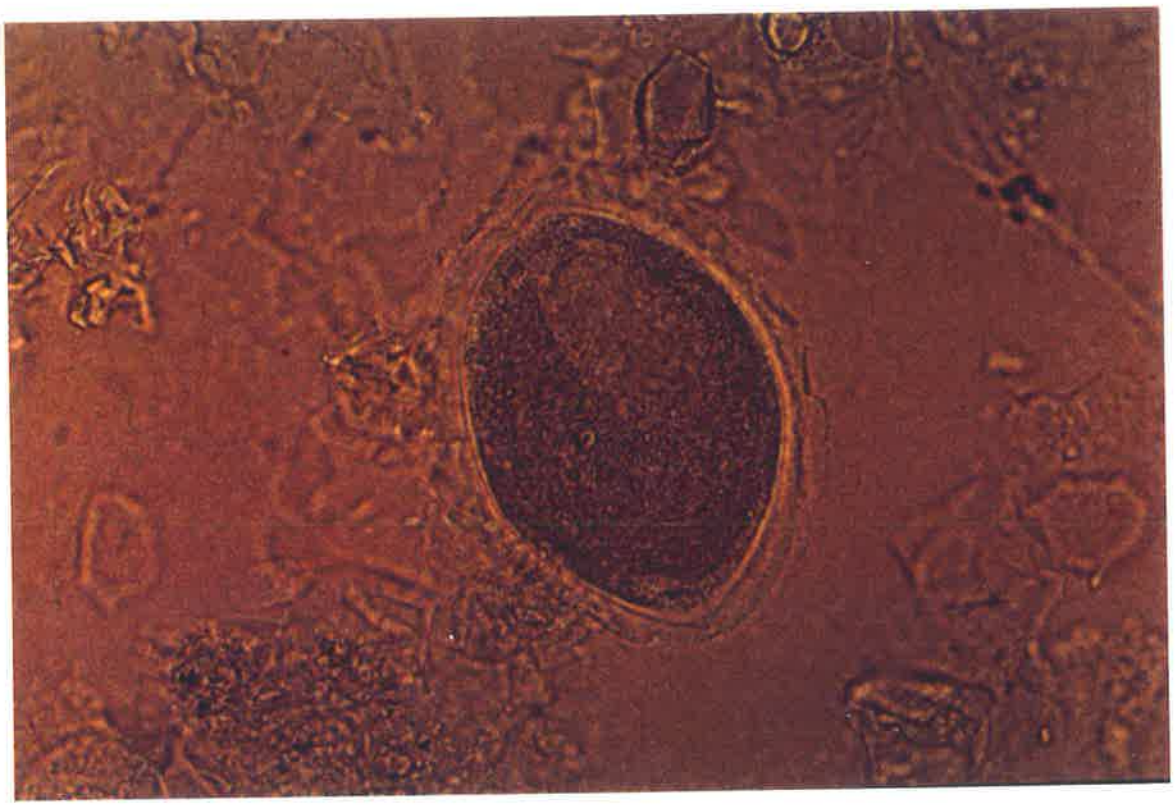
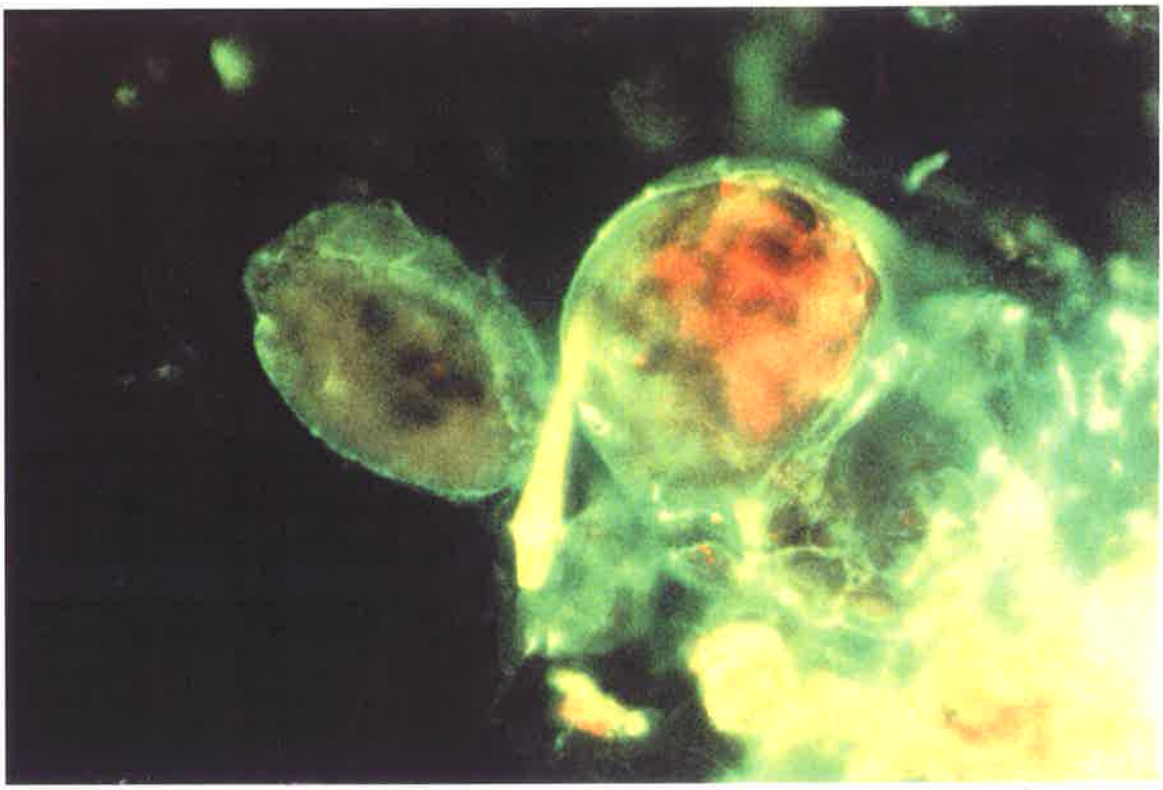


Plate 5.2: Positive immunofluorescence staining of rumen ciliates (entodiniomorphs) by pooled immune samples.

Fig.1 A group of entodiniomorphid ciliates showing complete peripheral staining

Fig.2: A phase photomicrograph of same specimens.

1



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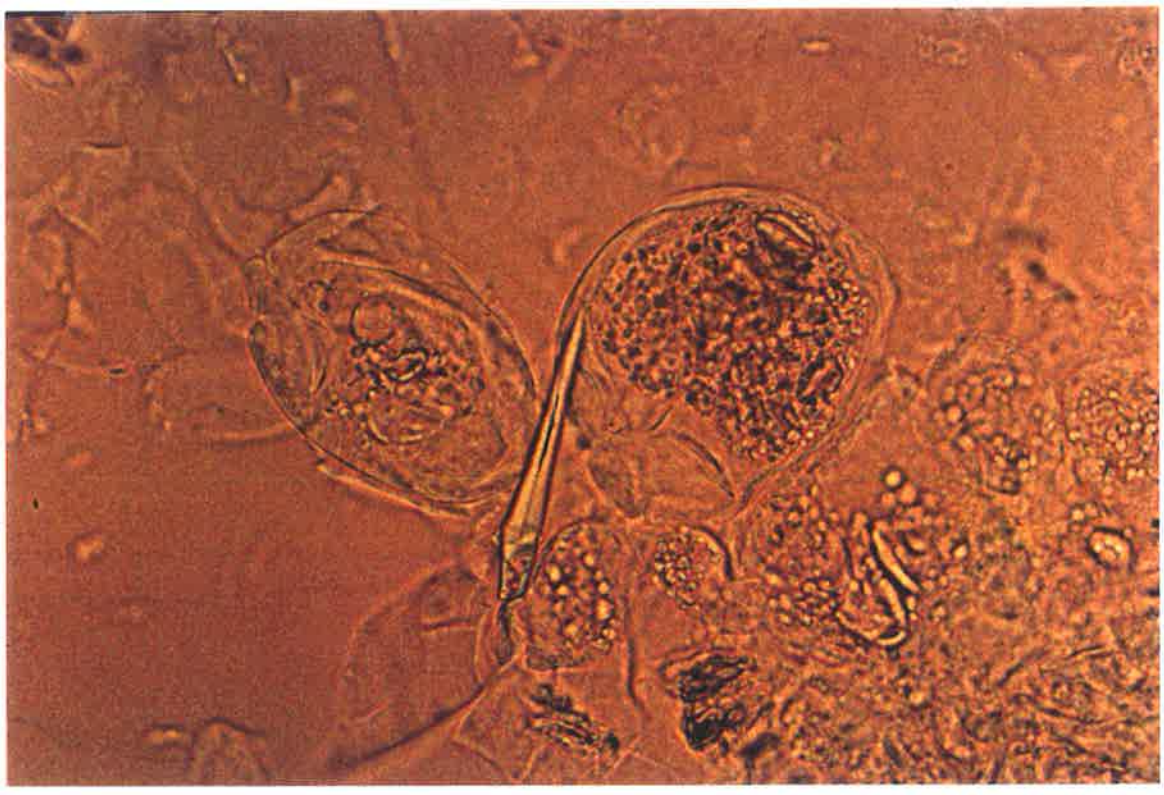
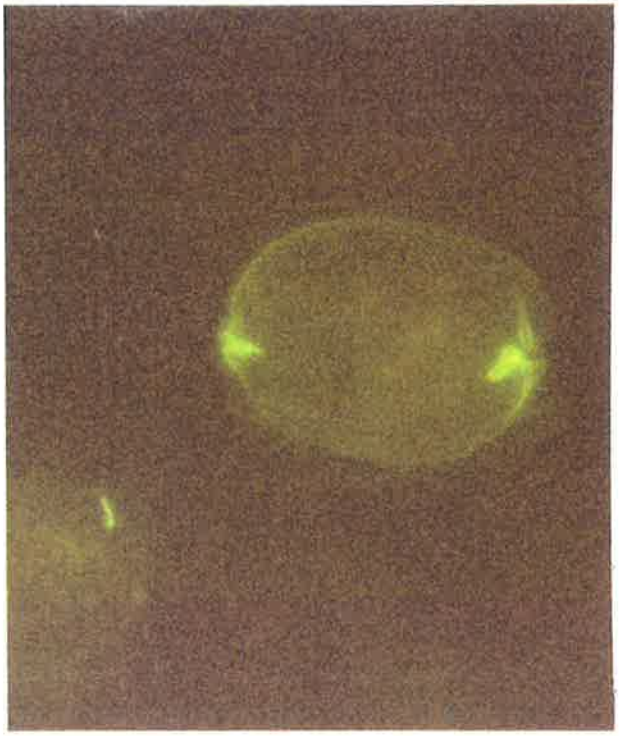


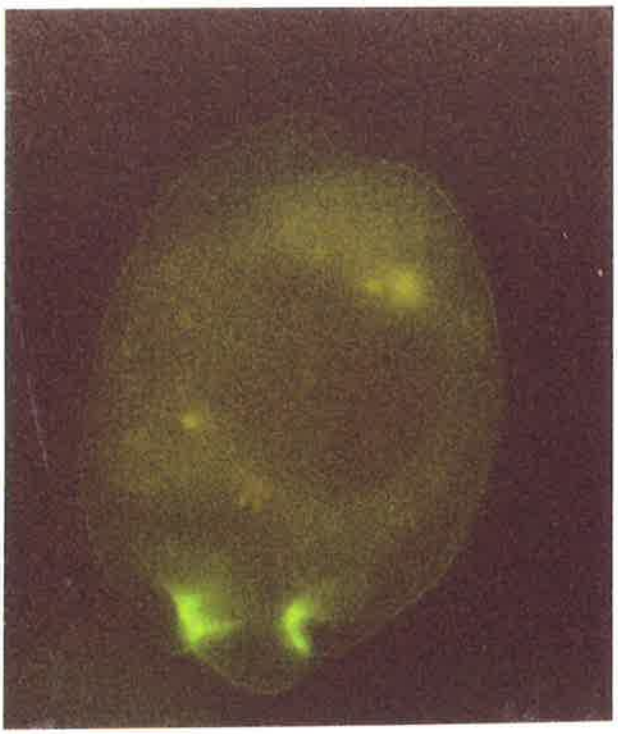
Plate 5.3: Type of immunofluorescence observed with preimmune samples.

Panel 1: An Entodinium with green fluorescence around the adoral region (thick arrow) and faint incomplete fluorescence around the periphery (thin arrow).

Panel 2: A smaller entodinium with fluorescence at both posterior and anterior ends.

Panel 3: An entodinium with an incomplete peripheral fluorescence.





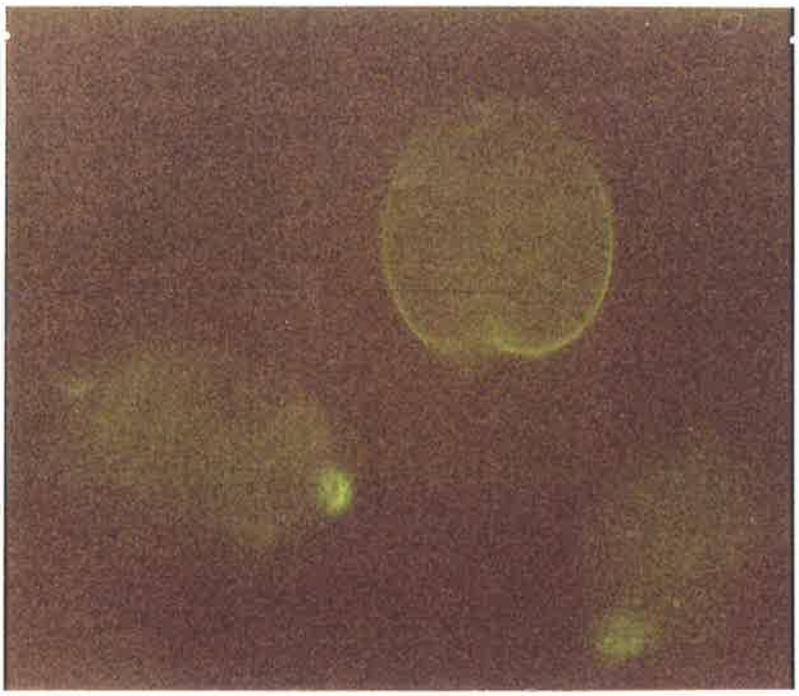


Plate 5.4: Entodinia incubated in negative controls showing a background autofluoresence

Similar studies conducted for the detection of IgG_2 and IgA antibodies in serum and milk and all of three classes (IgG_1, IgG_2 and IgA) in saliva did not produce significant staining (data not shown).

5.3.5.2 Evaluation by flow cytometry

The profile of forward and right angle light scatter by the rumen ciliates is shown in Fig. 5.4 (panel 1). The organisms exhibited a wide range of forward light scatter, consistent with the differences in size between the smallest entodiniomorphs and the largest species of entodiniomorphs (Polyplastrons) and the largest holotrichs. Fluorescence data was collected from the events occurring within the gate indicated in panel 1.

When cells were incubated only with an irrelevant mouse monoclonal antibody followed by fluorescein-conjugated goat anti-mouse immunoglobulin (negative control), the ciliates exhibited a relatively monodisperse peak of autofluorescence (Fig, 5.4, panel 2). Ciliates labelled after primary incubation in either preimmune serum (1:10) or immune serum (1:10) exhibited shifts in fluorescence intensity. Ciliates labelled with immune serum displayed a broad monodisperse peak of fluorescence, with a median peak clearly brighter than that obtained with preimmune serum. Nevertheless, preimmune serum produced some specific fluorescence with a small shoulder of relatively intensely labelled cells, indicating the presence of some natural antibodies against the organisms. Similar results were obtained in 3 separate experiments.

In order to compare the mean fluorescence intensity between cells incubated in preimmune and immune samples, the log values of fluorescence intensity of each sample dilution were converted to a relative scale (relative fluorescence intensity- refer Consort 30 user's guide for linear/log gain channel conversions). The calculated values are shown in Table 5.10. A significant difference ($P=0.0001$) in relative fluorescence intensity of cells was noticed between preimmune and immune sera respectively. In addition, dilution of preimmune and immune samples also produced significant difference ($P=0.0001$). However, it was noticed that the dilution did not have any significant effect beyond 1:200 dilution. Finally, the values of the ratio of immune to preimmune fluorescence intensity were compared (Table 5.10). At dilutions of 1:100, the ratio reached optimal values and at 1:10 dilution showed a decline suggesting an increase in the background noise. At dilutions higher than 1:200, the values became insignificant. Based on the histogram profiles and relative

fluorescence data analysis, antibody titre was determined as 1:200, which was found comparable to what was observed with the IFA test.

Table 5.10: Comparison of preimmune and immune serum samples at different dilutions

Dilution	Relative Fluorescence Intensity[a] Preimmune (mean±SD)[c]	Immune (mean±SD)[c]	RFI Ratio[b] Immune/Preimmune
1/10	7.35± .005	14.91± .015	2.01
1/100	3.72± .005	8.99± .010	2.42
1/200	3.27± .050	7.12± .080	2.18
1/400	3.00± .100	4.91± .105	1.64
1/800	3.15± .050	4.60± .100	1.46

[a] The binding of IgG antibodies from sera was detected using mouse monoclonal (McM_1) specific for sheep IgG_1 , followed by fluorescein-conjugated sheep anti-mouse IgG

[b] Relative Fluorescence Intensity Ratio

[c] Mean± standard deviation for duplicate experiments

Summary of analysis of variance showing the effects between preimmune and immune samples (treatment), the different levels of dilution and also the interaction between treatment and dilution

Source of Variation	Degree of Freedom	P-values
Treatment	1	0.0001
Dilution	4	0.0001
Interaction	4	0.0001

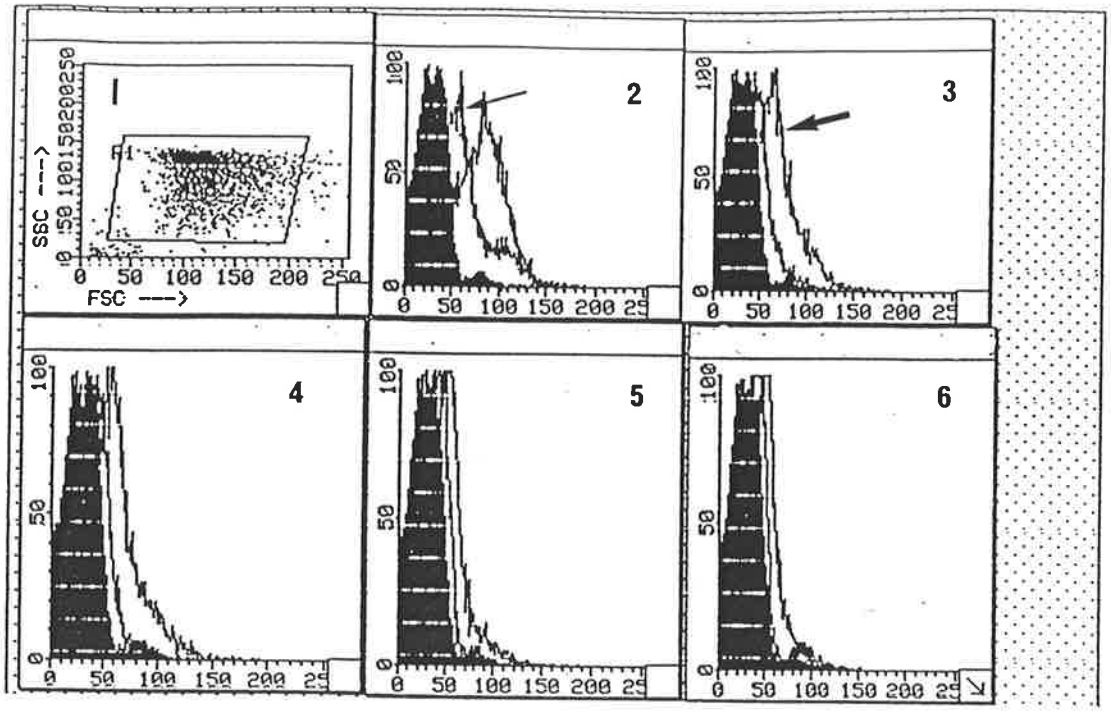


Fig. 5.4: Analysis of mixed rumen ciliates by flow-cytometry.

Panel 1- Forward (horizontal) and rightangle (vertical axis) light scattering by the mixed rumen ciliate population. The population gated electronically from investigation is indicated by the parallelogram.

Panel 2- Fluorescence profiles of mixed rumen ciliates labelled with control antibodies (shaded area) or with preimmune serum (thin arrow) and immune serum (thick arrow). Serum samples diluted 1:10.

Panels 3, 4,5 and 6- same as above but negative control is overlaid against histograms representing 1:100, 1:200, 1:400 and 1:800 dilutions of preimmune and immune sera respectively.

Note: Histograms in panels 2-6 represent frequency distribution of fluorescence in a population of 10,000 cells. The horizontal axis represents the fluoresence and the vertical axis the relative cell number in a maximal number of events in a single channel.

5.4 Discussion

The main aim in this part of the study was to examine whether rumen ciliate antigens can elicit specific antibodies in saliva and milk and to gain insight into the kinetics of the immune response to these antigens. It was by no means clear at the commencement of the work whether early contact with protozoa and presentation of the antigens of these organisms via the oral route would lead to tolerance or to specific humoral immunity. The sheep inoculated with purified mixed rumen ciliates showed rises in the titre of specific antibodies against these organisms in serum, saliva and milk. The pattern of antibody responses showed interesting similarities, and also certain differences. The most remarkable similarity was seen in respect of specific IgG responses. After primary immunization, the geometric mean IgG antibody titres rose steadily in all three of the body fluids examined in this study. The increases in saliva and serum were 5 to 5.5 fold, while the increase in milk was 16 fold (see Figures 5.2, 5.3 and Table 5.7). Booster immunization on day 24 produced a further two-fold rise in the titre in saliva (not significant statistically) and a nine-fold rise in the titres in milk. Similar rises were not observed in the serum. In absolute terms, when titres were expressed as geometric means, the titre in serum was 50 fold higher than in saliva and 3 times higher than in milk (Tables 5.6. and 5.7.).

The IgA antibody response to rumen ciliates followed a different pattern to the response of IgG antibodies. In the saliva, IgA was detected in only three out six animals in the period up to two weeks after primary immunization and the booster dose failed to elicit any additional response. No rise in IgA antibody was detected in the sera of any of the immunized sheep.

The published data concerning the time of appearance, the magnitude and the persistence of IgA antibodies in the serum of ruminants in response to any specific antigen are fragmentary and the response in saliva is virtually not known. The results of one of the studies examining the effect of intraperitoneal immunization in sheep reported that there was no significant increase in IgA antibody levels in serum (Sheldrake et al 1985a). The evidence from studies in humans suggests that an IgA antibody response may fail to occur in persons attaining a successsful response in the other immunoglobulin classes (Newcomb and Ishizaka, 1967), or that the appearance of IgA antibodies may be generally slow (Ogra et al., 1968). The latter authors also reported that inactivated vaccines failed to elicit IgA activity in the nasal and duodenal secretions. This observation has been

reinforced by the repeated reports of the failure of parenterally administered vaccines alone to stimulate mucosal immunity consistently in unprimed animals or humans (Pierce and Gowans, 1975; Svennerholm et al., 1980). In this respect, it appears that sheep also seem to show a similar trend, probably because only parenteral immunization was employed in this study. On the other hand, there are examples in which mucosal immune responses have been induced efficiently by using either systemic (Pierce and Gowans, 1975; Cox and Taubman, 1982) or combined systemic-local vaccination schedules (Husband, 1978; Husband et al., 1979) or local vaccinations (Mestecky et al, 1978; Linzer et al., 1981; Wachsmann et al., 1986). It has also been reported that parenteral vaccination alone could boost local secretory IgA antibody responses in intestinally primed individuals (Svennerholm et al., 1977). A notable exception involving a ruminant is the observation by Husband et al. (1979) that intraperitoneal immunization with a new antigen (OVA) in sheep led to an IgA specific antibody secreting cell response in mesenteric lymph. Based on observations from the foregoing studies, it may be suggested that these contradictory observations may reflect the design of the studies, particularly with respect to the species, antigen-dose response curve, the time course required to evaluate the desired sIgA response and the ability to evoke the IgA antibody responses repeatedly. The results suggest that previous exposure to the antigens and the nature of that antigen can greatly influence the secretory antibody responses and the choice of routes in any immunization protocol. These considerations also constituted a limitation in the present study, as there is a paucity of information on any of these areas in respect of rumen ciliate research.

In the light of foregoing conclusions, it was possible that the increased IgA antibody levels against rumen ciliates in the milk could have been due to natural priming against ciliate antigens and hormonal fluctuations during lactation. Studies in rodents have demonstrated that relocation of gut-derived IgA precursors to the mammary gland is under hormonal influence and only occurs in association with lactation (Weisz-Carrington et al., 1978). However, the control animals in Group I show clearly that this was not the case. Fig. 5.1 demonstrated that antibodies specific to both ovalbumin and rumen ciliates appeared only after immunization with the respective antigens. There was no possibility that the animals had been exposed previously to the control antigen (OVA). The appearance of specific IgA antibodies in milk following intraperitoneal immunization with OVA or rumen ciliates suggests that prior natural

mucosal exposure to antigen was not necessary to prime for a secretory antibody response.

In respect of specific IgM antibodies, it may be mentioned that neither the saliva nor the serum of any immunized sheep showed any detectable titres against rumen ciliates, whereas the milk of these animals after immunization had geometric mean titres as high as 1:70 two weeks after primary immunization (data not shown). However, the titres declined rapidly thereafter. IgM antibodies in ruminant salivary immunity may not be important, as the IgM isotype is secreted in saliva only in trace amounts (Pahud and Mach, 1970; Butler, 1983). The role of IgM antibodies in milk requires further investigation.

The current study, though not concerned directly with the investigation of the origin of specific IgG antibodies in the secretions of sheep, has produced data that support the concept that the presentation of antigen to extramammary lymphoid tissues results in an elevation of IgG (Chang et al., 1981) in both the serum and mammary secretions. The identification of the origin of these antibodies is beyond the scope of the current project, but clearly they could come either by transport from serum or by local production. The presence in milk of IgA antibodies against rumen ciliates could have similar origins.

The proportions of the IgG and IgA antibodies derived from each of these sources is hard to forecast from the currently available literature. There have been limited studies on the origin of antibody-containing cells in the ovine mammary gland following intraperitoneal immunization (Sheldrake and Husband, 1988). It has been concluded that the success of prior intraperitoneal immunization in stimulating an enhanced IgG_1-specific response to local antigen in the mammary gland probably results from recruitment of cells from the systemic lymphoid tissue that is primed by intraperitoneal immunization. In respect of IgA, the contribution of GALT to the local immune events in the mammary gland of sheep has been evaluated (Sheldrake et al., 1985a; 1988). These studies reported that no antigen-specific IgA-antibody containing cells of gut-origin were observed in the mammary gland, suggesting that in sheep the mammary gland does not form part of the IgA-dominated common mucosal immune system as it does in other species (McDermott and Bienenstock, 1979; Weisz-Carrington et al., 1979). On the other hand, this group has shown that the mammary gland can transport polymeric IgA from blood into the milk (Sheldrake et al., 1984). Thus, although there is no evidence of a cellular link between the gut and the mammary gland, there is nevertheless the opportunity for IgA and IgG produced in the gut to reach milk and saliva.

Irrespective of the precise origin of the antibodies detected in milk and saliva in the current study, the data confirm the potential for raising secretory IgG and IgA antibodies against rumen ciliates in sheep. Further studies are required to optimize the immunization protocol to achieve higher and more sustained antibody levels, in particular with respect to saliva. The study has confirmed the immunogenicity of rumen ciliates in sheep and raises the possibility that immunization, in particular to raise specific antibodies in saliva and milk, could be used to control the rumen ciliate population. If suitable preparations are used as vaccines, it might be possible to control specific ciliate populations.

The data presented in section 5.3.4 indicated that some natural antibodies against rumen ciliates could be detected by ELISA in all of the fluids examined in the study. It was not clear whether these titres reflected the presence of natural antibodies to rumen ciliates or the presence of antibodies that were cross-reacting with protozoal antigens. A third possibility was that some of these antibodies could be directed against bacteria that inevitably contaminated the ciliate preparations which were used to coat the ELISA plates (either adherent to the ciliates or ingested by them). Studies were therefore undertaken to address these questions. They demonstrated that (1) preimmune and immune samples of both serum and milk of immunized sheep contained antibodies binding to both mixed rumen bacterial and ciliate antigens, (2) absorption of the sera independently with bacterial and rumen ciliate antigens confirmed that there were antibodies in these samples reactive with both the rumen bacteria and the ciliates. However, these antibodies were found to be specific for their respective antigens. The absorption studies did not provide any information on the subcellular distribution of the antigens. The latter could be either cytoplasmic in origin or have been released from the cell surface. Soluble cytoplasmic proteins are the antigens most likely to bind to the plastic ELISA wells and it is probable that the assay was directed mainly against cytoplasmic antigens. In contrast, the antigens of major interest with respect to possible effects of antibodies on the viable organisms are those associated with the cell surface. Hence it is evident that the ELISA technique might not be a suitable method for measuring the levels of relevant anti-cell surface antibodies in blood or secretions.

In order to address this aspect, a conventional indirect immunofluorescence technique was employed to label viable ciliates. Drawbacks with this technique included the subjective nature of the visual assessment of antibody binding to the whole cells. Flow cytometry was therefore employed to evaluate quantitatively the interaction between

surface structures of rumen ciliates and the corresponding antibodies. Flow cytometric analysis offers a rapid estimate of the proportion of cells that bind antibody and an indication of the relative amount of antibody bound. In this technique, cells are dispersed in fluid suspension and flow in a narrow stream through a laser beam which excites fluorescence from labelled cells. Each cell generates optical signals that are measured and analysed. Cytometric system measures rapidly and accurately a large number of cells thus enabling statistical precision in cell counting and analysis of the distribution of biological properties among the different types of cells.

In view of the above features of flow cytometry, it was decided to use the technique to measure IgG and IgA antibody levels against ciliate surface antigens in preimmune and immune sera. This allowed the objective examination of 10,000 cells in a short time period and it also allowed measurement of the relative fluorescence intensity (proportional to the amount of bound antibody) of individual ciliates. The results complimented those obtained from the IFAT technique and it is interesting that the anti-ciliate antibody titres estimated by the two methods were comparable. It is clear from the results (Fig. 5.4) that pre-immune and immune sera both contained IgG (IgG_1) antibodies against rumen ciliates. However, the mean fluorescence intensity was greater with immune sera and titrations indicated that immunization increased the antibodies in the sera at least 200 fold.

In contrast to the labelling obtained with immune serum, rumen ciliate cells were not labelled by immune saliva. This result also contrasts with the detection of anti-ciliate antibodies in saliva by ELISA. It is possible that the method of storage of saliva might have affected the results of the study. Specimens of whole saliva were stored at - 80°C for almost one year before the immunofluorescence tests were done. It is likely that failure to incorporate glycerol as a preservative might have caused loss of activity of salivary antibodies. It has been reported that saliva specimens can be stored at - 80°C after the addition of 50 % glycerol which prevents any loss of antibody activity (Butler et al., 1990). However, further work using fresh saliva specimens is necessary to confirm that immunization induces salivary antibodies reactive with the ciliate cell surface.

Neither IFAT nor flow cytometry revealed IgA binding to the surface structures of ciliates. It has been reported that IFA detected activity almost exclusively in the IgG fractions (Duffus and Wagner, 1974; Wagner et al., 1974) of serum. It is not certain why the IFA technique has a bias towards detection of IgG activity. It appears that competition of different classes of

antibodies in the samples could be one of the major explanations. In the present study, serum IgA was not detected even by ELISA, but milk demonstrated significant levels of IgA antibodies. However, in proportion the total concentration of each isotype, the specific activity of IgA antibody was found to be almost 100 times less than that of IgG_1 antibody. In microtitre immunoassays, it has been shown that with hyperimmune cattle sera (which contains large amounts of IgG antibody) there is always an underestimation of the antibody activity of IgA (Butler et al., 1980a).

Similar comments may be made about the inconsistent detection of binding of IgG_2 antibodies to ciliates. It is not clear again whether the competitive inhibition among different classes of antibodies explains this inconsistency.

In conclusion, it may be stated that an indirect immunofluorescence test and flow cytometry confirmed the presence of surface active antibodies to rumen ciliates in the immune samples of serum and milk respectively. The antibody was mainly of the IgG isotype. It may be necessary to deplete IgG levels in these fluids in order to get an accurate estimate of IgA antibody activity in any of these fluids. In order to demonstrate the potential of these antibodies in the control of live ciliates, another investigation was undertaken to examine their structural fate and biological activity in the rumen fluid. The study of these areas warranted purification of immunoglobulins, in particular the sheep IgA, from external secretions of sheep. A new method of purification of this immunoglobulin from bronchio-alveolar washings (lung secretions) is described in the following section.

Section 6.0

Isolation and Purification of Sheep IgA from Bronchio-alveolar Washings and Colostrum.

6.1 Introduction

An important aim of this project was to examine the action of secretory antibodies on rumen ciliates. The stability of immunoglobulins and antibody activity in the rumen environment is therefore important. In this section, sheep IgA was purified in preparation for studies on the proteolytic effects of rumen fluid. In proteolytic studies, the purity of immunoglobulins merits high priority in order to measure specifically the proteolysis of immunoglobulins and to characterize the products of degradation. Many workers have described methods for preparing immunoglobulins of domestic ruminants from both serum and exocrine secretions (Aalund et al., 1965; Pahud and Mach, 1970; Mach and Pahud, 1971; Butler and Maxwell, 1972; Tewari and Mukkur, 1975 and Ungar-Waron et al., 1978). Most of these studies were directed mainly towards either purification of IgG or IgM. There have been also studies relating to the purification of ruminant IgA. It has been reported that lacteal secretions are not ideal as starting material for the preparation of ruminant polymeric IgA because this protein has size and ionic characteristics which overlap with dimeric IgG1 (Butler et al., 1980b). These workers attempted to separate IgA from IgG1 in fractions obtained from gel-filtration / DEAE cellulose chromatography by the use of affinity chromatography on protein A-sepharose columns. Fractions containing up to 85% IgA were obtained, but the remaining 15% of the protein was made up of IgG1 dimers. The same authors pointed out difficulties associated with purification of SIgA from external secretions of the intestinal, nasal and salivary mucosae. However, they were able to prepare purified SIgA by a simple method from the bronchio-alveolar washings of cattle, which do not contain significant amounts of IgG dimers. When these preparations were analysed by enzyme-linked immunosorbent assay, contamination with IgG monomers was limited to a maximum of 2-5%. Some other workers (Chin et al.,1986) have used this method, with slight modifications, for the preparation of ovine IgA with which to raise monoclonal antibodies. It is not clear whether the ovine IgA prepared in this manner had any contaminants of IgG.

In order to prepare highly purified sheep IgA, the conventional methods of ion-exchange and gel-filtration chromatography were chosen and the starting materials were the IgA-rich fractions of colostrum and lung washings.

Fast Protein Liquid Chromatography system (FPLC) is well known for its very high resolving power and it has been used in many rapid purification procedures to obtain a range of biomolecules including immunoglobulins (Stemmer and Loos, 1984; Sampson et al., 1984; Juronen et al., 1991). The FPLC system was employed to improve the purity of samples following gel-filtration chromatography.

Further purification steps were needed to achieve the required level of purity. In particular, it was necessary to remove IgG contaminants after the final FPLC step. Protein G, a type III bacterial IgG Fc receptor isolated from certain group C or G Streptococci, has been shown to possess higher binding affinity towards immunoglobulin G of ruminant species than Staphylococcal protein A (Akerstrom and Bjorck,1986). During the last decade certain immunochemical applications using protein G have been introduced (Akerstrom et al., 1985; Nilson et al., 1986;). There appears to be no published literature on the application of Protein G-affinity columns as an adjunct in the purification of IgA from ruminant body fluids. The use of a Protein-G affinity column in the final scheme resulted in good purity of sheep SIgA.

6.2 Materials and Methods

6.2.1 Preparation of sheep colostrum

The colostrum was obtained manually from two sheep at the first milking after delivery. Both samples were pooled and diluted with an equal volume of sterile saline. The mixed diluted sample was centrifuged for 15 minutes at 10,000g to remove both the cells and the colloidal fat. Casein was precipitated by acidification to pH 4.6 with 1N HCL and the supernatant was stored at -20°C after neutralization.

6.2.2 Preparation of bronchio-alveolar washings (BAW)

Lungs were obtained from freshly slaughtered sheep, packed in ice and transported to the laboratory. They were lavaged on arrival with 500ml aliquots of ice-cold sterile saline (pH 7.2) and the lavaged fluids were centrifuged at 10,000g for 30 minutes. Debris was discarded and the supernatant was concentrated to 1/25th of its original volume in an Amicon cell equipped with a PM 30 membrane. The supernatant was

precipitated with ammonium sulphate to 40% (W/V). The precipitate was redissolved in a buffer containing 25mM Tris-HCL, 132mM NaCL, 8.3mM NaN3 and 0.1mM EDTA (TESA) and dialysed against the same buffer to remove ammonium sulphate.

6.2.3 *Isolation of immunoglobulins*

6.2.3.1 *DEAE-Sephacel Ion-Exchange Gel-filtration Chromatography on Colostrum*

Frozen colostral supernatant was thawed and proteins were diluted in 25mM isotonic phosphate buffer (pH 7.4). The solution was then applied to a DEAE- Sephacel ion-exchange column (75ml) which had been equilibrated previously with the same buffer. The column was eluted successively with 25mM isotonic buffer, and then with the same buffer containing a NaCl concentration of (1) 50mM (100ml), (2) 100mM (100ml) and (4) 125mM (100ml), pH 7.4. All of the elutions were performed at 4°C and 4ml fractions were collected.

6.2.3.2 *Gel chromatography on lung washings*

The processed bronchio-alveolar lavage fluid was chromatographed on S300 columns equilibrated with TESA buffer in the cold room at 4°C. Protein loads of 20mg were applied per column (2.5cm x 90cm) per run. The eluate rate was maintained at 20ml hr -1.

6.2.3.3 *Fast Protein Liquid Chromatography*

The FPLC system consisted of a G250 gradient programmer, two P-500 dual piston pumps, a V-7 injection valve, a solvent mixer, a sample loop of 10 ml, a UV-1 monitor, a Rec-482 recorder, a Frac 100 fraction collector and a polyanion SI/17 ion-exchange column (Pharmacia Fine Chemicals-AB, Sweden). For elution, two buffers were used to generate a linear gradient (1) Buffer A contained 0.02MTris-Hcl, pH 8.0 (2) Buffer B contained 0.05M Tris-HCL with 0.5M NaCL. Prior to chromatography, pooled IgA rich fraction of lung-washings from the S300 gel-filtration was dialysed overnight against buffer A and filtered through a 0.2µm millipore filter. The sample was then applied to the Polyanion SI-17 column at a flow-rate of 1ml/min. The column was eluted successively with a 0 to 100% salt-gradient. Peak fractions were pooled and analysed by SDS-PAGE/ Western blot.

6.2.3.4 Protein G- Sepharose 4 Fast Flow affinity chromatography

A column containing Protein G- Sepharose 4FF (a recombinant form; Pharmacia LKB Biotechnology, Sweden) was used in this study. The affinity column was made as per manufacturer's instructions and equilibrated with the phosphate buffered saline, pH 8.25. After column equilibration, either FPLC samples of lung washings or S300 column-samples of colostrum were applied to the affinity column and washed with the same buffer to remove non- adherent immunoglobulins. The 'fall through' peak was invariably found to be about 5-10 ml, depending on the quantity of protein in the sample.The bound immunoglobulin G was eluted with 3M NaSCN, which was subsequently removed by dialysis against phosphate buffered saline, pH. 7.3. The respective eluted fractions were millipore-filtered in a lamina-flow hood and stored sterile at -80°C for future use.

6.2.4 Antisera

Monospecific rabbit anti-ovine IgG1, IgG2, IgA and IgM were obtained from Dr.A.J. Husband (Department of Pathology,University of Sydney, NSW). Mouse monoclonal antibody (F34B4) raised against ovine IgA was obtained from Dr. J.C.Chin (Central Veterinary Laboratory, Department of Agriculture, Sydney 2167). Rabbit anti-bovine serum albumin, alkaline phosphatase conjugated goat-anti rabbit and rabbit anti-mouse whole immunoglobulins were prepared by Dr.P. Ey (Department of Microbiology and Immunology, University of Adelaide, South Australia).

6.2.5 Double-radial Immunodiffusion in Agarose gel

Double radial immunodiffusion was performed on microscope slides (7.5 x 5.0 cm) precoated with 0.2% agarose. The gel was prepared by dissolving 1.0% agarose in phosphate buffered saline, pH 7.3, and poured directly onto slides and cooled. Wells were punched and aspirated in seven-hole patterns using a plexiglass template. Fifteen microlitre aliquots of antisera (rabbit-anti-sheep) monospecific for heavy chains of the respective immunoglobulin classes were applied to the centre wells and test samples were added to the peripheral wells. The slides were incubated in a moist chamber for 48 hours at room temperature, washed for 30 minutes in physiological saline and then for an additional 20 minutes in distilled water. The gels were then fixed, dried, and stained with a dye-mixture of amido black and Coomassie blue.

6.2.6 *Dot- blot immunoassay on nitrocellulose*

The relevant samples were dotted onto a nitrocellulose paper. The paper was dried thoroughly and blocked with 1% skim milk powder in 0.05% Tween saline for 30 minutes, washed twice with Tween saline and incubated with the corresponding class specific antisera. Following primary incubation, the nitrocellulose paper was washed again 3 times with Tween saline and reincubated with the second antibody which was either alkaline-phosphatase conjugated goat anti-rabbit or rabbit anti-mouse whole immunoglobulin, depending on the primary antisera used.

At the end of second incubation, the nitrocellulose paper was further washed 4 times in Tween saline containing 1mM $MgCl_2$ and 1μM $ZnCl_2$, 3-5 mins per wash and a final wash in 100ml saline for 2 mins. Bound conjugate was detected using a solution containing nitroblue-tetrazolium, phenazine-methosulphate and 5-bromo-4- chloro-3-indolylphosphate, which was prepared immediately before use. The positive reaction was detected as a coloured dot against the white nitrocellulose paper background.

6.2.7 *Electrophoresis and Western Blotting.*

In order to determine the purity of isolated immunoglobulins and other proteins, sodium dodecyl sulphate / polyacrylamide gel electrophoresis (SDS/PAGE) was performed under both non-reducing and reducing conditions, using the buffer systems of Laemmli (1970). Immunoglobulins from the respective chromatographed fractions were subjected to SDS/PAGE in 6%, 8%, and 12% acrylamide/BIS gels respectively. Molecular weight markers were used as reference, wherever necessary. Following electrophoresis, gels were either stained with 0.06% Coomassie blue G-250 in 3.5% perchloric acid ($HClO_4$) or electroblotted onto nitrocellulose in a transfer buffer (0.025M Tris/Hcl, 0.192M glycine pH 8.3)/ 20% methanol) at 40mA overnight at ambient temperature. The nitrocellulose sheets were then blocked in 100 ml of 1% skim milk powder made in 0.05% Tween-saline. The transferred proteins were probed subsequently by incubating with the relevant primary antisera and appropriate alkaline phosphatase conjugated secondary antibodies.

6.2.8 *ELISA based immunoglobulin distribution assay (EBDA).*

An indirect ELISA was developed to assay the distribution of different classes of immunoglobulins in partially-purified colostrum and bronchio-alveolar lavage fluid respectively. Flexible 96-well microtitre plates were coated with the test samples of a fixed dilution [diluted with a buffer containing 0.5M Tris-HCL, pH 7.5; 0.15M NaCl; 0.008M NaN3 (TSA)] and were left overnight at 4°C. The trays were then blocked with 0.05% Tween 20-saline/0.01% bovine serum albumin (TS/BSA) and incubated with the serial twofold dilutions of relevant antisera specific for corresponding classes (or subclasses) of sheep immunoglobulins (IgA, IgG_1, IgG_2 and IgM) for 1 hour at 37°C.

At the end of 1st incubation, the trays were washed with TS/BSA and further incubated with goat anti-rabbit Ig conjugate, (diluted 1/1000 with an enzyme diluent) for 1hour at 37°C. Finally at the end of the second incubation, plates were washed again with Tween-saline and to each well was added substrate (nitrophenyl phosphate) in diethanolamine buffer. Colour development was measured with a Titertek Multiskan (Flow Laboratories). Wells sensitized with either colostral IgA, IgG, IgM or TSA were included as positive and negative controls and to check specificity. The absorbance values two standard deviation greater than the mean of the negative control was taken as the threshold for positivity in test samples. The end point titre of a sample was taken as the last dilution giving an absorbance either equal to or greater than this threshold value.

6.3 Results

On DEAE-Sephacryl ion-exchange column with stepwise fractionation, the colostral immunoglobulins were eluted with 0.05M, 0.1M and 0.125M phosphate buffer, pH 7.3 respectively. The distribution of immunoglobulins in the respective fractions were detected by double-immunodiffusion technique (data not shown) and the IgA component was found to be eluted with 0.1M phosphate buffer. These fractions were passed through a Sephacryl S300 column and the gel filtration profile is shown in Fig. 6.1.

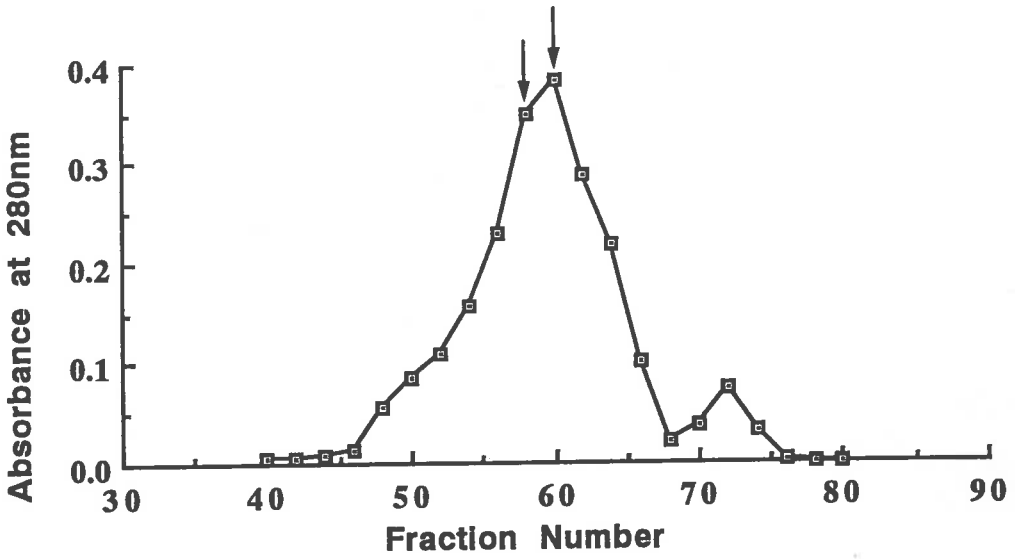


Fig.6.1. Gel filtration of colostral proteins. The eluents containing IgA from the DEAE-Sephacryl ion-exchange column were passed through a Sephacryl S300 column. The IgA-rich fractions in the gel-filtration profile are indicated by arrows.

The fractions 59 and 60 corresponding to major peak were inferred as IgA and the shoulder in the descending limb of the elution profile was identified as possible dimers of IgG. The fractions 59 and 60 were pooled and assayed by ELISA based distribution assay (EBDA). The results are shown in Table 6.1.

Table 6.1 Enzyme-linked immunosorbent assay on fractions 59 & 60

Rabbit antiserum (1/200 starting dilution) diluted serially two-fold	Coating antigen (Fractions 59 & 60 pooled) fixed dilution (1/60)
	Reciprocal titres
Anti-IgA	17,750
Anti-IgG_1	5,500
Anti-IgG_2	1,760
Anti-IgM	8,150

It was observed that pooled fractions of 59 and 60, although contained very high titres of IgA, were found to be still contaminated by proteins immunoreactive with anti-sera of other classes of ovine immunoglobulins, in particular, IgG_1 , IgG_2 and IgM. These fractions were used subsequently in Protein G chromatography to separate colostral IgA from contaminating immunoglobulins.

The elution profile from the Sephacryl S300 column of proteins precipitated from bronchio-alveolar lavage fluid by ammonium sulphate is shown in Fig. 6.2.

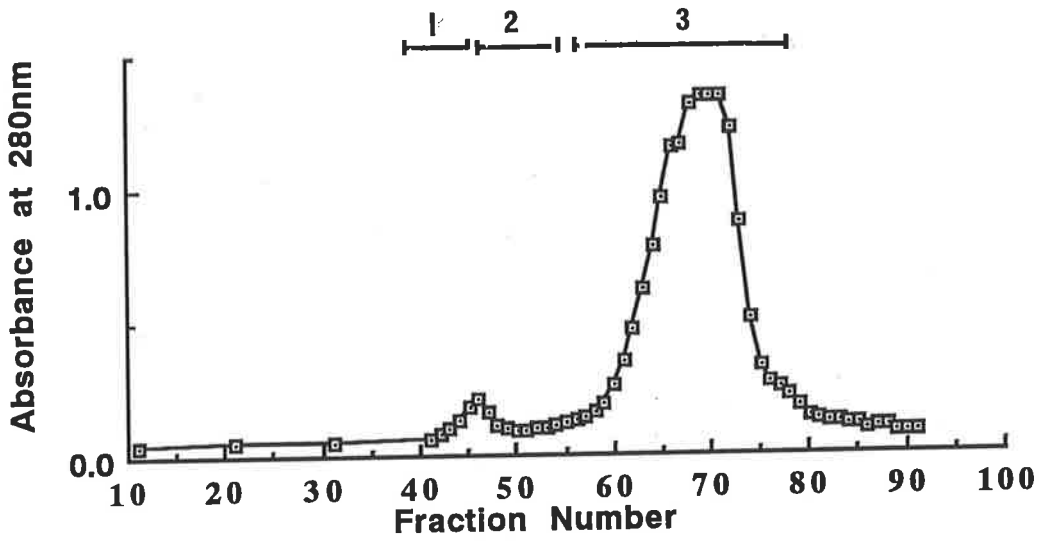


Fig.6.2: Elution Profile from the Sephacryl S300 column of proteins precipitated from bronchio-alveolar lavage fluid by ammonium sulphate. Pooled fractions for further analysis are indicated.

The fractions 40-48, 49-55 and 56-85 were pooled separately (1, 2 and 3 respectively). Pools were analysed by double radial immunodiffusion, dot-blot, EBDA and SDS-PAGE analytical techniques. Ouchterlony analysis showed the presence of IgA in each of the pools. IgM was not detected in any of the pools (Plate 6.1a). The dot immunoassay confirmed the presence of IgA in all of the pools, but also suggested the possible presence of IgM in pools 1 and 2 respectively (Plate 6.1b). Samples of pools 1 and 2 were mixed and assayed by EBDA. The results are shown in Table 6.2.

Table 6.2 Enzyme-linked immunosorbent assay on mixed samples of pools 1 and 2 from bronchio-alveolar washings (BAW).

Rabbit antiserum	Coating antigen (Pools 1 and 2) 1/20 fixed dilution
1/100 starting dilution	Reciprocal Titres
Anti-IgA	12,000
Anti-IgG1	600
Anti-IgG2	200
Anti-IgM	-----

It was noticed that mixed samples of pool 1 and 2 contained high titres of IgA with very low tires of subclasses of IgG. However, no IgM was detected by this assay.

The samples were further analysed by SDS-PAGE, which showed traces of albumin in pools 1 and 2 respectively (Plate. 6.2a). An immunoblot confirmed the presence of albumin and also indicated the presence of traces of IgG in the IgA-enriched fractions from the S300 gel-filtration columns (Plate. 6.2b).

In order to separate IgA from IgG and albumin, the pools 1 and 2 of the bronchio-alveolar fractions were further fractionated using the FPLC system. The purity of the samples after FPLC were analysed by both SDS-PAGE (Plate. 6.3a) and Dot-blot analyses (Plate. 6.3b). A clear separation of albumin was observed in FPLC eluents. However, traces of IgG remained in the albumin-free fractions. Also it may be observed in Plate 6.3a that lanes 2 and 3 corresponding to sheep colostral IgA-rich fractions from S300 gel filtration showed only contaminants of IgG but essentially no albumin. The albumin free samples of bronchio-alveolar lavage

Plate 6.1a: Double-immunodiffusion analysis examining the purity of IgA in lung washings. (a) a complete precipitin line showing the presence of IgA in all of the pooled samples when reacted against rabbit anti-sheep IgA (1/4 dilution) monospecific for ∞-chain. The precipitin line for wells 3, 4 (pool 1) and 5, 6 (pool 2) near the centre well fused with the lines of IgA, suggesting the presence of secretory component (SC) in these samples (b) Double-immunodiffusion showing the absence of precipitin lines for any of the pools when reacted against rabbit anti-sheep IgM (1/5 dilution) monospecific for μ chain. In both cases, the respective pools (Fig. 6.2) were concentrated to 0.5 mg/ml and used neat in duplicate wells- pool 1 in wells 3 & 4, pool 2 in 5 & 6 and pool 3 in 1&2 accordingly.

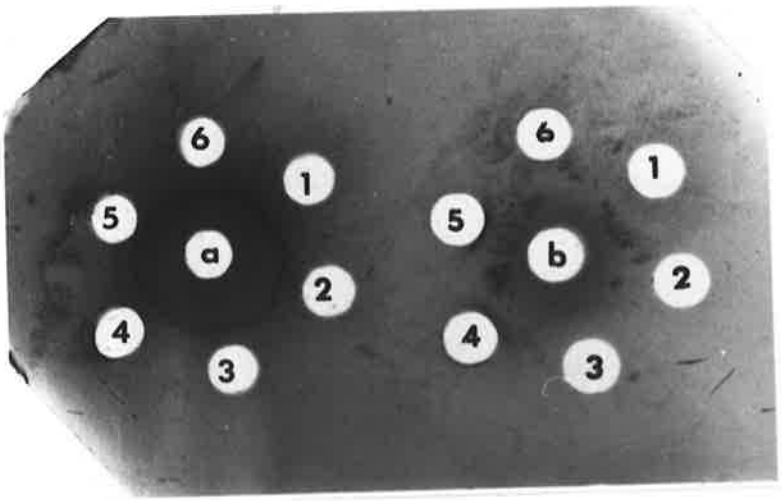
Plate 6.1b: A dot immunoassay for the detection of different classes of immunoglobulins in S300 column chromatography fractions of lung washings. A serial dilution assay of the respective pools (1, 2 and 3) have been shown. The method was as described in the text, with the respective samples of each pool dotted onto the nitrocellulose paper starting with the neat samples serially diluted two-fold up to 1/256 dilution. Rabbit anti-sheep IgA (1/2000 dilution), rabbit anti-sheep IgM (1/2500), mouse anti-sheep whole immunoglobulins (1/4000) were used as primary antibodies to detect the respective classes of immunoglobulins. The antigen-antibody complex was visualized with goat anti-rabbit and rat anti-mouse whole immunoglobulins (1/600 dilution).

Panel A- Row 1, positive (+ve) control (normal colostrum-casein-free); Rows 2, 3 and 4 showed the relative presence of IgA in pools 1. 2 and 3 and Row 5, rabbit IgG as system control. Anti-serum used was rabbit anti-sheep IgA

Panel B- Rows 1 and 4, (+ve) control (normal colostrum-casein-free); Rows 2 and 3, Pools 1 and 2 showed the presence of IgM. Anti-serum was rabbit anti-sheep IgM.

Panel C- Row 1, (+ve) control (normal colostrum-casein-free); Rows 2, 3 and 4 showing the relative presence of three immunoglobulin classes in pools 1.2 and 3 respectively. Anti-serum was mouse anti-sheep whole immunoglobulin (unlabelled). This anti-serum would have detected all Ig classes. The results showed that pool 3 contained immunoglobulins, which was inferred to be IgG on the basis of its elution volume corresponding to the major symmetrical peak which followed the smaller peaks in gel-filtration elution profile (Fig 6.2).

6:1a



6.1b

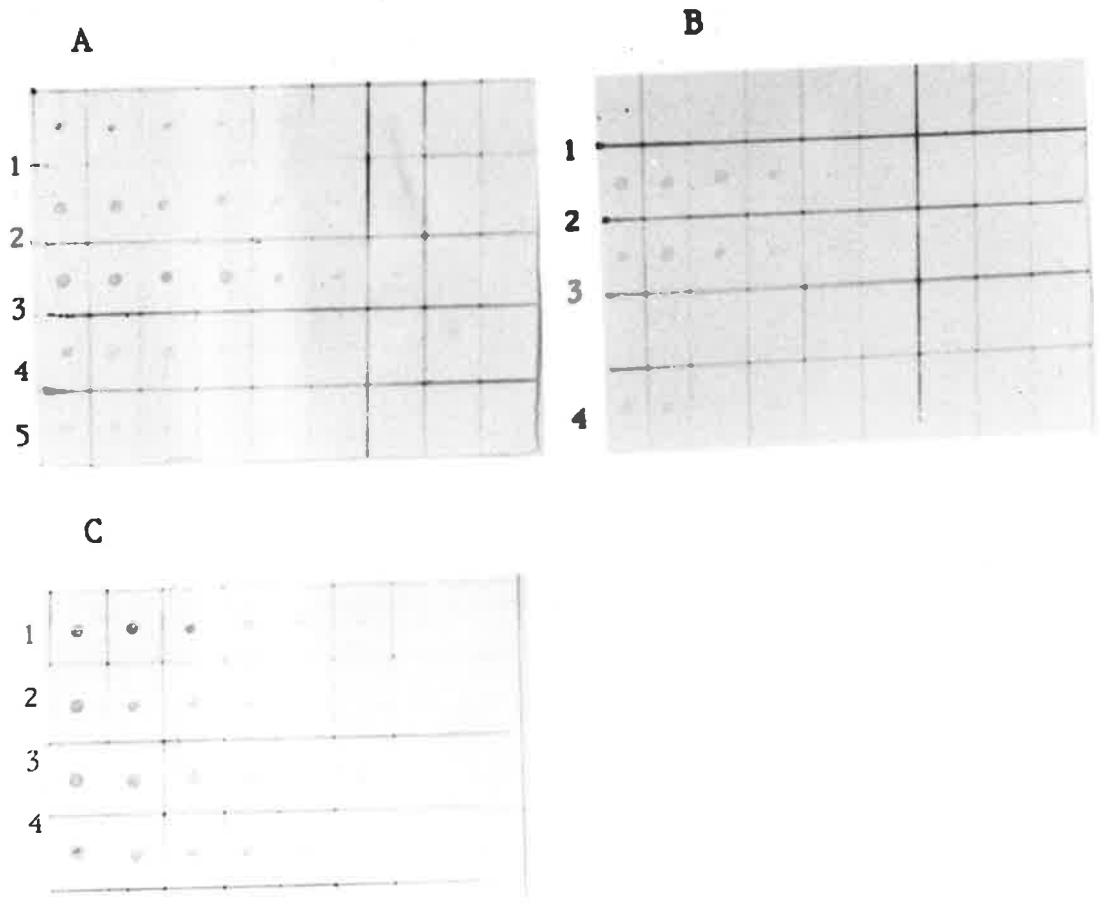
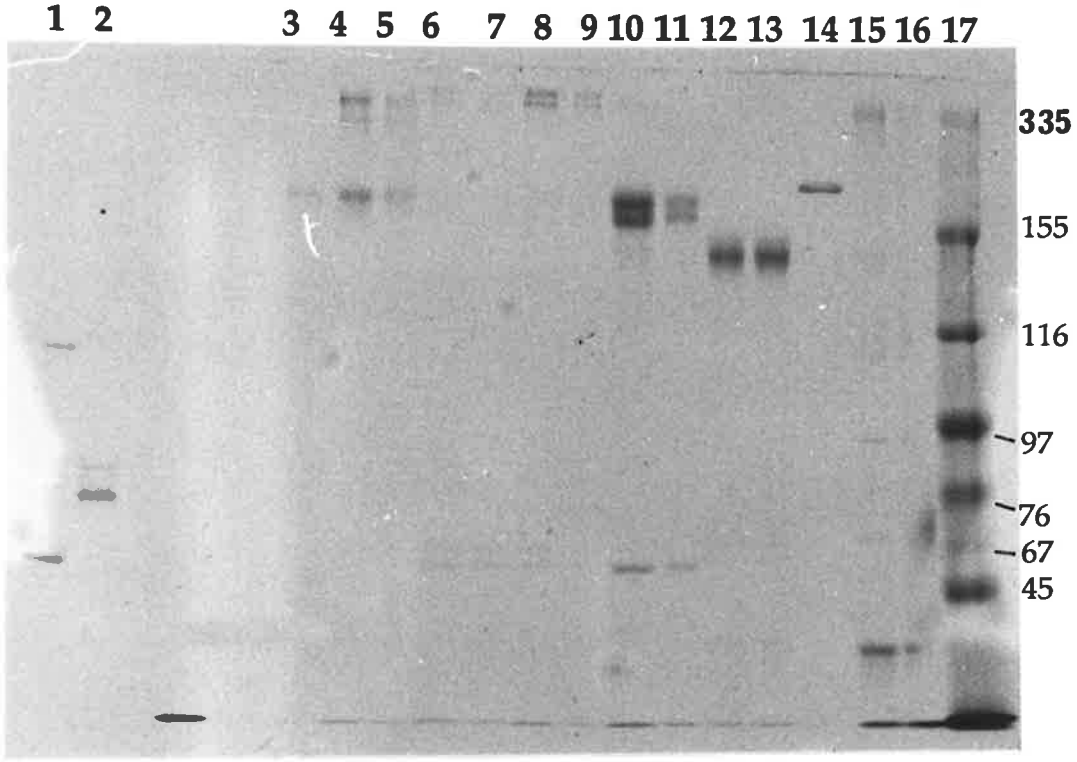


Plate 6.2a: SDS-PAGE on 8% polyacrylmide gels under non-reduced conditions. Resolved protein bands were stained with Coomassie blue. Lane 1, Bovine serum albumin showing monomers and dimers-molecular marker; Lane 2, human transferrin- marker; Lane 3, sheep IgG-marker; Lanes 4&5, partially purified colostrum of sheep containing IgA, IgG monomers and dimers; Lanes 6&7, partially purified bronchio-alveolar samples of pool 1; Lanes 8&9, duplicate samples of pool 2 both showing the presence of IgA with traces of both IgG and albumin; Lanes 10&11, samples of pool 3 showing the predominance of IgG and albumin in the later fractions (Fig. 6.2); Lanes 12&13, rabbit IgG-markers; Lane 14, rat IgG-marker; Lane 15, partially purified rat polymeric IgA; Lane 17, Molecular mass markers- Markers used were thyroglobulin (reduced) (335 kDa), mouse IgG_1 (155 KDa), phosphorylase B (97 kDa), bovine serum albumin (67 kDa) and ovalbumin (45 kDa). Also β-galactosidase (116 kDa) and human transferrin (76kDa)

Plate 6.2b: An immunoblot for further demonstration of the contaminants albumin and IgG in IgA enriched fractions from S300 columns: Left panel: Lanes 1&2, Bovine serum albumin probed with rabbit anti-bovine serum albumin (see arrow head); Lane 3, shows the absence of albumin in sheep colostral IgA; Lanes 4 and 5 IgA rich pools 1 and 2 of S300 column eluents (lung washings) respectively with bands indicating the presence of albumin, when probed with rabbit anti-bovine serum albumin; Lanes 6 and 7 (latter divided lengthwise in two-halves-see left half), pool 3 (lung washings) with albumin; Lanes 7 (right half), 8,9 and 10 showing the relative presence of IgA in pools 3, 3, 1 and 2 (lung washings) respectively when probed with rabbit anti-sheep antibody monospecific for ∝ chain; Lower bands in 8,9 and 10 suggesting the presence of secretory component (see thin arrow); Lane 11, sheep colostral IgA visualized with the same anti-serum (rabbit anti-sheep IgA); Lane 12, rabbit IgG as system control; Right panel is the same immunoblot as that in the left panel, re-incubated with rabbit anti-sheep IgG to show the contaminating IgG (thick arrow); Lanes 3,4,5,6,7,8,9,10 correspond to pools 1,2,3,3,3,1,2 of S300 column eluents (lung washings) respectively; Lane 11 corresponds to sheep colostrum.

6·2a


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
335
155
116
97
76
67
45

6·2b

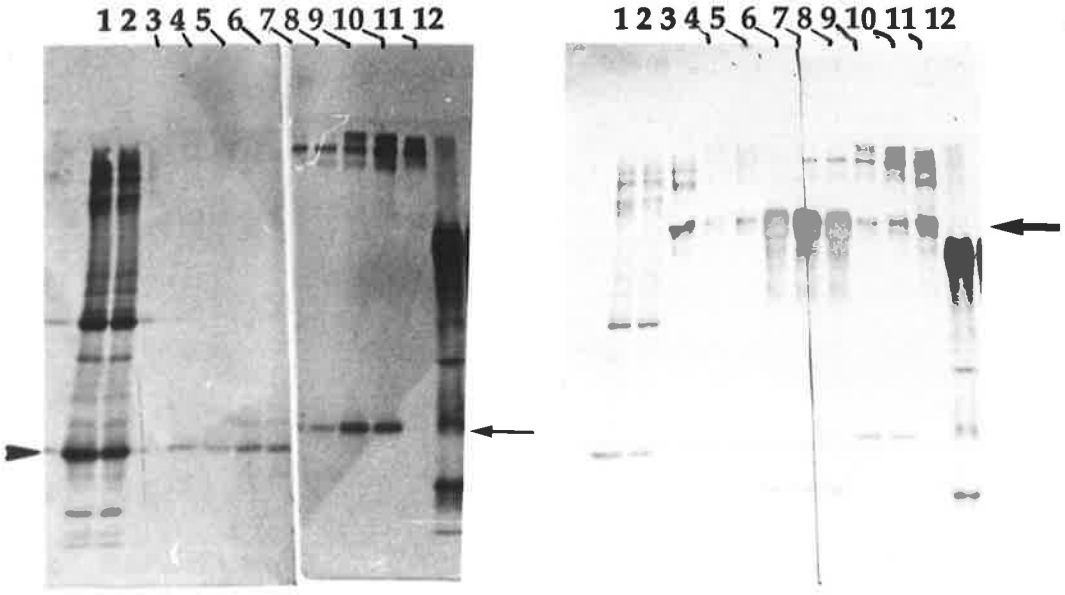
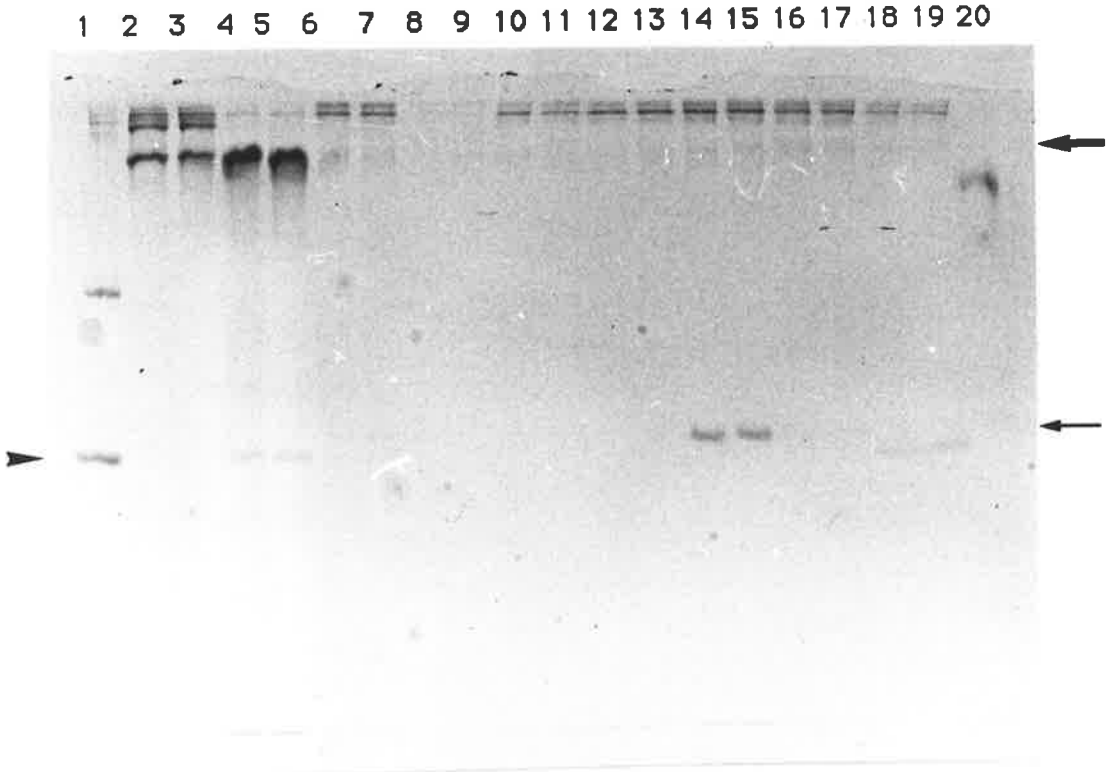

1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11 12

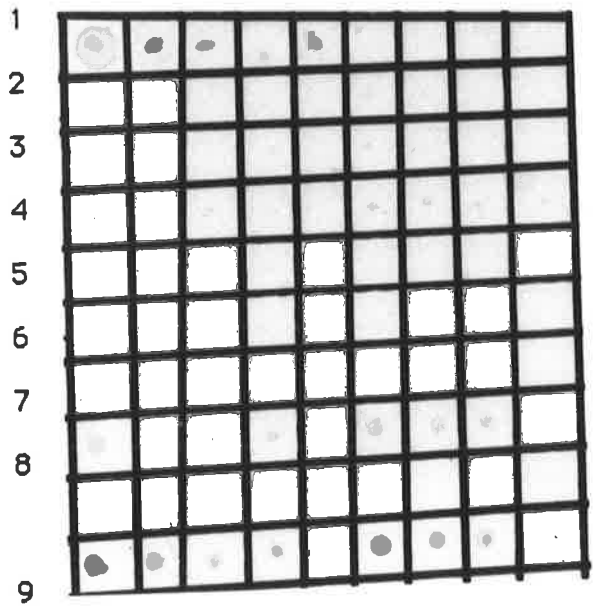
Plate 6.3a: SDS-PAGE (8% polyacrylamide gel under non-reducing conditions stained with Coomassie blue) on lung washings (S300-column and FPLC eluents) and sheep colostrum (S300-column fractions): Lane 1, bovine serum albumin monomers (see arrow head) and dimers; Lanes 2 & 3, sheep colostral IgA with IgG in S300 eluents (thick arrow); Lanes 4 & 5, IgG rich fractions (pool 3) of S300 column (lung washings) with traces of IgA and albumin; Lane 6, IgA rich fractions (pool 1) of S300 column with traces of IgG monomers and albumin (lung washings); Lane 7, IgA rich fractions (pool 2) of S300 column with traces of IgG and albumin (lung washings); Lanes 8 & 9, FPLC unbound-fractions free of any proteins (S300 column-lung washings); Lanes 10,11,12 & 13, FPLC eluents of pools 1 & 2- IgA with traces of IgG devoid of albumin (lung washings); Lanes 14, 15,16 & 17, FPLC eluents of pools 1 & 2, -IgA with traces of IgG and secretory component SC (thin arrow); Lanes 18 & 19, Lung washings (pools 1 & 2) prior to FPLC-, IgA with traces of IgG and albumin and Lane 20, purified sheep IgG as marker.

Plate 6.3b: A dot-blot immunobinding assay demonstrating the absence of albumin in lung washings after elution by FPLC: Rows 1 and 9, BSA as positive (+ve) control; Rows 2, 3 & 4, samples prior to FPLC elution in a 2 fold serial dilution up to 1:256 showing the presence of albumin at different levels; Rows 5, 6 & 7, FPLC eluted samples free of albumin; Row 8, rabbit IgG as system control. Albumin was detected using rabbit anti-bovine serum albumin.

6.3a



6.3b



fluid and colostral IgA fractions were used directly in Protein G affinity chromatography. The samples after being loaded into Protein-G affinity columns were washed with phosphate-buffered saline (PBS-pH 8.25). Bound immunoglobulins were eluted with 3M sodium thiocyanate (Na SCN-pH 7.4). The purity of immunoglobulins, both in the ' fall-through ' and the acid eluted-fractions, were analysed by SDS-PAGE (6% polyacrylamide gel) under non-reduced conditions. This was further examined by Western blot (immunoblot), where proteins transferred from the polyacrylamide gel (duplicate) were incubated with either mouse monoclonal specific against sheep IgA or rabbit anti-sheep IgA (heavy chain-specific). Virtually no difference was seen between these reagents, except that the mouse monoclonal antibody did not stain the secretory components (SC), whereas the polyclonal rabbit anti-sheep IgA stained both IgA and SC (see Plate. 6.4). Lanes corresponding to ' fall-through ' samples were found to contain IgA but were completely devoid of any IgG both in lung and colostral samples indicating an effective binding of sheep IgG to Protein-G affinity columns. At the same time, the lanes corresponding to thiocyanate-eluted fractions still showed some IgA, suggesting some weak binding of this isotype to Protein-G Sepharose column.

Plate. 6.5 represents SDS-PAGE analysis of the above samples on a 12% polyacrylamide gel under reducing conditions. The reduced gel confirmed the presence of secretory component. However, it remains to be clarified how much of it was derived from secretory IgA. The results from the non-reduced gel (Plate 6.4) indicate that at least some of the secretory component was free in the secretions.

Plate 6.4: An immunoblot analysis of lung washings and colostrum after Protein G chromatography: Panel: A-Incubation with mouse monoclonal antibody against sheep IgA; Panel: C-Incubation with rabbit anti-sheep IgA, monospecific for ∞ chain; Panels A & C-left to right: Lane 1, FPLC eluted IgA rich samples (lungs); Lane 2, ' fall-through ' samples after Protein G chromatography showing only IgA (Panel A) or IgA plus the presence of secretory component (Panel C-thin arrow); Lane 3, thio-cyanate eluted IgG eluted samples showing some IgA contamination; Lane 4, IgA rich colostrum after S300 filtration; Lane 5, 'fall-through' sample of colostral IgA after protein G chromatography, with distinct bands of IgA; Lane 6, thio-cyanate eluted samples showing traces of IgA; Right panels (B & D)- Same immunoblots as Panels A and C, after reincubation in rabbit anti-sheep IgG to show the distribution of IgG in the respective lanes: left to right- Lane 1, IgG in lungs sample before Protein G chromatography; Lane 2, ' fall-through 'sample free of IgG (lungs); Lane 3, thio-cyanate eluted IgG rich sample with traces of IgA; Lane 4, IgG dimers (large arrow head) and monomers (small arrow head) in IgA rich colostrum before Protein G chromatography; Lane 5,' fall-through ' sample rich in IgA without any traces of IgG; Lane 6, thio-cyanate eluted IgG rich colostral samples (monomers and dimers) with traces of IgA.

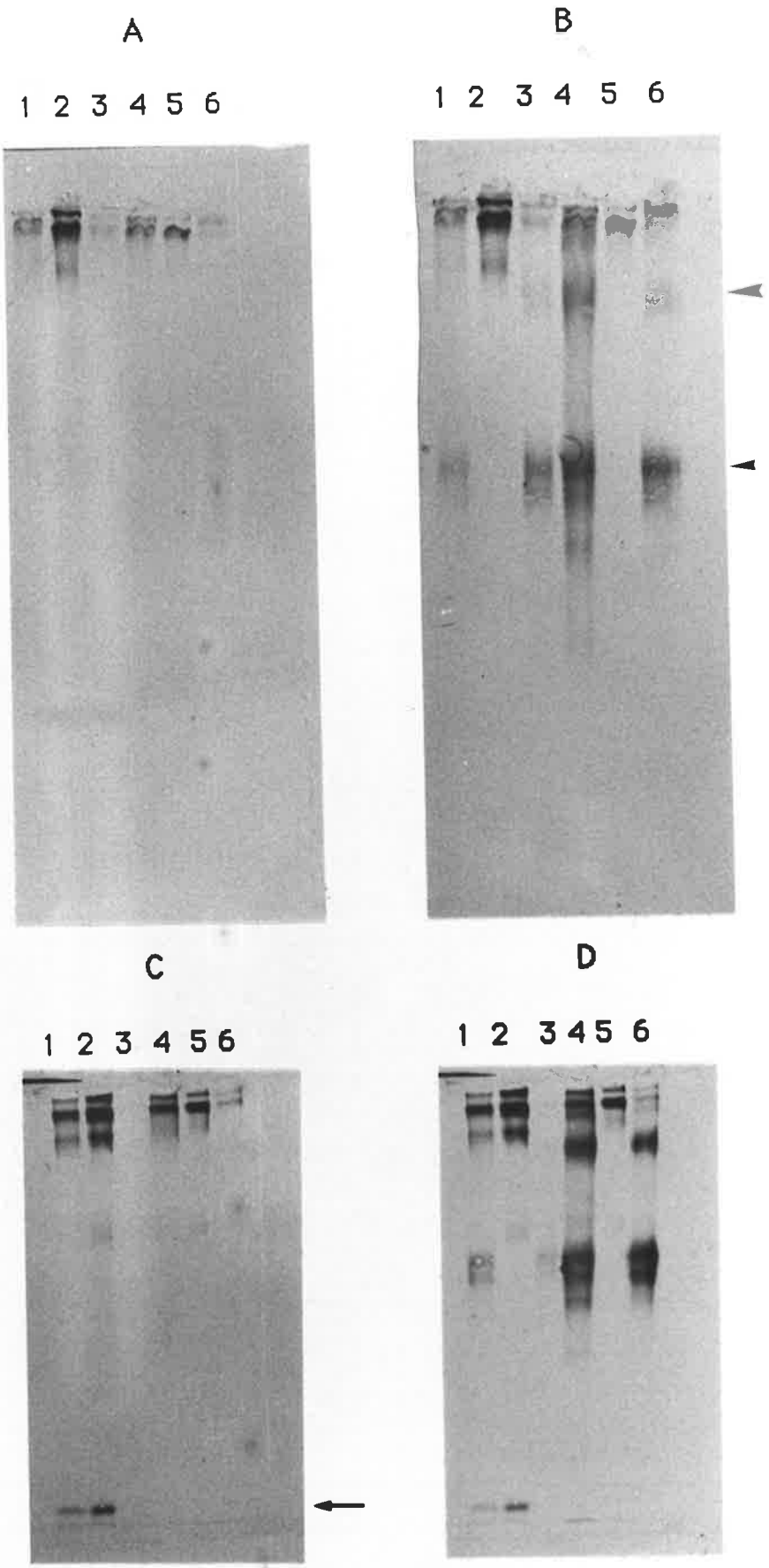
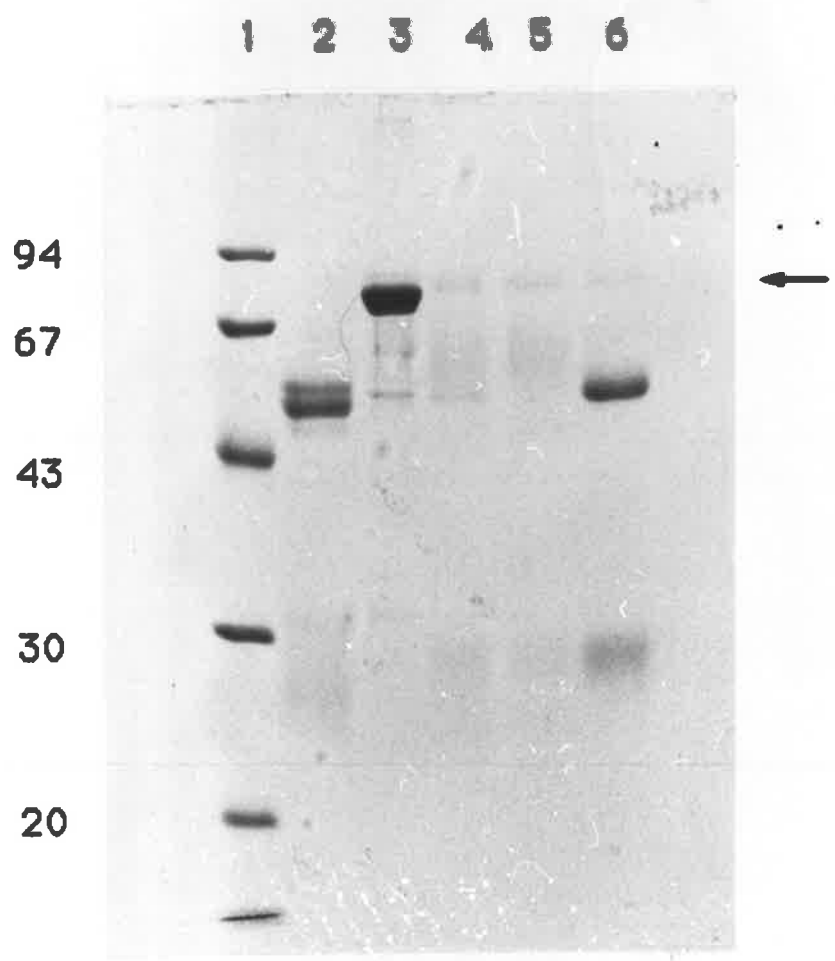

A
1 2 3 4 5 6
B
1 2 3 4 5 6
C
1 2 3 4 5 6
D
1 2 3 4 5 6

Plate 6.5: SDS-PAGE (12% polyacrylamide gels under reduced conditions) on protein G-affinity purified sheep IgA to demonstrate the heavy, light chains and secretory component: Lane 1, pre-stained molecular mass markers in kilo-daltons (kDa); Lane 2, rabbit IgG as molecular marker with heavy and light chains; Lane 3, human transferrin as molecular marker; Lanes 4 & 5, purified sheep IgA (lung washings) showing bands corresponding to secretory component (thin arrow), heavy and light chains respectively; lane 6, purified Colostral IgA with traces of SC and intense heavy and light chain bands respectively.


1 2 3 4 5 6
94
67
43
30
20

6.4 Discussion

DEAE-Sephacryl ion-exchange and Sephacryl S300 gel filtration chromatographic techniques were employed initially in an attempt to purify IgA from sheep colostrum. It proved difficult to separate IgA in the major peak (S300 gel-filtration, Fig. 6.1) eluents from a protein which was immunoreactive with anti-sera specific for IgG subclasses (see Table 6.1). When the same samples were analysed by Western blot, these contaminating proteins were identified and confirmed as monomeric and dimeric IgG (see Plate 6.4- Panels B and D). Furthermore, the results from ELISA based distribution assay also revealed the presence of IgM in the IgA rich gel-filtrates of colostrum (Table 6.1). Higher polymers of IgG has been reported in bovine serum and secretions (Sullivan et al., 1969). It has been observed that lacteal secretions are not ideal as starting material for the preparation of ruminant polymeric IgA because this protein has size and ionic characteristics which overlap with dimeric IgG_1(Butler, 1980b). These workers attempted to separate bovine IgA from dimeric IgG_1 in fractions obtained from gel-filtration and DEAE-cellulose chromatography by the use of affinity chromatography on protein A sepharose columns. However, it was not possible to remove dimeric IgG_1 completely from the final preparations. The same authors reported on the success of purifying bovine SIgA from the bronchio-alveolar washings which do not contain significant amounts of IgG dimers. In the present study, having failed to obtain purified IgA from S300 gel-filtration eluents of colostrum, it became necessary to consider sheep lung washings as an alternative source for the prepartion of IgA. This source has been used previously by Chin et al., (1986) based on procedures described by Butler et al., (1980b). Lung washings contain monomeric IgG but relatively little dimeric IgA. Difficulties were experienced in removing albumin and IgG monomers from IgA rich fractions of bronchio-alveolar washings using S300 gel-filtration. When the eluents from S300 gel-filtration were subjected to FPLC, although the albumin was removed, IgG monomers still remained in the FPLC eluted preparations.

Certain bacterial surface proteins bind with high affinity to Fc portions of various classes and subclasses of immunoglobulin from a variety of different species. The differential binding of IgG from different mammalian immunoglobulins to Staphylococcal protein A has been studied (Goudswaard et al., 1978; Myhre and Kronvall, 1981). These studies reported that Protein A had a low capacity for binding ruminant IgG and it was pointed out that Streptococcal protein G had a higher

binding affinity to ruminant IgG subclasses (Myhre and Kronvall, 1981). The ability of various sources of protein G to bind sheep IgG isotype has been demonstrated (Faulmann et al., 1989). There is apparently no literature on the use of Protein G columns to remove IgG contaminants in secretion during purification of ovine IgA.

At the initial stages of the study, the IgA enriched fractions of bronchio-alveolar washings from FPLC, equilibrated in phosphate buffered saline (PBS) at pH 7.2, were loaded onto a Protein G-4 Fast Flow affinity column pre-equilibrated with the same buffer at pH 7.2. After being loaded and washed with the same buffer, the ' fall-through ' fractions were examined for purity of IgA and still found to be contaminated with IgG (data not shown). Since the binding of immunoglobulins to affinity columns is very much pH dependant, an attempt was made to equilibrate the column with PBS at pH 8.3 and after loading of the samples, the column was washed with the same buffer at pH 8.3. This change in pH from 7.3 to 8.3 optimized the conditions for an efficient binding of sheep IgG monomers and dimers to protein G column. This finding was consistent with a recent study where it has been shown that the optimal conditions for binding IgG to protein G are 1.0M NaCl and pH 8.0 for human, mouse and goat (Pilcher et al., 1991). Similarly, Ey et al (1978) showed affinity of Protein A for various subclasses of mouse IgG is pH dependent. Even though the Protein G matrix bound sheep IgG monomers, it was not known whether the same column could bind sheep IgG dimers. It was shown in the present study that Protein G matrix bound effectively to sheep IgG dimers as well. Some degree of reactivity was also observed towards sheep IgA, as reflected in the immunoblot analysis (see Plate. 6.4). It was not clear whether this was due to nonspecific binding or whether Protein-G, similar to Protein-A (Harboe and Folling, 1974; Medgyesi et al., 1978), exhibits some binding affinity for other classes of immunoglobulins as well for IgG.

SDS-PAGE of non-reduced sheep colostral and lung samples illustrated clearly the dimeric nature of sheep IgA which correlated well with the mobility of molecular markers such as polymeric rat IgA and reduced thyroglobulin. (see Plate. 6.2a). The double immunodiffusion assay displayed double precipitin lines in pooled samples 1 and 2 of the S300 column chromatographed lung washings, suggesting the presence of secretory component (SC) as well as the IgA molecule (Plate 6.1a). The SDS-PAGE under reduced conditions provided further evidence for the presence of secretory component (see Plate 6.5). It was not possible to verify whether the detected SC was free or released component from SIgA,

because immunoblots (see Plates 6.2b and 6.4) suggested the possible presence of free SC in purified lung washings. It has been reported that reduced bovine SIgA released SC corresponding in mobility to free SC (Komar et al., 1975). If reduction releases SC, it is but logical to assume that it must be bound to the dimeric IgA seen in the non-reducing gels. The molecular weight of bovine SC has been reported to be ranging from 74-80 kDa (Butler, 1983). The zone correponding to SC in Plate 6.5 appeared similar in mobility to human transferrin, which has been reported to have a molecular weight ranging from 73 to 76 kDa (Roberts et al., 1966). However, it was not possible to determine the proportions of free and bound SC.

The ELISA results of colostral samples obtained after DEAE-Sepharose ion-exchange and S300 gel-filtration chromatography showed a protein immunoreactive with anti-sera specific for IgM. It was not clear whether this was a sign of cross-reactivity of this anti-sera toward α_2-macroglobulin (α_2-M), which is said to be a major contaminant in both IgM (Ungar-Waron et al., 1978) and secretory IgA preparations of colostrum (Butler et al., 1980b). Some of these aspects could not be investigated in the present study on account of non-availability of antisera specific for sheep SC and α_2-macroglobulins.

It may be concluded that Protein G affinity columns can be utilized effectively in removing sheep IgG monomers and dimers from preparations of sheep IgA. IgM may be a possible contaminant in samples of IgA from colostrum. However, the final product of Protein G affinity chromatography from bronchio-alveolar washings, contained IgA almost completely devoid of any other contaminants. This has been demonstrated for the first time in this study.

Section 7.0
Investigations into the Effect of Incubating SheepIgG and IgA in Rumen Fluid

7.1 Introduction

The main aim of this project has been to study secretory antibody responses in sheep, with a view to developing immunization strategies to manipulate the ciliate population in the rumen microflora of sheep. If such strategies are to succeed, then immunoglobulin and antibody activity must survive for a significant period in the environment of the rumen. The classical studies of Porter (see review: Cohen and Porter, 1964) on the proteolysis of immunoglobulins by enzymes such as papain and pepsin indicated relative resistance to degradation and also the generation of defined fragments which retained antigen binding activity. The studies in this chapter observe directly the effects of rumen fluid on immunoglobulin and other proteins and also its effects on antibody activity.

IgA is the major isotype in the saliva of ruminants. It is also well-represented in the intestinal secretions and milk of these animals. The usefulness of antibody in these secretions cannot be discounted, particularly its potential for modulating the rumen microbial population. It has been demonstrated in other species that secretory IgA is more resistant to proteolysis than IgG (Brown et al., 1970; Underdown, 1972; Underdown and Dorrington, 1974). However, the susceptibility of IgA to proteases of major pathogenic organisms has been reported (Kilian, 1981; Molla et al., 1986; 1988; Grenier et al., 1989; Heck et al., 1990). It appears that similar studies examining the proteolytic effect of ruminal microbial enzymes on ruminant SIgA have not been conducted in great detail. Such studies are warranted in the context of the overall project, which envisages an effector role for these molecules allowing manipulation of the rumen ciliate population.

In general, the binding by antibody fragments is not avid as that by the whole molecule. It has been said that for practical purposes, the Fab fragment does not bind to cells (Parham, 1983). Fragments like Fab/c consisting of one Fab and one Fc, retain the effector functions, (e.g., complement fixation, protein A binding of the intact IgG) but have a single combining site and cannot therefore cross-link antigen molecules. For cell-surface molecules, the bivalent binding of antibody can induce patching, capping and endocytosis of the antigen molecules, which is not

always desirable. In extreme cases of antigenic modulation, binding of bivalent antibody can result in long term loss of an antigen from the cell surface (Boyse et al., 1974).

The aims of the present investigation were to determine whether there is any fragmentation or degradation of purified IgG or SIgA when these molecules are incubated in rumen fluid and what effect this has on antibody activity.

7.2 Materials and Methods

7.2.1 Immunoglobulins/ Proteins

The proteins chosen to study proteolysis were purified sheep SIgA, IgG, casein and bovine serum albumin(BSA). The preparation and purification of SIgA has been described in Section 6.0. Purifed sheep IgG was donated by Dr. D. Auclair, Department of Animal Sciences, University of Adelaide, South Australia). Casein and BSA were purchased (Sigma Chemicals).

7.2.2 Preparation of rumen fluid for incubation

Samples of rumen fluid were obtained 3-5 hours after the morning (08.00 hours) feed from two rumen-fistulated wethers maintained on a shed-ration containing 800g pellets (65% digestibility and 7.8% crude protein) and 150g of oaten hay once daily. The fluid was strained through a layer of defined aperture nylon (300μm) and maintained at 39°C throughout the process of the experiment. Oxygen-free carbon-dioxide (Hungate, 1969) was used to bubble strained free rumen fluid (SRF) and McDougall's buffer to purge air from vessels. The composition of McDougall's buffer is shown in Table 7.1. The containers were capped and left in the water bath at 39°C before aliquoting into incubation-vials.

7.2.3 In vitro rumen incubation systems

7.2.3.1 For colorimetric assay of proteolysis

Two systems were used in this study. One was designated as System I which had a total reaction volume of 5ml [(4 ml SRF and 1 ml protein solution) and the other one was referred to as System II which had a final volume of 0.4ml, (350 μl SRF + 50 μl protein solution)]. 10 ml and 1 ml screw-capped containers were used in these systems respectively. Three proteins were used,viz., casein, BSA and sheep IgG.

System I was used to incubate these proteins for up to 12 hours and System II was used for limited incubation periods up to 4 hours in rumen fluid respectively. The enzymic activity and the digestion products were assayed by colorimetric assays and SDS-polyacrylamide electrophoresis.

Table 7.1: Composition of McDougall's buffer

Salt	g/litre
$NaHCO_3$	9.8
$Na_2HPO_4.12H_2O$	9.3
NaCl	0.47
KCl	0.57
$CaCl_2$ anhyd.	0.04
$MgCl_2$ anhyd.	0.06

N.B: The buffer solution was made according to the above composition, adding the $CaCl_2$ last; the solution was saturated with CO_2 at 39°C until the solution became clear.

7.2.3.2 For radiometric assay of proteolysis

In addition to ovine IgG, casein and BSA, sheep SIgA purified by procedures described in Section 6.0 was also included. The radiometric assay was found necessary in view of the limited amounts of sIgA available. Only System II was used. The digestion products were quantitated by release of acid soluble counts from radiolabelled proteins and analysed by SDS-PAGE under non-reducing conditions. The incubation time was not extended beyond 4 hours in this experiment.

7.2.4 Colorimetric assays

A colorimetric assay for protein using Coomassie Brilliant Blue G250 was employed to measure protein digests based on a method described by Sedmak and Grossberg (1977). The Coomassie G250 dye was prepared as a 0.06% solution in 3% perchloracetic acid (PCA-w/v) and was filtered through Whatman No 1 filter paper to remove any undissolved material. To construct the standard, each protein, viz., casein, BSA and sheep IgG was prepared in saline. The assay consisted of adding 0.5ml of G250 solution to 0.5 ml of protein solution, mixing immediately and determining the absorbance at 620 nm with a Carl Zeiss-

immediately and determining the absorbance at 620 nm with a Carl Zeiss PM QII spectrophotometer against a 1:1 mixture of saline and dye. Protein concentrations were verified by calculations based upon the molar extinction of the respective proteins at 280 nm. In assaying proteins, after incubation in the rumen fluid using System I, the precipitation was performed using trichloroacetic acid (6% TCA) with sodium de-oxycholate (0.0125% DOC) as a carrier. After centrifugation, the pellets were washed in 1:1 mixture of ether/ ethanol, redissolved in 50 µl of 0.1N NaOH and diluted to 1 ml with normal saline before mixed with the dye for reading the absorbance.

7.2.5 *Preparation of ^{14}C-labelled proteins*

Four proteins, viz., casein, BSA, sheep IgG and sheep SIgA were labelled with ^{14}C-formaldehyde (Amersham Ltd.), based on procedures described by Means and Feeny (1971) and Wallace (1983) for the reductive alkylation of proteins. For protein concentrations of 10mg/ml, 0.015 vol. of freshly prepared sodium borohydride solution (0.5mg/ml) was added, followed a few seconds later by the addition of 0.05 vol. of ^{14}c-formaldehyde (0.1mg/ml). All of the procedures were carried out on ice and the mixture was left at 4^{o}C for 1 hour and then dialysed against distilled water. The specific activity of ^{14}C-formaldehyde used in these experiments was 0.5 Ci/g and the proteins were labelled to a specific activity of 3.6 - 7.4 µCi/g. At this concentration, approximately 29 % of the ^{14}C-formaldehyde became protein-bound during 90 minutes reaction with casein as substrate. Increasing the reaction time to 24 hours did not show any improvement in the percentage of labelling of any of the proteins used in the study. A similar order of incorporation with other proteins was observed. The specific radioactivity of ^{14}C-labelled proteins varied from 3.6 µCi/g with sheep SIgA to 7.4µCi/g with BSA. Sheep IgG had an activity of 4.1µCi/g whilst that of casein was 6.8µCi/g.

7.2.6 *SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE was performed on digestion products, under reduced and non-reduced conditions, essentially as described earlier (Section 6.0). Proteins were precipitated from the reaction mixtures using DOC and TCA, micro-centrifuged for 5 minutes, and the pellets resuspended in 1 ml of ethanol:ether mixture (1:1). After further micro-centrifugation for 5 minutes, the pellets were dissolved in relevant sample buffer before loading into gels.

7.2.7 *Measurement of proteolysis*

In system I, the reaction mixtures, incubated at 39°C, contained proteins at a concentration of 2mg/ml in McDougall's buffer, pH 7.3. In system II, casein and BSA were maintained at 1.25mg/ml, and sheep IgG and IgA at 0.02mg/ml respectively. In the experiment using System I for colorimetric analysis, samples were removed from the reaction mixtures at 0, 1, 2 and 3 hours (a short incubation period) and at 0, 4, 8 and 12 hours (an extended incubation period). They were centrifuged immediately in a micro-centrifuge (12,000g, 2 min) to remove micro-organisms and the supernatants were frozen immediately in liquid nitrogen before being stored at -20°C until further analysis. In the colorimetric assays, frozen supernatants were thawed and the digestion products was subjected to a precipitation procedure with DOC and TCA and the final pellet was dissolved in 0.1N NaOH for colorimetric assays or in 1x sample buffer for SDS-PAGE. In the experiment using System II for radiometric analysis, the incubation times were 0, 2 and 4 hours. Here, the whole samples were removed at the prescribed time points and after the removal of microorganisms, the undigested protein/larger fragments were precipitated with DOC and TCA. Pellets were dissolved in 1x sample buffer for SDS-PAGE under non-reducing conditions. The appearance of ^{14}C in acid-soluble fractions of the supernatant was used as an alternative to express the percentage recovery of acid-insoluble digestion products.

7.2.7.1 *Maintenance of protease activity in the rumen fluid in vitro*

The maintenance of proteolytic activity in rumen fluid held at 39°C in vitro for various periods of time was followed using casein as the substrate. Casein was added at two different concentrations, (2mg and 1.25mg/ml) into the systems used in the study. After preincubating the rumen fluid for prescribed periods, casein was added and samples were removed exactly after 1 hour later, precipitated with DOC and TCA and examined by colorimetric assay.

7.2.8 *Measurement of the effects of proteolysis in rumen fluid on activity of anti-ciliate antibodies*

7.2.8.1 *Incubation of immune samples with rumen fluid*

The vials containing samples of immune milk whey (casein-free), obtained 14 days after immunization with rumen ciliates, were mixed with protozoa-free rumen fluid (PFRF) in the ratio of 1:7 and 1:15 and

used in triplicate for incubation for various periods of time. The reaction mixture had a final volume of 0.4 ml and a final substrate concentration of 20-40 μg /ml of IgG (total IgG values for day 14 was 0.34 mg/ml-see Table 5.7). The incubations were performed at 39°C under anaerobic conditions for up to a maximum 4 h incubation period, with an interval of 2 h between each time unless indicated otherwise. At the end of each time point, the respective reaction mixtures were removed, centrifuged at 12,000 g for 2 minutes to remove microorganisms and the supernatants were immediately frozen in liquid nitrogen before being stored at -20°C for further use. The maintenance of protease activity in the incubated rumen fluid was monitored simultaneously by using casein (Sigma Chemicals) as the substrate, at a final concentration of 1.25mg/ml of reaction mixture. Four sets of vials in duplicate containing protozoa free rumen fluid (PFRF) were set in the water bath and maintained anaerobically at 39°C at the beginning of the experiment. To each set of vials, casein was added at 1 h intervals within the 4 h incubation period and at the end of ensuing 1 h incubation, the samples were removed and assayed for the presence of casein utilizing Coomassie G250 dye as described by Sedmak and Grossberg (1977).

7.2.8.2 *Assays for residual antibody activity*

Samples of milk-whey from ewes that had been immunized with rumen ciliates (see Section 5.0), after being subjected to incubation in rumen fluid for various periods of time at dilutions of 1:8 and 1:16, the residual antibody activity was assessed by binding to purified rumen ciliates (see 5.2.9.2) and was measured by flow cytometry (see 5.2.9.3).

7.3 Results

7.3.1 *Measurement of proteolysis of immunoglobulins in rumen fluid*

Standard curves for the Coomassie blue dye-binding assay were constructed for casein, BSA and sheep IgG using dilution series of known concentrations. The stock solutions were diluted with normal saline to obtain a dilution series containing NaOH at a final concentration of 0.0025N. In the preliminary studies it was verified that there is no interference in colour development with NaOH at the concentrations used in the assay. The latter was incorporated in the standard assays because of its intended use as a solubilizing agent for the material precipitated with TCA from the digests of protein in rumen fluid. In the resultant assay, NaOH would be present at a concentration of 0.0025N. The standard curve for each protein and the quadratic

regression for the estimation of proteins in the experimental medium are shown in Fig. 7.1.

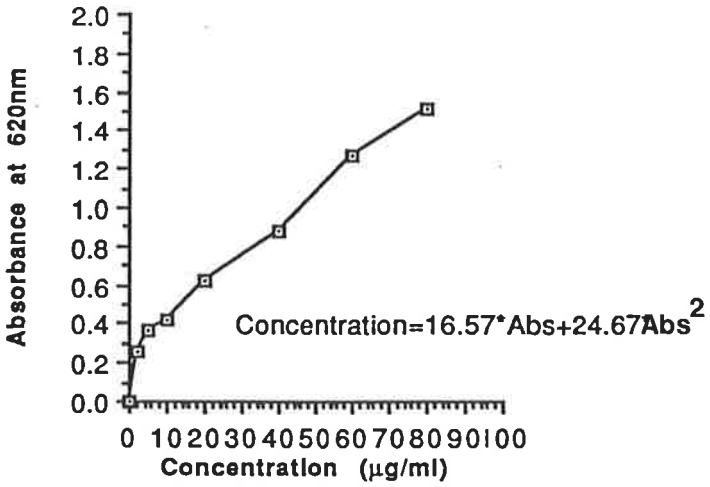
The results of the colorimetric analysis of protein degradation by rumen fluid in System I are illustrated in Figures 7.2 and 7.3. The protein concentration in acid precipitable material remaining at the end of each incubation period was measured by the Coomassie brilliant blue dye-binding assay. The Figures demonstrate clearly the fast digestion of casein which degraded completely to acid-soluble products within 1 hour after incubation in rumen fluid. The other two proteins, BSA and sheep IgG were found to be relatively resistant to proteolysis. The recovery of acid precipitable protein was calculated for each protein from the quadratic regressions shown in Fig. 7.1, using the corresponding absorbance values. The values thus obtained were used in the calculation of percentage of recovery. The data are presented in Table 7.2.

Table 7.2-Estimation of concentration of acid precipitable protein and its recovery at various periods of incubation in rumen fluid (Concentration in mg/ml is average of four values with standard errors- System I)

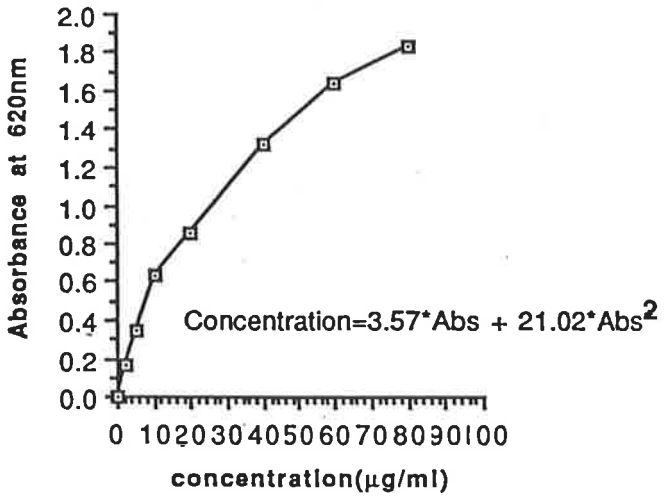
Time	Casein		BSA		Sheep IgG	
Short-incubation	Conc (mg/ml)	% recovery	Conc (mg/ml)	% recovery	Conc (mg/ml)	% recovery
0	1.630±0.026	81.5	1.755±0.022	87.8	1.779±0.028	88.9
1	0.113±0.005	5.7	1.687±0.037	84.4	1.705±0.041	85.3
2	0.085	0.0	1.713±0.041	85.7	1.739±0.025	86.9
3	0.055	0.0	1.685±0.043	84.3	1.684±0.048	84.2
Extended-incubation						
0	1.588±0.035	79.4	1.741±0.027	87.1	1.882±0.030	94.1
4	0.041	0.0	1.710±0.043	85.5	1.787±0.035	89.3
8	0.054	0.0	1.549±0.030	77.5	1.548±0.033	77.4
12	0.040	0.0	1.361±0.022	68.1	1.371±0.026	68.6

Fig: 7.1 The standard curves of absorbance versus concentration for casein, BSA and sheep IgG. They were constructed using absorbance values obtained with the Coomassie brilliant blue dye-binding assay applied to a dilution series of known concentrations of the respective proteins, according to a method described by Sedmark and Grossberg (1977). The values are averages of three replicates.

Casein



Bovine serum albumin (BSA)



Sheep IgG

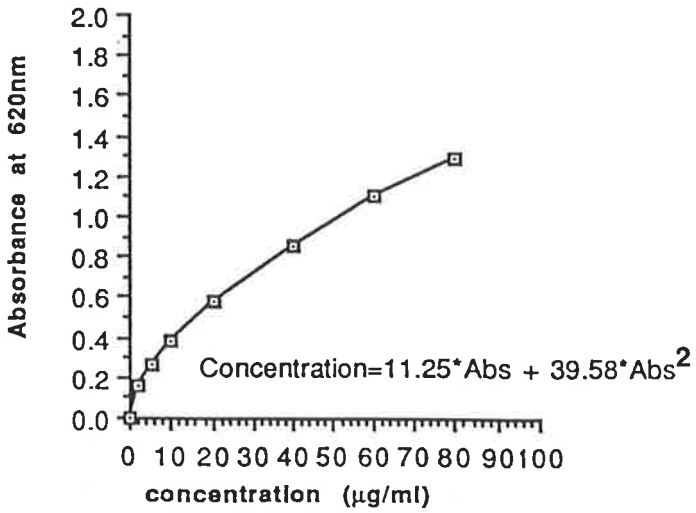
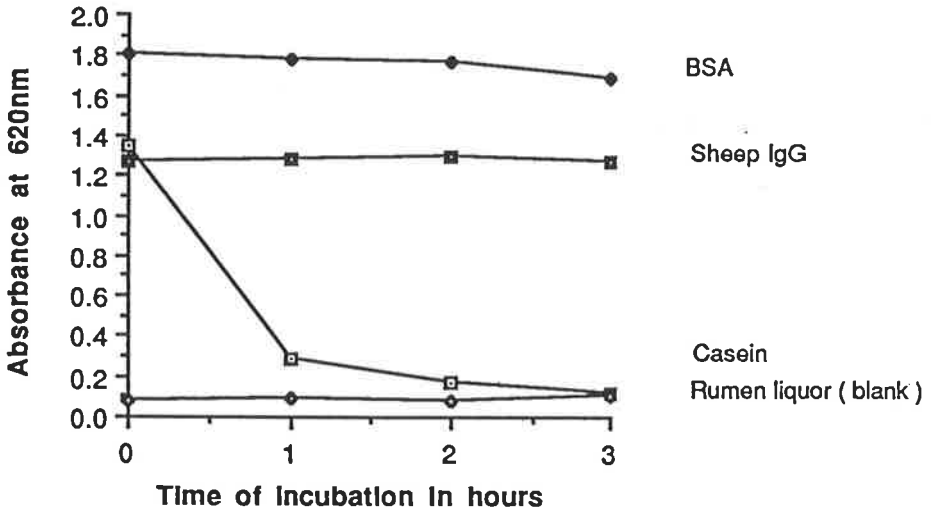


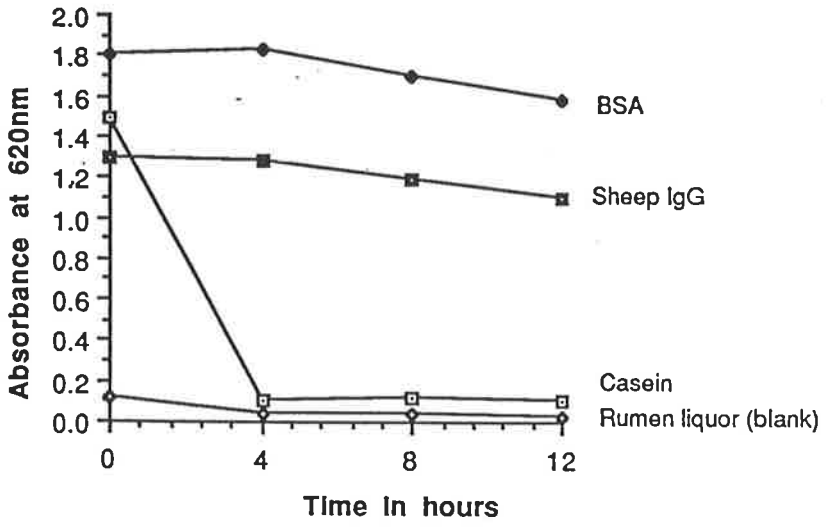
Fig. 7.2 Colorimetric assay on products of short-term incubation. Casein, BSA and sheep IgG were precipitated with sodium deoxycholate and trichloroacetic acid, washed with ethanol-ether mixture 1:1 and dissolved in 0.1N NaOH. Colorimetric assay involved the addition of equal volume of the respective protein and G250 solutions and reading the absorbance at 620nm against a 1:1 mixture of saline and dye containing the same concentration of NaOH (0.0025N) in the final assaying medium.

Fig. 7.3 Colorimetric assay on products of extended incubation. Proteins were precipitated with sodium deoxycholate and trichloroacetic acid, washed with ethanol-ether mixture 1:1 and dissolved in 0.1N NaOH. Colorimetric assay involved the addition of equal volume of protein solution and G250 solution and reading the absorbance at 620 nm against a 1:1 mixture of saline and dye containing the same concentration of NaOH (0.0025N).

Colorimetric assay on products of incubation



Calorimetric assay on products of extended incubation



During the first 3-4 hours, the recovery of casein was almost reduced to zero when assayed by colorimetry. In contrast, only approximately 4-5% of BSA or sheep IgG was rendered acid soluble. Even after 12 hours incubation in rumen fluid, BSA and sheep IgG remained approximately 72-78% acid precipitable by TCA respectively. On SDS-PAGE without reduction (see Plate 7.1), all three proteins (viz., casein, BSA and sheep IgG) yielded single bands at 0 time. No detectable native casein, or low molecular weight degradation products, was visible after as little as 1 hour incubation in rumen fluid. However, in cases of both BSA and IgG, distinct single bands representing the undegraded molecule were present at least up to the end of the first 4 hours of incubation (see Panels A and B, Plate. 7.1). Even after incubation in rumen fluid for 12 hours, much of the degraded BSA and IgG was detected as distinct bands of MW 25,000-50,000 (relative to immunoglobulin heavy and light chains). Interestingly, sheep IgG showed faint bands with mobilities equivalent to heavy chains and light chains from the start of incubation. These bands were found even in samples incubated in the McDougall's buffer, indicating the presence of these fragments along with the whole molecule, in the original preparation used in the study.

The digestion products of BSA and IgG at 8 and 12 hours contained multiple bands of lower molecular size smaller than the original intact molecules (see Panel B, Plate 7.1). It may be observed for IgG that there is a faint band with slightly greater mobility than undegraded IgG. This probably corresponds to the $F(ab)_2$ fragment (thick arrow). In addition there are two other bands, one just below the heavy chain zone (large arrow head) and the other just above the light chain region (small arrow head). It is probable that the former may be either pFc or Fab of the sheep IgG subclasses.

To identify some of these fragments, protein digests were subject to SDS-PAGE under reduced conditions. In general, on reduction, IgG yields two bands, one at about 50,000 and one at 25,000, $F(ab')_2$ and Fab fragments yield a doublet of bands at about 25,000, and Fc fragments yield one band

Plate 7.1 Analysis of products of digestion of defined proteins after incubation with rumen fluid (System I) by SDS-PAGE (12% gel) under non-reduced conditions. The gels were stained with Coomassie blue to identify protein bands.

Panel A: Lane 1, casein after 12 hours in incubation buffer; Lanes 2-5, casein after incubated 0, 4, 8 and 12 hours in rumen fluid; Lanes 6, 7 and 8 casein after 1 hour incubation in rumen fluid, where the rumen fluid had been pre-incubated for 4, 8 and 12 hours respectively before addition of casein; Lane 9, sheep IgG after 12 hours in incubation buffer; Lane 10, rumen liquor at 0 hour.

Panel B: Lane 1, BSA after 12 hours in incubation buffer; Lane 2, sheep IgG after 12 hours in incubation buffer; Lanes 3-4, rumen liquor after 4 and 8 hours incubation respectively; Lanes 5-8. BSA after 0, 4 , 8 and 12 hours incubation in rumen fluid; Lanes 9-12, sheep IgG after 0, 4, 8 and 12 hours incubation in rumen fluid.

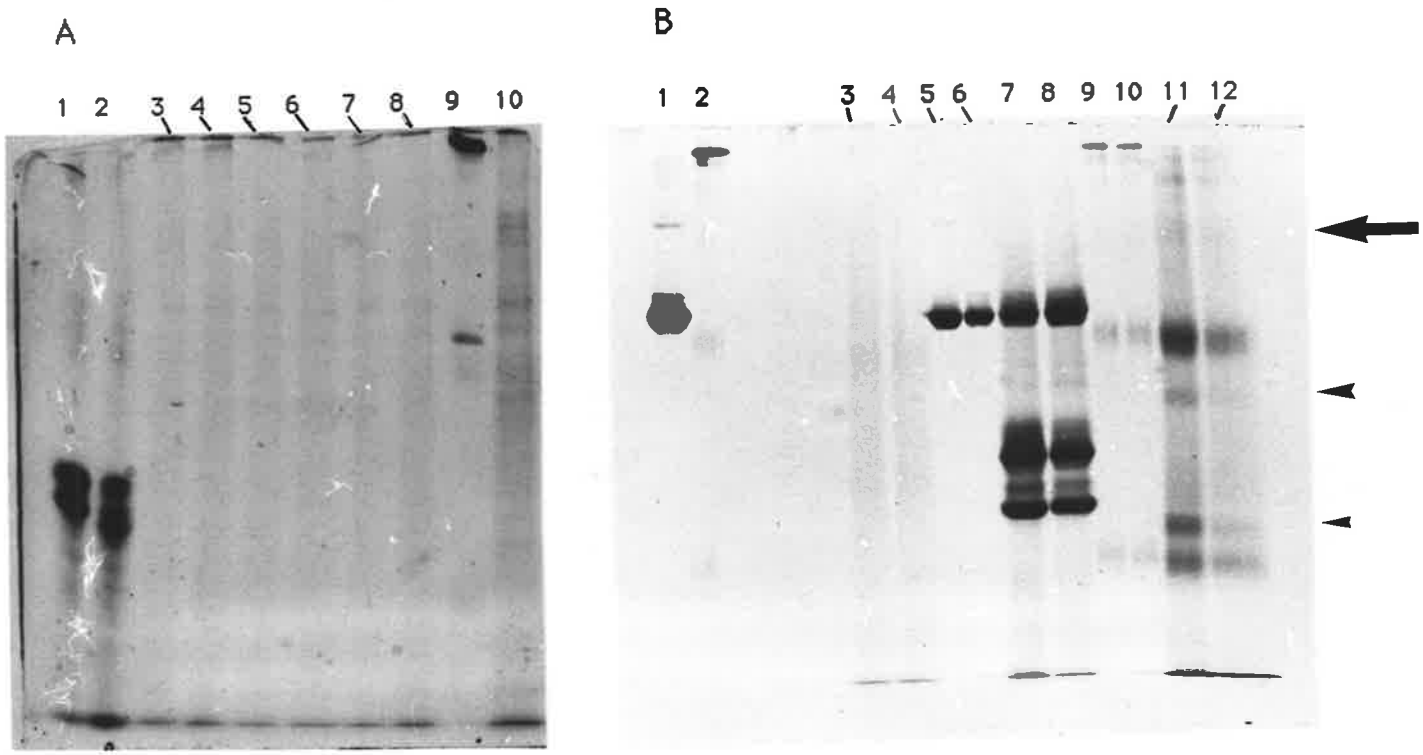
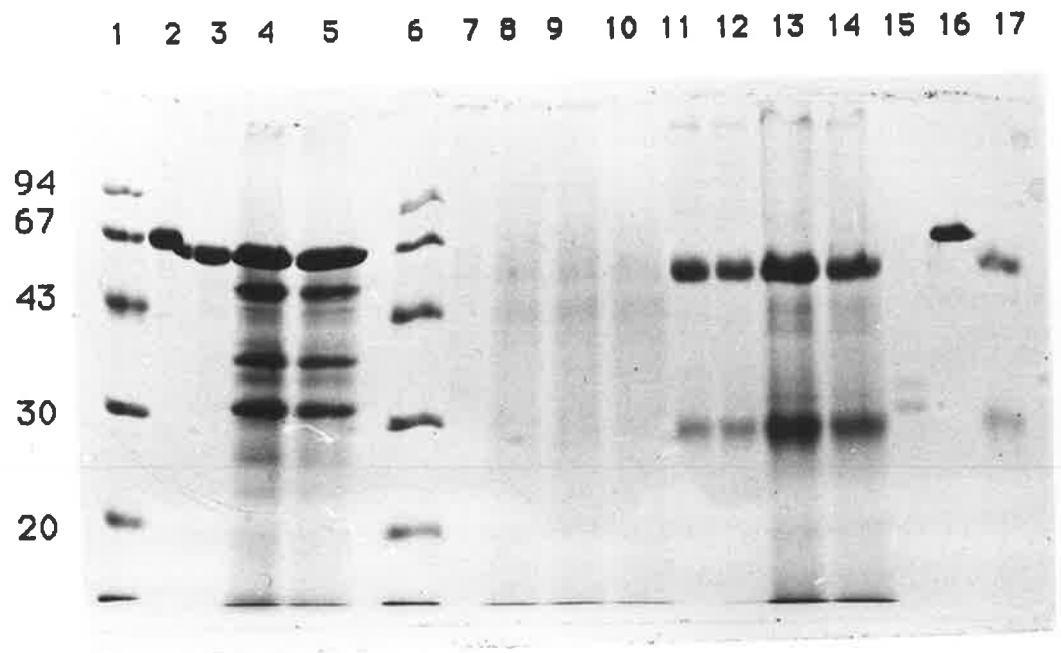

A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9 10 11 12

Plate 7.2 SDS-PAGE (12% gel) showing products of incubation under reduced conditions (System I). Lanes 1 and 6 contain molecular mass markers. The numerals on the left are $M_r \times 10^{-3}$ values for the standards; Lanes 2-5, BSA after 0, 4, 8 and 12 hours incubation; Lane 7, rumen liquor after 0 hour incubation; 8-10, casein after 4, 8 and 12 hours; 11-14, sheep IgG after 0, 4, 8 12 hours incubation; Lanes 15, 16 and 17 correspond to casein, BSA and sheep IgG after 12 hours in the incubation buffer.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
94
67
43
30
20

that runs at about 25,000, but normally behind the Fab doublet. The results in Plate 7.2 further testified the intactness of sheep IgG after 0 and 4 hours incubation by resolving into two distinct bands after reduction, the uppermost around 50,000 molecular size region (heavy chain) and the lowermost around 30,000 (see Lanes 11 and 12). Fragments in Lanes 13 and 14 were not resolved from heavy and light chains.

To examine the maintenance of proteolytic activity in vitro during these incubations, samples of casein digests were obtained after 1 hour incubation with rumen fluid which had been preincubated previously for various periods of time (viz., 4, 8 and 12 hours respectively). They were analysed by SDS-PAGE (System I) and by colorimetric assay (System II). No visible casein bands was detected in any of the 1 hour digests obtained in system I (see lanes, 6, 7 and 8 of panel A- Plate 7.1), indicating proteolytic activity is conserved for long periods in vitro. Similarly in System II, the presence of such activity was demonstrated up to 4 hours (see lanes 5,6 and 7 of panel B- Plate 7.3). In the latter system (System II), proteolytic activity was not detectable in rumen fluid that had been preincubated for more than 4 hours (data not shown). Thus it was inferred that with system II, the incubation times should not be extended beyond 4 hours, to ensure optimal enzymic activity.

The proteolytic activity of the rumen fluid was found to be associated with the microbial biomass rather than with cell free rumen liquor. Incubation of casein with the supernatant after high speed centrifugation to remove bacteria led to minor loss of acid precipitable material even after 8 hours of incubation at 39^{o}C (data not shown)

The proteolytic studies on ^{14}C-labelled proteins were limited to a maximum of 4 hours of incubation in` rumen fluid under anaerobic conditions using system II. The time-course of digestion of ^{14}C-labelled proteins in rumen fluid in vitro showed that while casein was completely hydrolysed within 2 hours, digestion of BSA, sheep IgG and IgA by the rumen micro-organisms was incomplete after 4 hours. This was observed by measuring the release of radioactivity into the acid-soluble fractions after specified periods of incubation in rumen fluid. The data are shown in Table 7.3.

Table 7.3 Radioactivity in the incubated samples

Protein	Sample fractions	Radioactivity (dpm)[a] Time of incubation in hours		
		0	2	4
Casein	Acid soluble	250	7152	7592
	Total	7440	8256	8080
	% recovery	97.1±1.23	13.4±0.89	6.0±0.99
BSA	Acid-soluble	423	810	1024
	Total	9720	9551	10576
	% recovery	95.6±1.88	91.5±1.63	90.3±1.10
Sheep IgG	Acid-soluble	510	525	1003
	Total	9100	9753	9988
	% recovery	94.3±1.06	94.6±1.34	89.9±1.79
Sheep IgA	Acid-insoluble	118	216	480
	Total	5400	5280	5920
	% recovery	97.8±1.45	95.9±1.88	91.8±2.29

[a]dpm: disintegration per minute

Note: Percentage recovery is the average of four values with standard errors

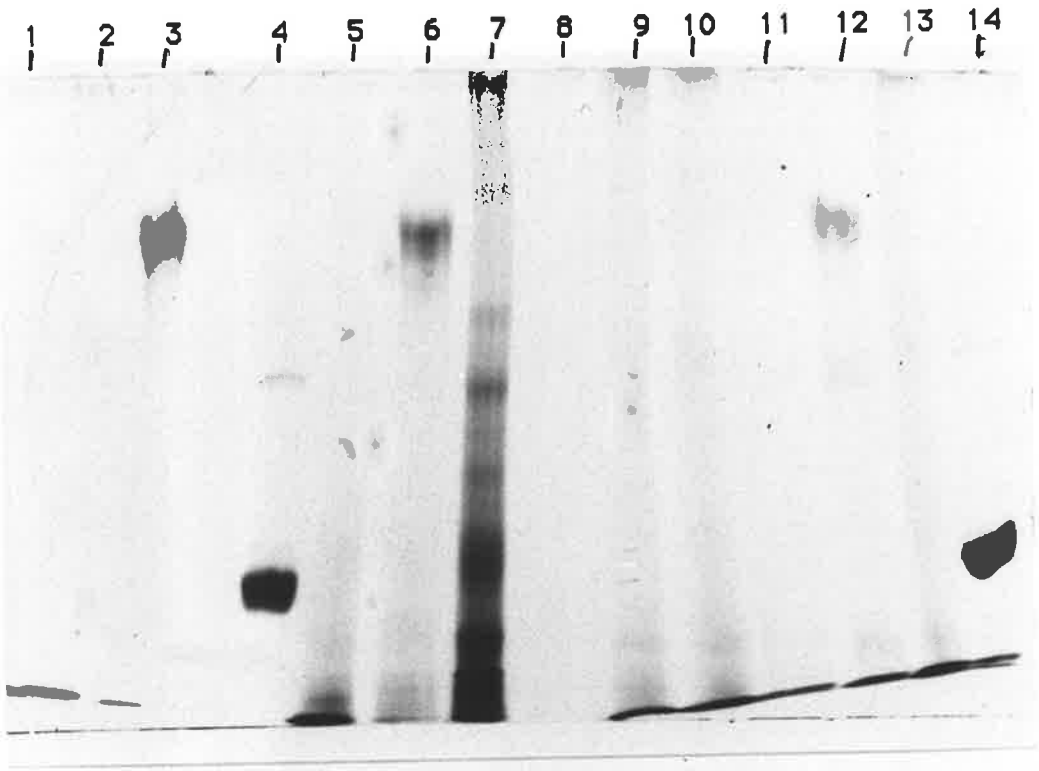
The above findings were found consistent with the earlier colorimetric assay measurements on similar proteins, but they also provided evidence that sheep IgA is also highly-resistant to digestion in the rumen fluid. However, chemical modification by formaldehyde might have caused structural changes in some of these proteins and the consequent increased resistance to proteolysis. The native proteins were therefore incubated with rumen fluid under the same conditions and the protein digests were examined by SDS-PAGE under non-reduced conditions (see Plate 7.3). It showed clearly the intact bands corresponding to IgG , IgA and BSA indicating the resistance of these proteins to proteolysis for the period of 4 hours incubation.

Plate 7.3 SDS-PAGE comparing products of incubation of sheep IgG, IgA, BSA and Casein

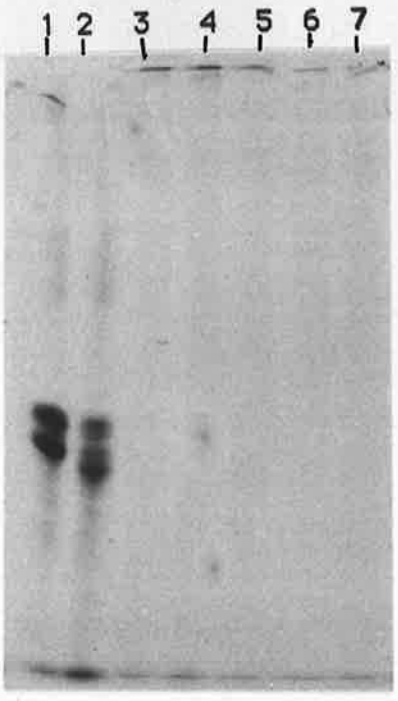
Panel A- An 8% gel showing products of incubation of sheep IgG, IgA and BSA under non-reduced conditions: Lanes 1 and 2, rumen liquor after 0 hour incubation demonstrating absence of any detectable soluble protein in the original rumen fluid used in the study.; Lanes 3, 6 and 12, sheep IgG after 0, 2 and 4 hours incubation in rumen fluid; Lanes 4 and 14, BSA after 0 and 4 hours incubation in rumen fluid; Lane 7, sheep IgA and co-precipitated microbial protein after 0 hour incubation; Lanes 9, 10 and 13, sheep IgA after 0, 2 and 4 hours incubation in rumen fluid; Lanes 5 and 8, rumen liquor after 2 and 4 hours incubation.

Panel B- 12% gel showing casein digests under non-reduced conditions; Lane 1, casein after 4 hours in incubation buffer; Lanes 2-4, casein after 0, 2 and 4 hours incubation in rumen fluid; Lanes 5-7, casein digests for 1 hour in rumen fluid that had been preincubated for 2, 4 and 5 hours respectively prior to addition of casein.

A



B



7.3.2 *Effects of rumen fluid on the activity of anti-ciliate antibodies from milk whey*

7.3.2.1 *Demonstration of residual antibody activity by flow cytometry*

Antibody activity in milk-whey samples after incubation with rumen fluid was estimated by measuring residual binding to ciliates in comparison with binding of unincubated whey antibody (Fig. 7.4). Incubations were performed at 2 dilutions of casein-free whey (1:8 and 1:16). In all cases, the fluorescence profiles of specifically labelled cells were compared with the fluorescence of cells labelled using irrelevant monoclonal antibodies in place of specific mouse anti-IgG or IgA monoclonal antibodies. At both dilutions, unincubated whey contained antibodies which caused a shift in the mean fluorescence of ciliates relative to the fluorescence of the non-specifically labelled control cells. Pre-incubation of whey for 2 hours in rumen fluid led to a reduction in the mean fluorescence intensity of the labelled ciliates and the appearance of a peak of essentially unlabelled cells. Incubation of whey in rumen fluid for a further 2 hours led to further reduction in binding to ciliates as measured by mean fluorescence intensity. However, even after 4 hours pre-incubation at both substrates concentration in rumen fluid, specific antibody was still detectable in the whey preparation. It proved not possible to detect binding to ciliates of IgA antibodies from whey in this assay.

The shape of the fluorescence profile of the labelled cells is of interest. The monodisperse peak of specific fluorescence in cells labelled with undegraded whey antibodies could indicate different amounts of antibody against different ciliate species and / or differences in antibody bound by different ciliates in relation to size (i.e., surface area). The biphasic shape of the fluorescence profiles of cells labelled with partially degraded whey antibodies probably reflects the greater ease of detection of residual antibody binding to large ciliates (by virtue of their larger surface area) relative to binding to the smaller species.

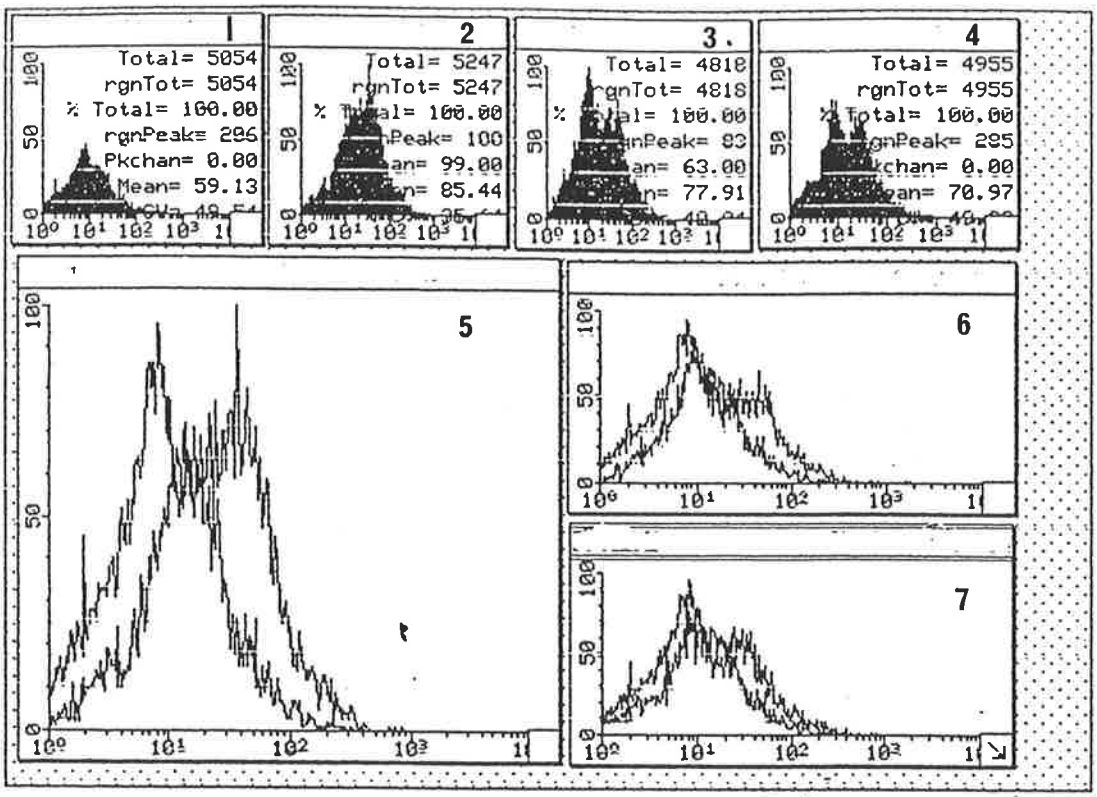
Fig.7.4: Flow-cytometry analysis of survival of anti-ciliate IgG antibody activity in milk samples (free of casein) incubated in rumen fluid.

Block A- Top four panels (1,2,3 and 4) fluorescence profiles of ciliates labelled with an irrelevant mouse monoclonal (1), an immune milk after 0hr incubation (2), after 2 hr incubation (3) and after 4 hr incubation (4) respectively; Milk (casein-free) dilution 1:8; Vertical axis, number of cells labelled in each channel; horizontal axis, log fluorescence intensity (arbitary units). Mean= mean fluorescence intensity of the total events measurred.

Panels 5,6 and 7, overlays of fluorescence profiles of ciliates labelled with reagent control or milk (casein-free) IgG antibodies incubated for 0 (panel 5), 2 (panel 6) and 4 (panel 7) hours with rumen fluid prior to estimation of residual binding activity.

Block B- As for Block A- fluorescence profiles for 1:16 dilution of milk.

A


1
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rgnTot= 5054
% Total= 100.00
rgnPeak= 206
Pkchan= 0.00
Mean= 59.13
2
Total= 5247
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3
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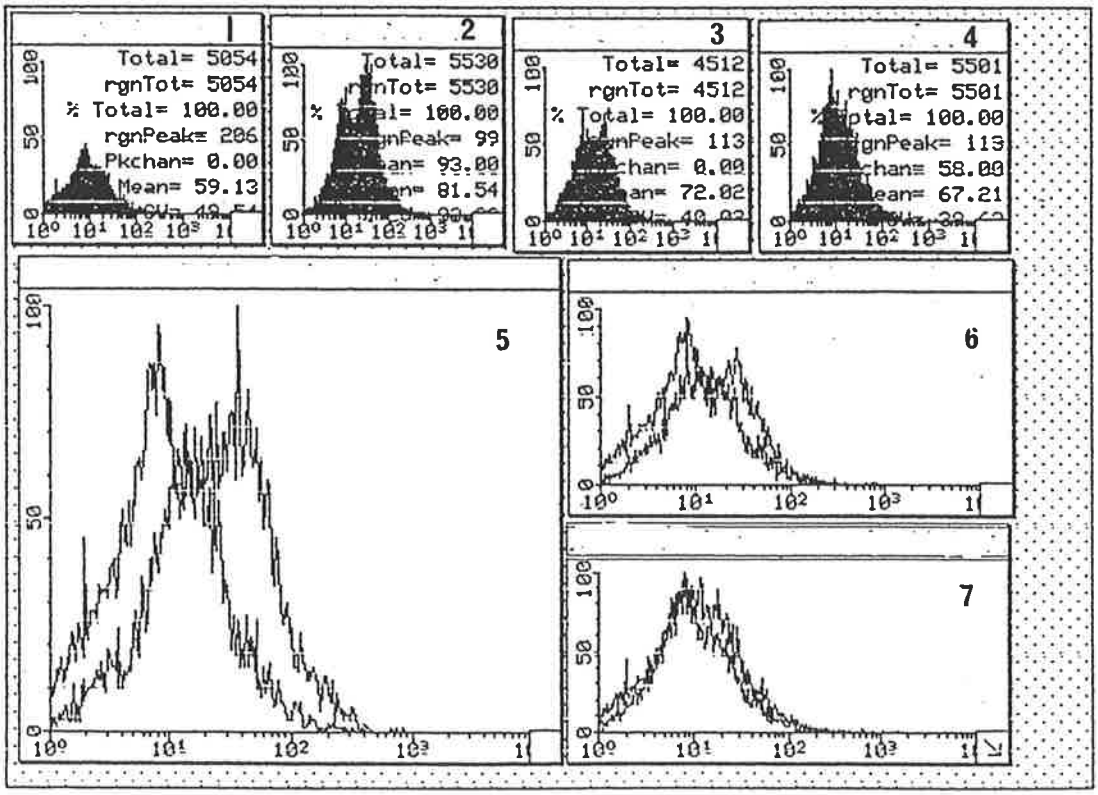
rgnTot= 4818
4
Total= 4955
rgnTot= 4955
5
6
7

B


1
Total= 5054
rgnTot= 5054
% Total= 100.00
rgnPeak= 206
Pkchan= 0.00
Mean= 59.13
2
Total= 5530
3
Total= 4512
rgnTot= 4512
% Total= 100.00
4
Total= 5501
rgnTot= 5501
5
6
7

7.4 Discussion

In order to make a better prediction on the possible effector functions of sheep IgG and IgA antibodies in rumen fluid, an investigation was undertaken on the effect of incubating immunoglobulins in proteolytic rumen fluid. The investigation was carried out on three levels. Firstly, the resistance of IgG to proteolysis into acid soluble products was compared with casein and BSA, using a colorimetric assay for residual protein. The assay depends on the binding of Coomassie brilliant blue and it is reported to be suitable for measuring concentrations of polypeptides with molecular weights greater than 3000 kDa. (Sedmak and Grossberg, 1977). Sheep secretory IgA was not included in this analyses, because insufficient purified protein was available. However, it was possible to compare the proteolysis of SIgA and other proteins by the use of isotypically labelled materials.

The second level of analysis was to investigate the nature of the residual acid insoluble polypeptides after various periods of incubation in rumen fluid by analysis under non-reducing and reducing conditions, using SDS-PAGE. In this way it was possible to examine clevage of the proteins and the individual polypeptide chains. These changes could occur without the release of acid soluble products and would not be detected by the calorimetric assay. Clevage could affect antibody activity and also the valency of antibodies and therefore their ability to cross-link or agglutinate.

Thirdly, the effect of proteolysis on the binding activity of antibodies against surface antigens of rumen ciliates was examined by flow cytometry, using an indirect immunofluorescence technique. The results obtained confirm that immunoglobulin is relatively resistant to the rumen micro-environment and that antibody activity can survive for several hours at least under simulated conditions in vitro.

The efficacy of an in vitro rumen incubation system like system II for prolonged incubation required characterization. In this study, the system was used in parallel with System I, a conventionally used in vitro rumen system. Using System I, proteolytic activity was essentially unchanged over a period of 12 hours. However, the enzymic activity declined in system II (mini-in vitro) when rumen fluid was incubated for more than 4 hours. The apparent reasons for those differences could not be determined in this study. Since the reaction times for antibody-antigen interactions and their resultant effector functions can be expected to occur well within a period of 4 hours, it was still considered possible to

use System II. The need for the choice of System II was necessitated by limitations on the availablity of purified sheep SIgA and the adaptation to radiometric technique.

The protein substrate concentrations used in systems I and II were within the range reported in some other studies (Brock et al., 1982; Wallace, 1983;). A concentration of 2 mg/ml was chosen for casein, BSA and sheep IgG in system I. It has been reported that at this concentration, the rate of casein hydrolysis is maximal (Wallace, 1983). The same author observed that the value greatly exceeded the K_m for casein, which is approximately 0.1-0.2mg/ml. Even in system II, casein and BSA were used at 1.25mg/ml. Brock et al., (1982) have used one percent (wt/vol) azocasein in their proteolytic studies. At this level, azocasein was at saturating concentration of substrate for the protease activity of mixed suspensions of rumen microorganisms. Wallace, (1983) reported that some substrate inhibition occurred when casein was used at 2mg/ml with certain types of rumen bacterial organisms., but it is possible that other proteins have K_m values higher than casein. The level of protein substrate concentration chosen was a compromise and the comparisons drawn among proteins should not be assumed to have been done at V_{max}.

Another important point is that the concentration of ^{14}C-labelled sheep IgG and IgA was kept at 0.02-0.03 mg/ml to meet the criteria used in the residual antibody-binding activity studies, where instead of saturating concentrations of the substrates, an enzyme excess might be expected. Regardless, no apparent differences were seen at least for the first 4 hours with regard to the susceptibility of sheep IgG and IgA to rumen proteolytic enzymes.

The ruminal proteolytic activity is mainly confined to two groups of microbial organisms, viz., bacteria and protozoa. It has been reported that the specific activity of the bacterial proteinases is 7 to 10 times higher than that of protozoa when either whole cells or cell extracts are assayed (Brock et al., 1982). The contribution of protozoa is therefore probably limited primarily to bacterial engulfment and degradation, a role amply documented by Coleman, (1980). Nevertheless, protozoa do possess proteinases (Forsberg et al., 1984). It has been emphasized that the proteinase activity for mixed rumen microbial organisms is 42.5% greater under anaerobic conditions over a 2 hour incubation time than the activity for the same preparation under aerobic conditions (Brock et al.,1982). For this reason, all experiments were conducted under anaerobic conditions.

The level of interference from microbial protein has not been ascertained in the present study. Microbial protein in the incubation assays was coprecipitated in one of the 0 hour incubation samples which contained purified sheep IgA (see Lane 7, Fig.7.6). It has been shown in other studies that microbial protein does not interfere with proteinase activity at concentrations up to 8 mg/ml of assay volume (Brock et al., 1982). It may also be noticed that rumen liquor, which was used as blanks in the proteolytic assays were virtually free of any other unidentified soluble proteins when the original bacterial protein was spun and loaded into the gel (see Plate. 7.3).

The colorimetric assay employed in this study was based on methods described by Sedmak and Grossberg, (1977). It was found that the curve for bovine serum albumin (BSA) was essentially linear from 0.5 to 50 μg of protein. The dose-response curves obtained for casein and sheep IgG were parallel to the BSA curve, but, as expected, the absorbance varied with the individual protein. The major advantage of G250 dye assay is that free amino acids and very small peptides do not react under the conditions prevailing in this assay. The assay per se is rapid, sensitive and useful in monitoring rumen protease activities using casein as substrate.

The rumen fluid used in the proteolytic studies contained mixed microbial population. In the radiometric assay, the level of incorporation of radioactivity into the microbial fractions was always found insignificant (data not shown). This was consistent with the observation that reductive methylation of proteins with ^{14}C-formaldehyde results in very low incorporation into microbial fractions (Wallace, 1983). Furthermore, it has been observed by the same author that the ^{14}C-labelled products of proteolytic digestion were not re-incorporated into acid-insoluble material during a 5 h incubation with rumen fluid. Thus the appearance of ^{14}C- in acid-soluble fractions was used as an alternative to measure its loss from acid-insoluble material in proteolytic measurements. The percentage recovery of sheep IgG and IgA obtained in this study, demonstrated clearly the resistance of both immunoglobulins to proteolysis in the rumen fluid during the first 4 hours of incubation. Even though there was no apparent cleavage of any of these immunoglobulin classes during this period, it was unclear whether more subtle clevage of the polypeptides was occurring.

Electrophoretic studies confirmed the resistance of these immunoglobulins to rumen proteolysis. Very little clevage of the molecules or the individual polypeptide chains was evident up to first 4

hours. Even with more extended incubations, proteolysis was limited and appears to yield some classical fragments, such as (Fab')2 and Fab. Bovine serum albumin was also relatively resistant to proteolysis, perhaps due to its compact globular structure.

Overall, the study demonstrated resistance of two major sheep immunoglobulin classes, IgG and SIgA to proteolysis in the rumen fluid at least during the first 4 hours of incubation. It is an observation made for the first time using rumen fluid as the source of proteolytic enzyme. It provides a basis to support the hypothesis that these immunoglobulins can survive in the rumen fluid. The rumen fluid used for studies on survival of antibody activity, contained the bacterial flora, but not the protozoa component. The latter was excluded to avoid premature binding of ciliate specific antibodies to ciliate antigens. The data from these studies indicate that the antigen binding functions of the immunoglobulin molecules is also preserved for a considerable time in rumen fluid. This was demonstrated directly for antibodies against ciliate surface antigens. Flow cytometry was used for the first time in the study of rumen ciliates and it provided an objective and quantitative comparison of antibody survival. The results indicate that antibodies can survive for several hours in simulated rumen conditions; long enough to interact with ciliates during the oral and rumen phases of the rumination process.

Section 8.0

Effects of Immune Serum, Saliva and Milk on Rumen Ciliates

8.1 Introduction

The presence of specific antibodies to rumen ciliates has been demonstrated in the serum, saliva and milk of lactating ewes (See Section 5.0). It has also been shown by flow-cytometry that these antibodies remain functional and still bind to rumen ciliates after 4 hours of incubation in rumen fluid (see Section 7.0) . In keeping with the aims of the present study, it is also imperative to investigate the effect of these antibodies on the ciliate population. This would not only reaffirm the antigenicity of the rumen ciliates to the host ruminant but would also provide evidence to support the concept that local immunity can influence rumen ciliate population and that this might be exploited to manipulate rumen function to improve productivity.

While the immunofluorescence and flow cytometry techniques served to detect the presence of antibodies against surface structures of rumen cilates, there are no published in vitro/in vivo techniques which permit the study of the effect of antibodies on live organisms, particularly under simulated rumen conditions. An attempt was made therefore, to observe the possible consequences that may result from the interaction of specific antibodies with ciliate surface antigen. Procedures were developed to observe and quantitate such effects when the ciliates were subjected to immunological reactions. The most effective way to record these results was found to be a video-phase-contrast microscopy, providing a record on a videotape.

8.2 Materials and Methods

8.2.1 Preparation of ciliates suspensions

The general procedures for the collection of rumen contents and the preparation of strained rumen fluid (SRF) have been described under Section 5.2. The required concentration of organisms in the test systems was achieved using either fresh SRF or freshly-prepared SRF diluted in McDougall's buffer (v/v: 1:1). These preparations were bubbled with 100% carbondioxide (CO_2), sealed with a rubber bung and maintained at 39°C in a water bath for 1 hr before transfer to reaction vials.

8.2.2 *Preparation of serum, saliva and milk samples for the incubation*

Frozen serum from the experimental animals was either unheated or inactivated at 56°C in a water bath for 30 minutes. Samples of saliva stored at - 80°C were thawed and used directly in the study. Similarly, frozen fat-free milk samples were thawed and either used directly or after the removal of casein by precipitating with 0.1M HCL and neutralizing with Tris-buffer. All the samples (serum, saliva and milk) were flushed with 100% (CO_2) and pre-incubated to 39°C in screw-capped vials (1ml).

8.2.3 *Experimental Design*

The immune samples (collected 2 weeks after immunization) from the six experimental ewes were pooled and diluted serially twofold in McDougall's buffer. The protocol shown in Table 8.1 was followed to set up the incubation systems:

Table 8.1 Experimental protocol

Proportion of Reaction Mixture			Concentration of Ciliates	Final dilutions Dilutions of the Body Fluids
Body Fluid	:	S.R.F[a]		
50µl	:	50µl	$5x10^4$/ml	1/2, 1/4, 1/8, 1/16, 1/32, 164, 1/128, 1/256

[a]S.R.F: Strained rumen fluid

8.2.3.1 *Experiment 8a*

Initially, incubation studies were conducted in screw-capped vials (in duplicate), containing an equal volume of SRF and the appropriate dilution of the immune samples. The final concentration of ciliates was $5x10^5$ ciliates/ml and the mixtures were incubated at 39°C for 45 minutes in an anaerobic jar filled with 100% CO_2. At the end of incubation, 10µl aliquots of the mixture from each vial were loaded by capillary action into haemocytometer chamber to view motility under a conventional phase-contrast microscope. In subsequent experiments, the reaction mixtures contained $5x10^4$ ciliates/ml. Five replicate vials were prepared for each

time point, to ensure the reliability of the observations. The percentage of motile ciliates was calculated, wherever possible. In order to calculate this, both the motile and immotile cells were counted directly in 20 fields (4 fields per replicate, 5 replicates per time point). The number of organisms in each field ranged from 20-40 at any one time when viewed under x250 magnification. The cells subject to incubation with either the preimmune serum or with McDougall's buffer were all considered 100% motile, based on several repeated observations during which no evidence of lysis or immobilization was noticed at any occasion. In the present study, following treatment of living rumen ciliates with immune serum, both entodiniomorphs and holotrichs showed a marked reduction in motility.Ciliates immobilized by antibody suffered no appreciable distortion except for the presence of a few sluggishly motile forms, particularly holotrichs. In each experimental series, control tubes in triplicate containing an equal volume of SRF (containing the ciliates at the precribed concentrations) and McDougall's buffer (1:1) were included to assess the survival of organisms in the absence of serum or secretions.

8.2.3.2 Experiment 8b

In order to minimize errors that might have arisen from delays due to the inevitable time lag in counting the many samples in the preceding study, a separate experiment was conducted at a selected level of serum dilution(1/16). The percentage of motile organisms was calculated from a total of 40 different fields examined on 5 separate replicates (8 fields per replicate and 5 relicates).

8.2.3.3 Recorder-set-up

The behaviour of the organisms observed in the above study, as viewed by the phase-contrast microscope, was projected field by field onto a TV screen via a video-recorder. The projected image for each field of view, containing 20-40 ciliates, was monitored and continually recorded (Videorecorder, Model SVHS-EIS, National Panasonic Ltd., Tokyo). The edited videotape, accompanied by an audio-presentation, is made available herewith as part of the thesis. The transcript of the audio-presentation is attached (See appendix I).

8.3 Results

All cells with a definite sign of motion were classified as motile. This included not only those exhibiting translocation but also those displaying activity of ciliary tufts associated with the ad-oral zone (a region

around the oral cavity) in entodiniomorphid species like Polyplastrons. The motility of organisms cultured in McDougall's buffer alone or in theMcDougall's buffer plus pre-immune fluids was excellent throughout the period of incubation, all ciliates retaining vigorous ciliary function. No toxic effects of sera or secretions were noted in these control incubations.

The results in Table 8.2 illustrate the effects of serum from animals immunized with rumen ciliates on the motility of the purified organisms in vitro. In general, the antiserum had least effect on small entodiniomorphid ciliates (E.nanellum and E. exiguum), which remained active at dilution of antibody that immobilized most other species. Some holotrichs exhibited sluggish movements for a time before they became immotile.

Among the different species of rumen ciliates investigated- the holotrichs, Isotricha spp. and entodiniomorphs- Entodinium caudatum, Entodinium longinucleatum, Entodinium bursa, Diplodinium spp., Polyplastron multivesiculatum showed greatest reduction in motility, although in Polyplastrons the movement of ad oral cilia persisted sluggishly until the end of incubation. All of these species were found consistently in the rumen contents of the two fistulated wethers which were used as the source of rumen ciliates.

The results in Table 8.2 indicate that the effect of the immune serum on motility was essentially constant up to a dilution of 1:64 (Experiment 8a). Beyond this dilution, the proportion of motile organisms as a percentage of total organisms was indistinguishable from negative controls. The behaviour of rumen ciliates was examined as a function of time and serum concentration. At lower dilutions of immune serum (1:2 to 1:8), the effect on motility was rapid and maximal immobilization was observed within 20 minutes. At higher dilutions, organisms incubated in immune samples demonstrated active motility at early time periods. However, at the end of 45 minutes, dilutions of immune serum up to 1:64, induced levels of immobilization similar to those shown in Table 8.2 were noticed between preimmune and immune samples. By this criterion, an immobilization titre of 1:64 was obtained for the pooled immune serum samples.

This dilution is considerably less than the titre of the serum when assessed for surface binding by IFAT or flow cytometry (1:200 in each case). The findings suggest that the abrupt cut-off at 1: 64 may reflect the rather insensitive nature of " motility " as an index of cell function - lesser degrees of impairment of function may escape notice by visual inspection. In a record experiment (Experiment 8b), where the length of exposure of

ciliates to antibody were controlled more carefully, the extent of immobilization was essentially identical to that observed in Experiment 8a.

An important question arises as to whether antibody caused immobilization of the ciliates or whether it was cytotoxic. Attempts were made to assess viability using routine dye exclusion methods (trypan blue and eosin), but the technique was not satsifactory when applied to these organisms. However, the possibility that immobilization resulted from complement-mediated killing was investigated. Immobilization occurred to a similar degree whether or not complement in the antiserum was inactivated by heating at 56°C for 30 minutes. Furthermore, the antiserum was also treated with methylamine as well as heating at 56°C, in order to definitively inactivate all traces of complement activity. Again, there was no reduction in the immobilizing activity of the antiserum. Because pre-immune serum also did not reduce motility, it is concluded that antibody alone, rather than complement or toxic factors in serum, was responsible for the effects of immune serum on ciliate motility.

When incubations of ciliates ($5x10^4$ cells/ml) were carried out in the presence of saliva (1:2 to 1:32) or milk (1:2 to 1:256) respectively, no significant differences in motility between immune and preimmune samples were noticed. However, at dilutions between 1:2 to 1:4, with milk, occasionally a few organisms (holotrichs) were observed to move around with a tail like structure which trailed behind these organisms. In addition, a certain degree of clustering was observed (data not shown).

Table 8.2 Effect of different dilutions of preimmune and postimmune sera on rumen ciliates

Pooled serum Motility/ Immotility	Final serum dilution	Motility of organisms after incubation at 39°c for 45min.		Percentage of	
		Motile	Nonmotile	Motile	Nonmotile
		Mean ± s.e (n=20)			
Experiment 8a					
Postimmune	1/2	4.15±0.45	20.8±1.0	16.6	83.4
Postimmune	1/4	4.70±0.61	22.8±1.3	17.1	82.9
Postimmune	1/8	4.05±0.57	22.5±1.4	15.3	84.7
Postimmune	1/16	4.75±0.46	24.3±1.5	16.4	83.6
Postimmune	1/32	5.10±0.59	21.5±1.3	19.2	80.8
Postimmune	1/64	5.75±0.65	22.7±1.5	20.2	79.8
Postimmune	1/128	all	none	100	0
Postimmune	1/256	all	none	100	0
Preimmune	all dilutions	all	none	100	0
McDougall's buffer				100	0
Experiment 8b					
		Mean ± s.e (n=40)			
Postimmune	1/16	3.70±0.35	23.2±0.94	13.8	86.2
Preimmune	1/16	all	none	100	0
McDougall's buffer		all	none	100	0

8.4 Discussion

The effect of antibodies on rumen ciliates has been investigated for the first time in this study. The antibodies used were raised against a mixed population of rumen ciliates obtained from two rumen-fistulated wethers. Fresh samples of ciliates were obtained for each experiment and the organisms were always comprised of entodiniomorphs and holotrichs. An anaerobic incubation system was designed specifically for the purpose of observing ciliate behaviour. The validity of the system was demonstrated (at least for a period of 4 hours) by the maintenance of an active population of cells in negative controls which had either equal volumes of strained rumen fluid (SRF) and McDougall's buffer (MB) or SRF and preimmune samples, respectively.

Under the conditions adopted to determine the percentage of motile and non-motile cells in the samples, the identification of all of the individual species was found to be very difficult for certain reasons: (1) when the organisms were collected soon after feeding the host animal, the ciliates were so full of storage polysaccharides that no internal details were visible and it was difficult to see the skeletal plates which are one of the keys for proper identification of species. (2) Similarly, with an increase in time after feeding, the amount of polysaccharide decreases and staining is required to observe the plates and the nuclei. Adoption of staining techniques was not compatible with studies on the effect of antibodies on rumen ciliates. Therefore, the identification of genera or species was made *in situ* based on the morphological features which were visible by phase contrast microscopy.

Although the subject of immune responses to protozoan parasites is being studied extensively with protozoan parasites, very useful observations have also been made on the effect of serum on free-living ciliates. It has been described that when the actively-swimming organisms belonging to the genus Glaucoma had been in contact with heated immune sera, they showed a rapid loss of motility and a characteristic shrinkage of the outer pellicle (Robertson 1939). They agglutinated progressively and settled to the bottom of the vessel. In some other studies it was reported that when healthy Paramecia were mixed with appropiate dilutions of homologous antiserum, they were immobilized within a period of 2 hours. The antigens involved are major surface proteins which are type specific and allow identification of Paramecia into a series of immobilization antigen types (Bernheimer and Harrison, 1940; Beale, 1948). In the present study with the rumen ciliates, although the loss of motility was observed clearly under the microscope,

neither agglutinative reactions nor clustering were seen with any of the immune samples, except occsionally with the milk samples. One of the possible explanations may be the nature of the ciliated organisms used in the study. For example, with holotrichous Glaucoma species, rapid agglutination occurred and the titre levels ranged from about 1:800 to 1:3000 with the various sera (Robertson, 1939): whereas with the Paramecia, immobilization is the main reaction with titres of approximately 1:1000 (see review by Beale, 1957; Harrison and Fowler, 1946). In the present study, following treatment of living rumen ciliates with immune serum, both entodiniomorphs and holotrichs showed a marked reduction in motility, the latter showing shrinkage during immobilization. It has been mentioned that the reaction of Paramecia in specific antiserum resulted in the development of a gelatinous and sticky product towards the tip and between the cilia (Harrison and Fowler, 1946). Frequently, part of this product was extruded into the medium and some of it got swept to the rear to form a "sea-anchor" arrangement (probably shed immune complexes) which trailed behind the organism as long as latter retained motility. Tail-like structures were occasionally observed with the rumen holotrichs when they were incubated with immune milk samples in the present experiments. By treating living Paramecia with antibody labelled with fluorescein, Beale and Kacser (1957) showed that specific antibodies bound to the tips of cilia and they were also found deposited on the whole surface of the organisms. These authors concluded that the antigen concerned with the immobilization reaction, is a diffusible substance originating from the surface of the Paramecia, and also from the cilia. The distinct fluorescence which was observed in the routine indirect immunofluorescence assays of the surface membranes of both the entodiniomorphs and holotrichs seems to suggest that rumen ciliates too possess similar features on their surface. In addition, a bright halo was observed covering the periphery of holotrichs, suggestive of the binding of antibodies to the tips of the cilia (see Plate 5.1).

It is not known whether, after immobilization, recovery of motility of rumen ciliates was possible. It seems not unreasonable to suppose that the cilia and other surface structures can shed the accumulations of antigen-antibody product and then resume normal activity. The shedding of these accumulations has been reported in free-living ciliates, such as Tetrahymena, Colpoda and Paramecia (Robertson, 1939). In the case of Tetrahymena and Colpoda species that had regained motility, the organisms did not react with antibody of the same kind as

that which caused the immobilization. This suggests that the relevant surface antigens were modulated by antibody but studies were not extended to examine whether the antigens were re-expressed. Although the importance of a similar phenomenon cannot be ignored in rumen ciliates, there are practical difficulties in setting up an experimental system to sustain these organisms under anaerobic conditions for longer than 4-5 hours. Until such a system is designed which allows optimal survival of these organisms for periods of at least 24 hours without any non-specific loss of motility, it would be hard to investigate modulation and re-expression of surface antigens. Further work is also needed to devise ways of assessing ciliate viability in order to study the cytotoxicity of antibodies.

In the estimation of the percentage of motile ciliates, a method was developed for quantitative evaluation of ciliate motility that would provide information on individual cells which are not motile. Objective methods to assess motility were difficult to devise, owing to the relatively large and very variable size of the organisms (20-200m) and the variety of species present in SRF. Procedures were developed to ensure that always a fixed volume of incubation mixture was loaded into a haemocytometer chamber, previously warmed to 39°C immediately before use. The phase-contrast field of view, containing 20-45 organisms, was selected at random for each aliquot and the counting on each replicate completed with a time lag of 1 minute from the time of pipetting the sample from each vial. No difficulty was experienced in counting non-motile organisms, other than the normal variation in number from field to field. Occasionally a few sluggish forms, particularly holotrichs, were encountered. They were counted as motile. No evidence of agglutination was observed. Counting was repeated on several fields per replicate and it was possible that motile organisms in adjoining fields might have been counted more than once. The time required for such counts was long in Experiment 8a, with 8 different dilutions (each replicate 5 times). Because this introduced a considerable time lapse, which might have caused considerable non-specific loss of motility due to exposure of the organisms to atmospheric conditions, a simpler experiment (8b) was performed using one selected dilution level (of 1:16). The proportion of immobilized organisms was found to be similar, regardless of the very much reduced time required to count the organisms (see Table 8.2). Within the limits of the subjective estimate, the percentage of nonmotile organisms appeared to be a true reflection of the effect of the antibodies of the immune sample.

An immobilizing effect similar to what has been observed with the heated immune sera was produced by both unheated preimmune and immune sera (results not shown). However, it is probable that the two phenomena were not the same. The immobilization caused by unheated preimmune and immune sera might have been due to either (1) the direct effect of complement activation by the alternative pathway or (2) complement fixation by the classical pathway. Heat inactivation effectively removed the possibility of the direct effect of complement on the organisms, but this was re-enforced by further treatment of the serum with methylamine. The effect of unheated pre-immune serum could have been due to the presence of natural antibodies (see Section 5.0). Those antibodies without inactivation of the complement might have caused complement mediated killing of the cells. In contrast, the amount of natural antibodies in pre-immune serum was insufficient to cause immobilization after complement inactivation.

Immune saliva and milk samples did not show any retarding effect on the motility of the rumen ciliates . These results must be interpreted carefully. In the first instance, complete immobilization is a rather insensitive criterion for the effects of antibodies on the behaviour of rumen ciliates. In the rumen microenvironment, even quite subtle effects of antibody on motility or on the efficiency of the cilia could alter the competitiveness of the protozoa in the overall rumen microflora and lead to loss of niche and attrition of the ciliate population (Shedlofsky and Freter, 1974; Kilian et al., 1988). The time for which ciliates could be incubated with these samples could not be extended beyond 4 hours, because beyond this period, the individual organisms even in the negative control samples started dying. It was assumed that under the test incubation system, the conditions must have become unfavourable for further survival of the organisms. Attempts to drop the concentration of cells below $5x10^4/ml$ to $5x10^3/ml$ in the incubation mixture resulted in non-specific loss of motility in the controls. In view of these findings, longer term effects of incubation with antibodies in saliva and milk could not be investigated satisfactorily.

Within the established conditions, the reason for the low level of activity of milk antibodies to ciliates may be explained on the basis of titres of anti-ciliate antibodies registered in the secretions of the lactating ewes. Titres for IgG antibodies and for IgA antibodies were1:320 and 1:80 respectively (see Section 5.0). In a study on the effect of antibodies on Cryptosporidium, bovine colostrum with a titre of 1:10 against the oocyst wall and 1:40 against the sporozoites, failed to alter the course of

cryptosporidiosis, when administered orally to humans (Saxon and Weinstein, 1987). Subsequently, it has been shown in a recent study that bovine colostral whey containing a titre of specific antibodies as high as 1:200,000 conferred protection to challenge of neonatal calves (Fayer et al., 1989a) with $5x10^6$ oocysts. When incubated with cell concentrations of $5.6x10^6$ sporozoites/ml, the same hyperimmune colostrum produced virtually complete neutralization of sporozoites in 10 minutes (Perryman et al., 1990). In contrast to rapid sporozoite neutralization by polyclonal antibodies in hyperimmune colostral whey, sporozoite neutralization mediated by a combination of monoclonal antibodies in hybridoma culture supernatants proceeded in a time-dependent manner. At higher concentrations, these antibodies reduced the intensity of infection produced by challenge with oocysts. These studies illustrate clearly the need for high titres of antibodies to demonstrate a protective action against the target organisms.

In the present study, it may be noted that although immunization with rumen ciliates elicited enhanced IgA and IgG antibody responses in the milk of lactating ewes, the titres may not be sufficiently high to demonstrate either gross agglutination or immobilization. Notwithstanding this observation, other effects of these antibodies on the surface antigens of ciliates should be given due consideration, because very clear surface binding of milk antibodies to rumen ciliates was demonstrated through flow-cytometry studies (see Section 5.0). Furthermore, some evidence of the formation of gross antigen-antibody precipitates and shedding of complexes from ciliates incubated in immune milk whey has been observed in the current experiments. The neutralizing ability of ruminant milk antibodies on Cryptosporidium sporozoites has been reported in a recent study (Riggs et al., 1989). These properties of milk make it possible to suggest that the observed titre in the present study might be adequate to function as protective ' preformed ' immunity. That is, if lambs receive antibody from milk before the entry of rumen ciliates, the establishment of these organisms in the rumen might be prevented or delayed.

Some technical aspects of salivary antibodies also require consideration. Storage of saliva without preservatives such as glycerol has been shown to reduce antibody life (Butler et al., 1991). Further, these authors observed that there are difficulties experienced in the assessment of humoral immunity in regional body fluids. Firstly, it is not clear how salivary antibody can be equated to that in serum. Serum is homeostatically-regulated while the rate of salivary flow varies from

animal to animal and with the form of salivary stimulus. It should be remembered that salivation was induced by pilocarpine in these studies and that this would lead to the production of a dilute secretion. This measure had to be taken in order to overcome the practical restraints experienced in the collection of unstimulated saliva from sheep.Secondly, exocrine body fluids may contain degradative factors such as proteases of either host or bacterial origin, which are either absent or inhibited in serum. Milk is also an exocrine secretion. Therefore, similiar conditions may prevail in milk as well as in saliva. There are no published studies in which systematic comparisons have been made with regard to the stability of antibodies in the respective secretions. In the present study, although specimens of saliva were always stored at -80^{o}C, preservatives were not incorporated to avoid further dilutions of the stimulated saliva. In the light of the foregoing, it could be only surmised that the mode of storage of saliva might have led to some loss of antibody activity.

It must be emphasized that while considering the effect of salivary antibodies on rumen ciliates, the confounding effect of whole saliva should be given due consideration, particularly the enzymes and the presence of salivary agglutinins. Two types of enzymes need special mention, lipase and lysosomal enzymes. Though ruminant saliva does not seem to have the major enzyme amylase unlike in the monogastric animals and humans, the secretion of a lipase (pregastric esterase) in the pharyngeal region of neonatal ruminants has been reported (Moore and Noble, 1975). In human milk, there is considerable evidence that non-immune human milk kills trophozoites of Giardia lamblia mediated by bile-salt stimulated lipases (Gillin et al., 1985; Hernell et al., 1986). While this aspect has not been investigated in the milk of sheep, it is possible that pregastric esterases in saliva could also have a similar effect on rumen ciliates.

In another study, the lysosomal enzymes of human crevicular fluid has been shown to inhibit the attachment of certain bacterial organisms (Streptococci and bacteriodes) to simulated tooth surfaces (Cimasoni et al, 1987). Neither the presence of similar enzymes in ruminant saliva nor their similar effects on rumen ciliates have been examined. Another most important component of whole saliva is the presence of salivary agglutinins. Apart from the ability of the latter to react with bacterial surface structures and block their adherence (Gibbons and van Houte, 1975), their complexing with the immunoglobulins has been reported in human saliva and amniotic fluids (Eggert, 1980). There

is paucity of information on interactions between rumen cilates and these components of ruminant saliva. Further, the recognition of these interactions in secretions containing non-mucin glycoproteins, immunoglobulins and mucins illustrates the difficulties inherent in elucidating the effects of salivary antibodies on rumen ciliate population. It is of great interest that in other sites inhabited by a mixed commensal microflora, nonspecific factors and competition with other microorganisms increase the effectiveness of secretory antibodies in preventing colonization (Shedlofsky and Freter, 1974; Kilian et al., 1988). Studies of this nature were beyond the scope of the present study.

The overall impact of the study was, however, to demonstrate the potential of specific-serum antibodies to immobilize rumen ciliates. It does not preclude the possible protective role that antibodies of milk and saliva could play following immunization against rumen protozoa. Possible effects of these secretory antibodies on individual species, and the long term impact on the rumen ciliates of exposure to secretory antibody were not demonstrated due to limitations of the test systems employed. Other ways of assessing the effects of antibodies on ciliate behaviour were considered. One such is the subject of another study, designed to obtain an insight into the effect of antibodies on the predatory behaviour of rumen ciliates. This is described in the accompanying section.

Section 9.0

Effect of Antibodies on Engulfment of bacteria by Rumen Ciliates

9.1 Introduction

It has been demonstrated in many studies that the rumen ciliates prey heavily on rumen bacteria and utilize them for their metabolic functions (Coleman, 1964; 1967a; 1967b; 1972; 1975; Coleman and Hall, 1969). In all of these studies, radiometric assays using isotypically-labelled bacteria have been used to obtain the relevant data on the uptake of bacteria by rumen protozoa,. As the interruption of this process could achieve one of the ultimate objectives of the present efforts, it was considered necessary to investigate the effect of antibodies on the engulfment activity of rumen ciliates on bacteria. Therefore, short experiments were designed (up to 40 minutes duration) with mixed rumen ciliates and [^{35}Sulphur-methionine-labelled] Escherichia coli to determine whether antibodies in immune samples could cause reduction in the predatory activity of ciliates.

9.2 Materials and Methods

9.2.1 Source of protozoa for engulfment study

As before rumen fluid from two rumen-fistulated wethers maintained on sheep-pellets (800g pellets- 65% digestibility +7.5% crude protein) and 1kg oaten straw fed once daily) was used as the source of protozoa. It was usually collected 3-4 hours after the morning feed. The collected rumen fluid was taken to the lab within ten minutes, strained through a defined-aperture nylon (300μm) to remove plant debris and the strained rumen fluid (SRF) bubbled with 100% CO_2 for 2-3 minutes and maintained in a water bath at 39°C before drawing samples for engulfment studies.

9.2.2 Enumeration of protozoa in strained rumen fluid (SRF)

The method for enumeration of protozoa using haemocytometer is described in Section 4.0. The concentration of entodiniomorphs ranged between 0.8×10^6 - 1.2×10^6 per ml and that of holotrichs was between 4×10^4 - 8×10^4 per ml.

9.2.3 *^{35}Sulphur-methionine labelling of Escherichia coli*

Samples of E.coli were collected from overnight culture in liver-broth (L-broth) checked for absorbance (1.0), enumerated using a haemocytometer and adjusted to (2.25×10^{10} per ml). 1ml of these samples was subcultured in 10 ml of L-broth for 2 hours at 37°C. Samples were grown to an absorbance of 0.5 and centrifuged at 2000g for 5 minutes. The bacteria were then washed x 3 with minimal medium, resuspended in 5 ml minimal medium lacking sulphate and incubated for 1 hour at 37°C (shaking). At the end of 1 hour starvation, [^{35}S]-methionine (Amersham Searle, 1.05 Ci per ml) was added to a final concentration of 5μCi per ml and incubation continued with shaking at 37°C for 30 minutes. At the end of incubation, the labelled samples were centrifuged at 2000g for 5 minutes, the pellets were washed x3 with L-broth and they were then resupended in 1 ml of L-broth containing 15 % glycerol. The suspensions were then aliquoted into 6 Eppendorf tubes (150 μl per tube) and placed in dry ice for 3 hours before being stored at -20°C until further use.

9.2.4 *Pre-immune and immune sera*

Frozen samples of pre-immune sera (collected from six experimental ewes) were thawed and pooled and 1 ml aliquots were inactivated at 56°C in a water bath for 30 minutes. Samples of immune sera (collected 2 weeks after priming) were also treated in a similar manner. Both sera were diluted serially twofold in McDougall's buffer (see Table7.1) and flushed with 100% CO_2 and pre-incubated to 39°C before being used in the reaction mixture.

9.2.5 *Incubation system for engulfment studies*

The reaction mixture contained 1:1 volume of SRF containing ciliates (Entodinia 4×10^5- 6×10^5 per ml; holotrichs 2×10^4 -4×10^4 per ml) and heat-inactivated serum dilution in 1 ml rubber sealed screw-capped vials. A 1: 1 (v/v) mixture of SRF containing ciliates at similar concentrations and McDougall's buffer served as the standard to monitor the normal uptake of bacteria. Prior to the addition of SRF to the reaction vessels, the incubation mixture was added with L-cysteine-hydrochloride (neutralized before use) to a final concentration of 0.01% and incubated at 39°C in anaerobic jars filled with 100 % CO_2. Incubation was continued for a set period of time as shown in Table 9.1. At the end of each incubation period, 10μl of ^{35}S-labelled E.coli (10^8 per ml) was

inoculated into the reaction vessels and engulfment was allowed to occur for set time periods as shown in Table 9.1. Separate vials in duplicate were used for each time point. Samples of labelled E.coli were always flushed with 100 % CO_2 and pre-incubated to 39°C before inoculation.

Table 9.1 Experimental protocol

Dilution of serum	Set time for pre-incubation in minutes prior to inoculation of ^{35}S-E.coli	Set time for engulfment of ^{35}S-E.coli in minutes
1:2	0, 10, 20, 40	0, 5, 10, 20, 40
1:8	as above	as above
1:16	" "	" "
1:32	" "	" "
1:64	" "	" "
1:128	" "	" "

9.2.6 *Measurement of uptake of bacteria by rumen ciliates*

Whole samples, incubated for various period of time after the addition of ^{35}S-E.coli, were rapidly centrifuged at 4°C first at 300g for 1min to separate all protozoa and then at 2000g for 3 minutes to recover the residual free bacteria. The ciliates were washed twice in phosphate buffered saline (PBS-pH 7.2) and resuspended in 1ml of scintillant to determine the incorporated radioactivity. The residual bacteria were washed twice in the same buffer before resuspension in 1ml of scintillant for estimation of radioactivity. Samples of supernatant remaining after removal of the residual bacteria were similarly mixed with 1ml scintillant for estimation of ^{35}S- label which was free in the medium.

9.2.7 *Statistical analysis*

The significance of differences between uptake of different sample groups was determined by one way analysis of variance for independent samples.

9.3 Results

The engulfment of bacteria by the ciliates was measured by the uptake of ^{35}S-labelled E.coli of known specific activity. Experiments were carried out initially to select the required time of exposure of rumen ciliates to antibody and to choose an appropriate time over which to assess the uptake. In Fig.9.1, A and B show the effect of various dilutions

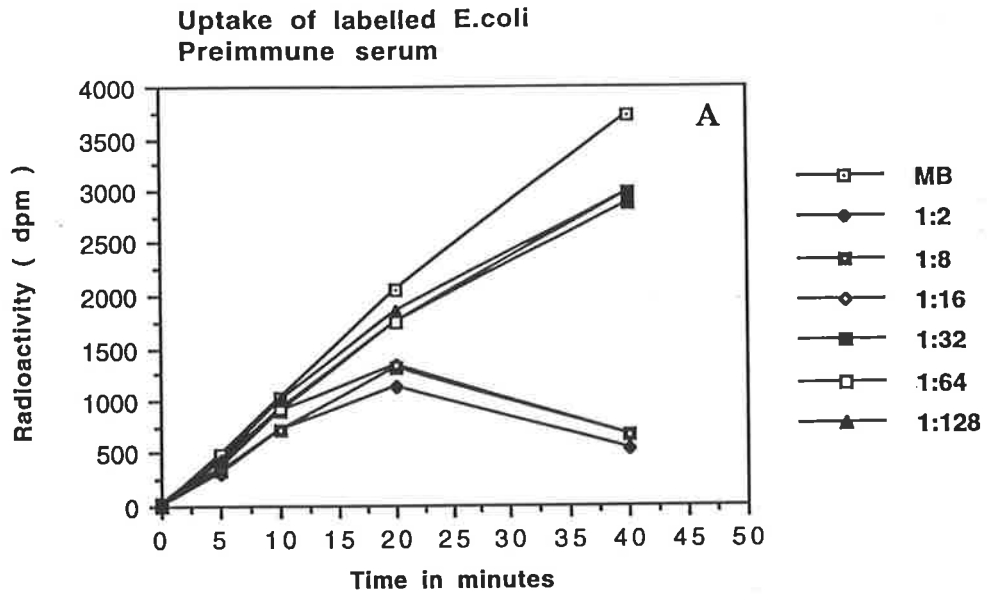
of pre-immune and immune sera on this process at zero pre-incubation time, i.e., 0 exposure to antibody prior to the inoculation of labelled E.coli. When the amount of radioactivity incorporated into protozoa was plotted against time, it was observed to be essentially linear over the 40 minute incubation period in the standard incubation buffer (McDougall's buffer). Ciliates incubated in higher dilutions (1:32 to 1:128) of pre-immune serum exhibited approximately linear uptake for the first 10 minutes and thereafter showed a slight deviation up to the end of 40 minute period. Ciliates incubated in lower dilutions (1:2 to 1:16) of the same serum showed a linear uptake for the first 10 minutes, although at a lower rate than the control organisms. Beyond 10 minutes (i.e., 20 minutes from commencement of exposure to serum) there was a decline in uptake. Ciliates incubated in immune serum, at all of the dilutions except 1:128, showed the lowest uptake during the first 10 minutes and thereafter no further net increase in radioactivity.

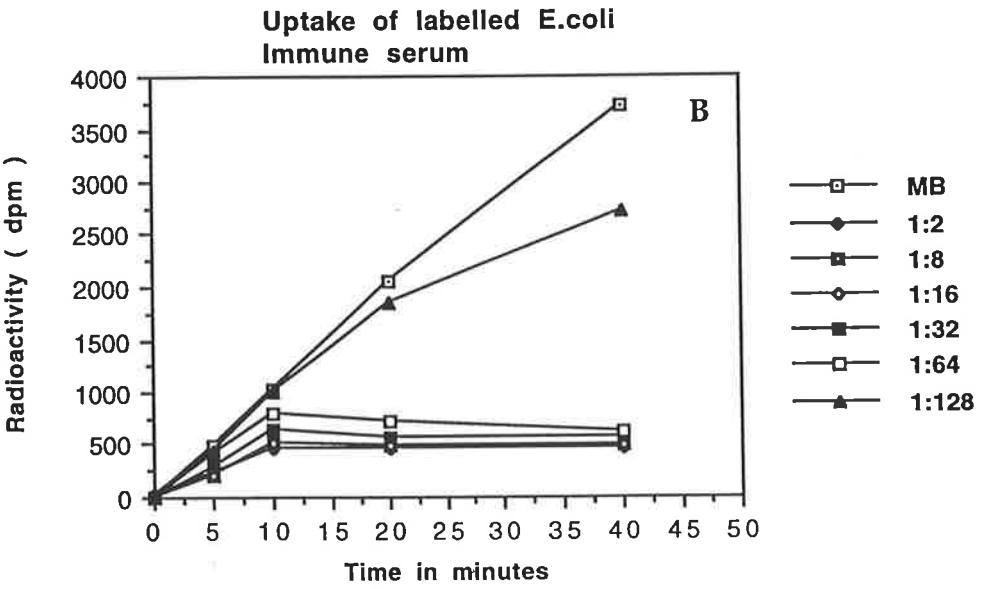
When the uptake of bacteria by ciliates was investigated after pre-incubation for 10 minutes in pre-immune serum, similar trends were noticed as those observed for zero pre-incubation time (Cf: Fig.9.1-A and C). However, organisms pre-incubated in immune serum (i.e., 10 minute exposure to antibody prior to the inoculation of E.coli) demonstrated less uptake. The curve corresponding to 1:128 dilution became less steep and even the curves for 1:32 and 1:64 dilution started merging with those corresponding to lower dilutions (Cf: Fig.9.1-B and D).

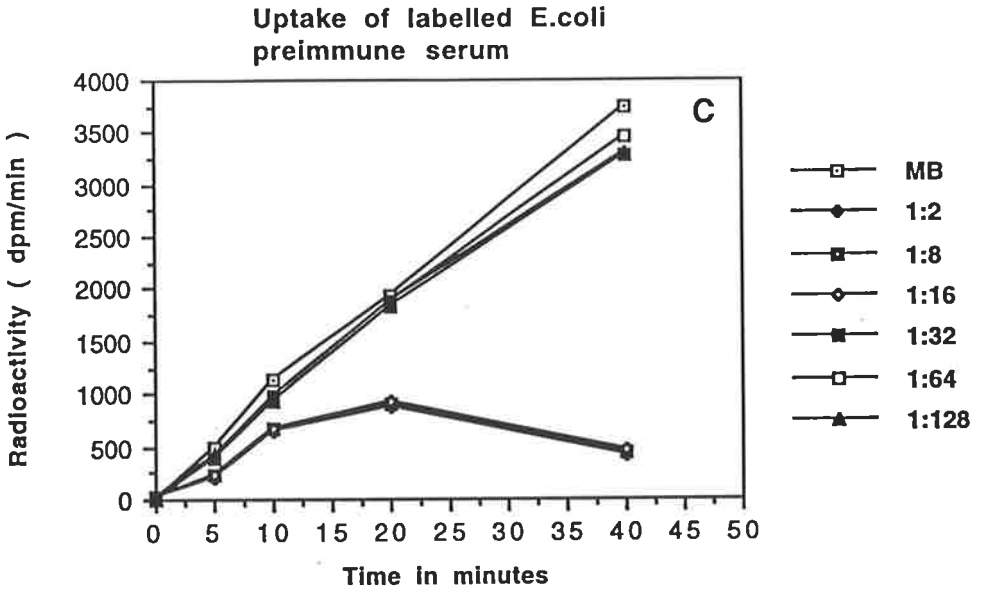
When the labelled E.coli was inoculated after the pre-incubation period of 20 minutes, the uptake by ciliates incubated in standard buffer continued to appear linear over the 40 minute engulfment period. Ciliates incubated in various dilutions of pre-immune serum showed a similar trend, although at a slightly lower rate than that observed with the shorter pre-incubation times. The level of uptake by ciliates incubated in immune serum was observed to be lowest at all of the dilutions used in the study other than 1:128 (Fig.9.1-D and F).

When ciliates were subjected to pre-incubation of 40 minutes, the kinetics of uptake in standard buffer lost its linearity over 40 minute engulfment time. Ciliates incubated in higher dilutions (1:32 to 1:128) of pre-immune serum exhibited comparable trends like those organisms in standard buffer (see Fig 9.1 G) whereas ciliates in immune serum showed a profile similar to what was observed after 20 minute pre-incubation (Cf: Fig. 9.1-F and H).

Figure 9.1 Effect of various dilutions of pre-immune and immune sera on the kinetics of uptake of bacteria after various pre-incubation time: A and B, zero preincubation (page 182); C and D, 10 min preincubation (page 183); E and F, 20 min preincubation(page 184); G and H, 40 min preincubation(page 185). MB, McDougall's buffer.


Uptake of labelled E.coli
Preimmune serum
A
Radioactivity (dpm)
4000
3500
3000
2500
2000
1500
1000
500
0
0 5 10 15 20 25 30 35 40 45 50
Time in minutes
MB
1:2
1:8
1:16
1:32
1:64
1:128


Uptake of labelled E.coli
Immune serum
B
Radioactivity (dpm)
4000
3500
3000
2500
2000
1500
1000
500
0
0 5 10 15 20 25 30 35 40 45 50
Time in minutes
MB
1:2
1:8
1:16
1:32
1:64
1:128


Uptake of labelled E.coli
preimmune serum
C
Radioactivity (dpm/min)
4000
3500
3000
2500
2000
1500
1000
500
0
0 5 10 15 20 25 30 35 40 45 50
Time in minutes
MB
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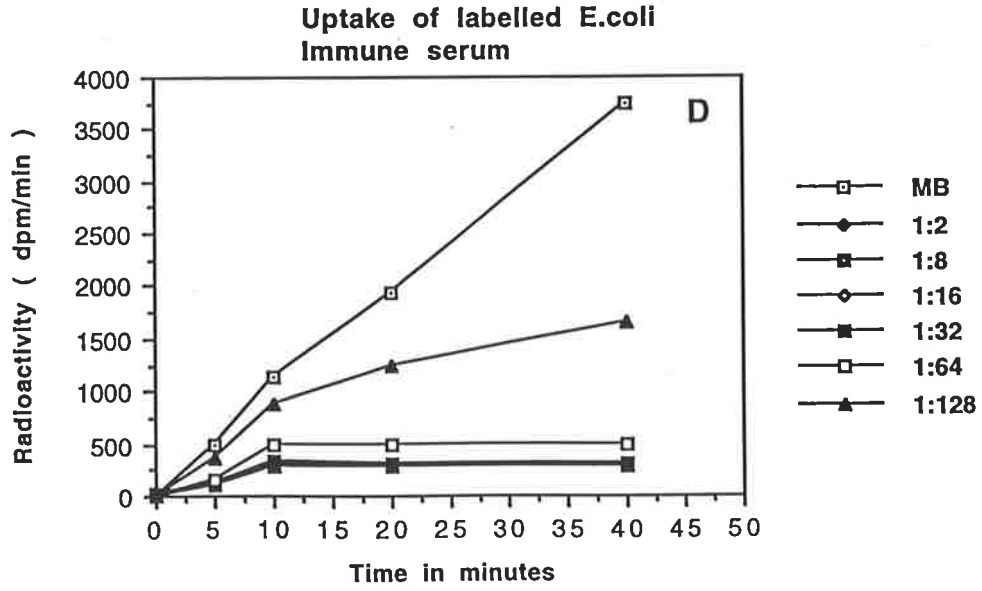
1:8
1:16
1:32
1:64
1:128


Uptake of labelled E.coli
Immune serum
D
Radioactivity (dpm/min)
4000
3500
3000
2500
2000
1500
1000
500
0
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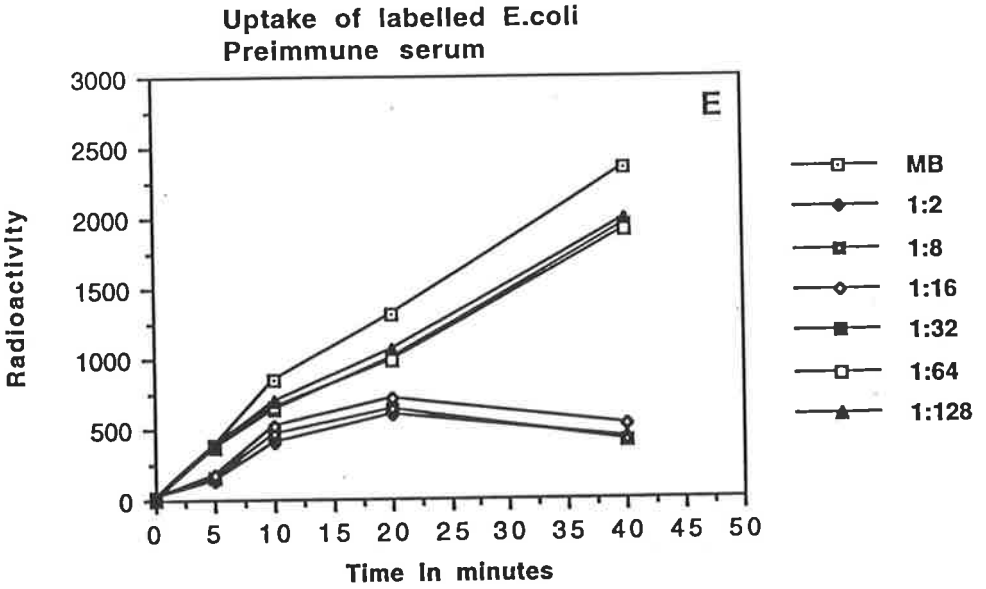
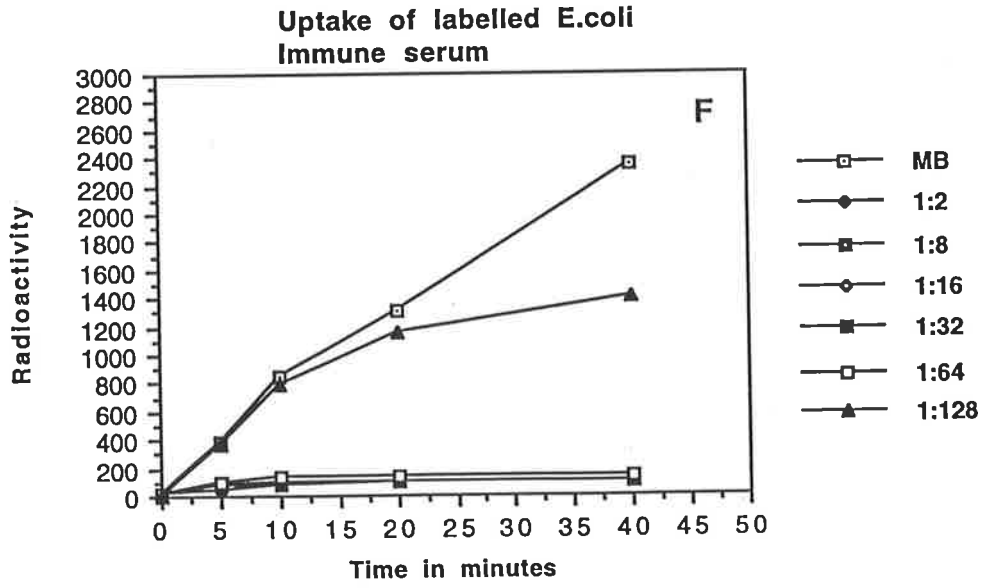
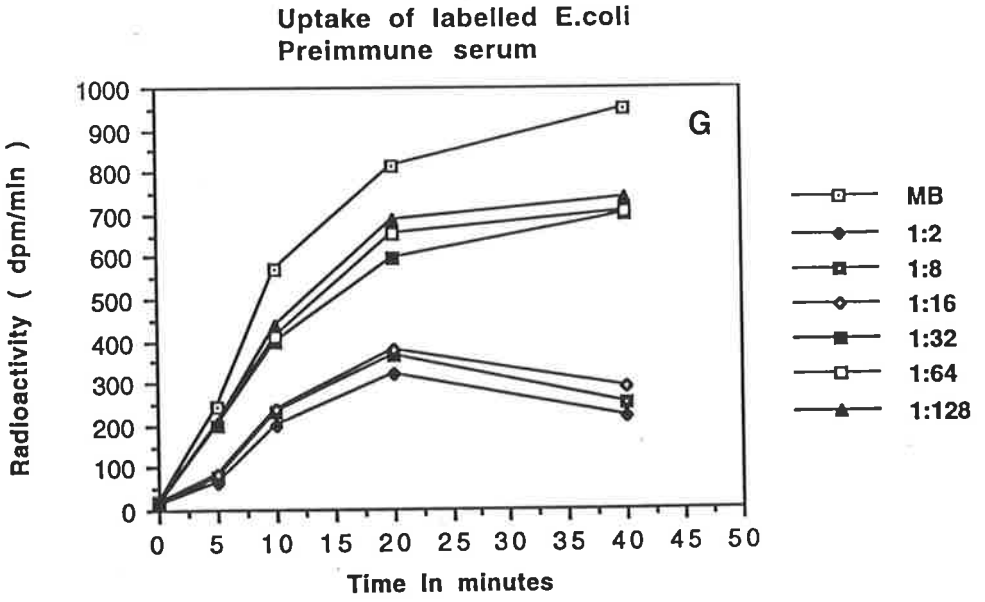
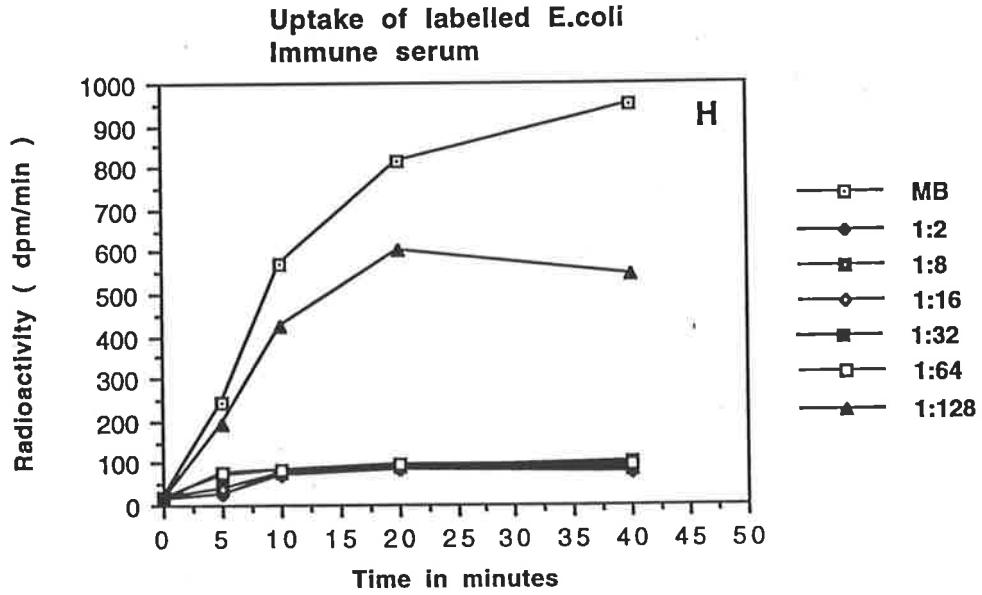
Time in minutes
MB
1:2
1:8
1:16
1:32
1:64
1:128


Uptake of labelled E.coli
Preimmune serum
E
Radioactivity
3000
2500
2000
1500
1000
500
0
0 5 10 15 20 25 30 35 40 45 50
Time in minutes
MB
1:2
1:8
1:16
1:32
1:64
1:128


Uptake of labelled E.coli
Immune serum
F
Radioactivity
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0 5 10 15 20 25 30 35 40 45 50
Time in minutes
MB
1:2
1:8
1:16
1:32
1:64
1:128


Uptake of labelled E.coli
Preimmune serum
G
Radioactivity (dpm/mln)
Time in minutes
MB
1:2
1:8
1:16
1:32
1:64
1:128


Uptake of labelled E.coli
Immune serum
H
Radioactivity (dpm/mln)
Time in minutes
MB
1:2
1:8
1:16
1:32
1:64
1:128

It may be reiterated that under the assay conditions used in the study, no significant differences were observed in radioactivity counts among any of the lower dilutions (1:2; 1:8 and 1:16) of preimmune serum after incubation for 0, 10 and 20 minutes respectively. With the immune serum, even those differences could not be detected. Therefore, 1:16 dilution represented the lowest dilution tested on a subsequent study. At dilutions of 1:16 and above (i.e., up to 1:64), significant differences ($P< 0.01$) were observed between pre-immune and immune sera with respect to the uptake of labelled E.coli. Dilution of 1:64 represented the highest limit which demonstrated clearly the effect of higher level of antibody in the immune serum in comparison to that of pre-immune serum. At dilution above 1:64, i.e., 1:128, no significant differences in uptake were observed between pre-immune and immune sera. Based on the foregoing observations, experiments were conducted with selected dilutions (1:16 and 1:64) of preimmune and immune sera to assess uptake by ciliates after pre-incubation for 20 minutes.

When the amount of radioactivity incorporated into protozoa was plotted against time, kinetics similar to what was seen during the preliminary experiments were observed (see Fig 9.3). The curve representing the standard buffer (McDougall's buffer) reflected a linear uptake of bacteria by mixed rumen ciliates. The data on the incorporation of radioactivity into the different fractions of the incubation mixture are shown in Table 9.2. After a maximum of 40 minutes of engulfment, over 33% of the labelled bacteria disappeared, of which approximately 30% was found in the ciliate pellets and the remaining 3% was assumed to have lost the label into the cell-free supernatant.This has been illustrated graphically in Fig 9.2

Under the conditions used in the study, there is little incorporation of ^{35}S-E.coli into the protozoal fraction at zero minute incubation. No non-specific association of killed (boiled) ciliates with the radio-labelled bacteria at either 4^{o}C or 39^{o}C was observed (data not shown). Neither uptake by viable ciliates was observed at 4^{o}C (data not shown).

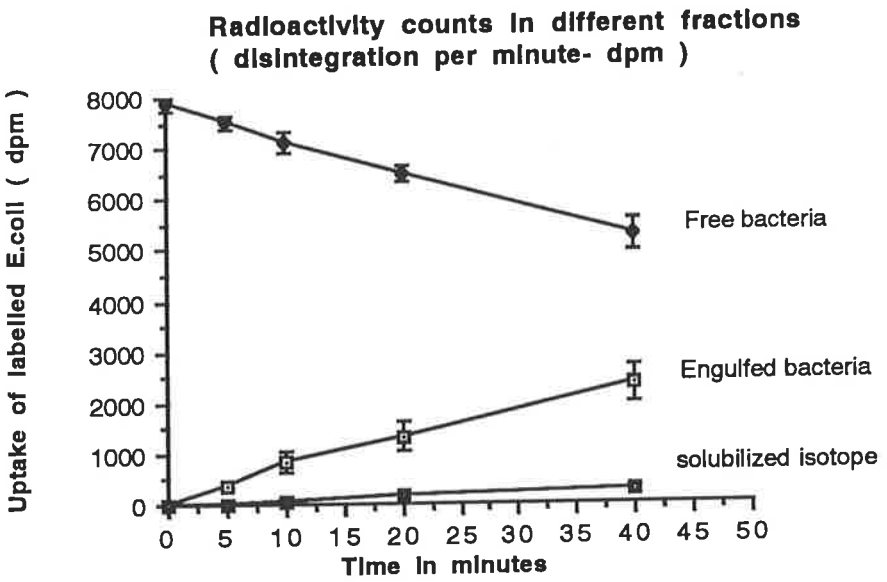


Fig. 9.2 Radioactivity counts in different fractions- uptake of labelled E.coli by rumen ciliates in standard in vitro rumen incubation buffer (McDougall's buffer). Experiment was carried out with 10^8 E.coli/ml (8000 dpm)

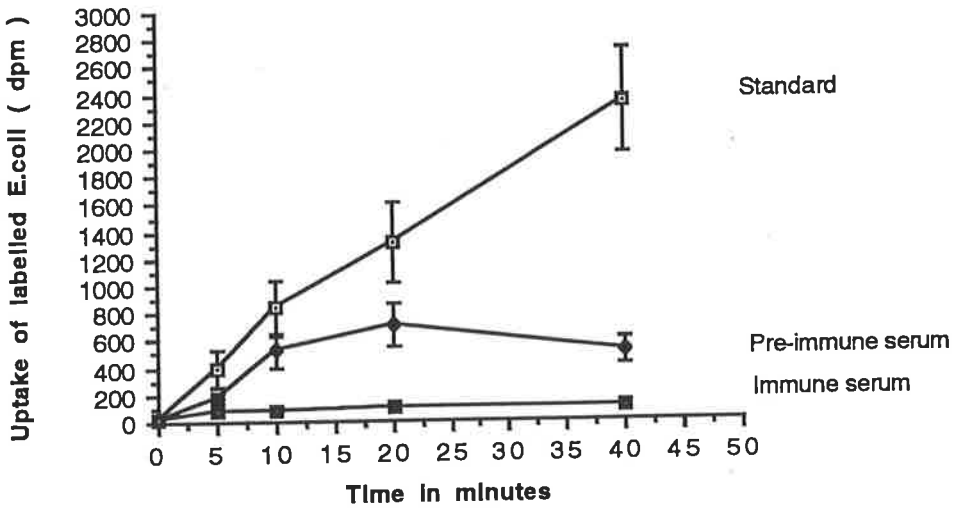


Fig. 9.3: Effect of selected dilution of sera on predation after 20 minute pre-incubation. The profile represented the predatory pattern exhibited by these organisms when they were pre-incubated with a selected dilution of serum sample (1:16) for 20 minutes and thereafter exposed to ^{35}S-E.coli for periods up to 40 minutes.

Table 9.2 Uptake of labelled E.coli by rumen ciliates after 20 minutes of incubation in pre- immune and immune sera. Sera used at a dilution of 1:16

Incubation mixture	Fraction	Radioactivity[a] Period of engulfment in minutes				
		0	5	10	20	40
		Mean±SE[b]				
SRF:MB[c]	Protozoa	20±2.6	387±66.6	838±103.4	1315±145.6	2340±188.7
	Bacteria	7880±61.4	7524±65.0	7103±102.3	6489±77.5	5299±154.8
	Supernatant	10±1.5	25±1.2	59±3.4	134±6.5	267±15.8
SRF:PS[d]	Protozoa	16±1.6	168±19.1	520±66.1	708±78.8	510±55.5
	Bacteria	7756±51.0	7734±76.5	7145±112.3	7135±97.5	7239±144.8
	Supernatant	20±1.5	32±1.6	79±3.4	114±4.5	300±15.8
SRF:IS[e]	Protozoa	13±0.5	74±8.8	85±12.4	94±19.8	96±14.5
	Bacteria	7980±40.8	7767±66.5	7721±90.3	7868±66.8	7667±99.0
	Supernatant	9±1.5	65±9.2	80±3.4	84±6.5	307±15.8

[a]dpm- disintegration per minute; [b]Mean±SE- average of eight duplicate values with the corresponding standard errors.[c]MB- McDougall's buffer; [d]PS- preimmune serum; [e]IS- immune serum.

It may be noticed that ciliates pre-incubated for 20 minutes in 1:16 dilution of preimmune serum, demonstrated approximately a linear uptake up to first 10 minutes, although at a lower rate. Beyond 20 minutes (i.e., 40 minutes from commencement of exposure to serum, there was a decline. Ciliates in immune serum at the same dilution (i.e. 1:16) registered lowest uptake for the first 10 minutes. Beyond 10 minutes (i.e., 30 minutes from the commencemnt of exposure to serum) there was no further net increase in radioactivity. It is interesting to observe that the radioactivity measured in the cell-free supernatant (i.e., solubilized isotope) remained at similar levels at the respective time points in the incubation mixtures (see Table 9.2).

A similar study conducted with 1:64 dilution of pre-immune and immune sera exhibited a profile as shown in the accompanying Fig. 9.4

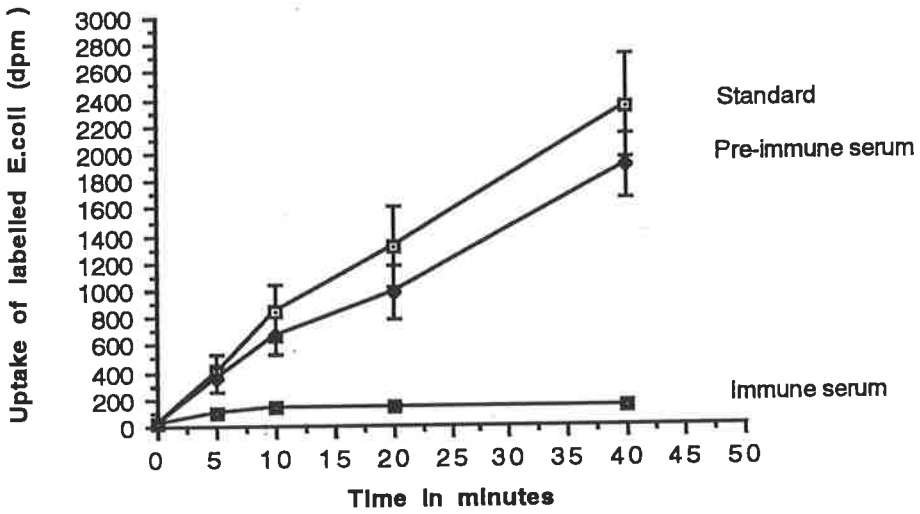


Fig. 9.4: Effect of selected dilution of sera on predation after 20 minute pre-incubation. The profile represented the predatory pattern exhibited by these organisms when they were pre-incubated with a selected dilution of serum samples (1:64) for 20 minutes and thereafter exposed to ^{35}S-E.coli for periods of up to 40 minutes.

It may be noticed that at higher dilution (1:64), ciliates incubated in preimmune serum demonstrated a linear uptake approximating the standard, whereas ciliates in immune serum exhibited no net increase in radioactivty beyond 10 minutes indicating that predatory activity nearly ceased after 10 minutes as it was observed with 1:16 dilution. It suggested

also that during the pre-exposure time of 20 minutes, the antibodies had the optimal effect on the ciliates regardless of the dilution levels at least up to 1:64 dilution of immune serum.

The effect of time of exposure to antibody of various dilutions of pre-immune and immune sera on uptake of labelled-bacteria by ciliates was illustrated clearly when the uptake for 20 minutes was compared for three different dilutions between pre-immune and immune sera after various periods of pre-incubation (see Fig.9.5).

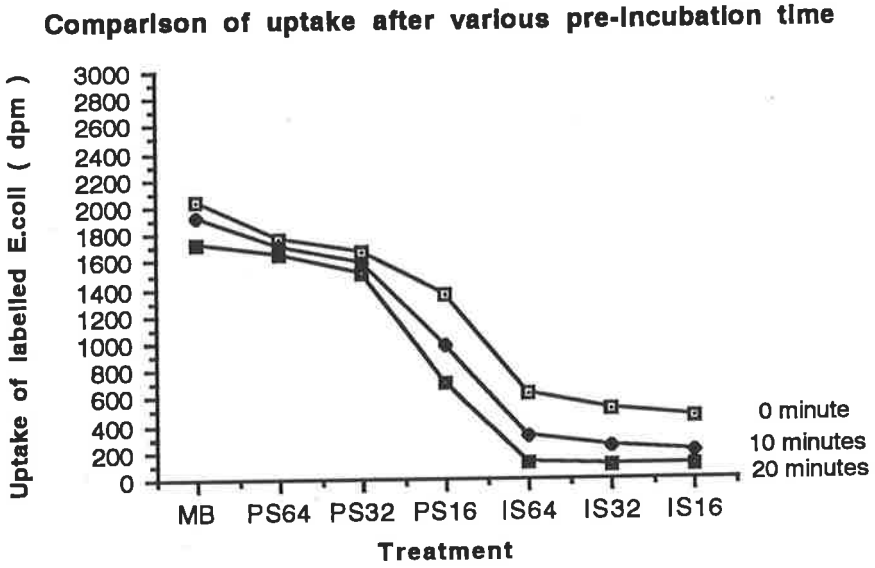


Fig. 9.5: Effect of treatment on the 20 minute uptake of bacteria by ciliates after various pre-incubation time. MB-McDougall's Buffer; PS16, preimmune serum (1:16 dilution); PS 32, pre-immune serum (1:32); PS64, preimmune serum (1:64 dilution); IS16, immune serum (1:16 dilution); IS32, immune serum (1:32) and IS64, immune serum (1:64 dilution).

With pre-immune serum (1:16 dilution), the uptake declined rapidly with increase in pre-incubation time. No such effect was observed at higher dilutions of 1:32 or 1:64 of the same serum, whereas with the immune serum, the uptake was significantly ($P<0.01$) lower than that of pre-immune serum in all of the dilutions at any examined time point. Furthermore, after pre-incubation for 20 minutes in immune serum, the ciliates exhibited lowest uptake for the three dilutions used in the analysis.

9.4 Discussion

The main objective in this investigation was to assess the effect of antibodies specific to rumen ciliates in restricting the predatory behaviour of a mixed population of these organisms in a rumen environment. It was mentioned in Section 1.0 that engulfment of bacteria by rumen ciliates results not only in the loss of bacterial protein but also in reduced efficiency of nutrient utilization in ruminant animals. The data provided evidence that the immune serum antibodies markedly restricted the predatory activity of rumen ciliates. This suggests the potential benefits such effects can have both on the availability of bacterial protein as well as on efficiency of utilization of nutrients by the host animal.

The bacterium Escherichia coli was used to measure the effect of antibodies on the uptake of bacteria by rumen ciliates. It was chosen because it can be grown aerobically and labelled uniformly with ^{35}S by growth in ^{35}Sulphur-methionine. This isotope ^{35}S gets incorporated very rapidly and efficiently into target cells. The assay is very sensitive to small differences in labelled cells. In the assays it was found convenient to measure the levels of incorporation of ^{35}S-E.coli into protoza with great precision.

The experiments were conducted initially to examine the normal uptake of bacteria in standard incubation buffer and to assess the effect of various dilutions of pre-immune and immune sera on this process. When the ciliates were allowed to engulf labelled E.coli over an incubation period of 40 minutes without any prior exposure to antibody, it was observed that there was a linearity in uptake of labelled bacterial cells over the stipulated period of incubation in McDougall's buffer (see Figures 9.1 A-B). When the kinetics of uptake of bacteria in the same buffer was examined with a change in time of pre-incubation (i.e., 10 and 20 minutes), still a similar trend was seen. However, pre-incubation period of 40 minutes exhibited a curvilinear uptake over the following engulfment period of same duration(Fig.9.1-G and H). This suggested that with an increase of total time (i.e., exposure + incubation), the ciliates could become physiologically inactive in the in vitro system used in the study resulting in a progressive decline in the uptake of bacteria. In these studies, pre-incubation time of 20 minutes was chosen to ensure the physiological vigour of the rumen ciliates and also to allow optimal reaction time for the antibody as observed in the initial studies.

Various dilutions of pre-immune and immune sera showed different kinetics of uptake of labelled E.coli. At zero pre-incubation, ciliates incubated in higher dilutions (1:32 to 1:128) of pre-immune serum approached nearly the values obtained for those organisms incubated in the standard buffer, suggesting that these dilutions did not exert any significant effect on the ciliates to curtail their predatory activity. However, the lower dilutions (1:2 to 1:16) appeared to have had an inhibitory effect on the predatory functions of these organisms. It was noticed that in these dilutions, after 20 minutes of engulfment, there was a decline in uptake. It suggested the possibility of the presence of natural antibodies in the pre-immune sera (see Section 5.0; sub-section 8.4). However, the amount of natural antibody might have been insufficient to cause a marked reduction in predatory activity, instead producing a gradual arresting of the activity of these organisms over a 40 minute engulfment period. This might have also occurred on account of a situation, where the organisms would have still continued to engulf bacteria, may be at slower rate, but in addition certain proportion of ingested ^{35}S-E.coli would have been digested and the resultant products along with the ^{35}S-label would have been released back into the medium. This might have caused a decline of radioactive counts of the protozoal fractions. It has been reported in studies relating to the fate of engulfed bacteria, the digested products started appearing in the reaction medium as early as 30 minutes after ingestion (Coleman, 1975). The differential counts made in respect of major three fractions, namely, protozoal pellets, bacterial pellets and cell-free supernatant of the reaction mixture did not show any evidence to this possibility.

The type of profiles obtained for ciliates incubated in immune serum demonstrated clearly the effect of antibodies on the predatory activity of these organisims. This was evident in all of the dilutions except 1:128 at 0 pre-incubation. The effect of immune serum became more and more pronounced with an increase of exposure time to antibody (10 and 20 minutes pre-incubation). The amount of radioactivity incorporated into protozoal fractions reached the lowest level after 20 minute pre-incubation (Cf Fig.9.1-D and F). This indicated that the lag time required for antibodies present in immune samples to complete immunological reactions, was between 10 and 20 minutes. It is interesting to observe that after pre-incubation for 10 and 20 minutes, in dilutions of 1:2 to 1:64 , no net increase in radioactivity was seen beyond 10 minutes of engulfment. It indicated that the predatory activity

persisted only for the first 10 minutes of incubation regardless of either a change in pre-incubation time or dilution. Even at the highest dilution (1:128) used in this study, the immune serum appeared to have exerted an inhibitory effect on the predatory behaviour of ciliates. The findings of the study provided additional support for the hypothesis that antibodies raised against rumen ciliates can act in rumen fluid and inhibit predation of bacteria by these organisms. Other assays, such as effects of antibody on adherence of ciliates to the rumen wall or to digesta, and on the in vivo populations, are vital but could not be accomodated in this project.

Section 10.0

Research Perspectives.

The role of protozoa in ruminant nutrition is contentious but there is significant evidence to suggest that at times with animals receiving a high energy/low protein diet, the protozoa are detrimental to the host animal in that they reduce the Protein/Energy ratio of absorbed products.

The experiments reported in this thesis are the first to apply an immunological approach to the manipulation of rumen microorganisms as a means of enhancing the productivity of ruminant animals. Such an approach is not only novel but is attractive in that vaccination is a safe means of manipulating the metabolism of animals. As such it is likely to be more successful than chemical intervention.

The positive aspects of the study included (1) the demonstration of the presence of antibodies to rumen ciliates in the internal and external secretions of sheep, (2) the prospects of boosting specific antibodies to rumen ciliates in the saliva and milk of these animals, and (3) the survival and effector activity of these antibodies on live organisms in rumen fluid.

The nature of the findings suggests an immediate need to demonstrate the effect of protozoa-specific antibodies under in vivo conditions, (though in vitro studies with immune sera confirmed immobilization of the rumen ciliates and the reduced predatory behaviour of this population on bacteria) and also to investigate various means of maintaining a sustained antibody response preferably in saliva to have full benefits of a technique to control the protozoal population under field conditions. The future research could possibly take, as a matter of choice, some of the following directions:

(a) immunization of gestating ewes to raise protozoa-specific antibody levels in milk and examine its effects on the establishment of rumen protozoa and on the growth rate of new-born lambs

(b) study the effects of repeated immunization of lambs with defined protozoa on the kinetics of both protozoa-specific antibodies in saliva and the population dynamics of protozoa

These studies would confirm whether or not an immunological approach to regulation of rumen protozoa may be a practical way to improve the nutritional status and performance of ruminants.

It may then become necessary to characterize protozoal antigens and to proceed to their production by recombinant DNA techniques as a prelude to large scale assessment of vaccination against these rumen microbes. A need may arise even to involve genetic modification of enteric bacteria to express protozoal antigens thereby continuously stimulate the gut immune system. It need not necessarily be the rumen mucosa but even the lower part of the digestive tract, because the current popular concept is that continuous gut stimulation offers the greatest potential to establish sustained antibody responses in the external secretions. Such studies may necessitate more specific humoral immune response studies with selected lymphocyte sub-populations and their kinetics to enhance the magnitude of the responses.

An immunological approach to microbial "management" could be extended to manipulation of individual species or groups of organisms, once more-specific effects of these become known. Such groups might include for example the holotrichs, large entodinia, or even bacteria in a particular niche, e.g. lactate-producers.

Such studies may precipitate the need for development of monoclonal antibodies against individual species or groups. This requirement in itself may involve a vigorous line of research in terms of production of monoclonal antibodies and the consequent targetting of individual populations to gain more knowledge about degradation of plants cell-wall components, establishment of new bacterial spectra, fungal proliferation, regulation of end-products, and the net fermentation patterns and protein/ energy transactions in the rumen. The judicial use of monoclonals may even allow establishment of pre-determined microbial populations for specific requirements of production in keeping with the prevailing feeding conditions and production systems.

The present study is just a beginning. It is exciting and holds great promise for the future. At this juncture, the need for further studies of antigen localization/trapping, cell migration patterns of immunocompetent cells, cytokine production, and antibody formation in the different segments of the lymphoid system (both systemic and mucosal systems) must be emphasized.

These studies may involve in vitro experiments which are useful to determine the individual factors and cells that may be required for cell activation, proliferation, and differentiation but also in vivo experiments which can provide insights into the lymphoid microenvironment.

The interface of disciplines is always unchartered water because it is fraught with difficulties, but it can also be very fruitful. This thesis represents a tentative foray into such an area, but has already identified research programmes with great potential for manipulating microbe/host interactions and thereby the productivity of the world's ruminant animals.

Section 11.0

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Section 12.0

Appendices

Appendix I

Effect of Antibodies on the Motility of Rumen Ciliates

The recordings in this videotape show the behaviour of rumen ciliates when they were subject to incubation in 8-fold dilutions of normal and immune neat inactivated serum samples. The fields of view are numbered 1-10 and will be explained in the following sequence

Field-1: highly motile rumen ciliates of two major groups, entodiniomorphs and holotrichs in the normal incubation buffer without serum. Entodiniomorphs- mainly small cells of mixed species, but larger sized Genus Polyplastron are seen showing their natural slower movements under low-power (x80).

Field-2: showing the same organisms (in incubation buffer only) at x250. Holotrichs are mainly of the genus Isotricha with a vestibulum at the end of the body. In this field entodiniomorphs may be differentiated into medium and smaller sized groups. The incubation medium appears fluid and the organisms are freely swimming.

Field-3: This is another part of the field in the incubation buffer at x250 magnification. Note again the rapid movements of most of the ciliates, also the long nucleus of Entodinium longinucleatum, the highly variable caudal spines of some species of entodiniomorphid ciliates and the gracefully gliding of Isotricha spp.

Field-4:This is a low power view of the ciliates after incubation in the preimmune serum for 45min. The pattern of motility for all species is essentially identical to that shown of ciliates suspended in buffer only

Field-5: Certain forms of entodinia with caudal spines and fast moving smaller entodinia are seen at x250 in the sample incubated in preimmune serum. Occasionally holotrichs are seen darting across the field.

Field-6: A cluster of entodinia with caudal spines and smaller entodinia after incubation in the preimmune serum (x250).

Field-7: A specimen of Polyplastron multivesiculatum exhibiting wave like ciliary motion in the adoral region. By the side is an Entodinium longinucleatum with a remarkable long macronucleus and cytoproct. Incubation was in preimmune serum (x250).

Field-8: This sample was incubated in immune serum. At low power (x80) most ciliates are seen to be immobilized. Isotrichs are dead and shrunken and a few are sluggishly moving around; Larger entodiniomorphs appear moribund but adoral ciliary motion in Polyplastrons is still visible and only a few smaller entodinia are moving in relatively normal fashion.

Field-9: Ciliates in immune serum at x250. Holotrichs from Genus Isotricha, Entodinia include E.longinucleatum, different forms of entodinia with the characteristic caudal spines. Note in particular the immobilization of all but a few smaller entodinia.

Field-10: A typical field from a preparation of ciliates at magnification x250, used in the counting of motile and immotile organisms to evaluate the percentage motility after incubation in preimmune or immune sera respectively.