



INTESTINAL SURVIVAL AND ABSORPTION  
OF EPIDERMAL GROWTH FACTOR IN THE SUCKLING NEONATE.

A thesis submitted to the University of Adelaide,  
South Australia, for the degree of  
Doctor of Philosophy.

by

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Dedicated to my parents

Shirley-Anne and Allen Ewart Gale

and my husband

Christopher James Angley.

**STATEMENT**

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge it contains no material that has been previously published by any other person, except where due reference is made in the text. I consent to this thesis being made available for photocopying and loan if applicable.

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THESIS ABSTRACT

In addition to meeting the requirements for protein and calories, milk contains a wide spectrum of growth factors which stimulate growth and differentiation of various tissues both in vitro and in vivo. These growth factors include insulin, epidermal growth factor (EGF), and insulin-like growth factors. Human milk appears to be particularly rich in EGF, the concentrations exceeding adult serum levels by 1000-fold in day 1 milk and 100-fold in milk expressed at later stages of lactation. This has led to the hypothesis that milk-derived EGF may play an important role in neonatal growth and development.

The physiological role of milk-derived EGF in the suckling neonate may only be assessed after quantitative studies on both the survival and absorption of orally-administered EGF in the neonatal intestine. In this thesis, the fate of orally-administered EGF was examined both in the human infant and in an experimental animal model, the newborn lamb. Since a quantitative evaluation of EGF metabolism is not feasible in the human neonate, the intestinal survival and absorption of EGF was assessed by a study of the relationship between the level of dietary EGF intake and urinary EGF excretion in premature infants. This indirect method provided a non-invasive, yet ethically practical evaluation of EGF uptake. A quantitative examination of both the intestinal survival and the rate of intestinal absorption of EGF was examined using the newborn lamb as a model of the human infant.

The results of the human study were found to be consistent with the hypothesis that EGF crosses the gastrointestinal wall to enter the general circulation.

Premature infants receiving higher levels of EGF in their diet showed greater urinary EGF excretion after the second postnatal week. Whilst this finding could also be explained by an indirect effect of some breast milk component on rates of endogenous EGF synthesis, they are nonetheless consistent with the absorption of intact EGF across the neonatal intestine.

The lamb was used as an experimental animal model of the human infant to study both the intestinal survival and absorption of EGF. To assess intestinal survival, reentrant catheters were inserted into the mid-small intestine of suckling lambs. A minimum of 15-30% of intragastric  $^{125}\text{I}$ -labelled EGF reached the mid-small intestine as immunologically intact EGF, providing strong evidence that a substantial proportion of milk-derived EGF would reach the small intestine intact.

Intestinal uptake of unlabelled EGF was measured in vivo using the autoperfused lamb intestine. In lambs of age 1-18 days, immunologically intact EGF was absorbed into blood but not lymph at rates that showed no correlation with the age of the lamb. The rates of absorption were low, resulting in a mean venous blood concentration of 0.02nM following infusion of 50 $\mu\text{g}$  EGF into a 20cm segment of gut. Given these low concentrations plus the known rapid clearance of EGF from plasma by various organs including the liver, it appears that very little luminal EGF would reach peripheral tissues intact in the neonatal lamb.

The studies in lambs therefore suggest that the direct growth-promoting actions of milk-derived EGF in the suckling infant are likely to be confined to the gastrointestinal tract and possibly the liver. Any actions in other tissues

are more likely to be mediated by secondary responses to the actions of EGF in the gut and the liver.

CHAPTER ONE

HISTORICAL REVIEW



## 1.1 Introduction.

This review includes information published prior to January 1985 at the time of commencement of work for this thesis. More recent work published since January 1985 relevant to this dissertation will be cited in the appropriate discussion sections of each chapter. The topics covered in this historical presentation of the literature will include a description of the nature of growth factors in milk, changes in their concentration during lactation, differences between species and the site(s) of synthesis of milk growth factors. Additional topics include the intestinal survival and absorption of growth factors and other milk proteins from the neonatal gastrointestinal tract and the evidence for growth factors reaching peripheral neonatal tissues in biologically active form. Since the aim of this thesis is to examine the significance of milk-derived epidermal growth factor (EGF) in the suckling neonate, the literature review will focus upon the known biological effects of EGF in isolated cells in tissue culture, organ culture and in vivo. Finally, a general discussion of the possible biological roles of milk growth factors will be included with particular reference to EGF.

## 1.2 Definition of Growth Factors.

Polypeptide growth factors are a group of hormone-like proteins involved in the regulation of growth processes. Growth factors stimulate anabolic responses in target cells through binding to specific, high affinity receptor molecules located, as an intrinsic membrane protein, on the plasma membrane of responsive cells. This interaction leads to internalization of the growth factor-receptor complex by receptor-mediated endocytosis, which ultimately leads to an

increase in both cell size (hypertrophy) and cell number (hyperplasia), through a stimulation of various anabolic pathways. Anabolic cellular pathways, including nutrient uptake, ion transport, DNA synthesis, RNA synthesis and protein synthesis are stimulated together with a co-ordinate inhibition of catabolic pathways, such as protein degradation (Ballard et al, 1982b; James and Bradshaw, 1984).

Numerous growth factors have been detected in a wide variety of tissues and biological fluids, although detailed characterization has been hindered by the relative low abundance of these substances in biological systems. A list of growth factors identified up until early 1985 is given in Table 1.1, showing the extent of characterization of each substance.

### 1.3 Mechanisms of Growth Factor Action.

Considerable research is being devoted to identification of the mitogenic signal(s) following interaction of a growth factor with its receptor, an area covered in detail in several reviews (Heldin and Westermark, 1984; James and Bradshaw, 1984). Here only a brief discussion of this area will be presented highlighting major points presented in the literature prior to 1985. Certain general characteristics appear to be common to the activity of all polypeptide growth factors: (a) initiation of the response by formation of a specific, high-affinity cell surface receptor complex, (b) generation of an intermediate signal that is responsible for at least a part of the overall mechanism, (c) internalization of both the ligand and its receptor largely through the mediation of clathrin-coated pits, and (d) the ultimate degradation of the ligand

and often the receptor by the action of lysosomal enzymes.

The nature of the signal initiated by the formation of the cell-surface complex and the role (if any) of internalization in the process of information transfer are largely undetermined. The discovery that tyrosine-specific protein kinase activity is an inherent part of several growth factor receptors may provide insight regarding second-messenger formation (Carpenter et al., 1979a). The EGF receptor contains as part of its integral structure, a tyrosine-specific protein kinase that is capable of phosphorylating both the receptor itself and a variety of other substrates (Carpenter et al., 1979a). Similar protein kinase activity has also been reported for the insulin (Kasuga et al., 1982) and platelet-derived growth factor receptors (Ek et al., 1982). While it appears likely that tyrosine-kinase activity inherent in the structure of several specific growth factor receptors will play some role in the propagation of the initial plasma membrane signal, this activity does not appear sufficient to provide the complete basis for activity as shown by studies with the EGF receptor (Schreiber et al., 1981).

The importance of the internalization of the ligand-receptor complex by receptor-mediated endocytosis in the process of information transfer is not as yet determined. While there is little doubt that this process is of importance in removing cell-surface ligands through lysosomal degradation, it has been speculated that this process is also important in the regulation of cell sensitivity to the growth factor by the resultant decrease in receptor number at the plasma membrane (down-regulation). It is difficult to determine whether this phenomenon is of



importance in vivo since it is not possible to determine when, if ever, sufficient quantities of growth factor are present at the cell surface to cause a significant change in receptor number. However, it has been shown that chronic exposure to insulin will produce changes in receptor number as evidenced by decreases in insulin receptor numbers in genetically obese animals and certain human patients (Soll et al., 1974 and 1975).

It is also possible that the internalization of the receptor and/or ligand acts specifically to propagate the biological response once inside the cell. Experiments with monoclonal antibodies have shown that if internalization is required, then it cannot be the ligand itself that transfers the information to the interior of the cell (Schreiber et al., 1981). In support of this, Heumann et al. (1981) have shown that nerve growth factor injected directly into cells does not elicit any response. These data specifically argue against any response model based on the occupation of intracellular receptors by internalized growth factor. It should be noted however, that in several tissues growth factor receptors have been identified on various organelles (Bradshaw and Rubin, 1980). Those associated with the Golgi apparatus are not likely to be involved in degradation, recycling of internalized receptors or the de novo synthesis of new receptors. However, receptors associated with other organelles, in particular the nucleus, are not as easily explained. These receptors may represent (a) translocated cell-surface receptors, (b) receptors synthesized de novo from the same gene and directed independently to the nucleus site or (c) genetically distinct receptor molecules. The last two possibilities are unlikely if the internalized

ligand is not directly involved; therefore, translocation of receptors from the plasma membrane to intracellular organelles as a result of ligand occupation, represents the only likely possibility if intracellular receptors are involved in the mechanism of action.

#### 1.4 Synergistic action of growth factors.

Growth and proliferation of tissues appears to be modulated by a complex interplay of growth-promoting agents. For example, the EGF receptor serves as a binding site not only for EGF but also for other polypeptide growth factors. Transforming growth factor- $\alpha$  secreted by murine sarcoma virus-transformed cells has been shown to bind to EGF receptors and induce proliferation (Marquardt et al., 1983). There is also evidence to suggest that polypeptide growth factors may act synergistically to regulate the growth and differentiation of target cells. A transient pre-exposure of cultured fibroblasts to platelet-derived growth factor has been shown to result in over a 10-fold increase in the sensitivity of the cells to the mitogenic effects of EGF (Wharton et al., 1983). In the same series of studies, the presence of either insulin or plasma in the culture medium enhanced the stimulatory effect of EGF on DNA synthesis in quiescent cultures of the fibroblast cell line, IOT 1/2.

Other agents may also effect the action of growth factors on cells. For instance, the tumor promoting phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate inhibits the binding of EGF to cellular receptors on various cell types (Shoyab et al., 1979; Brown et al., 1979). The inhibition of binding correlates well with the biological activity of various tumor promoters on mouse skin and in cell culture (Shoyab et al., 1979; Brown et al., 1979; Weinstein et

al., 1980). Similar inhibition of EGF binding to its membrane receptor has been documented where fibroblasts were treated with either vasopressin (Rosengurt et al., 1981), cyclamate or with saccharine (Lee, 1981). Thus, it appears that many agents may be capable of interacting with the specific receptors of growth factors, thereby modulating the binding and cellular response to the particular growth factor.

### 1.5 Epidermal growth factor: Discovery and characterization.

EGF, the protein of particular interest in this thesis, represents the best characterized of all polypeptide growth factors discovered. EGF was first detected some 30 years ago as an incidental observation during the course of studies on nerve growth factor. Extracts of the submaxillary gland of the mouse, when injected into newborn animals, induced precocious eyelid opening and incisor eruption (Cohen, 1959; Cohen 1962), due to direct stimulation of epidermal growth and keratinization (Cohen and Elliott, 1963; Cohen, 1965). The factor responsible for these effects was isolated (Cohen, 1962) and found to be a low molecular weight (6,000 Da), heat stable, nondialyzable polypeptide that accounted for approximately 0.5% of the protein content of the submaxillary gland. Even though EGF is concentrated a thousand-fold over plasma levels in the male mouse submaxillary gland, its isolation and purification were difficult until the observation that the elution of EGF by P-10 gel-filtration was retarded beyond the column volume. This unusual property of the peptide permitted a high degree of purification which, when followed by a DEAE cellulose column, allowed the isolation

of a homogeneous peptide (Savage and Cohen, 1972). EGF, isolated by this procedure, has 53 amino acids in a single peptide chain with three disulphide bridges (Savage, Inagami and Cohen, 1972), as shown in the schematic diagram in Figure 1.1. In the mouse submaxillary gland, EGF exists as a 74,000 molecular weight complex formed from two EGF molecules and two molecules of a binding protein of 30,000 molecular weight (Cohen and Taylor, 1974). In addition to binding EGF, the 30,000 molecular weight component possesses arginine esterase activity. An analogous binding protein with arginine esterase activity also exists for nerve growth factor in the mouse submaxillary gland (Greene et al., 1969). The esterase activities of these two growth peptides may activate precursor molecules by proteolytic action.

Human EGF, was first detected (Starkey et al., 1975) and isolated (Cohen and Carpenter, 1975) from human urine. A new aspect of the biology of EGF emerged with the report by Gregory (1975) that urogastrone, a gastric antisecretory hormone isolated from human urine, was identical to human EGF. Human EGF/urogastrone also has 53 amino acids as a single polypeptide chain and shares 70% sequence homology with mouse EGF. A comparison of the amino acid sequences of both human and mouse EGF is given in Fig. 1.2. The mouse and human polypeptides show similar mitogenic and gastric acid inhibitory properties and compete for the same receptor sites in a variety of animal tissues. Indeed mouse and human EGF can bind to either human or mouse receptors with near-equal affinity and specificity (Cohen and Carpenter, 1975). This suggests that both peptides belong to a family of mitogenic, acid-inhibitory polypeptides that show some interspecies structural variations, but are probably near-

identical in the active site regions responsible for receptor binding and biological activity (Das, 1982).

By January 1985, EGF-like activity had been reported in various species including the human, rat, mouse, rabbit, pig and sheep. In the human, EGF has been detected in a number of body fluids including urine, blood, saliva, milk and gastric juice (Cohen and Carpenter, 1975; Gregory, 1975; Starkey et al., 1975; Gregory et al., 1979; Hirata and Orth, 1979a and 1979c; Carpenter, 1980; Hirata et al., 1980; Beardmore et al., 1983; Moran et al., 1983; Oka and Orth, 1983; Petrides et al., 1984b; Read et al., 1984; Uchihashi et al., 1984). EGF has also been located in a variety of human tissues including the submandibular gland, Brunner's glands in the first part of the duodenum, thyroid gland, jejunum, kidney, pituitary and adrenal glands (Elder et al., 1978b; Heitz et al., 1978; Hirata and Orth, 1979a and 1979b).

In the mouse, EGF is present in many biological fluids and tissues in addition to the well-documented submaxillary gland (Cohen, 1962; Savage and Cohen, 1972; Van Noorden et al., 1977; Hirata and Orth, 1979a; Steidler and Reade, 1981; Nexo et al., 1984; Perheentupa et al., 1984), including saliva, urine, milk and kidney tissue (Bynny et al., 1974; Hirata and Orth, 1979a; Murphy et al., 1979; Beardmore and Richards, 1983; Perheentupa et al., 1984; Petrides et al., 1984a). Rat EGF has been detected in milk, urine, the brain, kidney, submandibular and Brunners duodenal glands (Moore, 1978; Oka et al., 1983; Fallon et al., 1984; Olsen et al., 1984; Thornburg et al., 1984). In other species, EGF has been detected in sheep urine (Waters et al., 1982), pig milk (Read et al., 1984) and the

submaxillary gland of the rabbit (Turkington, 1971).

There is some evidence for the existence of tissue-specific forms of EGF, differing in biological function. EGF from different tissues/body fluids of a particular species has been reported to vary in molecular weight, amino acid composition, isoelectric point, chromatographic properties, immunological behaviour or biological potency (Hirata and Orth, 1979d; Hirata et al., 1982; Assoian et al., 1984; Olsen et al., 1984). Future research of growth factor structure/function relationships should delineate the importance of these tissue-specific forms of growth factors in the control of growth and differentiation of responsive target tissues.

The presence of EGF in such a wide variety of biological fluids and tissues suggests that the growth factor may act in either a paracrine or autocrine mode, rather than in an endocrine manner. Endocrine hormone-like substances such as glucagon, testosterone or insulin are elaborated in a specific cell type and are transported to distantly removed target cells by means of the bloodstream. Paracrine agents differ in that the released hormone or polypeptide growth factor reaches its nearby target cell by diffusion while in autocrine interactions, the target cell is the same as that actually producing the factor (Sporn and Todaro, 1980). It appears that polypeptide growth factors act mainly in a paracrine or autocrine mode, rather than as endocrine factors (James and Bradshaw, 1984).

The study of EGF action in animals has been greatly hindered in the past by a lack of sufficient quantities of the purified growth factor. Since the concentrations of EGF in biological fluids and tissues are too low for the

purposes of large scale purification of this growth factor, recombinant DNA techniques have been employed to produce the quantities of EGF required for research purposes. Recombinant human EGF is now produced in yeast (Urdea et al., 1983; Brake et al., 1984) or *Escherichia coli* (Smith et al., 1982) transformed with plasmids containing the chemically synthesized gene for human EGF. This potentially unlimited source of human EGF should prove invaluable in the future years of research on EGF.

#### 1.6 Growth Factors in milk.

Breast milk or alternatively bovine milk-based formula usually represents the sole nutritional source to the human infant during the first few months of extra-uterine life. For many years, milk was thought of as merely a vehicle for nutrition, meeting the infant's requirements for protein, fat and carbohydrates. However, the presence of components such as enzymes, immunoglobulins and other defence agents, hormones and growth factors suggests other purposes for milk in addition to its nutritive value (Brambell, 1970; Koldovsky, 1980; Watson, 1980). The presence of growth-promoting agents in milk was first reported by Klagsbrun in 1978, although reports of the stimulatory effects of colostrum on the growth of the intestinal mucosa in Beagle puppies had been published earlier (Heird and Hansen, 1977). In 1978, Klagsbrun detected growth-promoting activity in human milk based on the ability of milk to stimulate DNA synthesis in human fibroblasts in vitro. Human milk, at a concentration of 1%, was as potent as 5% human serum in stimulating DNA synthesis in human fibroblasts, suggesting that milk could substitute for serum as a source of growth factors for cultured cells. Further

studies by Klagsbrun and Neumann (1979) showed that the milk of other species, including the cow, sheep and goat, also contained mitogenic activity which was highest in colostrum collected within 24 hours after birth. In addition to increasing DNA synthesis and cell division, other anabolic actions of milk in cultured cells include a stimulation of protein synthesis (Read et al., 1984) and a co-ordinate inhibition of protein degradation (Ballard et al., 1982b). Ballard et al (1982a) demonstrated that protein degradation in mammalian cells in vitro could be inhibited by the addition of small quantities of bovine colostrum to the culture medium.

Since the initial recognition of the growth-promoting effects of milk in cultured cells, several studies have been directed at the identification of the specific growth factors responsible for the activity. Carpenter (1980) demonstrated that 70% of the mitogenic activity in human milk on mouse fibroblasts and 93% of the activity on human fibroblasts could be neutralised by addition of specific antibodies to human EGF, thereby identifying EGF as a major growth factor in human milk. Subsequent measurements of EGF, either by homologous radioimmunoassay (Beardmore et al., 1983; Moran et al., 1983) or by radioreceptor assay (Read et al., 1984) have confirmed that human milk is very rich in EGF (30 - 111  $\mu\text{g}/\text{l}$ ), relative to the normal plasma concentration (less than 0.2  $\mu\text{g}/\text{l}$ , Hirata et al., 1980). EGF has also been detected in the milk of a number of other species as mentioned before, such as the mouse (Bynny et al., 1974; Beardmore and Richards, 1983), rat (Oka et al., 1983; Thornburg et al., 1984) and the pig (Read et al., 1984).



A number of other growth factors also contribute to the growth-promoting activity of milk. Insulin was identified as an important growth factor in bovine milk by Ballard and co-workers in 1982 following its initial identification in bovine milk by Koprowski and Tucker (1973). The passage of bovine milk through an anti-insulin affinity column abolished the ability of this milk to inhibit intracellular protein degradation in cell lines that are known to be particularly sensitive to insulin (H35 or MH<sub>1</sub>C<sub>1</sub> hepatoma cell lines). (Ballard et al., 1982a). Brown and Blakeley (1984) published the partial purification and characterization of a 35,000 Da growth factor present in goat colostrum, which they called colostalic basic growth factor. A similar factor was subsequently identified in the colostrum of cows and sheep. The factor was shown to be present only in colostrum, being undetectable in later milk. At the same time, Shing and Klagsbrun (1984) achieved partial characterization of the major activity in bovine colostrum that stimulated DNA synthesis in mouse fibroblasts, and designated this factor as bovine colostrum growth factor. Bovine and goat basic colostrum factors were also found to be chemically and functionally similar to a growth factor previously isolated from human platelets, platelet-derived growth factor (Brown and Blakeley, 1984). Other growth-promoting agents have been detected in bovine milk. In 1984, Jahnke and Lazarus detected a bombesin immunoreactive peptide in the whey of whole or skim bovine milk in nanomolar concentrations. Bombesin, a tetradecapeptide of molecular weight 1,620 Da, was first isolated from amphibian skin (Erspamer and Melchiorri, 1973), but now has been found in various mammalian tissue

extracts (2-5).

Many growth-promoting agents have also been identified in human milk. Somatomedin-C or insulin-like growth factor-I was partially purified from human milk in 1984 by Baxter and colleagues. In addition, Baxter et al (1984) detected a large molecular weight insulin-like growth factor binding protein. In the same year, Petrides et al., (1984) isolated 3 polypeptides from human milk on the basis of their ability to stimulate anchorage-independent growth of human neonatal skin fibroblasts in culture and of rat kidney fibroblasts in semisolid media. These proteins were therefore named as transforming growth factors. They were found to compete with mouse EGF for specific EGF receptors located on human placental membranes and to possess molecular weights similar to that determined for EGF (6000 Da), but were subsequently found to differ in amino acid composition (Petrides et al., 1984b). Another type of growth factor, colony-stimulating factor, was partially purified from human milk in the previous year by Sinha and Yunis (1983). This factor is an anionic high molecular weight protein of 40-70 KDa that was found to stimulate bone marrow proliferation in vitro. Other growth factors that have been detected in human milk include erythropoietin (Bielecki et al., 1972), insulin (Cevreska et al., 1975; Read et al., 1984), and nerve growth factor (Wright and Gaul, 1983).

A list of the growth factors identified in the milk of various species is given in Table 1.2. The identification of additional growth factors in milk since early 1985 has occurred and as laboratory techniques for the detection and isolation of relatively low abundance substances continue to improve, more growth factors will probably be identified in

milk in the future years ahead.

### 1.7 Differences between species in the growth factor profile in milk.

The concentrations of various growth factors in milk have been found to vary considerably between species. For instance, EGF is present in very high concentrations in human, rat and mouse milk but not in the milk of ruminants. Possibly the balance of growth-promoting agents in the milk of a particular species is tailored to suit the species-specific needs of the recipient suckling young.

Of the known growth factors in human milk, EGF may represent the major activity. Human milk is very rich in EGF, the concentration in mature milk (30-111  $\mu\text{g}/\text{l}$ , Read et al., 1984) exceeding that in plasma (0.2  $\mu\text{g}/\text{l}$ , Hirata et al., 1980) by more than 100-fold. Furthermore, both Carpenter (1980) and Shing and Klagsbrun (1984) reported that EGF was responsible for over 70% of the mitogenic activity of human milk in cultured mouse or human fibroblasts. Other growth factors that are present in human milk probably contribute to a lesser extent to the overall growth-promoting activity. Insulin concentrations (0.5-2.8  $\mu\text{g}/\text{l}$  in mature milk) are only several times greater than those in plasma and are low relative to the concentrations required for growth-promoting activity in cultured cells (Read et al., 1984). The concentration of insulin-like growth factor-I in mature milk (6-8  $\mu\text{g}/\text{l}$ ) is relatively low being only approximately 2-3% of the level measured in the serum of normal human subjects (Baxter et al., 1984). The significance of other known milk growth factors, such as colony-stimulating factor, erythropoietin, nerve growth factor and transforming growth factors are less obvious

because their concentrations have not been measured accurately.

In contrast to its high concentrations in human milk, EGF is undetectable in the milk of several ruminants including the goat, cow and sheep (Brown and Blakeley, 1984; Shing and Klagsbrun, 1984). Notwithstanding this, it is known that bovine and human colostrum are approximately equipotent in the ability to stimulate growth of some types of cultured cells (Read et al, 1984), indicating the presence of different sets of growth factors in ruminant and human milk. A platelet-derived growth factor-like peptide as well as insulin-like growth factors appear to be the major growth factors in ruminant milk (Brown and Blakeley, 1984; Shing and Klagsbrun, 1984).

Another growth factor that is present in human but not bovine milk or colostrum is colony-stimulating factor. Human colostrum or mature milk was shown to contain this factor by the detection of a proliferative response of mouse or human bone marrow cells in culture when extracts of human milk (or colostrum) were added to the medium. Since no proliferation was detected when the bovine milk or colostrum was added, it was concluded that bovine milk lacked this factor (Sinha and Yunis, 1983).

In conclusion, the available evidence indicates that the concentrations of various growth factors vary considerably in milk from different species, human milk representing a particularly potent source of EGF. The significance of these differences will no doubt become apparent when the physiological role for each growth-promoting agent is delineated in the future.

#### 1.8 Changes in the Growth-Promoting activity of milk during

lactation.

The concentration of several growth factors present in human, bovine and murine mammary secretions have been shown to change with duration of lactation. In bovine and human milk, mitogenic activity is maximal in day 1 colostrum, declining sharply during the first week and thereafter remaining at a constant level. This trend has been observed for growth-promoting activity determined in cultured cells (Steimer and Klagsbrun, 1981; Read et al., 1984; Shing and Klagsbrun, 1984) as well as for concentrations of EGF (Beardmore et al., 1983; Read et al., 1984; Shing and Klagsbrun, 1984), insulin (Cevreska et al., 1975; Falconer et al., 1984; Read et al., 1984), insulin-like growth factor-I (Baxter et al., 1984), and bovine colostrum growth factor (Brown and Blakeley, 1984), the latter being undetectable in mature milk. In one study the EGF concentration in human colostrum was reported to be similar to the concentration measured in later milk (Moran et al., 1983). However, they defined colostrum as milk expressed during the first week and may therefore have missed the changes detected within that period (Beardmore et al., 1983; Read et al., 1984).

In spite of the fall in growth factor concentrations during the first few days of lactation, mature human milk still contains potent mitogenic activity. Although EGF and insulin levels in mature human milk (approximately 50 and 4nM respectively) are only 10% of those in colostrum, they are still considerably higher than the concentrations measured in serum, 100-fold and several fold greater respectively (Read et al., 1984). Moreover, Read and colleagues (1984) have shown that when expressed on a

protein basis, growth factor concentrations are virtually identical in colostrum and mature human milk. Given the fact that protein concentration correlates inversely with the volume of milk produced (and consumed), Read and colleagues (1984) have suggested that the total growth factor delivery to the human infant remains nearly constant throughout lactation.

It would appear that the ruminant receives most of its growth factors from colostrum, rather than throughout lactation. Although growth factor activity has been detected in mature bovine (Steimer and Klagsbrun, 1981) and ovine milk (Falconer et al., 1984), PDGF-related substances are present only in colostrum (Brown and Blakeley, 1984; Shing and Klagsbrun, 1984). In support of this concept, mature bovine milk lacks growth-promoting activity in assays of DNA synthesis in mouse fibroblasts (Shing and Klagbrun, 1984).

The pattern of EGF concentration in mouse milk throughout lactation differs considerably from the human and ruminant. In the mouse, low levels of EGF are present in mammary secretions just after birth, rising to maximum levels during mid-lactation and then declining towards weaning (Beardmore and Richards, 1983). This pattern may not represent a general pattern for all growth-promoting agents in mouse milk, in view of the observation that prolactin concentrations are maximal on days 2-3, fall thereafter until day 6 and then remain steady until weaning (Richards and Beardmore, 1984).

The significance of species-specific patterns of growth factors will remain elusive until the biological roles of milk growth factors are clearly defined. It is plausible

that these differences reflect either the varying needs of the neonate, the mammary gland, or the sites of synthesis of milk growth factors in the different species. These possible biological functions of milk growth factors are reviewed in the following sections, with particular emphasis on EGF.

#### 1.9 Site(s) of synthesis of milk growth factors.

The origin(s) of polypeptide growth factors in milk is not yet established. Milk growth factors could be: (1) derived entirely from the systemic circulation; (2) liberated from cells present in milk such as macrophages, lymphocytes, neutrophils or epithelial cells; (3) synthesized locally by the lactating mammary gland itself or (4) derived from a combination of these potential sources. Considerable evidence supports the hypothesis that milk growth factors are derived at least in part from the systemic circulation. Colostral growth factors present in ruminant milk appear to originate from the circulation. The high levels of colostrum growth factors in pre-colostrum and colostrum together with the rapid falls in levels during lactation are characteristic of the concentration of several blood-derived proteins in mammary secretions (McClelland et al., 1978; Healy et al., 1980). In addition, calculations of insulin arteriovenous differences across the mammary glands and milk production rates in dairy cows indicate that insulin in bovine milk can be derived entirely from the blood (Beck and Tucker, 1978). In support of the findings of Beck and Tucker, Cevreska et al., (1975) found that insulin concentrations in human milk correlated closely with maternal serum levels.

In the case of EGF, it is also clear that circulating

EGF can be transported from the systemic circulation into the milk. Blakeley et al., (1982) demonstrated significant uptake of this peptide by the goat mammary gland when  $^{125}\text{I}$ -labelled EGF was infused into the arterial system supplying the gland. Similar results were obtained by Gresik et al., (1984) in a study where milk samples were collected from lactating mice 2-6 hours after the intravenous administration of a tracer dose of  $^{125}\text{I}$ -labelled EGF. The milk was found to contain relatively high levels of radioactivity of which 35-46% was able to be precipitated by trichloroacetic acid and 26% by a specific anti-mouse EGF antiserum. In other experiments, Gresik et al., (1984) administered a 10 $\mu\text{g}$  bolus intravenous dose of EGF to lactating mice and determined that compared to control mice, the EGF concentration in milk was doubled 4 hours after administration.

The effects of sialoadenectomy (removal of salivary glands) on the concentration of EGF in mouse mammary secretions have been documented by Gresik et al., (1984). Milk collected from lactating mice that had undergone sialoadenectomy 6 months earlier contained 50% less EGF compared with control milk collected from intact lactating mice. This observation supports the conclusion that plasma EGF contributes to the level of EGF present in milk (Blakeley et al., 1982).

Transport of EGF from the blood to the milk across the mammary epithelium may occur by means of specific receptors. In 1983, Taketani and Oka demonstrated the presence of specific, functional receptors for EGF in mouse mammary epithelial cells (Taketani and Oka, 1983b). Two types of EGF receptors were found to exist; a low-affinity and a



high-affinity receptor. Given the low circulating plasma concentration of EGF (1  $\mu\text{g/l}$ , Bynny et al., 1984), it is likely that the high affinity, rather than low affinity receptor would be involved in this transport process. High affinity EGF receptors in the placenta have been postulated to play a similar role in transporting plasma EGF into the fetal circulation (Richards et al., 1982).

The alternative origin of milk growth factors, namely local synthesis in the mammary gland, has proven more difficult to establish. Given that the majority of polypeptide growth factors probably act in a paracrine or autocrine manner (James and Bradshaw, 1984; Heldin and Westermark, 1984) together with the evidence that EGF stimulates cell proliferation in cultured mammary gland epithelial cells (Turkington, 1969a and 1969b; Papadimitriou et al., 1977; Tonelli and Sorof, 1980; Imagawa et al., 1982; Taketani and Oka, 1983a and 1983b; Fitzpatrick et al., 1984a and 1984b), it is plausible that local synthesis within the gland itself may occur. However, immunohistochemical techniques have so far failed to localize EGF in the mammary gland tissue (Van Noorden et al., 1977). Presumably, if EGF was being synthesized in large amounts by the mammary gland, it would be packaged in secretory vesicles in a similar fashion to casein granules. In contrast to the mammary gland, submandibular salivary gland EGF has been localized by immunocytochemistry to granules in the specialized granular-tubular cells of the ducts (Van Noorden et al., 1977). Comparisons between these two tissues suggest that either (1) EGF is not synthesized in the mammary gland or (2) EGF is present in a precursor or high-molecular weight form and is not detected by the

immunocytochemical localisation technique or (3) EGF is synthesized but not stored in the mammary gland.

#### 1.10 Survival of growth factors in the neonatal intestine.

If milk growth factors are to influence neonatal development they would need to survive digestion in the infant gastrointestinal tract. The intestinal survival of milk proteins in the gastrointestinal tract is affected by numerous factors including the maturity of the gut, the resistance of the particular protein to proteolytic digestion, and the presence of enzyme inhibitors ingested simultaneously with the milk protein. The maturity of the gastrointestinal tract directly affects protein digestion. In the adult, protein digestion begins in the stomach as food is mixed with gastric juice containing hydrochloric acid and pepsin. Approximately one fifth of the swallowed protein is digested in the stomach and the remainder by pancreatic enzymes and intestinal hydrolases in the lumen of the upper gut and in the brush border of the intestinal mucosa. Enzymatic decomposition of proteins therefore yields amino acids and small peptides, which are absorbed swiftly by passive diffusion through aqueous pores or by active transport mechanisms in the brush border membrane. Once inside the epithelial cell, proteinases hydrolyse the small polypeptides further into individual amino acids (Jacobson, 1979). Compared to the adult, intestinal protein digestion is diminished in the neonate due in part to a higher pH (Takeuchi et al., 1981) and lower concentration of pepsin (Furihata et al., 1972) in gastric secretions, allowing proteins to pass intact to the small intestine (Johnston 1968; Jones, 1972; Abrahamson et al., 1979).

It is of interest to note that EGF, a growth factor

present in the milk of various species, is also known to be a potent inhibitor of gastric acid secretion, thereby limiting the digestion of proteins (Gregory et al., 1978). In addition, only very small amounts of pancreatic enzymes can be detected in the rat duodenum or jejunum during the first 2 weeks following birth (Robberecht et al., 1971).

The second major factor affecting the survival of ingested milk proteins in the gastrointestinal tract is the resistance of a particular protein to proteolytic attack. Growth factors such as insulin, EGF, insulin-like growth factors and colostrum platelet-derived growth factor-like factors are known to be very stable in acid conditions (Gregory and Willshire, 1975; Gregory et al., 1978; Carpenter and Cohen, 1979a). Moreover, a number of growth factors including EGF are relatively resistant to proteolytic degradation (Cohen and Taylor, 1974).

With regard to the third factor influencing intestinal protein digestion, milk has been shown to contain trypsin inhibitors that could conceivably reduce the rate of growth factor degradation in the intestine of infants fed milk compared with other diets (Laskowski and Laskowski, 1951).

Several studies have provided evidence that growth factors are able to survive digestion in suckling neonates. Indirect evidence supporting the intestinal survival of milk growth factors was published by Heird and Hansen (1977). They examined the effect of either suckling or artificial feeding with simulated bitch milk on the intestinal mucosa of newborn Beagle puppies. The mucosal mass, DNA and protein content and enzyme activities of the intestine were higher in puppies suckled from birth than in counterparts receiving artificial feeds. Not only do these results

suggest that colostrum contains growth-promoting agents specific for intestinal mucosa, but they also provide evidence that the colostral growth factor(s) remain biologically active in the intestine after oral ingestion.

More recently, Thornberg and colleagues (1984) have shown that orally-administered EGF survives intestinal digestion in newborn rats. Sixty nanogram doses of  $^{125}\text{I}$ -labelled mouse EGF were administered orally to 13-day old suckling rats. Thirty to sixty minutes after intragastric administration the rat pups were sacrificed, the stomach and intestinal flushings were collected and the radioactivity characterized to determine the extent of degradation of EGF. Degradation was assessed by several criteria including molecular weight, immunoreactivity and receptor binding capacity. According to all three criteria, over 90% of radioactivity recovered from the stomach and intestinal contents represented intact EGF, indicating that at least some of the EGF had reached the small intestine in a biologically active form.

#### 1.11 Absorption of growth factors and proteins in neonatal intestine.

Intestinal absorption of intact proteins into the circulation is very much restricted under most circumstances (Jacobson, 1979). The two most important factors controlling the absorption of intact macromolecules are firstly, the efficiency of digestion in both the lumen and the enterocyte and secondly, the impermeability of the brush border membranes to aqueous polypeptides of molecular weight above approximately 200 Da. The importance of the former is illustrated by studies involving direct infusion of proteins into the lumen of the small intestine. Warshaw and

coworkers (1971) were able to measure intact albumin (66,000 Da) in the portal vein and intestinal lymph of adult rats following direct delivery into the lumen of the small intestine, where the levels would be undetectable following oral administration. Similarly, Warshaw and colleagues (1971) found that intraluminal but not oral administration of insulin induced a hypoglycemic response in adult rats. These studies demonstrate that the efficiency of luminal digestion prior to the site of intestinal absorption can to some extent determine the quantities of food proteins reaching the circulation intact. Various factors in addition to enzymic activity would influence the efficiency of luminal digestion, including the rate of gastric emptying, peristalsis, intestinal transit time, gut mucosal surface area and the thickness and composition of the mucus coat (Patt, 1977; Bjarnason and Peters, 1984; Walker, 1978). Any perturbation of these factors, such as in intestinal disease, could therefore result in increased absorption of macromolecules. In the case of the newborn, the apparent increased survival of milk proteins associated with immaturity of the gastrointestinal tract could result in a higher rate of intestinal absorption of growth factors in the suckling infant compared to the adult. The ability of the newborn animal to absorb intact proteins across the gut wall during suckling may also be enhanced by a markedly enhanced pinocytotic capacity in the enterocyte of the neonatal intestine compared to that in the adult (Henning, 1981).

In addition to luminal digestion and the rate of pinocytosis, the permeability barrier of the brush border membrane constitutes the other major regulator of intestinal

protein absorption. Here also, the newborn appears to differ from the adult. The postnatal absorption of intact immunoglobulins in the suckling young of several species, including humans, ruminants and rodents has been well documented (Comline et al., 1951; Leece and Morgan, 1962; Asplund et al., 1962; Healy, 1977; Cabello and Levieux, 1981). After several days to weeks, depending on the species, the infant intestine appears to mature into the adult form, a process referred to as "closure", such that intact protein is no longer absorbed in measureable quantities (Weström et al., 1984). The mechanism of transfer of intact proteins prior to closure appears to vary in different species, the newborn ruminant demonstrating non-specific bulk transfer of proteins for 12 to 48 hours postnatal (Baintner, 1986), while the rodent intestine shows a permeability that is specific to immunoglobulins but lasts until weaning (Henning, 1981). In the human, immunoglobulins may be transferred exclusively via the placenta, with very limited postnatal protein absorption in the intestine (Baintner, 1986).

Evidence for the intestinal absorption of biologically active growth factors in the newborn has been obtained from studies involving the oral administration of insulin. Hypoglycaemic effects of high doses of orally-ingested insulin have been observed in mice (Kelly, 1960), rats (Mosinger et al., 1959; Kidron et al., 1982), piglets (Asplund et al., 1962) and newly-born calves (Pierce et al., 1964). Erythropoietin and prolactin also appear to cross the intestinal mucosa intact in infant rats (Carmichael et al., 1978; Grovenor and Whitworth, 1983). In the case of EGF, Cohen and Taylor (1974) observed in early studies that

orally-administered EGF could induce precocious eyelid opening in newborn mice. More recently, Thornburg and colleagues (1984) demonstrated, in experiments mentioned previously in this review, that a small proportion of intragastric  $^{125}\text{I}$ -labelled EGF could be recovered from peripheral organs such as the liver and the lung of suckling rats in an immunoreactive form. This immunoreactive material had not, however, retained the ability to bind to EGF receptors. While these studies have provided some evidence for the intestinal absorption of intact growth factors in the newborn, quantitative analysis is lacking.

#### 1.12 The Biological Effects of EGF.

EGF has been demonstrated to elicit biological responses in intact animals, organ cultures and various mammalian cells in vitro (Carpenter and Cohen, 1979a). Although a large number of studies have been published in the literature regarding the biological effects of EGF, particularly in vitro, the precise role(s) of EGF in mammalian physiology and pathology is not yet understood. In this section of the review, the documented evidence investigating the biological effects of EGF on the growth and development of various tissues will be given.

##### 1.12(a) Biological effects of EGF on fetal and neonatal development.

EGF appears to be an important growth factor in the growth and development of the fetus and neonate of various mammalian species. In the mouse, both EGF and its specific receptor have been detected in fetal and neonatal tissues (Nexo et al., 1980; Adamson et al., 1981; Adamson and Meek, 1984). EGF levels were measured in these studies by means of antibody and receptor binding techniques (Nexo et

al., 1980). While specific antibody binding studies detected EGF in the fetal tissues at 14½ days of gestation, receptor-binding studies showed that an appreciable amount of receptor-active EGF was present in the tissues as early as 11½ days of gestation. In support of these findings, Adamson et al., (1981) found that EGF was able to bind to and promote anabolic responses in fetal mouse tissues in vivo and in vitro. Binding activity was found in the fetal mouse lung, limbs, liver, brain, parietal endoderm, heart and trophoblast. Furthermore, Adamson and Meek (1984) later reported that the number of EGF receptors in fetal mouse tissues increased during gestation until parturition, although the receptor affinity tended to decline. EGF may therefore initially stimulate proliferation in embryonic cells and then induce differentiation as the tissues mature (Adamson and Meek, 1984).

The presence of human EGF in maternal and fetal blood as well as in amniotic fluid suggests a role for EGF in human fetal development (Ances, 1973; Barka et al., 1978; Ladda et al., 1979). It is presumed to play a role in human fetoplacental growth and development through its mitogenic action and by increasing placental and fetal membrane hormone secretion (Benveniste et al., 1978; Bahn et al., 1980; Huot et al., 1981; Hirata et al., 1982; Lai and Guyda, 1984; Mitchell and Casey, 1984). These actions are thought to be mediated by EGF binding to specific high affinity receptors present in human amnion, chorion, decidua (Rao et al., 1984), and placenta (O'Keefe et al., 1974; Hock and Hollenberg, 1980; Hirata et al., 1982; Carson et al., 1983; Lai and Guyda, 1984).

#### 1.12(b) Biological effects of EGF on skin and corneal



epithelium.

EGF has been shown to be a potent mitogen for a variety of cultured cells of ectodermal and mesodermal origin (Carpenter and Cohen, 1979). It is not surprising therefore, that tissue and cells derived from skin are EGF-responsive since skin develops from surface ectoderm and underlying mesenchyme during early fetal development (Moore, 1973). For example, keratinocytes derived from skin in cell culture respond to the addition of EGF to the culture medium (Sun and Green, 1977). EGF markedly stimulates their proliferation and leads to enhanced keratinization and squame production (Green, 1977). EGF has also been shown to delay the ultimate senescence of these cells, thereby increasing their culture lifetime (Rheinwald and Green, 1977). Owing to these effects, culture of epidermal keratinocytes, particularly those of human origin, has been greatly improved (Green et al., 1979). Single cultured cells can now generate in vitro stratified colonies that ultimately fuse and form an epithelium that is a reasonable approximation of the epidermis.

EGF both in vivo and in organ culture promotes the proliferation of the basal cell layer of various epithelia (Cohen, 1965; Cohen and Taylor, 1974). This can be observed in fetal, neonatal and adult tissues (Cohen, 1965). Early studies demonstrated that in vivo EGF can accelerate the opening of the eyelids and tooth eruption in the neonate (Cohen, 1959; Cohen, 1962). This was later found to be the consequence of a more generalized effect, namely an enhancement of epidermal keratinization that affects the upper layers of the epidermis (Cohen and Elliott, 1963; Cohen and Savage, 1974) and results from an increased

proliferative rate of the basal cell layer. Histological examination of the tissues of these animals showed enhanced keratinization and an increase in the thickness of the epidermis not only in the eyelid area but also in the back skin and epithelium lining the mouth (Cohen and Elliott, 1963), results that have been confirmed by biochemical measurements (protein, RNA, DNA) of pure epidermis obtained by trypsinization of standard areas of skin from 5-day-old control and EGF-treated rats (Angeletti et al., 1964; Mann and Fenton, 1970).

Similar effects of EGF have been reported in the back skin or anterior shank skin from 9-day-old chick embryos maintained in organ culture (Cohen, 1965). While the thickness of the epidermis in control cultures remained almost unchanged, a marked increase in the number of epithelial cell layers was seen by day 3 when the tissues were exposed to EGF. If the cultures were allowed to grow for 9 days, although feather development was observed in control cultures, in cultures exposed to EGF feather formation was inhibited and the subepithelial layer keratinized. Essentially similar results were obtained when the skin was derived from the anterior shank region of 11-day-old chick embryo, although in this case scale formation, rather than feather formation, was observed in control cultures and inhibited in EGF-treated cultures (Cohen, 1965).

The inhibitory effect of EGF on either feather or scale development in embryos maintained in culture was not pursued further until the studies of Moore and colleagues on hair and wool growth. Moore and colleagues (1981a) found that daily subcutaneous injections of high doses of EGF from

birth reduced the length, growth rate and diameter of hair fibres of the first hair coat of the neonatal mouse. A more striking example of the action of this growth factor was observed in the skin of the Merino sheep. Subcutaneous infusion of (2-3 mg) or more of EGF in wethers for 28 hours resulted in the rapid shedding of the entire fleece (Moore et al., 1981b). In addition, infusion of large quantities of EGF for 10 days into foetal lambs at 110-115 days of gestation has been reported to induce shedding of the wool fibres, changes in the epidermis and in the sebaceous and sweat glands (Thorburn et al., 1981a and 1981c). Similar reports on the inhibition of wool growth by high quantities of EGF have also appeared in the literature (Moore et al., 1982); Hollis et al., 1983; McDonald et al., 1983; Moore et al., 1984; Panaretto et al., 1984).

The inhibition of wool or hair growth is related to the quantity of EGF administered, the mode of administration and the period of delivery. Oral administration was found to be ineffective on wool shedding possibly because the concentration of EGF required for this effect was not achieved due to either limited intestinal absorption from the oral dose and/or effective plasma clearance of absorbed EGF. Subcutaneous infusion of EGF appears to be the most effective mode of administration to cause inhibition of wool growth (Moore et al., 1982).

It is not yet known whether EGF causes inhibition of wool fibre growth by a direct or indirect mechanism, since there is evidence supporting both types of action. Inhibition of wool growth may result from the binding of EGF to follicular cells, as it has been shown that iodinated EGF is selectively concentrated in the rat epidermis (Covelli et

al., 1972) and specifically binds to cell membrane receptors in the skin (O'Keefe et al., 1974). On the other hand, the actions of EGF in the skin may be mediated or potentiated by circulating hormones such as thyroxine and growth hormone, which are known to influence wool growth (Wheatley et al., 1966; Wallace, 1979; Moore et al., 1984), to interact in vivo with EGF (Hoath et al., 1983).

EGF can modulate the growth of many epithelial tissues in vivo but it would appear that its effect is also dependent upon the nature of the underlying connective tissue (Steidler and Reade, 1980). In a study of the in vivo effects of EGF on newborn mice, EGF injected subcutaneously for up to 14 days following birth increased the epithelium and keratin of both the skin and oral mucosa but not the respiratory epithelium. These findings suggest that underlying connective tissue influences the mitogenic effects of EGF in either a direct or indirect manner (Steidler and Reade, 1980).

EGF may not only enhance the growth and differentiation of immature epithelium but may also be involved in repair and maintenance mechanisms of epithelial tissues. This has led to the hypothesis that EGF may be involved in the wound healing process. Not only does EGF increase epidermal cell mitosis, it appears to stimulate the activity of a number of epidermal enzymes including ornithine decarboxylase (Blosse et al., 1974) and to modify the biosynthesis of fibronectin, a cell-surface high molecular weight glycoprotein produced by many types of differentiated cells including fibroblasts, endothelial cells and hepatocytes. While the biological function of fibronectin is not clearly defined, it appears to play an important role in cell adhesion to various

surfaces and is therefore implicated in the wound-healing process (Carpenter and Cohen, 1979a).

In vivo studies have now provided direct evidence that EGF can accelerate wound healing. Topical administration of EGF to a back wound in mice was found to enhance the rate of wound closure both in control and sialoadenectomized mice (Niall et al., 1982).

Corneal epithelium, like skin epithelium, is extremely sensitive to the hyperplastic effect of EGF. In organ culture, both chick and human fetal corneas respond to EGF with a marked hyperplasia of epithelial cell layers (Cohen and Savage, 1974; Savage and Cohen, 1973). However, in accordance with the phenotypic expression of this tissue, the upper cell layers do not keratinize. Likewise, although the topical application of EGF in vivo to normal rabbit eye does not appear to affect the morphology of the corneal epithelium significantly, if the cornea is partially denuded of its epithelium, the topical application of EGF markedly stimulates the regeneration process, and a transitory hyperplasia of the epithelial layer can be observed once the denuded area is covered (Savage and Cohen, 1973; Frati et al., 1972).

#### 1.12(c) Biological effects of EGF on the lung.

Sundell and colleagues examined the possibility that EGF could induce growth and maturation of the fetal lung epithelium. It was shown that a constant infusion of EGF into fetal lambs for 3-5 days stimulated epithelial growth in many sites, including upper and lower airways. In addition, fetal administration of EGF between 123-130 days of gestation EGF appeared to afford protection against the development of hyaline membrane disease (Sundell et al.,

1975 and 1980), suggesting a stimulation of epithelial differentiation as well as growth. However, similar studies by Thorburn et al., (1981b) produced negative results. A study by Catterton and colleagues showed that intramuscular or intraperitoneal injection of approximately 5µg EGF into 24-day-old rabbit fetuses induced accelerated maturation of the lung (Catterton et al., 1979). The lungs of the EGF-treated rabbits at 27-days-old compared with saline-treated controls showed greater lung distensibility and stability on deflation associated with an increase in the number of type II alveolar cells, approaching the number seen in newborn rabbits at term.

The mechanisms of action of EGF on the growth and maturation of the fetal lung are unknown. The presence of high-affinity receptors for EGF in the fetal rabbit lung (Devaskar, 1982) suggest a direct action, although the responses to EGF that have been observed in isolated lung cells (Keller and Ladda, 1981) differ substantially from those in vivo (Sundell et al., 1975; Catterton et al., 1979; Sundell et al., 1980). For example, Keller and Ladda (1981) found no effect of EGF on surfactant production by isolated type 2 cells derived from an adult rabbit.

Differences in response to EGF in vivo and in vitro suggest either that lung cells in culture have lost their ability to respond to growth factors and do not therefore reflect the in situ situation, or that the mechanism of EGF action is at least partly indirect. The former could result from cellular damage, senescence in culture or because cells were isolated from the adult, rather than the neonatal or fetal respiratory tract which is less differentiated and possibly more responsive (Sundell et al., 1980).

Notwithstanding the possible loss of responsiveness in culture, considerable evidence is available to support the alternative explanation of an indirect action on lung growth and development. A number of different hormones are known to influence the maturation of the fetal and neonatal lung, including adrenocorticotrophin, glucocorticoids, thyroid hormones, prolactin and sex hormones (Liggins, 1968 and 1969; Kikkawa et al., 1971; Kotas and Avery, 1971; Wang et al., 1971; Wu et al., 1973; Platzker et al., 1975; Smith et al., 1975; Torday et al., 1975; Hamosh and Hamosh, 1977; Erenberg et al., 1979; Sundell et al., 1979; Gross et al., 1980; Crone et al., 1983; Khosla et al., 1983; Mullon et al., 1983; Smith and Sabry, 1983; Vilos et al., 1983; Das et al., 1984; Sadiq and Devaskar, 1984). For example, administration of thyroid hormones or glucocorticoids to fetal or newborn animals has been shown to promote pulmonary maturation. The mechanisms by which these hormones affect lung development is not clear, although a number of studies suggest indirect mechanisms involving the regulation of other substances and/or their specific receptors including EGF and its receptor. Thyroid hormone administration has been reported to increase the circulating serum levels of EGF in humans (Uchihashi et al., 1984) and the levels of EGF in the submandibular glands of mice (Gresik et al., 1981). Also thyroid hormone may also regulate the EGF receptor system since treatment of hypothyroid rats 96 hours prior to sacrifice increased the level of EGF binding in the liver to that found in controls (Mukku, 1984). Glucocorticoids were also shown to regulate the number of EGF receptors present in tissues; an infusion of glucocorticoids into fetal rabbits was found to increase

the number of pulmonary EGF receptors (Sadiq and Devaskar, 1984). These observations suggest that EGF may influence lung growth and maturation indirectly either by modifying the circulating levels of some of these hormones or the tissues responsiveness to them. Alternatively, the direct actions of EGF on lung tissue could be modified by the synergistic actions of other growth factors and hormones. Indeed the lack of responsiveness to EGF in culture may be overcome by the simultaneous addition of these other factors.

Given the vast number of agents that have been reported to affect lung maturation, it would appear that a complex interaction of these agents regulates lung development in vivo. While the precise role for EGF is not yet clear, evidence suggests that it is a vital growth factor in fetal and neonatal lung development.

1.12(d) Biological effects of EGF on the gastrointestinal tract.

The cells of the epithelial lining of the alimentary tract have a high rate of turnover (Quastler and Sherman, 1959). Various hormones and growth factors have been suggested to control intestinal development and function in vivo (Sharp et al., 1980; Robinson et al., 1982) and shown to regulate the growth responses of intestinal cells in organ culture and in cell culture in vitro (Quaroni and May, 1980). EGF has been shown to exert a number of diverse biological effects on the alimentary system in a variety of mammalian species. The first documented biological effect of EGF on the alimentary tract was discovered by Cohen in 1962 when daily injection of EGF into newborn mice caused premature eruption of the incisors at 6-7 days instead of



the normal 8-10 days. The second effect of EGF on the gut was reported by Bower and colleagues in 1975, when EGF was shown to inhibit gastric acid secretion. Since these earlier studies, EGF has been shown to exert many biological effects on the tissues of the gut in vivo, in organ culture systems and in vitro. In addition, the detection of EGF at various sites of the gastrointestinal tract and the identification of receptors in gut tissues suggest a role for EGF in alimentary physiology.

EGF has been localized by immunohistochemical techniques at various sites along the alimentary tract including the submaxillary gland (Creamer et al., 1961; Cohen, 1962; Hirata & Orth, 1979a) and Brunner's glands located in the first part of the duodenum (Creamer et al., 1961; Elder et al., 1978b; Heitz et al., 1978; Hirata and Orth, 1979a) and jejunum (Hirata and Orth, 1979a). The presence of EGF has been detected in various alimentary fluids including saliva (Server et al., 1976; Gregory et al., 1979; Hirata and Orth, 1979b; Murphy et al., 1979; Nexo et al., 1984; Petrides et al., 1984), gastric juice (Gregory et al., 1979) and pancreatic juice (Hirata et al., 1982).

The hypothesis that EGF has a role in gastrointestinal physiology is also strengthened by the identification of specific receptors for EGF at various sites in the gastrointestinal tract. Receptors for EGF have been located in the rat small intestine (Forgue-LaFitte et al., 1980; Blay and Brown, 1983), in the rat liver (O'Keefe et al., 1974) and in the gastric glands of the guinea-pig (Forgue-LaFitte et al., 1984). Also, binding of EGF by mouse pancreatic acini (Logsdon and Williams, 1983) and rat

pancreatic acini (Korc and Matrisian, 1983) has shown to be specific, saturable and reversible.

Trophic effects of EGF have been observed in most tissues of the alimentary system in vitro, in organ culture systems and in vivo. The mitogenic effects of EGF have been detected as proximal as the oral mucosa; subcutaneous injection of EGF in newborn mice for up to 14 days following birth increased the thickness of the epithelium and keratin of the oral mucosa (Steidler and Reade, 1980). Other proximal alimentary structures have also been reported to be affected by EGF. The rate of DNA synthesis measured in the tongue and oesophagus of adult mice was found to be stimulated by the presence of EGF (Scheving et al., 1979).

In addition to the well-documented inhibitory effect of EGF on the level of gastric acid secretion (Gerring et al., 1974; Bower et al., 1975; Gregory, 1975; Koffman et al., 1977; Elder et al., 1978a; Gregory et al., 1978), EGF also exhibits trophic effects on the stomach. EGF was found to stimulate DNA synthesis in the gastric mucosa of the adult mouse (Scheving et al., 1979), and to increase the activity of ornithine decarboxylase in the stomach within 4 hours of subcutaneous administration of EGF to neonatal mice (Feldman et al., 1978). Endogenous EGF present in the submandibular gland and saliva may play a role in the maintenance of the gastrointestinal mucosa and in gastric cytoprotection. The elimination of the major salivary glands in the rat resulted in a decrease in (<sup>3</sup>H)-thymidine uptake and DNA content of the gastric oxyntic mucosa (Skinner et al., 1984). Also, the removal of submandibular salivary glands of rats resulted in the gut becoming more prone to damage by bile salts (Skinner and Tepperman, 1981). Moreover, the

administration of exogenous EGF to experimental animals (rats, guinea pigs) promotes the healing of either mechanically-induced (Koffman et al., 1977) or reserpine-induced gastric ulcers (Gregory et al., 1978). The role of endogenous EGF present in human saliva is not yet determined, and the efficacy of exogenous EGF as an antiulcer agent in man remains to be established (Hollenberg, 1979).

Reports of the mitogenic effects of EGF on the epithelial cells of the small intestine are numerous. The effects on the small intestine have been studied in vivo and in vitro in various mammalian species including the rat and the mouse. EGF was found to promote differentiation of fetal mouse duodenal absorptive cells in organ culture isolated from 15-day-old fetuses (Beaulieu and Calvert, 1981). Also, EGF appeared to stimulate DNA synthesis or mitosis in mouse duodenal and jejunal mucosa (Chabot and Hugon, 1980), in rat duodenal mucosa (Dembinski et al., 1982), in mouse duodenal, ileal and colonic crypt cells (Al-Nafussi and Wright, 1982) and in rat jejunal and ileal crypt cells (Al-Nafussi and Wright, 1982).

EGF administration in vivo has been shown to promote maturation of the neonatal gastrointestinal tract (Malo and Menard, 1980; Calvert et al., 1982; Dembinski et al., 1982; Malo and Menard, 1982; Li et al., 1983; Oka et al., 1983; Majumdar, 1984). However, the responses in experimental animals appear to depend upon a number of factors. Firstly, age affects the responsiveness of the intestine to EGF. Chabot and colleagues (1983) administered EGF to adult mice by intraperitoneal injection and found that this regimen did not modify the protein, DNA content

nor enzyme activities of the small intestine. In support of this, Oka et al., (1983) reported that subcutaneous administration of EGF stimulated the functional maturation of the intestine of 2-week-old but not 3-week-old rats, whose intestinal maturation was virtually complete. Secondly, the health of the experimental animal affects EGF-responsiveness of target tissues. Evidence suggests that EGF can still exert a trophic effect on the gut of adult rats who have experienced gastrointestinal damage such as the atrophy associated with starvation (Dembinski et al., 1982). Thus it would appear from these studies that an immature or poorly functioning intestine is particularly responsive to EGF.

Thirdly, the mode of administration affects the nature of the response to EGF. For example, intramuscular or subcutaneous injection of EGF to two-week-old rats increased the body weight, intestinal weight and rate of protein and DNA synthesis in the intestinal mucosa but had limited or no effect on intestinal enzyme activities (Oka et al., 1983; Majumdar, 1984). In contrast, intravenous administration of EGF to suckling week-old mice increase the activities of sucrase, trehalase, glucoamylase, lactase and other intestinal enzymes along the entire small intestine (Malo and Menard, 1980, 1982). EGF was also found to promote intestinal growth and differentiation in fetal mice following the introduction of the growth factor into the maternal peritoneal cavity (Calvert et al., 1982). Since oral, intravenous and subcutaneous modes of EGF administration appear to exert varying effects on the intestine, it is plausible that both oral and systemic EGF play an important role in regulating growth and maturation

of the gastrointestinal tract in vivo.

The observation that other factors such as glucocorticoids and thyroid hormones enhance the maturation of the immature gut (Martin and Henning, 1982), together with the evidence that these hormones may regulate the number of EGF receptors (Mukku, 1984) has led to the hypothesis that the effects of cortisol and thyroxine may be mediated by EGF in vivo. Conversely, there is also evidence to suggest that the in vivo actions of EGF are mediated by classical hormones such as thyroxine. Indeed, infusion of EGF into fetal sheep for 3-14 days caused an increase in the weight of the adrenal glands as well as other neonatal organs (Thorburn et al., 1981).

Thyroid hormone has been shown to enhance the maturation of the fetal or neonatal gut by altering the activities of several intestinal enzymes, promoting the achievement of adult levels. For example, thyroxine administration to neonatal rats caused a decrease in jejunal lactase activity (Boyle et al., 1982), an increase in sucrase and maltase activity (Martin and Henning, 1982), and enhanced the maturation of several colon glycosidases (Litin et al., 1983). However, thyroid hormone does not appear to have a mitogenic effect on the proliferation of epithelial cells of the intestine (Litin et al., 1983; Arsenault and Menard, 1984).

As mentioned above, the maturation effects of hormones such as thyroxine may be mediated by EGF. In a study to determine if thyroid hormones regulate the EGF receptor, Mukku (1984) compared the binding of iodinated EGF to liver membrane preparations from euthyroid and hypothyroid rats. Binding to liver membranes of hypothyroid rats was only 30-

40% of that measured in the control membrane preparation, while treatment of hypothyroid animals with triiodothyronine 96 hours prior to sacrifice restored EGF binding to nearly control levels. These results suggest that thyroid hormones may regulate the EGF receptor system. Furthermore, the content of EGF in mouse submandibular glands increases after administration of thyroid hormones (Gresik et al., 1981) and human patients with hyperthyroidism show elevated levels of serum EGF (Uchihashi et al., 1984). Thyroid hormones may therefore regulate both the binding of EGF to its receptors, and synthesis and/or secretion of EGF in vivo.

Glucocorticoids have been shown to stimulate maturation of the neonatal intestine in vivo (Koldovsky et al., 1970) although similar effects could not be demonstrated in vitro (Arsenault and Menard, 1984). The lack of effect in vitro may be explained if the effects of corticosteroids are mediated by secondary agents such as EGF. Glucocorticoids such as cortisol and the synthetic derivative cortisone acetate have been shown to exert similar effects as the thyroid hormones on intestinal enzyme levels (Martin and Henning, 1982; Kedinger et al., 1983; Litin et al., 1983; Arsenault and Menard, 1984).

From the various studies of gastrointestinal maturation, it may be concluded that the development of the small intestine is likely to be modulated by a number of interacting substances such as growth factors and some classical hormones. These substances may act synergistically, or exert their actions through other mediators. It is also apparent that the absence of an individual factor does not prevent maturation of the intestine from ultimately occurring in the developing

neonate.

Whilst a substantial number of studies have investigated the effects of EGF on the small intestine, up until 1985 very little data existed on the effect of EGF on the developing or adult colon. In 1980, Scheving et al., (1980) found that EGF increased DNA synthesis in the mouse colon, maximal stimulation occurring at 8 and 12 hours after the injection. Similarly, Al-Nafussi and Wright (1982) observed that EGF stimulated colonic crypt cell proliferation in mice 8 hours after injection, but found that in the case of the rat, the colon was unresponsive to EGF. Other workers, however, have found no effect of EGF on the colon of mice (Feldman et al., 1978) or rats (Johnson and Guthrie, 1980). Since DNA synthesis in the gastrointestinal tract exhibits circadian variation and the stimulatory effect of EGF has been shown to depend upon the particular time at which it is administered in vivo (Scheving et al., 1979; 1980), failure to consider this fact could lead to a conflict of results in intact animals.

In support of the in vivo findings of EGF action on the colon, Sadiq and colleagues (1984) demonstrated the presence of plasma membrane-bound EGF receptors in the fetal rabbit colon as well as the stomach and small intestine. Interestingly, the fetal colon displayed the highest specific binding and receptor density of all tissues studied, confirming earlier findings of Scheving et al., (1980). Scheving and colleagues observed a consistent and certainly more dramatic effect of EGF on colonic rectal DNA synthesis compared to that in other regions of the intestine. Therefore, EGF seems to have an accentuated effect on certain phases of the maturative process of the

colon.

EGF may have a role in the development of other gastrointestinal organs such as the exocrine pancreas and the liver. Not only has EGF been detected in pancreatic juice (Hirata et al., 1982) and extracts from the human pancreas (Hirata and Orth, 1979), it has also been reported to enhance pancreatic growth in newborn rats (Dembinski et al., 1982) and to stimulate basal and cholecystokinin stimulated release of amylase from cultured acini (Logsdon and Williams, 1983). In support of a direct action in pancreas, murine EGF has been shown to bind to mouse pancreatic acini in vitro (Logsdon and Williams, 1983).

Substantial evidence supports a role for EGF in hepatic growth and differentiation. As early as 1965 it was observed that exogenous EGF administration caused an accumulation of fat stored primarily as triglyceride in the liver of experimental animals (Heimberg et al., 1965); later, EGF was demonstrated to cause bile duct proliferation in vivo (Farebrother and Mann, 1970). In adult rat hepatocytes in vivo or in primary culture, EGF stimulates DNA synthesis (Richman et al., 1976) and has been reported to induce hepatic hypertrophy and hyperplasia (Bucher et al., 1978). In newborn rats, EGF induces thymidine incorporation into developing liver cells and enhances mitosis (Richman et al., 1976; Draghi et al., 1980). EGF receptors have been found on hepatocyte membranes (O'Keefe et al., 1974), and an EGF-receptor complex has been isolated from hepatocytes using a glutaraldehyde/sodium borohydride crosslinking technique (Sahyoun et al., 1978). Electron microscope evaluation of isolated hepatocytes in culture demonstrates that hepatocytes have the capacity to



internalize EGF in vitro (Carpentier et al., 1981). Evidence for the capacity of the liver to sequester EGF from the systemic or portal circulations in vivo has been supplied by studies of St. Hilaire et al. (1983). A bolus dose of  $^{125}\text{I}$ -labelled EGF was administered to intact adult rats by ingestion into either the hepatic or portal vein. Ninety-nine percent of the intraportal dose was taken up by the liver in 3 minutes whereas 58% of the intrahepatic dose appeared in the liver in 10 minutes. Of the EGF sequestered by the liver from the portal vein, approximately one fifth appeared in the bile as immunoreactive EGF within 90 minutes.

1.12(e) The Biological effects of EGF on the mammary gland.

The presence of EGF in the milk and colostrum of various mammalian species (see Section 1.6) is consistent with a role for EGF in mammary gland physiology. The EGF in milk may be related to the needs of the suckling neonate and/or may occur as a consequence of a primary function involving the stimulation and regulation of mammary gland proliferation during lactation. In this section of the review evidence supporting the role of EGF in the growth and development of the mammary gland will be given.

As early as 1969, the proliferative effect of EGF on mammary gland epithelial tissue maintained in organ culture was reported by Turkington (1969). Since then other reports have shown that EGF is a mitogenic factor for mammary cells *in vitro* (Papadimitriou et al., 1977; Tonelli and Sorof, 1980; Imagawa et al., 1982; Taketani and Oka, 1983a). Taketani and Oka (1983a) found that EGF stimulated cell proliferation but inhibited functional differentiation of

mouse mammary cells in culture, thereby acting as a regulator of hormone-dependent growth and differentiation. The hypothesis that EGF is an important growth-regulator of the mammary gland is strengthened by the demonstration of saturable specific EGF receptors in both normal and malignant mammary tissues (Taketani and Oka, 1983b; Fitzpatrick et al., 1984a and 1984b).

The role of EGF in the development of the mammary gland has been demonstrated in vivo by Okamoto and Oka (1984), who found that the mammary gland of lactating sialoadenectomized mice was smaller in size and produced less milk compared with that of normal mice. When 5µg EGF was injected daily into sialoadenectomized mice, the capacity of the mothers to nurse their offspring was restored to that of control animals. Given that the salivary glands are a rich source of EGF in mice (Cohen, 1962), the results of Okamoto and Oka (1984) suggest that endogenous EGF produced in the submandibular gland is vital to the development of the mouse lactating mammary gland. In addition, it would appear that the mammary gland is capable of sequestering EGF from the systemic circulation. In both lactating mice (Gresik et al., 1984) and goats (Blakeley et al., 1982), EGF can be transported from the circulation across the mammary epithelium and secreted in the milk. (see section 1.9).

EGF may not only be involved in the growth of the normal mammary gland but also that of the tumor-bearing gland. In recent years, several lines of evidence have implicated EGF in the process of neoplastic transformation (Turkington, 1969b; Ross et al., 1976; Carpenter and Cohen, 1979; Harrison and Auersperg, 1981). Fitzpatrick et al. (1984b) have suggested that EGF may also be important in

regulating the growth of some mammary gland cancer cells in vivo because of its mitogenic action on some mammary gland cell lines in vitro. In these studies a positive correlation was found to exist between the level of EGF binding and the percentage of malignant cell types present in the sections of various tumors, suggesting a role for EGF in the development and progression of breast cancer.

1.12(f) Other Biological effects of EGF.

In addition to its effects in skin, breast, lung, gut and liver, EGF has been shown to promote growth of other tissues including kidney, brain and bone. Several groups have demonstrated mitogenic responses to EGF in isolated kidney cells (Carpenter and Cohen, 1978; Hollenberg, 1975), results that are indicative of a direct EGF action in kidney. A direct action on kidney, a tissue of mesodermal origin, is not surprising in view of the known mitogenic actions of EGF in a wide variety of ectodermal or mesodermal cells (Carpenter and Cohen, 1979). With respect to the brain, EGF has been shown to be mitogenic for rat brain astrocytes in tissue culture (Leutz and Schachner, 1981; Vellis and Herschman, 1982). In addition, a role in motor function has been suggested as a consequence of the immunohistochemical detection of EGF in the extrapyramidal components in the rat brain in situ (Fallon et al., 1984). EGF has also been implicated as a factor involved in the development of bone by studies demonstrating a stimulation of DNA synthesis in osteoblast-like cells in vitro (Ng et al., 1983) and in organ culture (Canalis and Raisz, 1979).

In summary, the many biological responses to EGF in a variety of cell and organ culture systems, together with the many observations of EGF action in vivo strongly suggest a

role for EGF in mammalian growth and development. In view of the known actions of EGF on crucial neonatal tissues such as the gut and the lung, EGF may be useful in the treatment of preterm infants. Indeed, the well-being of the infant at birth largely depends upon the functioning capacity of the respiratory system and gastrointestinal tract. This being so, the effects of EGF on postnatal development of these crucial tissues have received considerable attention as part of a search by neonatologists for any agents that can be used to stimulate growth and maturation of these tissues, particularly in premature infants.

To conclude, future research directions will include the identification of clinical applications of EGF to improve the growth and development of human infants and to aid in the treatment of poorly-functioning tissues in the adult as a result of disease processes.

#### 1.13 Biological Role(s) of Milk-derived EGF.

The high concentrations of growth factors in milk are suggestive of a role in the regulation of the growth and maturation of infant tissues. Certainly it is established that immature tissues respond to growth factors, as described in earlier sections of this review. However, the importance of milk, rather than endogenous synthesis, as a source of growth factors in the infant has not been clarified. The presence of high concentrations of growth factors in milk cannot be taken as proof of a role in infant development because, for reasons discussed earlier, growth factors in milk may at least partly reflect an involvement in the maintenance of the lactating breast.

It is difficult to establish from an epidemiological standpoint the significance of milk growth factors to the

human infant. Clinical observations have shown that normal term infants receiving artificial diets grow at comparable rates at the gross level as counterparts receiving breast milk (Fomon et al., 1971; Holly and Cullen, 1977; Swiet et al., 1977; Evans, 1978). In fact, some studies have shown that artificially fed infants gain weight more rapidly than those receiving breast milk (Taitz, 1971; Neumann and Alpaugh, 1979) although these were not pair-feeding experiments. However, while normal healthy breast-fed infants do not appear to have an advantage over infants receiving alternative diets, preterm or sick infants may receive beneficial effects from breast milk. For example, the incidence of gastrointestinal diseases is greater amongst infants receiving artificial formulas than infants who are breast fed (Myers et al., 1984). Although the reason for this observation is not known, it is plausible that growth factors in breast milk may offer a direct luminal protective or trophic effect to the neonatal intestine. It is concluded that while epidemiological studies indicate that dietary growth factors do not appear essential to infant survival and overall body growth, particularly in healthy term infants, adequately controlled trials are not available (or indeed feasible) in human infants to assess the importance of exogenous growth factors. Clearly, quantitative studies on the intestinal digestion, absorption and biological responses to oral growth factors in experimental animal models of the human infant are needed to clarify this point.

In order to establish a biological role for any milk growth factor, it is necessary to demonstrate that biologically active amounts of the growth factor (1) are

present in milk, (2) survive gastrointestinal digestion, (3) are absorbed intact into the circulation (for direct action on peripheral tissues) and (4) reach the target tissues. Of the known growth factors in human milk, EGF would appear to represent a major activity. In this section of the review, the evidence supporting a role for milk-derived EGF in infant development will be discussed in relation to the four above criteria. Some of the studies mentioned hereafter have already been discussed in previous sections of this review. However, since the results of these particular studies are pertinent to the following discussion, they are also included in this section.

1) Concentrations of EGF in human milk.

The concentration of EGF in human milk is very high relative to that required for mitogenic responses in isolated cells. The concentration of EGF required for mitogenic effects to occur in vitro is approximately 0.1nM (Ballard and Gunn, 1984), whereas the concentration of EGF in human colostrum is approximately 50 nM and in mature milk is of the range 5-20 nM (Read et al., 1984). Given that the concentration of EGF in mature human milk is in the range of 5-20 nM or 30-111  $\mu\text{g/l}$ , a 5 Kg. human infant consuming 500 ml. milk per day would ingest up to 10  $\mu\text{g}$  EGF per kilogram. In view of the fact that mitogenic responses were observed in the gastrointestinal mucosa of rats following oral administration of 30  $\mu\text{g}$  EGF/kg/day (Dembinski et al., 1982), it is conceivable that the amount of EGF consumed daily by suckling human infants could exert a direct luminal trophic effect on the gut of the infant.

It appears that endogenous production of EGF may be very low in the fetal and neonatal period (Kasselberg et

al., 1985; Van Noorden et al., 1977). Thus, exogenous milk-derived EGF may be the infants' major source of EGF. A low rate of endogenous production, combined with the presence of large amounts of EGF in the diet suggests that milk may well provide an important source of EGF to the suckling infant. In contrast, human milk does not appear to be such an important source of other milk growth factors such as insulin-like growth factors, where the concentration in milk is low relative to that in the infant at birth (Read et al., 1984).

2) Gastrointestinal survival of milk EGF.

Evidence has been given in previous sections of this review to suggest that a significant amount of orally-administered EGF would survive digestion in the suckling neonate. To reiterate, Thornburg and colleagues (1984) have provided qualitative evidence that EGF remains intact for long periods in the neonatal rat intestine. Thirty to sixty minutes following intragastric infusion of  $^{125}\text{I}$ -labelled EGF in neonatal rat pups, over 90% of radioactivity recovered from the stomach and intestinal flushing represented intact immunoreactive EGF, indicating that a proportion of EGF reaches the intestine undegraded. Furthermore, studies of the effect of oral EGF on neonatal development in vivo also support the hypothesis that EGF survives intestinal digestion in the infant. For example, Cohen and Taylor (1974) reported that oral EGF could induce precocious eyelid opening and premature incisor eruption in newborn mice. Whilst qualitative evidence exists for EGF survival, as yet quantitative studies have not been reported.

3) Gastrointestinal absorption of milk EGF.

The evidence for intestinal absorption of EGF has also

been stated in the previous sections of this review. Notwithstanding this evidence, there is little information pertaining to the quantitative aspects of intestinal uptake of milk-derived EGF. It is therefore uncertain whether milk EGF crosses the intestinal barrier in amounts sufficient to exert direct effects on distant target tissues such as the neonatal gut and lung.

4) Clearance of EGF absorbed by the neonatal intestine.

The concentration of EGF reaching distant target tissues not only depends upon both the survival and absorption of EGF but also the rate of clearance from the circulation. The route of intestinal absorption will directly affect the rate of EGF clearance in the neonate. If EGF is absorbed exclusively into blood, the portal circulation, is must first survive hepatic clearance before entering the systemic circulation. However, if EGF is absorbed into the lymphatic circulation it will bypass the hepatic circulation.

The rate of clearance of EGF from the general circulation in vivo is likely to be very rapid according to the evidence reported in the literature. From studies involving a bolus intravenous dose of radiolabelled EGF to adult dogs, Elder et al (1978a) reported that the circulating half-life for EGF was 1-4 minutes, EGF being undetectable in the blood after 10 minutes. They also reported that a small but detectable amount of the administered radioactivity (3-6%) appeared in the canine urine as immunoreactive EGF. In support of this, Nexo et al., (1981), have also reported that circulating EGF is rapidly cleared into the kidney and subsequently concentrated in urine. Up until 1984, therefore, it was



generally considered that urinary EGF originated from plasma EGF filtered by the kidney. However, a significant proportion of urinary EGF now appears to have a renal origin (Olsen et al., 1984).

As mentioned above, if EGF is absorbed into the portal circulation, it must first survive passage through the liver in order to then enter the general circulation. St. Hilaire et al., (1983) examined the fate of EGF in the portal circulation and found that 99% of a bolus dose of  $^{125}\text{I}$ -labelled EGF administered to rats by intraportal injection was taken up by the liver in 3 minutes, of which a small percentage of intact EGF appeared in the bile after 90 minutes. Burwen et al., (1984) supported these findings giving evidence for the existence of a non-lysosomal pathway to enable the transport of small amounts of intact EGF from the portal circulation to the bile. It would appear that the liver sequesters EGF from the portal circulation of which only a small percentage survives hepatic clearance to be secreted directly into the bile.

Since the route of absorption from the gut and the clearance rates of EGF are not yet established, the amount of orally-administered EGF reaching neonatal tissues such as the lung cannot be predicted. Therefore, as yet the importance of milk-derived EGF in the growth and development of crucial neonatal tissues cannot be evaluated without quantitative analysis of these parameters.

#### Overall Conclusion.

Provided it can be demonstrated that EGF survives digestion, it appears likely that milk-derived EGF could stimulate growth of the infant gastrointestinal tract, in view of the known biological responses to EGF administered

topically in the gut. If EGF is further shown to cross the intestinal barrier intact in biologically active amounts, it is also likely that milk EGF would play an important direct role in growth and maturation of peripheral tissues, notably lung.

Table 1.1 Representative polypeptide growth factors and related substances

A. Detailed Characterization

Epidermal growth factor (EGF)  
Insulinlike growth factors I & II (IGF I & II)  
Interleukin-2 (T-cell growth factor)(IL-2)  
Nerve growth factor (NGF)  
Platelet-derived growth factor (PDGF)  
Transforming growth factor (Type I or  $\alpha$ )(TGF)  
Insulin

B. Partial characterization

Cartilage-derived growth factor  
Colony-stimulating factors (CSFs)  
Endothelial-cell growth factors(ECGFs)  
Erythropoietin  
Eye-derived growth factor (EDGF)  
Fibroblast-derived growth factor (FDGF)  
Fibroblast growth factors (FGFs)  
Glial growth factor (GGF)  
Osteosarcoma-derived growth factor (ODGF)  
Thymosin  
Transforming growth factor (Type II or  $\beta$ )(TGF)

C. Initial characterization

B-cell growth factor  
Bone-derived growth factor  
Chondrocyte growth factor  
Endothelial-derived growth factors  
Macrophage-derived growth factors  
Neurotrophic growth factors  
Transforming growth factors ( $\gamma$ )

Table 1.2 Growth factors identified in the milk of various species

Growth factor	Major sources	Target tissue	Mol. wt (kDa)
Bombesin-like peptides	Various mammalian tissues	Various tissues, e.g. gut, lung	1-3
Bone-derived growth factor	Bone, cartilage	Calvariae, chondrocytes	6-8
Colony-stimulating factor (CSF)	Cultured mouse L-cells, human urine	Immature granulocytes, macrophages	40-70
Endothelial cell growth factor	Bovine hypothalamus	Endothelial cells	17-25
Epidermal growth factor (EGF)	Mouse submaxillary, human urine	Epidermal cells, fibroblasts	6
Erythropoietin	Plasma	Red cell precursors	27-62
Fibroblast-derived growth factor	Transformed fibroblasts	Fibroblasts	31
Fibroblast growth factor (FGF)	Bovine pituitary, brain	Mesodermal cells	16
Insulin	Pancreas	Most cell types	6
Insulin-like growth factor I (IGF-I)/somatomedin C	Serum, liver, fibroblasts	Most cell types	7.5
Insulin-like growth factor II (IGF-II), MSA	Human, rat serum, cultured BRL-3A rat liver cells	Most cell types	7.5
Interleukin-2	T-cell hybridoma cells	T cells	5
Nerve growth factor (NGF)	Mouse submaxillary gland	Sympathetic and sensory neurons	13
Platelet-derived growth factor (PDGF)	Human platelets	Fibroblasts, smooth muscle	30
Somatomedin B	Human plasma	Fibroblasts	5
$\alpha$ -Transforming growth factor ( $\alpha$ -TGF)	Various transformed cells	Epithelial cells, fibroblasts	6
$\beta$ -Transforming growth factor ( $\beta$ -TGF)	Many tissues	As $\alpha$ -TGF	23-25
$\gamma$ -Transforming growth factor ( $\gamma$ -TGF)	Transformed cells	Fibroblasts	11
Thrombin	Plasma	Fibroblasts	30

Figure 1.1 Amino acid sequence of mouse EGF with placement of disulphide bonds (Savage et al., 1973).

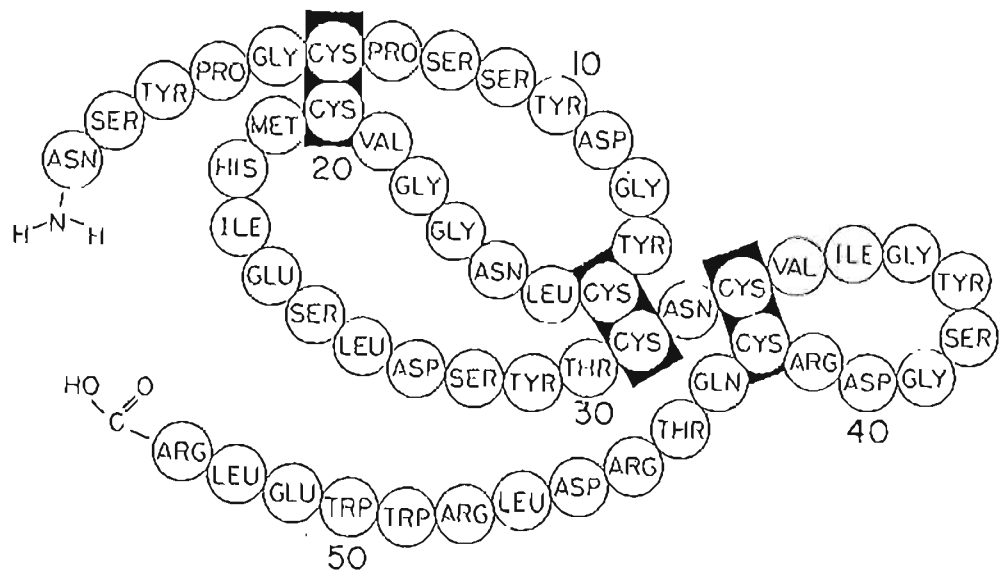


Figure 1.2 Amino acid sequences of mouse EGF and human EGF / urogastrone (Gregory, 1975).

Asn	Ser	Tyr	Pro	Gly	Cys	Pro	Ser	Ser	10 Tyr	Asp	Gly
Asn	Ser	Asp	Ser	Glu	Cys	Pro	Leu	Ser	His	Asp	Gly
Tyr	Cys	Leu	Asn	Gly	Gly	Val	20 Cys	Met	His	Ile	Glu
Tyr	Cys	Leu	His	Asp	Gly	Val	Cys	Met	Tyr	Ile	Glu
Ser	Leu	Asp	Ser	Tyr	30 Thr	Cys	Asn	Cys	Val	Ile	Gly
Ala	Leu	Asp	Lys	Tyr	Ala	Cys	Asn	Cys	Val	Val	Gly
Tyr	Ser	Gly	40 Asp	Arg	Cys	Gln	Thr	Arg	Asp	Leu	Arg
Tyr	Ile	Gly	Glu	Arg	Cys	Gln	Tyr	Arg	Asp	Leu	Lys
Trp	50 Trp	Glu	Leu	Arg	-----	EGF (mouse)					
Trp	Trp	Glu	Leu	Arg	-----	EGF (human)/urogastrone					



CHAPTER TWO

SCOPE OF THE THESIS

From the knowledge of EGF in January 1985, the following points are clearly established with respect to the potential role of milk-derived EGF in infant development:

- Human milk contains high concentrations of EGF.
- EGF will stimulate the growth and maturation of various infant tissues, including the gut and lung, both of which are crucial neonatal tissues.

What is unclear is:

- The proportion of orally-administered EGF that survives digestion to reach various sections of the gastrointestinal tract in a biologically active form.
- The proportion of EGF that is absorbed intact into the circulation, and the route of absorption (portal blood or intestinal lymph).
- The rates of clearance of EGF absorbed into the portal vein or intestinal lymph.

While qualitative information is available on the intestinal survival and absorption of EGF in suckling neonates, quantitative information is lacking. In this thesis both the intestinal survival and absorption of orally-administered EGF will be examined in a quantitative manner under conditions close to the physiological using the newborn lamb as an experimental animal model of the human infant. The fate of orally-administered EGF will then be determined in the human infant by an indirect, non-invasive, yet ethically feasible manner.

The results of the studies undertaken by the candidate are described in Chapters 5-8. A brief description of the contents of each of these chapters is given below.

#### Chapter Five.

In this chapter, the properties of recombinant hEGF are

compared with the naturally-occurring EGF present in human milk and urine. Since the concentration of hEGF in natural sources is too low for the purposes of large scale purification, and milligram quantities of EGF were required for the biological studies for this thesis, recombinant hEGF was considered as a potential substitute for milk-derived hEGF. Since milk-derived EGF had not been well characterized, it was necessary to prove that both the recombinant and the natural milk growth factor were identical before use in the in vivo studies.

#### Chapter Six.

Monoclonal antibodies and a specific polyclonal antiserum were developed for use in the development of specific radioimmunoassays required for the in vivo studies described in this thesis. In this chapter, the production and characterization of a specific polyclonal antiserum and a range of monoclonal antibodies directed against human recombinant EGF is presented. In addition, the hEGF binding site of each antibody was investigated using synthetic octapeptides homologous with the primary amino acid sequence of hEGF.

#### Chapter Seven.

The intestinal survival and absorption of milk-derived EGF in the suckling human infant cannot be assessed quantitatively by direct methods. However, the analysis of urinary EGF provides an indirect yet ethically practical measure of EGF uptake from the gut in premature babies. In this chapter, the urinary excretion of EGF in premature human infants receiving breast milk, a rich source of EGF, is compared with the urinary EGF excretion of counterparts receiving diets of low EGF content.

Chapter Eight.

Since direct measurements of EGF intestinal survival and absorption are not feasible in the human infant, these measurements were made in newborn lamb as a model of the human infant under conditions close to the physiological. To assess the survival of EGF in the neonatal intestine, chronic reentrant catheters were surgically implanted in newborn lambs to enable complete collection of small intestinal contents following gastric administration of EGF. Analysis of intestinal absorption of EGF was achieved by adaptation of an in situ autoperfused intestinal method to the newborn lamb.

CHAPTER THREE

ACKNOWLEDGEMENTS.

The work for this thesis has taken full advantage of the techniques and biochemical reagents already established or present in the laboratories of Dr. John Wallace (Biochemistry Department, University of Adelaide), Dr. John Ballard (CSIRO, Division of Human Nutrition), and Dr. Leanna Read (Waite Institute, Department of Animal Sciences, University of Adelaide), and therefore the work of fellow colleagues in these three research groups contributing to this thesis will be acknowledged below. In addition, the research for this thesis has been greatly enhanced by collaboration with other scientists and research groups, whose help is acknowledged under the appropriate chapter headings below. Any work not acknowledged in this section should be regarded as work completed by the candidate of this PhD thesis.

### Chapter Three.

Human recombinant EGF was kindly donated by Dr. Carlos George-Nascimento (Chiron Corporation, Emeryville, C.A., U.S.A.), and mouse EGF was supplied by Dr. J. Koch (Division of Molecular Biology, CSIRO (Australia, North Ryde, N.S.W., Australia). An initial supply of a specific polyclonal antiserum raised in rabbits against recombinant human EGF was provided by Dr. B. Kemp (Department of Medicine, Repatriation General Hospital, Heidelberg, Victoria, Australia), and a polyclonal antiserum raised in rabbits against mouse EGF was prepared by Ms. Lia Summer in 1984 in the laboratory of Dr. John Wallace. The radioreceptor assay for EGF was developed by Drs. Leanna Read and John Ballard. The tissue culture work for the preparation of cell lines required for receptor and biological assays was carried out by Ms. Paula Gravestock and Ms. Marina Ross.

### Chapter Five.

Human urinary and milk EGF used in this study were partially purified from adult male urine and human milk respectively by Ms. Lia Summer in 1984 in the laboratories of Dr. John Wallace and Dr. John Ballard.

### Chapter Six.

The preparation of the polyclonal antiserum and monoclonal antibodies was completed entirely by the candidate. For technical reasons, the fusion procedure in the production of monoclonal hybridoma cultures was completed by the candidate in the laboratory of Dr. N. Hoogenraad, (Department of Biochemistry, La Trobe University, Sydney, N.S.W., Australia), but the remaining work for the production of these antibodies was completed at the University of Adelaide.

The epitope mapping procedure involving the synthesis of synthetic octapeptides homologous with the hEGF sequence was completed entirely at CSL, Parkville, Victoria, Australia under the supervision of Dr. M. Geysen, Department of Biochemistry.

### Chapter Seven.

The individual urine specimens for the human infant study were recorded and collected by the candidate from the Neonatal Intensive Care Unit, Flinders Medical Centre, under the direction of Drs. Peter Marshall and Simon James. Specimens from the Neonatal Intensive Care Unit, Queen Victoria Hospital were collected by Ms. Linda Faull. The determination of creatinine concentration in the urine samples was greatly assisted by Ms. Judy Burgoyne.

### Chapter Eight.

Whilst the procedures for the surgery were primarily

developed by Dr. Leanna Read, the candidate became proficient at the surgical techniques and performed all the surgical experiments described in this thesis, with the aid of technical assistants Mr. Callum Gillespie, Mr. Jamie McNeil and Mr. Andrew Lord in Dr. Read's laboratory.

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CHAPTER FOUR

GENERAL MATERIALS AND METHODS.

## GENERAL MATERIALS AND METHODS.

This section describes materials and methods that are applicable to more than one of the following chapters.

### 4.1 Growth Factors.

Chiron Corporation generously provided milligram quantities of purified recombinant hEGF [Batch 9A02XX] for all of the biological studies undertaken in this thesis.

Recombinant human EGF was produced in yeast cells transformed with plasmids containing the chemically synthesized gene for human EGF [hEGF] (Urdea et al., 1983; Brake et al., 1984).

Mouse EGF [mEGF] was kindly supplied by Dr. J. Koch (Division of Molecular Biology, CSIRO [Australia], North Ryde, N.S.W., Australia). This preparation of mEGF was isolated from the submaxillary glands of adult male mice by the method of Savage and Cohen (1972).

For the preparation of EGF standard solutions of known concentration for routine use in EGF assays, reverse-phase HPLC techniques were used to accurately determine the protein concentration of EGF in stock solutions, (Burgess et al., 1982) which were then stored at  $-20^{\circ}\text{C}$  at a concentration of  $2 \times 10^{-4}\text{M}$  in 10 mM HCl. On the same day of assay, aliquots of these stock solutions were diluted in the appropriate assay buffer to give a suitable range of standards.

### 4.2 Radiolabelling of Growth Factors.

For the studies cited in the following chapters, both mEGF and hEGF were used as tracer radiolabelled ligands in both radio-receptor assays and radioimmunoassays. Both hEGF and mEGF were iodinated using either the iodogen method or the chloramine-T method. Human EGF was also labelled with

tritium for some studies by reductive methylation using tritiated-sodium borohydride.

Materials for radiolabelling procedures.

1, 3, 4, 6 - Tetrachloro - 3 $\alpha$ , 6 $\alpha$  - diphenyl glycouril (Iodogen) was obtained from Pierce and Warriner (U.K.) Ltd., Chester, England; carrier-free Na<sup>125</sup>I in NaOH [100 mCi/ml] and tritiated sodium borohydride [11.8 Ci/mmol] from the Radiochemical Centre, Amersham, Bucks., England. Sephadex G-50 fine was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; chloramine-T and sodium metabisulphite from BDH Chemicals Ltd.

(a) Iodination of EGF by the Iodogen method.

The procedure for the iodination of EGF using the Iodogen method was adapted from a method previously published by Fraker and Speck (1978). Iodogen [1, 3, 4, 6 - tetrachloro - 3 $\alpha$ , 6 $\alpha$ , diphenylglycouril] was first described by Fraker and Speck as a reagent for iodinating proteins and cell membranes. Virtually insoluble in water, iodogen mediates rapid iodination in the solid phase with aqueous solutions of iodide ions and proteins.

Iodogen was dissolved in dichloromethane at 40 $\mu$ g/ml and 100 $\mu$ l of this Iodogen solution was evaporated to dryness in the bottom of 1ml polypropylene tubes at room temperature under nitrogen (Markwell and Fox, 1978). These dried tubes were then stored desiccated for up to 6 months at -20°C.

Eighty microlitres of potassium phosphate buffer [0.05M, pH 7.6] containing 0.9% sodium chloride and 10 $\mu$ g EGF [1.6 nmol] were added to the iodination tube, followed by Na<sup>125</sup>I [0.5mCi, 5 $\mu$ l]. After 15 minutes at room temperature an excess of potassium iodide [20 $\mu$ l; 0.2M] was added and the contents of the iodination vial loaded immediately onto a

Sephadex G-50 fine column as described below. By this procedure, the iodination reaction was terminated by removing the solution from the insoluble film of iodogen. An excess of unlabelled iodide was added to prevent subsequent radiolabelling of albumin on the G-50 column, that could have occurred if any iodogen was dislodged from the reaction vial.

(b) Iodination of EGF by the chloramine-T method.

The method used was that of Von Obberghen-Schilling and Pouyssegur, (1983), but modified by substantially limiting the amount of chloramine-T used, thereby preventing excessive oxidation of the protein. Human or mouse EGF [10 $\mu$ g, 1.6 nmol] in 0.05M sodium phosphate [pH 7.4, 10 $\mu$ l] was placed in an iodination tube and 1 mCi carrier-free 50 $\mu$ M sodium iodide [ $^{125}$ I] in NaOH and 400 $\mu$ M chloramine-T in water [20 $\mu$ l] were successively added. This reaction was terminated after 45 seconds by the addition of 40 $\mu$ l 500 $\mu$ M sodium metabisulphite in H<sub>2</sub>O, and loaded immediately onto a Sephadex G-50 fine gel-filtration column for purification of the tracer.

(c) Reductive Methylation of EGF to prepare  $^3$ H-EGF.

The procedure was adopted from the method published by Tack et al., 1980. In a reaction vial placed on ice, 30 nanomoles of EGF was dissolved in 60  $\mu$ l borate buffer [150mM, pH 9] containing 0.2% formaldehyde. Approximately 0.08 $\mu$ mole or 1 mCi of solid tritiated sodium borohydride [11.8 Ci/mmole] was added, and the mixture was then left to react to completion for 30 minutes before the contents of the reaction tube were transferred to a Sephadex G-50-fine gel filtration column for purification of the tracer.

(d) Purification of Tracer by Gel Filtration.

A column of Sephadex G-50 fine [1 x 30cm] was

equilibrated before use with 0.05M sodium phosphate buffer [pH 7.4] containing bovine serum albumin [1% w/v]. Reaction vial contents from the iodination and reductive methylation of EGF were loaded onto the column, which was then run at a flow rate of 15ml/hour. Fractions [1ml] were collected and the radioactivity counted on either a gamma counter (in the case of the iodinated species) or Beta counter (in the case of the tritiated species).

(e) Determination of the percentage of radioactivity associated with EGF.

The fraction of radioactivity associated with protein was calculated by precipitation of the purified tracer with trichloroacetic acid. Ten microlitres from each of the fractions collected from each column run were added to tubes containing 450µl of 50mM sodium phosphate buffer [pH 7.4] / 0.5% bovine serum albumin. The tubes were then placed on ice following the addition of 50µl of 100% trichloroacetic acid, added to precipitate the protein from solution. The tubes were then centrifuged at 1500g for 30 minutes at 4°C, the pellets and supernatants separated, and the radioactivity determined in each. The percentage of radioactivity associated with protein was calculated from the ratio, radioactivity in the pellet: total radioactivity in the pellet and supernatant combined.

(f) Determination of the specific activity of each radiolabelled species.

The radioactivity associated with each of the fractions containing trichloroacetic acid precipitable protein was summed to calculate the specific activity of the radiolabelled protein. This calculation represents an approximation of the actual specific activity because it was

assumed that the amount of protein originally added to the reaction mixture was totally recovered in the fractions collected from the gel filtration purification step.

The specific activity of various preparation of  $^{125}\text{I}$ -labelled mEGF or hEGF using either the chloramine-T method or iodogen method were in the range of 50-95 Ci/g. The specific activity of tritiated human EGF was generally much lower than that of the iodinated moieties, being approximately 0.2 Ci/g.

#### 4.3 Specific Polyclonal antisera against mEGF and hEGF.

A polyclonal antiserum raised in rabbits against recombinant hEGF was generously provided by Dr. B. Kemp (Department of Medicine, Repatriation General Hospital, Heidelberg, Victoria, Australia). Since this supply of antiserum was limited and insufficient in quantity for all of the biological study requirements, another specific polyclonal antiserum against recombinant human EGF was prepared by the candidate. To prepare a polyclonal antiserum to human recombinant EGF a rabbit was injected at multiple intra-dermal sites over the mid-dorsal region, with hEGF [100 $\mu\text{g}$ ] dissolved in 2ml of a solution containing  $\text{KH}_2\text{PO}_4$  [10mM] and 0.9% [w/v] NaCl, pH 7.4 and emulsified with an equal volume of Freund's complete adjuvant (Commonwealth Serum Laboratories, Parkville, Victoria, Australia). The rabbit was immunized again 3 weeks [100 $\mu\text{g}$  hEGF], 8 weeks [50 $\mu\text{g}$ ] and 17 weeks [50 $\mu\text{g}$ ] after the first injection and blood samples collected every 2-5 weeks. Antiserum was collected 24 weeks after the primary immunization when the titre had reached a maximum.

A polyclonal antiserum raised in rabbits against mouse EGF was prepared by Ms. Lia Summer in the laboratory of Dr.

John Wallace. The immunization procedure was similar to that described above for the preparation of the hEGF specific antiserum.

#### 4.4 Materials for radioimmunoassays and radioreceptor assays.

Human gamma-globulin and PEG 6000 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Fetal calf serum was from Commonwealth Serum Laboratories, Parkville, Victoria, Australia, while tissue culture media were purchased from Gibco, Grand Island, NY, U.S.A. Bovine serum albumin was Sigma Fraction V, extracted by the method of Chen (1976) to remove growth factors; L-[4,5-<sup>3</sup>H] leucine [40-60 Ci/mmol] was purchased from the Radiochemical Centre, Amersham, Bucks, U.K. Normal rabbit serum and affinity purified sheep anti-rabbit antiserum was purchased from Silenus Laboratories, Hawthorn, Victoria, Australia.

#### 4.5 Radioimmunoassays for mEGF and hEGF.

During the time spent completing the experimental work for this thesis some aspects of radioimmunoassay methodology were improved. Therefore the method of fluid radioimmunoassay used in the first series of studies described in Chapter 5 (method 1), differs from the method used subsequently in Chapters 6, 7 and 8. In the initial method developed, method 1, 20% polyethylene glycol was added to each tube to precipitate the antibody from the incubation mixture. In the later method, method 2, the incubation mixture was pre-incubated with a second antibody directed against the first antibody before the addition of a lower percentage of polyethylene glycol (6%), yielding a more specific precipitation of immunoglobulin that improved assay accuracy and sensitivity.

Method 1. Fluid-phase radioimmunoassay for hEGF and mEGF.

The incubation volume of 250 $\mu$ l; consisted of 50 $\mu$ l antiserum [anti-hEGF or anti-mEGF], 150 $\mu$ l  $^{125}$ I-labelled hEGF or mEGF [8000 c.p.m., 0.1 ng] and 50 $\mu$ l of different concentrations of mEGF or hEGF (milk, urine or recombinant). Concentrations of anti-sera [anti-hEGF, 1:100,000; anti-mEGF, 1:20,000] were chosen such that 30-40% of added tracer bound to the homologous antibody in the absence of competing EGF. The specific polyclonal antiserum against hEGF used in this method was donated by Dr. B. Kemp. Antisera, tracers and growth factors were diluted in a solution containing phosphate buffer [25 mmol/l; pH 7.4], NaCl [100 mmol/l], EDTA [10 mmol/l] and bovine serum albumin [10 g/l]. Tubes were incubated for 1 h at 37°C then for 48 h at 2°C, after which a 0.5ml volume of ice-cold human gamma-globulin [1 mg/ml] in  $\text{KH}_2\text{PO}_4$  [0.1 mol/l; pH 7.4] was added to each tube, followed immediately by 0.75 ml ice-cold 20% polyethylene glycol 6000 in  $\text{KH}_2\text{PO}_4$  [0.1 mol/l; pH 7.4] to precipitate the antibody. Tubes were mixed thoroughly, left for 3-5 min. in ice and then centrifuged at 1500 g for 30 min. at 4°C. Supernatants were aspirated and the precipitates counted for gamma radioactivity. Radioactivity bound to tubes in the absence of antibody was subtracted from the total to obtain the antibody-bound radioactivity. No correction was made for radioactivity bound in the presence of excess amounts of unlabelled EGF.

Method 2. Fluid-phase radioimmunoassay for hEGF.

In Method 2, the specific polyclonal antiserum against human EGF was the antiserum prepared by the candidate. The final dilution of rabbit anti-human EGF antiserum required to bind approximately 50% of the tracer was 1:175,000. The



first phase of the radioimmunoassay was identical to that described in Method 1, except that tubes were incubated at 4°C overnight, rather than at 37°C for 1 hr plus at 4°C for a further 48 hrs. For the second phase, each tube received 1µl normal rabbit serum plus 2.5µl sheep anti-rabbit IgG serum, in a total volume of 100 µl of phosphate buffer. After thorough mixing 1 ml 6% w/v polyethylene glycol 6000 in phosphate buffer [100mM, pH 7.4] containing no albumin was added, and tubes were incubated for 1 hr at 22°C. Antibody-bound radioactivity was pelleted by centrifugation at 1500g for 30 minutes, the supernatant was discarded and the tubes counted for gamma radioactivity. As in method one radioactivity bound to tubes in the absence of first antibody was subtracted from the total to obtain the antibody-bound radioactivity, and no correction was made for radioactivity bound in the presence of excess amounts of unlabelled EGF.

#### 4.6 Radioreceptor assays for EGF.

A radioreceptor-assay for EGF was originally developed by Dr. Leanna Read and Dr. John Ballard using cultured AG2804 Simian virus 40-transformed human lung fibroblasts (obtained from the American Type Culture Collection, Washington DC, U.S.A.). In comparison with other cell lines in the laboratory, this cell line has a relatively high binding capacity for EGF and most importantly, it yields a relatively sensitive competitive assay compared to the other cell lines available.

In the first step of the assay, confluent monolayers of AG2804 fibroblasts were washed twice in Hank's salts, pH 7.4 containing 0.1 [w/v] bovine serum albumin over a period of 60 min at 37°C to remove any growth factors present from the

growth medium which is normally supplemented with 10% fetal calf serum. Fresh salts containing  $^{125}\text{I}$ -labelled EGF [approximately 0.8ng, 0.04 $\mu\text{Ci}$ ] and standard EGF or sample were then added in a total volume of 500 $\mu\text{l}$ . Plates were incubated at 24°C for 90 minutes before being washed five times at 4°C with Hank's salts without albumin. Monolayers were dispersed by trituration in 1ml of 0.5 M NaOH containing 0.1% [w/v] Triton X100 for the measurement of radioactivity and protein. Since the number of cells may vary in each well the results were standardized by normalizing the data in terms of cellular protein. The protein content of digested cell monolayers was measured by an Auto Analyzer adaptation of the Lowry method of Dulley and Grieve (1975), using bovine serum albumin as the standard.

CHAPTER FIVE

PROPERTIES OF SYNTHETIC-GENE RECOMBINANT  
HUMAN EPIDERMAL GROWTH FACTOR: COMPARISON WITH THE  
NATURAL GROWTH FACTOR FROM HUMAN URINE AND MILK.

The work presented in this chapter has already been published:-

Read, L.C., Summer, L., Gale, S.M., George-Nascimento, C., Ballard, F.J. and Wallace, J.C. 1986) Properties of synthetic-gene recombinant human epidermal growth factor: comparison with the natural growth factor from human urine and milk. J. Endocr., 109, 245-250.

ABSTRACT

The receptor-binding, immunological and biological properties of synthetic-gene human epidermal growth factor (EGF), produced by recombinant techniques in yeast have been compared with those of mouse sub-maxillary gland EGF and partially purified EGF from human urine and milk. The three forms of human EGF produced parallel concentration curves in radio-receptor assays using AG2804 Simian virus 40-transformed human lung fibroblasts and iodinated mouse or recombinant human EGF. Equivalent receptor-binding activities of urine and milk EGF were equipotent with recombinant human EGF in an homologous radioimmunoassay using recombinant human EGF antiserum with  $^{125}\text{I}$ -labelled recombinant human EGF, while none of the hEGF preparations were effective in competing for binding of  $^{125}\text{I}$ -labelled mouse EGF to mouse EGF antiserum. Urine, milk and recombinant human EGF stimulated protein synthesis and inhibited protein degradation in cultured AG2804 fibroblasts with identical potency. On a weight basis, mouse EGF was equipotent with recombinant human EGF in competitive binding to cell receptors and in effects on protein synthesis or protein degradation in AG2804 fibroblasts. It is concluded that recombinant human EGF is indistinguishable from the natural growth factor from urine or milk and shares similar biological properties with mouse EGF. Therefore, the use of the recombinant species as a substitute for human milk EGF in the biological studies described in this thesis is justified.

## INTRODUCTION

Biological studies of the nature presented in this dissertation require substantial amounts of purified growth factor. In the past, progress in the understanding of the physiological role(s) of EGF has been seriously impeded by a lack of sufficient quantities of growth factor. Although immunologically reactive EGF is detectable in a variety of human tissues and fluids, including serum, urine and milk (Gregory, 1975; Starkey et al., 1975; Hirata and Orth, 1979a and 1979b; Hirata et al., 1982; Elson et al., 1984; Read et al., 1984), concentrations are too low for the purposes of large scale purification. To overcome the problem of inadequate supplies of purified EGF, the gene for hEGF has recently been chemically synthesized and expressed in yeast (Urdea et al., 1983; Brake et al., 1984) and *Esherichia coli* (Smith et al., 1982). Synthetic-gene recombinant hEGF produced in yeast was generously supplied by Chiron Corporation for the present series of studies. The amino acid sequence for recombinant hEGF is homologous with the naturally occurring growth factor purified from human urine (Brake et al., 1984). However, since there is some evidence for the existence of tissue-specific forms of EGF, it was necessary to determine whether recombinant hEGF is identical to the naturally occurring milk-derived growth factor before use in the present studies of hEGF intestinal survival and absorption. EGF from different sources has been reported to vary in molecular weight, amino acid composition, isoelectric point, chromatographic properties, immunological behaviour or biological potency (Hirata and Orth, 1979a and 1979b; Hirata et al., 1982; Assoian et al., 1984; Olsen et al., 1984). In this chapter the

immunological reactivities, receptor-binding properties and biological potencies of recombinant hEGF produced in yeast are compared with those of urine hEGF, milk hEGF and mouse EGF (mEGF).

#### MATERIALS AND METHODS.

##### Growth factors.

1. Human EGF:- see Chapter 4, section 4.1.
2. Mouse EGF:- see Chapter 4, section 4.1.
3. Human urinary EGF:

Human urinary EGF was partially purified from adult male urine by Ms. Lia Summer in the laboratory of Dr. John Wallace using the procedure of Savage and Harper (1981) with fractionation on Bio-Gel P-10 as the final step. Fractions from the Bio-Gel P-10 column showing activity in EGF radioreceptor assays were pooled, freeze-dried and redissolved in HCl [10mM]. The purity of hEGF in the Bio-Gel P-10 pool was similar to that described by Savage and Harper (1981).

4. Human milk-derived EGF:

Partially purified human milk-derived EGF was also prepared by Ms. Lia Summer in the laboratory of Dr. John Wallace, using the procedure of Shing and Klagsbrun (1984), with fractionation on Sephadex G-100 as the final step. The purification achieved was similar to that described in that report. All radioreceptor assay activity eluted at the same column volume as an added mouse EGF [mEGF] standard. Active fractions were pooled, freeze-dried and redissolved in HCl [10mM].

##### Iodination of EGF.

Recombinant hEGF and mEGF were used as tracers in radio-receptor assays and radioimmunoassays. Both were

iodinated to a specific activity of 50Ci/g using the Iodogen method as described in Chapter 4, section 4.2.

#### Radioreceptor assays.

The EGF receptor binding properties of recombinant hEGF, purified mEGF and partially purified milk and urine hEGF were compared in radioreceptor assays as described in Chapter 4, section 4.6. In these radioreceptor assays, the various human or mouse EGF preparations were used as unlabelled ligands, with purified mEGF or hEGF as the  $^{125}\text{I}$ -labelled peptide.

#### Radioimmunoassays.

Radioimmunoassays were used to compare the antibody-binding properties of recombinant hEGF, purified mEGF and partially purified milk and urine hEGF. Two polyclonal antibodies were used in these comparisons, one raised against mEGF isolated from the submaxillary glands of adult male mice (prepared by Ms. Lia Summer, see Chapter 4, section 4.3) and the other an antiserum to recombinant hEGF (donated by Dr. B. Kemp, see Chapter 4, section 4.3). The radioimmunoassays were carried out as described in Chapter 4, section 4.5 [method 1], using EGF tracer and antiserum from the homologous species.

To ensure accurate comparisons between growth factors, radioimmunoassays and radioreceptor assays were carried out simultaneously using the same preparations of tracers and growth factors.

#### Measurement of rates of protein synthesis and degradation in cultured fibroblasts.

The biological potencies of recombinant hEGF, milk hEGF, urine hEGF and mEGF were compared in assays of protein synthesis and protein breakdown in confluent monolayers of



AG2804 fibroblasts, the cell line used for radioreceptor assays. AG2804 fibroblasts were selected so that the biological potency and receptor binding properties of the various EGF preparations could be compared in the same cell line. Secondly, it had previously been established in the laboratory of Dr. John Ballard that AG2804 fibroblasts show growth responses at lower concentrations of EGF than do non-transformed fibroblasts (Ballard and Gunn, 1984). Rates of protein synthesis and degradation were measured simultaneously by the methods described by Ballard and Gunn (1984). The rates of intracellular protein degradation were measured as the release of trichloroacetic acid-soluble radioactivity after labelling the cultures with [<sup>3</sup>H]leucine. For labelling, monolayers were incubated at 37°C in leucine-free Eagle's Minimal Essential medium (MEM) containing fetal calf serum [1%] and L-[4,5-<sup>3</sup>H]leucine [1 µCi/ml]. This medium was replaced after 18h with serum-free MEM containing leucine [2mM] but no isotope. After a 3-h incubation at 37°C to permit degradation of short-lived proteins, a further 4-h degradation period at 37°C commenced in the presence of MEM containing leucine [2mM] and different concentrations of recombinant hEGF, urine hEGF, milk hEGF or mEGF. Protein break-down rates are expressed as the percent of labelled protein converted to acid-soluble material over the 4-h period.

For measurement of rates of protein synthesis, the same protocol and identical solutions were used, except that [<sup>3</sup>H]leucine was included during the final 4-h period in the presence of growth factor rather than the earlier 18-h incubation. Rates of protein synthesis are calculated as the percent cellular protein synthesized in 4 h, i.e. %

$$\text{synthesized} = \frac{100 \times A}{B \times C}$$

where A is the incorporation of leucine in dpm per cell protein,

B is the specific activity of medium leucine [dpm per nmol],

C is the leucine content of cell proteins. [The value for C was taken as 0.777 nmol per  $\mu\text{g}$  cell protein, the average obtained in this laboratory for total protein isolated from several cell lines (Ballard, 1982)].

Potencies of EGF preparations in protein synthesis and degradation assays were expressed as the concentration that produced a response equivalent to 50% of that obtained with saturating concentrations of EGF (i.e. half-maximal effects).

## RESULTS

### Radioreceptor assays.

Radioreceptor assays were used for initial comparisons between recombinant hEGF, purified mEGF and the partially purified milk and urine hEGF preparations. The purpose was several fold; firstly, to compare recombinant hEGF with a purified preparation of EGF, mEGF, in receptor binding potency, and secondly to determine whether partially purified milk and urinary hEGF yielded competitive binding curves in the radioreceptor assay parallel to those obtained with the purified growth factors (recombinant hEGF and mEGF). Lastly, radioreceptor assays were used to obtain an estimate of the concentration of EGF in partially purified hEGF preparations from milk and urine, as a basis for further comparisons of immunological and biological potency.

Firstly, the receptor binding capacity of recombinant

hEGF was compared with that of a pure preparation of mEGF. In the competition curves obtained with  $^{125}\text{I}$ -labelled hEGF as the radioactive ligand, as shown in Fig. 5.1(a), half-maximal competition occurred at  $5\mu\text{g EGF/l}$  for both recombinant hEGF and mEGF indicating that the two pure preparations were equipotent in receptor binding activity. In support of this result, the two pure preparations of mEGF and recombinant hEGF were also shown to be equipotent in competing with mEGF tracer for receptor binding sites [see Fig. 5.1(b)].

In the experiment shown in Fig. 5.1(a), mEGF generated a competition curve that was non-parallel to that obtained with recombinant hEGF as the competing growth factor. However, this was not a general observation; in most instances it has been observed that the two growth factors compete identically for  $^{125}\text{I}$ -labelled hEGF tracer. The data shown in Fig. 5.1(a) was chosen for graphical illustration because the curves were obtained from an experiment where all four growth factor preparations were compared simultaneously.

Secondly, the receptor binding activity of partially purified hEGF prepared from either milk or urine was compared with that of recombinant hEGF to determine whether the three preparations of hEGF gave parallel competition curves and were therefore free from interfering substances. Serial dilutions of partially purified milk and urinary hEGF produced competition curves with  $^{125}\text{I}$ -labelled hEGF as radioactive ligand which were parallel to those of recombinant hEGF [see Fig. 5.1(a)]. Concurrently with the assays shown in Fig. 5.1(a), radioreceptor assays performed using  $^{125}\text{I}$ -labelled mEGF tracer gave competition curves for

both milk and urine hEGF preparations that were parallel to the curves obtained with either pure mEGF or recombinant hEGF. In the experiment shown graphically in Fig. 5.1(b), urine and milk hEGF were tested at only three concentrations. In other experiments where higher concentrations were also included, competition curves were indistinguishable from those obtained with mEGF as the competing growth factor. Again, the experiment used for graphical illustration was chosen because all four growth factor preparations were compared simultaneously.

Finally, estimates of the concentration of EGF in the two partially purified preparations of hEGF were obtained using the competition curves obtained with recombinant hEGF. From the competition curves of either experiments shown in Fig.5.1, it was estimated that the partially purified milk or urine extracts contained 1.0 mg EGF/l [0.16 $\mu$ M]. These estimates of EGF concentration were then used as a basis for further comparisons of immunological and biological potency. These results are summarized in Table 5.1.

#### Radioimmunoassays.

Using the estimates of EGF concentration in milk and urine hEGF preparations [1.0 mg/l; 0.16 $\mu$ M, see Table 5.1] obtained from the radioreceptor assays, radioimmunoassays were carried out to determine whether recombinant, milk and urinary hEGF would show equivalent activity on an immunological basis. In addition, the immunological behaviour of mEGF was also compared with that of the hEGF preparations.

Two polyclonal antibodies were used in immunological comparisons between the various EGF preparations, one raised against mEGF isolated from the submaxillary glands of adult

male mice and the other an antiserum to recombinant hEGF. In Fig. 5.2, concentrations of milk and urine hEGF are expressed as radioreceptor assay-equivalents, as derived from the competition curves in Fig. 5.1(a). It is apparent that milk or urine hEGF, standardized in this way, behave identically to recombinant hEGF in the homologous radioimmunoassay with half-maximal effects at 2 $\mu$ g/l in each case.

The three hEGF preparations showed similar, low reactivity to mEGF antiserum, inhibiting mEGF tracer binding by less than 20% at concentrations up to 1000  $\mu$ g/l [Fig. 5.2(b)]. In contrast, 50% reduction in mEGF tracer binding was observed with 2 $\mu$ g unlabelled mEGF/l. As expected from the low affinity of this antiserum for hEGF,  $^{125}$ I-labelled hEGF bound only 5% as well as mEGF tracer (results not shown).

Antiserum to hEGF showed less than 1% cross-reactivity to mEGF [Fig. 5.2(a)], 50% inhibition of hEGF tracer binding requiring greater than 2 mg mEGF/l compared with 2 $\mu$ g recombinant hEGF/l. It was also observed that concentrations of antiserum which bound 30-40% hEGF tracer bound less than 1% of mEGF tracer (results not illustrated). The results of the immunological comparison of the three hEGF preparations and mEGF are summarized in Table 5.1.

#### Protein synthesis and degradation in cultured fibroblasts.

The biological potencies of the four EGF species were compared in assays in protein synthesis and degradation in confluent monolayers of AG2804 fibroblasts, the cell line used for radioreceptor assays for EGF. The rate of protein synthesis and degradation was expressed as the percent of cellular protein synthesized or degraded in the cell line

during the four hours of exposure to the particular EGF species. The relatively short exposure time of 4 hours was chosen to avoid effects of down-regulation, a phenomenon which reduces cell response to growth factors that has been shown to occur in tissue culture after several hours of exposure to growth factors (Ballard and Gunn, 1984). Therefore due to the relatively short time period of exposure to EGF, the responses to EGF in the AG2804-transformed fibroblasts were found to be relatively small (maximum responses of approximately 25% of the control value in the absence of EGF, see Fig. 5.3). However, although responses to EGF were small, the reproducibility was high, as indicated by the standard error bars in Fig. 5.3.

Recombinant hEGF, urine hEGF and mEGF stimulated protein synthesis and inhibited protein breakdown with virtually identical dose-response curves, half-maximal responses occurring at 0.35 $\mu$ g recombinant hEGF/1, 0.3 $\mu$ g urine hEGF/1 or 0.2 $\mu$ g mEGF/1. Milk hEGF was assayed only at sub-maximal concentrations because it was considered that the preparation was impure, probably containing other growth factors in concentrations which were sufficiently high to contribute to the anabolic responses in AG2804 fibroblasts if EGF concentrations greater than 0.6 $\mu$ g/1 were used. Nevertheless, results in Fig. 5.3 suggest that milk hEGF has similar potency to the other EGF preparations both in stimulating protein synthesis and in inhibiting degradation.

#### DISCUSSION

In this chapter the immunological, receptor-binding and growth-promoting properties of recombinant hEGF are compared with those of the natural, previously characterized growth factor from human urine. The results obtained are evidence

that recombinant hEGF is indistinguishable from urine hEGF. First, equivalent receptor-binding activities of the two EGF preparations were equipotent in competitive binding to an antibody directed against recombinant hEGF. Recombinant hEGF and urine hEGF standardized in this way were also equally active in stimulating protein synthesis or inhibiting protein degradation in cultured fibroblasts. Secondly, recombinant hEGF had similar potency on a weight basis to mEGF, both in radioreceptor assays and in effects on protein metabolism in cultured fibroblasts. Since the work of other groups has indicated that urine hEGF and mEGF have comparable activities in radioreceptor assays (Hirata & Orth, 1979a; Savage & Harper, 1981) and in the stimulation of DNA synthesis in cultured cells (Hollenberg & Gregory, 1976), it is apparent that the biological properties of recombinant hEGF are equivalent to those of urine hEGF. Lastly, the polyclonal antibody raised against recombinant hEGF showed little affinity for mEGF and, conversely, recombinant hEGF bound very poorly to anti-mEGF antibody, as expected from previous studies comparing mEGF with urine hEGF (Gregory, et al., 1977; Starkey & Orth, 1977). Thus, on the basis of the receptor binding, immunological and biological properties tested, recombinant hEGF behaves identically to the natural growth factor from urine.

The biological studies presented in Chapters 7 and 8 on the role of milk-derived EGF in the neonate required substantial amounts of purified growth factor. Since milk-derived hEGF could not be purified in the quantities required for these studies, a recombinant form of hEGF produced in yeast was provided by Chiron Corporation in milligram amounts. However, before this recombinant form

could be used as a substitute for the naturally-occurring milk growth factor in biological studies, the two forms of EGF had to be shown to be identical homologous peptides. Therefore, the properties of recombinant hEGF were compared with those of partially purified milk hEGF.

The presence of relatively high concentrations of EGF in human milk has been known for several years (Beardmore et al., 1983; Moran et al., 1983; Read et al., 1984), but up until 1985, the structural or functional relationship between milk and urinary forms of hEGF had not been clearly established. However some evidence for the similarity of the two forms had been provided before the commencement of the present study. Beardmore et al, (1983) reported that following fractionation of human milk on Bio-Gel P-10, the major peak of immunoreactive EGF eluted at the same volume as urine hEGF, while Shing and Klagsbrun (1984) presented evidence that human milk contains a form of EGF with similar properties to those of urine hEGF, including molecular weight, isoelectric point and behaviour on anion exchange chromatography. From the studies presented in this chapter, no differences were detected in the receptor-binding, biological and immunological properties of recombinant, milk and urinary hEGF, supporting these previous findings. Since these studies have been completed, more conclusive evidence has been reported in the literature. Petrides and colleagues (1985) isolated several polypeptide growth factors from human milk, one of which was found to be nearly identical in amino acid composition to human EGF of molecular weight 6000 Da, the hEGF species previously isolated and sequenced from human urine (Gregory et al., 1975).



In the present study, the properties of recombinant hEGF were compared with the major 6000 Da EGF species partially purified from human urine and milk. In contrast to the findings of others, no other EGF species of higher molecular weight were detected in the preparations. Larger molecular weight forms of immunoreactive EGF have been found by conventional soft gel chromatography not only in milk and urine but also in serum, saliva and gastric juice (Gregory et al., 1979; Hirata and Orth, 1979a, 1979b; Hirata et al., 1982; Assoian et al., 1984; Elson et al., 1984).

In 1979, Hirata and Orth (1979a) reported their discovery of a high molecular weight moiety in human urine that appeared to have only about 26% of the biological potency (receptor-binding activity) of the 6000 Da hEGF. On sodium dodecyl sulphate polyacrylamide gel electrophoresis in the presence of a reducing agent, high molecular weight hEGF appeared to consist of two components of 28,000 and 33,000 Da (Hirata and Orth, 1979a). In the same year, Hirata and Orth (1979b) reported that the high molecular weight form could be converted by mouse EGF-associated arginine esterase to biologically active small molecular weight [6000 Da] EGF, suggesting that it might represent an EGF precursor. Since this early report, other research groups have purified this high molecular weight moiety to homogeneity and have partial sequence information (Mount et al., 1987; Tsukumo et al., 1987). Sequence analysis of the amino terminus of the intact molecule and of two tryptic fragments and by carboxy-peptidase Y analysis revealed the molecule to correspond to residues 828-1023 of the hEGF precursor, the sequence of the latter being predicted from the known hEGF mRNA sequence (Bell et al., 1986).

The identification of structurally distinct forms of EGF in various fluids and tissues is suggestive of the existence of tissue-specific forms of EGF. It would appear that minor EGF forms of varying molecular weights other than the major 6000 dalton peptide can be isolated from biological extracts using alternative or more extensive purification procedures than those used in the present study. Nevertheless, it can be concluded from this study that the major EGF species in human milk and urine has identical receptor binding, immunological and biological potency. In addition, recombinant hEGF is shown to have identical properties to the naturally-occurring hEGF species in milk and urine and thus it is considered a suitable substitute for milk hEGF in the biological studies of this thesis.

Table 5.1 Comparison of immunological, receptor-binding and biological properties of recombinant hEGF, urinary hEGF, milk hEGF and mEGF.

Property	Recombinant hEGF	Urine hEGF	Milk hEGF	mEGF
<b>Receptor Binding Capacity</b>				
----- Half-maximal competition with $^{125}\text{I}$ -hEGF	0.83nM	5ul/ml	5ul/ml	0.83nM
	parallel competition curves			
Half-maximal competition with $^{125}\text{I}$ -mEGF	0.5nM	3ul/ml	3ul/ml	0.5nM
	parallel competition curves			
Estimated EGF concentration from radioreceptor assay.		0.16uM	0.16uM	
<b>Antibody Binding Capacity</b>				
----- Homologous mEGF RIA Conc. required to displace 50% of radioactivity bound in the absence of unlabelled EGF	>0.16uM	>0.16uM	>0.16uM	0.33nM
Homologous hEGF RIA Conc. required to displace 50% of radioactivity bound in the absence of unlabelled EGF	0.33nM	0.33nM	0.33nM	>0.33uM
<b>Biological Potency</b>				
----- Protein synthesis nM for half-maximal effect	0.06nM	0.05nM	-	0.03nM
Protein degradation nM for half-maximal effect	0.06nM	0.05nM	-	0.03nM

Fig. 5.1. Epidermal growth factor (EGF) radioreceptor assays. AG2804 human lung fibroblasts were incubated with (a)  $^{125}\text{I}$ -labelled recombinant human EGF (hEGF) or (b)  $^{125}\text{I}$ -labelled mouse EGF (mEGF) together with different concentrations of unlabelled recombinant hEGF (●), urine hEGF (△), milk hEGF (▲) or mEGF (○). Values are means  $\pm$  S.E.M. of three determinations at each EGF concentration. Where S.E.M. lines are not shown, they fall within the area of the symbols.

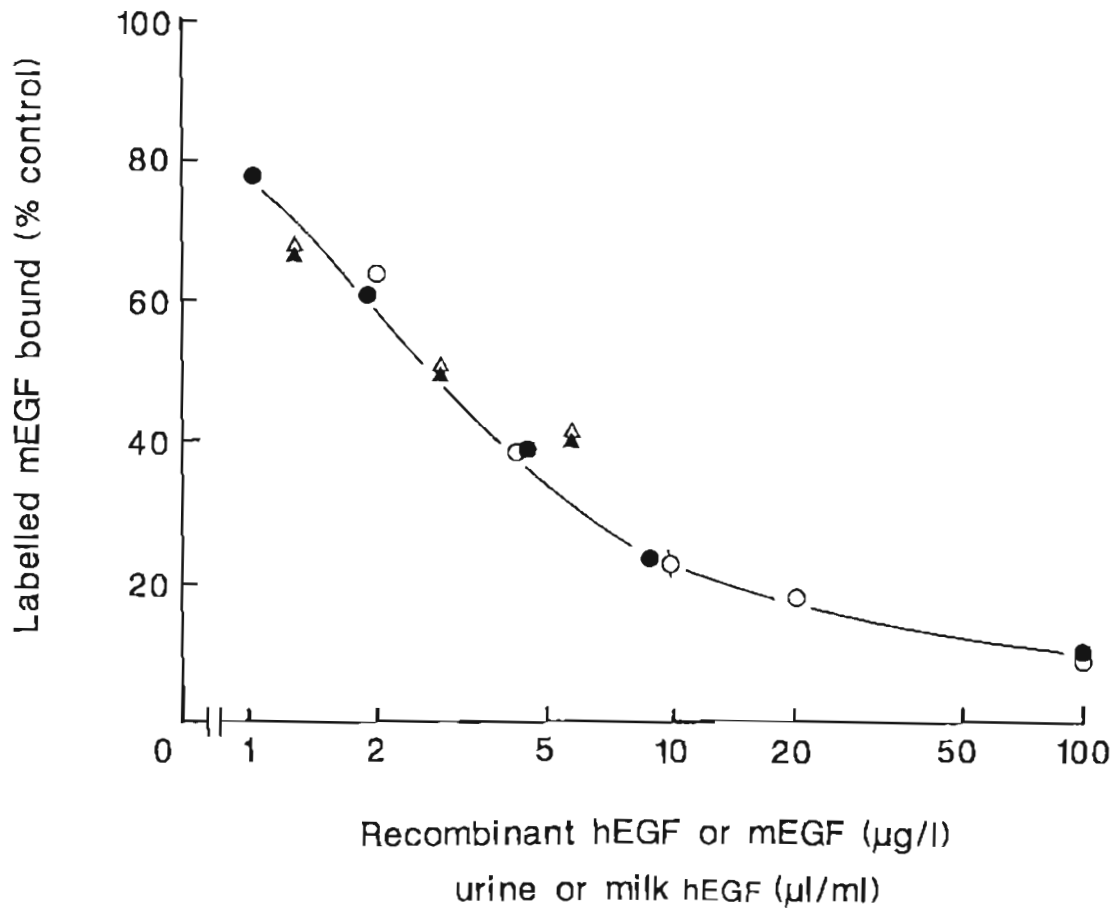
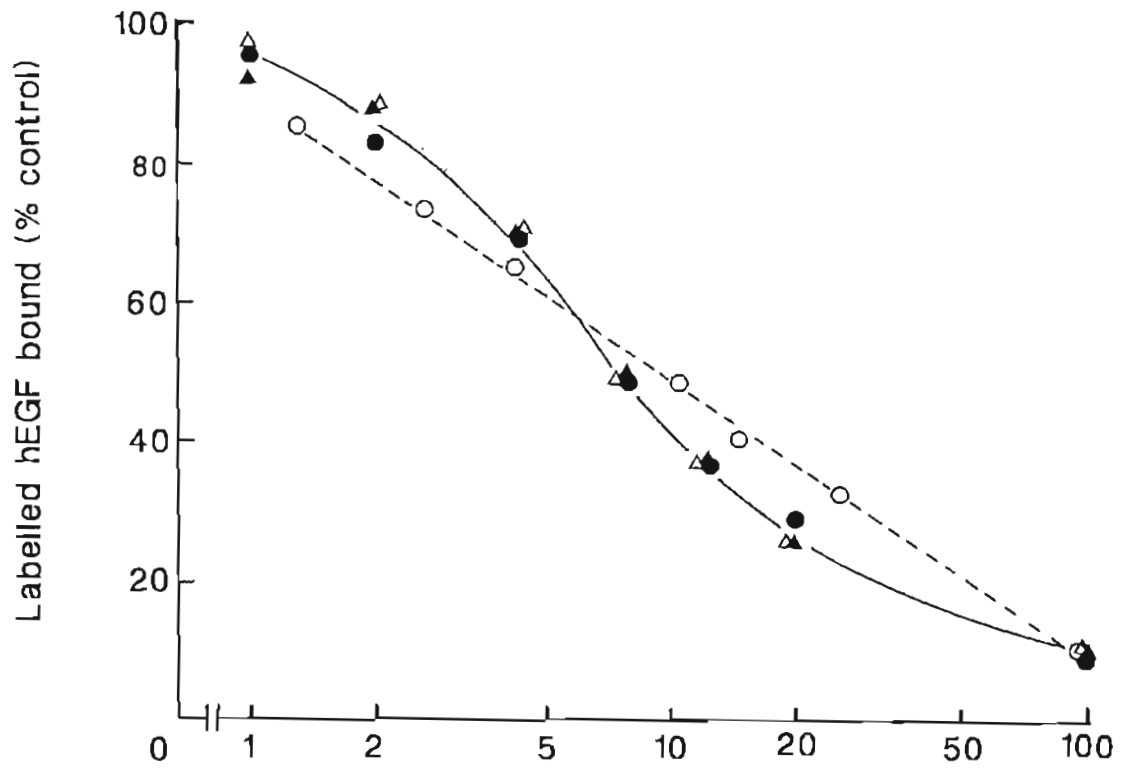
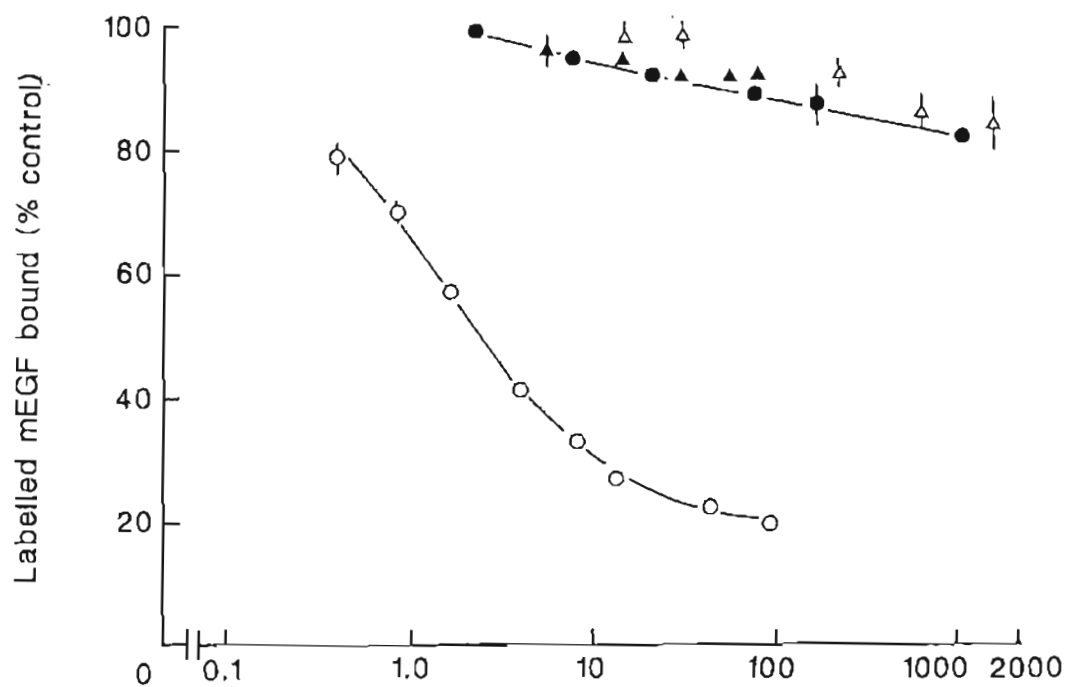
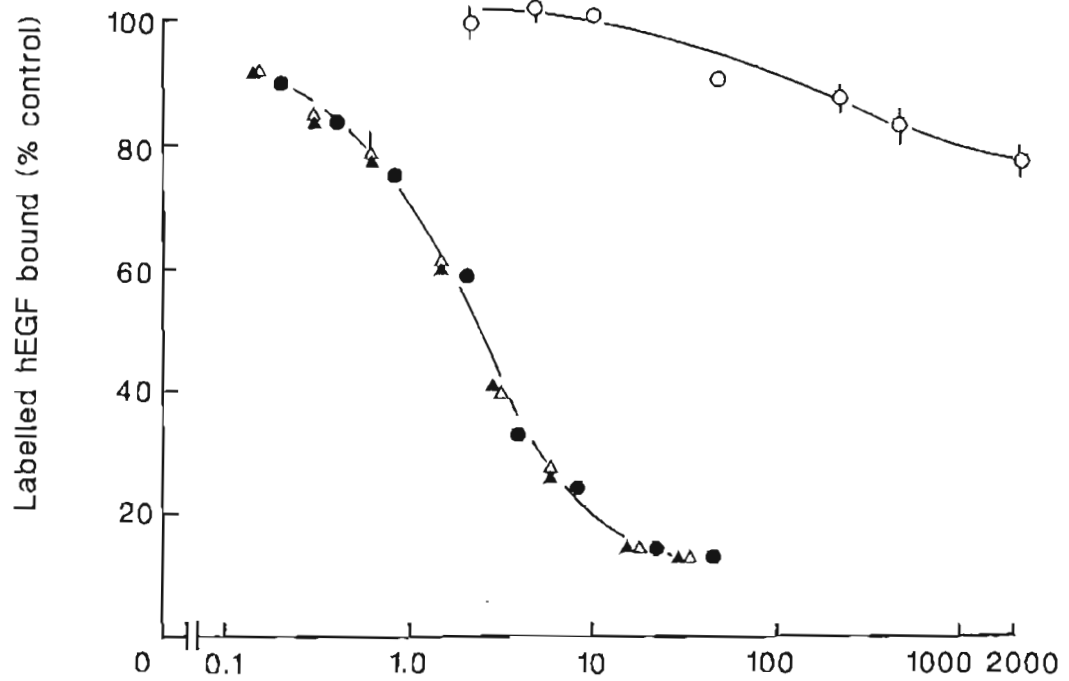


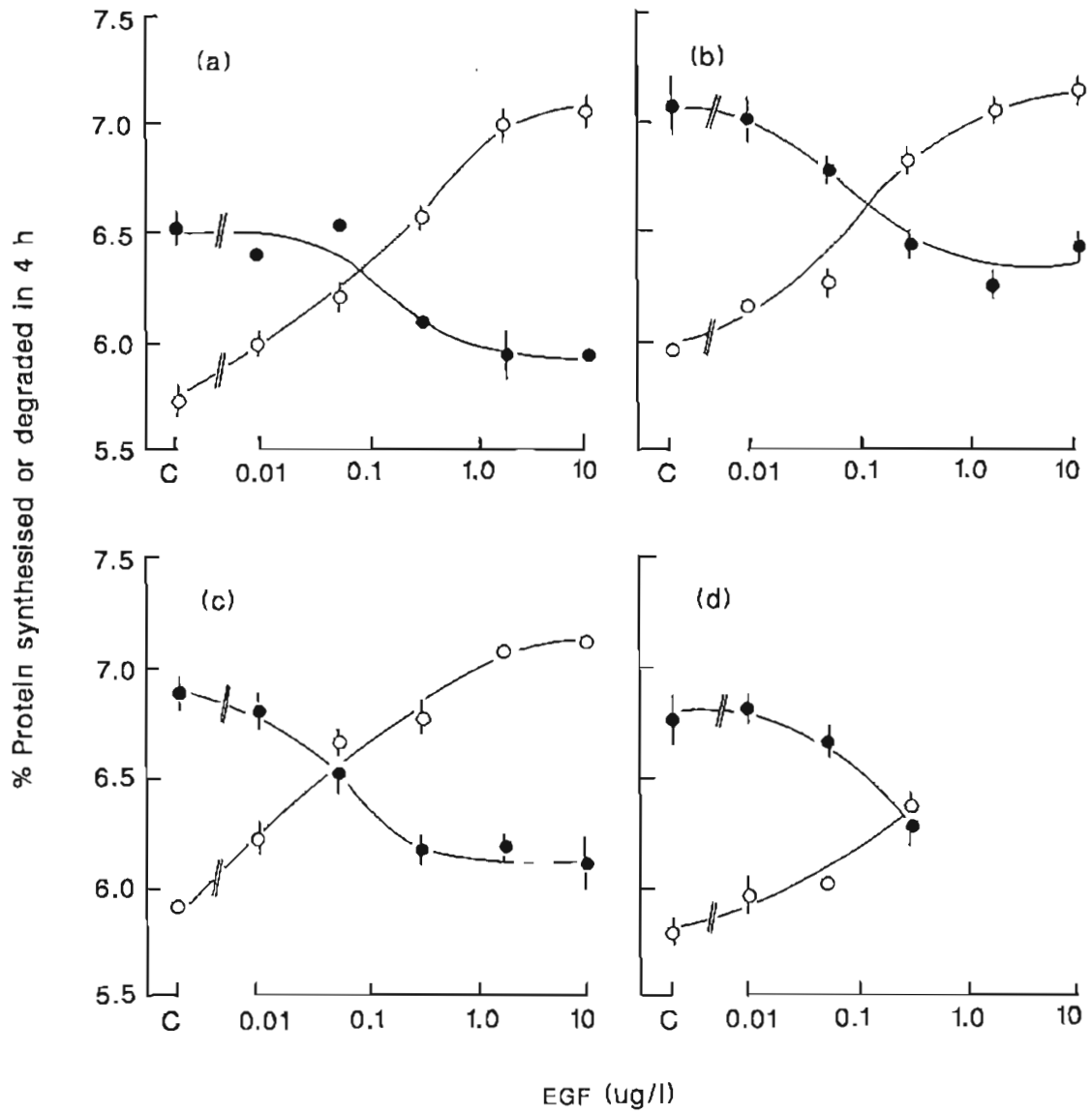
Fig. 5.2. Epidermal growth factor (EGF) radio-immunoassays. (a) Recombinant human EGF (hEGF) antiserum was incubated with  $^{125}\text{I}$ -labelled recombinant hEGF and the indicated concentrations of unlabelled recombinant hEGF ( $\bullet$ ), urine hEGF ( $\Delta$ ), milk hEGF ( $\blacktriangle$ ) or mouse EGF (mEGF) ( $\circ$ ). RRA equivalents represent concentrations of EGF in milk or urine extracts, estimated by radioreceptor assays shown in Fig. 5.1. (b) Mouse EGF antiserum was incubated with  $^{125}\text{I}$ -labelled mEGF together with unlabelled growth factors as in (a). Values are means  $\pm$  S.E.M.,  $n = 3$ . The S.E.M. falls within the area of the symbols where no lines are shown.



Recombinant hEGF or mEGF ( $\mu\text{g/l}$ )  
urine hEGF or milk hEGF ( $\mu\text{g RRA equivalents/l}$ )

Fig. 5.3. Protein synthesis and breakdown in AG2804 human lung fibroblasts. Effects of (a) recombinant human EGF (hEGF), (b) urine hEGF, (c) mouse EGF (mEGF) or (d) milk hEGF on the % cellular protein synthesized (○) or degraded (●) during the 4-h experimental period. Values are means ± S.E.M., n = 4. Where no S.E.M. lines are shown, they fall within the area of the symbol. Arrows indicate concentrations giving half-maximal effects. C, control.





**CHAPTER SIX**

PREPARATION OF MONOCLONAL ANTIBODIES AND POLYCLONAL  
ANTISERUM DIRECTED AGAINST SYNTHETIC-GENE RECOMBINANT

hEGF: CHARACTERIZATION OF MONOCLONAL ANTIBODIES  
AND COMPARISON WITH THE POLYCLONAL ANTISERUM

ABSTRACT

Fifteen monoclonal antibodies directed to species-specific determinants of human epidermal growth factor (hEGF) were obtained by fusing murine myeloma cells with BALB/c mouse splenocytes sensitized to recombinant hEGF. Also, a specific polyclonal antiserum was raised in a rabbit against recombinant hEGF. A highly sensitive, specific radioimmunoassay has been developed using either the polyclonal antiserum or monoclonal antibody #1. In addition nine of the fourteen antibodies characterized are shown to bind to discontinuous epitopes of the hEGF molecule, while the remaining antibodies recognize particular synthetic octapeptides homologous with the primary hEGF sequence. Three of the monoclonal antibodies are shown to bind to epitopes associated with the N-terminus of hEGF.

INTRODUCTION.

In Chapter Five it was concluded that recombinant human EGF is indistinguishable from the natural growth factor from urine or milk, and shares similar biological properties with mouse EGF. Since recombinant hEGF thus appears to be identical to the naturally occurring growth factor in human milk and is now available in milligram quantities, it is suitable for biological studies of EGF.

In Chapters 7 and 8 of this thesis, the intestinal survival and absorption of EGF in the suckling neonate is investigated in a quantitative manner in the newborn lamb and in an indirect manner in the premature human infant. These studies required methods to accurately and specifically measure human recombinant EGF in biological samples of blood, lymph and urine. Specific assays were developed using immunological reagents that were prepared by the candidate as described in this chapter.

Traditionally, the measurement of growth factors in biological systems has been achieved by the use of radioreceptor or radioimmunoassay techniques. While the radioreceptor assay is an invaluable tool for detecting biologically active EGF and may in some instances be more sensitive than the radioimmunoassay, the assay is not species-specific. In contrast to the radioreceptor assay, the radioimmunoassay is species-specific since antisera prepared against EGFs of various species show very little cross-reaction with heterologous EGF. Thus, the radioimmunoassay technique is useful when it is desirable to exclusively measure EGF from a particular species, such as required in the studies described in this thesis.

In addition, the receptor assay is also more subject to

factors that interfere with ligand binding, such as high salt concentration, low pH and proteolytic enzymes as well as interference by related growth factors (Hock and Hollenberg, 1980). For example, the radioreceptor assay for EGF using AG2804 fibroblasts is subject to interference by platelet-derived growth factor (PDGF) (Brown and Blakeley, 1983 and 1984). Although this particular problem of PDGF interference can be avoided by the use of a cell line that is unusually rich in specific EGF receptors, such as the human epidermoid carcinoma line (A-431), non-specific interference is still often seen when determining EGF concentration in biological specimens.

The radioimmunoassay has been employed extensively by researchers for the measurement of EGF in various biological fluids and tissue extracts in preference to the receptor assay for reasons such as stated above (Gregory et al., 1977; Starkey and Orth, 1977; Dailey et al., 1978; Gregory et al., 1979; Murphy et al., 1979; Mattila et al., 1985 and 1986). In a comparative study on the merits of using either a radioimmunoassay or radioreceptor assay to measure the amount of EGF present in the urine of human subjects, the immunoassay appeared to be the method of choice. The immunoassay was more precise and sensitive than the receptor assay, the limit of detection being 0.025 and 0.0125 pmol for the radioreceptor and immunoassay respectively (Nexo et al., 1981). Given the particular advantage of high specificity, the radioimmunoassay was considered the method of choice for the purpose of EGF quantitation for the biological studies presented in this dissertation.

The specific polyclonal antiserum available for the

study described in Chapter 5 was generously provided by Dr. B. Kemp (Department of Medicine, Repatriation General Hospital, Heidelberg, Victoria, Australia). However, a new source of specific antisera or antibody was required as the supply of antisera from Dr. Kemp was insufficient in quantity for the substantial number of routine radioimmunoassays anticipated with the biological studies. The production of monoclonal antibodies described in this chapter was undertaken to provide an unlimited supply of specific antibody for routine use in a human EGF radioimmunoassay. In addition, it was intended that a range of monoclonal antibodies directed against known epitopes of the EGF molecule be produced for the analysis of the integrity of EGF present in the biological samples. Following the production and isolation of a range of anti-hEGF monoclonal antibodies, the isotypes were determined and binding characteristics to hEGF and mEGF investigated using a fluid-phase radioimmunoassay technique. In addition, the binding of the monoclonal antibodies to 3 types of radiolabelled hEGF were compared. To detect the nature of the epitopes for each monoclonal antibody i.e. sequential or conformational, the reactivity of each monoclonal to a denatured, reduced form of both hEGF and mEGF, S-carboxymethylated hEGF and mEGF, was compared to the reactivity to native hEGF and native mEGF respectively.

The amino acid residues involved in the epitope of each monoclonal antibody were investigated using a strategy recently described by Geysen and colleagues (1984). This procedure involved the systematic synthesis of all the possible octapeptides homologous with the human EGF sequence onto solid support pins placed in the convenient

configuration of a 96-well plate. Interaction of the antibody with each of the peptides was monitored by means of an ELISA (enzyme-linked immunosorbent assay).

During the substantial time taken to produce this unique range of monoclonal antibodies, a polyclonal antiserum specific for human EGF was produced for routine radioimmunoassay. Consequently, the biological studies described in this thesis were undertaken using the radioimmunoassay developed with the polyclonal antiserum. The production and purification of the specific polyclonal serum is discussed in this chapter in addition to a description of the routine radioimmunoassay developed.

#### MATERIALS AND METHODS.

6.1. Growth Factors. - see Chapter 4, Section 4.1

6.2 Radiolabelled EGF. - see Chapter 4, Section 4.2

6.3 Preparation of Polyclonal Antiserum Against hEGF.

A polyclonal antiserum against hEGF was prepared in a rabbit using an intradermal immunisation schedule as described in Chapter 4, Section 4.3. Blood samples collected from the rabbit every 2-5 weeks were tested for specific antibody using a solid phase radioimmunoassay (SPRIA) as described below. Fifty millilitres of antiserum was collected 14 weeks after the primary immunization, and stored at 4°C in the presence of 0.1% sodium azide.

6.4 Preparation of Monoclonal Antibodies Against hEGF.

6.4(a) Immunization of Mice for Monoclonal Antibody Production.

In order to provide immune donor spleen cells for the subsequent production of antibody-producing hybrid cell lines, mice were immunized against hEGF over a total period

of 10-12 weeks. On three separate occasions four 6-week-old BALB/c female mice were immunized with 25  $\mu$ g of hEGF each. The primary and secondary immunizations were subcutaneous injections given six weeks apart, the primary immunization given in the presence of Complete Freund's adjuvant, the secondary in the presence of Incomplete Freund's adjuvant. Four weeks after the second injection, serum was obtained by retro-orbital bleeding and tested for anti-hEGF activity using the solid-phase radioimmunoassay (SPRIA) and the enzyme-linked immunosorbent assay (ELISA) described below. The two mice with the highest antibody titres were then injected intravenously in the tail with hEGF in 0.01M borate-saline, pH 8.0, three days before the spleens were removed and used for hybridization.

#### 6.4(b) Solid-phase Radioimmunoassay (SPRIA).

A solid-phase radioimmunoassay was developed for rapid identification of anti-hEGF activity in either serum or tissue culture supernatants taken from hybridoma colonies. Since the fluid-phase radio-immunoassay technique required 24 hours to complete it was not practical for use in the early stages of hybridoma screening in the production of monoclonal antibodies. In contrast, the SPRIA can be performed in under 3 hours if the plates are stored for later use after the first incubation step as described below.

Polyvinyl chloride round-bottom 96-well microtiter plates (Dynatech, Alexandria, VA) were coated overnight at room temperature with 75  $\mu$ l (60 ng protein) of affinity column-purified rabbit IgG directed against mouse IgG heavy and light chains (Silenus Laboratories, Hawthorn, Vic., Australia) in PBS (25mM  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 150mM NaCl, 10mM



EDTA, adjusted to pH 7.4 with NaOH). After three washes with PBS containing 5% fetal calf serum, 50  $\mu$ l of the culture supernatant to be tested was added to the wells and incubated at room temperature for 1 hour, followed by washing as before.  $^{125}\text{I}$ -labeled-hEGF (50  $\mu$ l, 10,000 cpm) in PBS containing 0.25% w/v bovine serum albumin was then added to each well and incubated at room temperature for 1 hour. After three washings, the wells were cut apart and the radioactivity in each well determined. The non-specific binding of  $^{125}\text{I}$ -hEGF was calculated by the determination of counts bound in the absence of antibody or antisera. This result was subtracted from the counts bound in the presence of the various concentrations of antisera.

#### 6.4(c) Enzyme-linked Immunosorbent Assay (ELISA).

An ELISA was developed to enable rapid screening of either serum or tissue culture supernatants in conjunction with the radioimmunoassay. In this assay, anti-EGF activity is detected after an interaction of the antibody with EGF unmodified by labelling techniques. In the radioimmunoassay, the anti-EGF activity is detected after interaction of the antibody with a labelled EGF species. The use of both assays in the screening process ensures that the antibody is not only specific for EGF but also recognizes EGF in both labelled and unlabelled (i.e. unmodified) forms.

Polyvinyl chloride round-bottom 96-well microtiter plates (Dynatech, Alexandria, VA) were coated overnight at room temperature with 100 $\mu$ l of a saturating concentration of polyclonal rabbit anti-hEGF antisera (1/1000 dilution of antisera using 0.1M Carbonate/Bicarbonate buffer, pH 9.6 as the diluent). Excess unbound antibody was removed by

washing the plates 5 times with PBS containing 0.05% v/v Tween 20. One hundred microlitres of hEGF (50 ng) in PBS containing 1% w/v bovine serum albumin was added to each well except for the negative control wells. Control wells were treated in the same manner in all assay steps as test wells except for the addition of hEGF. After one hour of incubation at 37°C, the plates were washed as before and 50µl of the culture supernatants to be tested were added to both a test and a control well, followed by a further one hour incubation at 37°C. The plates were washed again as before and 100µl of peroxidase-conjugated rabbit anti-mouse immunoglobulin (equivalent to 7ng IgG) (Nordic Immunological Laboratories, Tilburg, The Netherlands) in PBS containing 0.05% v/v Tween 20 was added to each well. Following a 1 hour incubation at 37°C, the plates were washed, and a 100µl of substrate solution applied to each well. The substrate solution (10mM Citrate, 2.5 mM O-tolidine dihydrochloride, and 0.025 mM EDTA) was activated just before use by the addition of 0.003% hydrogen peroxide. Within 5-10 minutes of adding the activated substrate solution, the reaction was terminated by the addition of 50 µl/well of 3M HCl. After termination of the reaction the absorbance of each well at 450nm was determined by a TITERTEK MULTISKAN (spectrophotometer) to allow quantitative analysis of the data.

#### 6.4(d) Cell Hybridization and Selection of Hybrid Clones.

Two independent fusion procedures were performed, one hybridization performed using the P3-NS1-Ag4-1 mouse myeloma cell line, the other with the nonimmunoglobulin secreting variant P3-X63-Ag8. On the day of the cell fusion (or hybridisation), the 2 myeloma cells lines were harvested

from tissue culture flasks in which they had been nurtured from frozen stocks of each lineage and maintained in cell culture for approximately a week. In addition, a single suspension of spleen cells were prepared from each of the two donor mice. Using sterile technique, the spleen of each mouse was removed and placed in a ground glass homogenizer in the presence of culture medium, homogenized to produce a single cell suspension, then centrifuged several times to remove cellular debris and to minimize the risk of microbial contamination arising from the primary culture. Splenocytes ( $1 \times 10^7$ ) were fused with  $1 \times 10^6$  myeloma cells using 38% w/v PEG-1540 (Baker Chemical Company, Phillipsburg, N.J.) at  $37^\circ$  for 6 minutes. Approximately  $5 \times 10^5$  spleen cells/well were plated into 96-well tissue culture plates (Costar) in RPMI 1640 tissue culture medium (Flow) containing 20% v/v fetal calf serum, insulin ( $1.3 \mu\text{M}$ ), pyruvate ( $0.6 \text{mM}$ ) and oxaloacetate ( $1 \text{mM}$ ) and supplemented with hypoxanthine ( $0.1 \text{mM}$ ), thymidine ( $1 \mu\text{M}$ ), and aminopterin ( $0.1 \mu\text{M}$ ). After two weeks of tissue culture, the aminopterin was deleted from the maintenance tissue culture medium. However, the culture medium was supplemented with hypoxanthine ( $0.1 \text{mM}$ ) and thymidine ( $1 \mu\text{M}$ ) for a total of 4-5 weeks after the time of the hybridization procedure. Supernatants from the wells in which colonies grew were screened for reactivity with hEGF using the SPRIA and ELISA assays. Subclones were derived by limiting dilution in microtitre plates (Oi and Herzenberg, 1980) and rescreened for reactivity with hEGF. Fourteen of the fifteen monoclonal antibodies were produced in ascites by injecting pristane-primed mice with  $5 \times 10^6$  cells of each particular hybridoma clone (Potter, 1972). The subsequent ascites

fluid was harvested two weeks following injection and stored at 4°C in the presence of 0.01% w/v sodium azide before purification of the antibodies from crude ascitic fluid.

6.4(e) Procedure for the Identification of each

Hybridoma Monoclonal Antibody Isotype.

Knowledge of the antibody class and subclass is required to determine the strategy of purification of each monoclonal antibody from crude ascites fluid (Goding, 1983). A double immunodiffusion technique was used to identify the immunoglobulin isotype for each of the hybridoma monoclonal antibodies (Ouchterlony, 1958). For this procedure, wells were cut in an agarose gel plate and filled with appropriate solutions of monoclonal antibody and mono-specific antisera against mouse immunoglobulin classes and subclasses, which then diffused through the gel and caused lines of precipitate at the line of junction.

To prepare the agarose gel plates for this procedure, glass microscope slides (50 mm x 75 mm) were washed with alcohol, air dried, then dipped in 0.1% w/v agarose in PBS/0.01% w/v sodium azide and air dried again. One percent agarose (A grade, CALBIOCHEM, CA, USA) w/v in PBS/azide solution was melted and poured onto the agarose coated slides. The agarose was allowed to set, and wells cut in a rosette pattern; monospecific antisera against mouse IgM, IgG $\gamma$ 1, IgG $\gamma$ 2a, IgG $\gamma$ 2b (Meloy Laboratories, Springfield, VA) were placed in the central well of separate rosettes, and the corresponding hybridoma tissue culture supernatants for each antibody and positive control immunoglobulin solutions were placed in the remaining peripheral wells. The slides were then incubated for 48 hours at 4°C in a humidified atmosphere, before being washed and stained for the presence

of protein. To wash the slides, they were repeatedly immersed in saline for 30 minutes followed by drying by overlaying with filter papers and weighted down with a heavy glass plate. The slides were then air dried and incubated in Coomassie Blue stain (0.1% w/v Coomassie blue R250, 50% v/v methanol, 7% v/v acetic acid) for 30 minutes followed by destaining overnight in ethanol, acetic acid and water (9:2:9 ratio). Prior to analysis, tissue culture supernatant samples from each of the positive hybridoma colonies were concentrated 10-fold by centrifugation in Centriflo CF-25 membrane cones (Amicon Corporation, USA) at 400g for approximately 10-20 minutes. Since all the antibodies were of the IgG class, purification of each antibody was possible utilizing protein A affinity chromatography techniques.

6.4(f) Purification of Ascitic Fluid and Polyclonal Serum by (Protein A)-Sepharose Affinity Chromatography.

The monoclonal antibodies were isolated from crude ascitic fluid by (protein A)-Sepharose affinity chromatography (Goding, 1978) to allow further characterization and estimation of the concentration of protein in each antibody stock solution. In addition, the polyclonal antisera was also purified in the same manner to enable subsequent estimation of IgG concentration using spectrophotometric techniques. A column containing (Protein A)-Sepharose (purchased from Pharmacia Fine Chemicals, Uppsala, Sweden) was equilibrated before use with a solution containing Tris-chloride (100 mM) and sodium chloride (150 mM, pH 7.2). The effluent was monitored continuously using spectrophotometric techniques at a wavelength of 280 nm.

Following equilibration, either serum or ascitic fluid was loaded onto the column and the column was allowed to develop at 1 ml/minute. After loading, the column was washed with at least 2 column volumes of the equilibration solution. Once the column had been washed by equilibration solution, the antibodies were eluted and collected from the column using a 100 mM glycine-hydrochloride solution of pH 3.0. The purified IgG solution was neutralised (pH 7.2) and the IgG precipitated with saturated ammonium sulphate solution, and stored in PBS containing 0.01% w/v sodium azide at 4°C. Since the immunoglobulins precipitated with ammonium sulphate are mostly IgG, an estimate of the IgG content was obtained by determining the total protein by extinction at 280 nm given that a 1% solution of IgG has an extinction of 14 at this wavelength (Kirschenbaum, 1978).

#### 6.4(g) Fluid phase Radioimmunoassay.

Since the wells of the microtiter plate in the SPRIA had to be cut apart manually, most of the radioimmunoassay work following final isolation of hybridoma cell lines was completed using the fluid-phase RIA procedure. The binding affinity for each anti-hEGF hybridoma antibody for hEGF was determined in a fluid phase RIA by using unlabelled hEGF to compete for the binding of the antibody to  $^{125}\text{I}$ -labelled hEGF. The fluid-phase RIA was carried out using a double-antibody technique as described in Chapter 4, section 4.5, method 2. For the first phase of the assay an amount of purified monoclonal antibody sufficient to bind approximately 50% of the tracer was incubated with  $^{125}\text{I}$ -labelled EGF (chloramine-T method) (5000 cpm, 0.05 ng), together with varying concentrations of unlabelled EGF standard. In the second phase of the assay, normal mouse

serum and rabbit anti-mouse IgG (Silenus Laboratories, Hawthorn, Vic, Australia) was used instead of normal rabbit serum and sheep anti-rabbit IgG as described in Chapter 4, section 4.5, method 2.

To detect reactivity of the antibodies with S-carboxymethylated hEGF and cross-reactivity with both S-carboxymethylated and native mouse EGF, these proteins were used instead of native hEGF in the first phase of the assay. When polyclonal rabbit anti-hEGF antisera was present as the first antibody, the second phase of the assay was performed as described in Chapter 4, section 4.5, Method 2. However, when mouse monoclonal antibodies were present as first antibody, normal mouse serum and rabbit-anti-mouse IgG were used in the second phase of the assay.

An application of the fluid-phase radioimmunoassay was used to examine the reactivities of each of the antibodies to 3 labelled species of hEGF, namely  $^3\text{H}$ -hEGF prepared by reductive methylation,  $^{125}\text{I}$ -hEGF prepared by the iodogen method and  $^{125}\text{I}$ -hEGF prepared by the chloramine-T method. In the initial phase of the radioimmunoassay, a constant amount of tracer hEGF was titrated against a concentration gradient of each of the antibodies tested. The second phase of the assay was completed as stated above.

#### 6.4(h) Preparation and Isolation of S-carboxymethylated hEGF and mEGF.

##### Materials.

All reagents used for the preparation of S-carboxymethylated EGF were of HPLC grade. Guanidine hydrochloride, 2,3-dihydroxy-1,4-dithiol-butane and iodoacetic acid were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K.; trifluoroacetic acid from Pierce

Chemicals, Rockford, Illinois; acetonitrile with 190nm cutoff from BDH Chemicals, Dagenham, Essex, U.K.; and butyl silica 2.1 mm x 3 cm reverse-phase column from Brownlee Laboratories, CA, U.S.A.

#### Method.

The procedure for the preparation and isolation of S-carboxymethylated EGF was adapted from the method of Gurd, 1967. The same two-step procedure was used for the modification of both mEGF and hEGF. In the first step of the preparation, the growth factor was fully reduced by the presence of a denaturing and a reducing agent. In the second step the reduced protein was S-carboxymethylated by the presence of excess iodoacetic acid. For the first step 20µg of EGF dissolved in 20µl 10mM HCl was incubated at room temperature with 200µl 6M guanidine hydrochloride, pH 8.5 and 20µl 0.1M 2,3-dihydroxy-1,4-dithiol-butane. After a reaction time of 15 minutes, a sample of this mixture was analysed by reverse-phase chromatography to test for complete reduction of EGF. In the second step, excess iodoacetic acid (final concentration 0.1M) was added to the reaction mixture and the pH adjusted to 8. After a minimum reaction time of 15 minutes at room temperature, the S-carboxymethylated EGF was separated from the reaction mixture by reverse-phase chromatography.

Mobile phases utilized for chromatography were 0.1% trifluoroacetic acid (pH 2.1) (buffer A) and 0.1% trifluoroacetic acid/100% acetonitrile (buffer B). The column eluates were monitored by UV absorbance at 280nm. The reaction mixture was loaded onto the column after equilibration of the column with 0.1% TFA/20% acetonitrile. After loading the mixture, the column was washed with 0.1%



TFA/20% acetonitrile at a flow rate of 0.2 ml/min until a constant absorbance reading of column eluate was achieved. To elute human EGF from the column an acetonitrile gradient of 20-60% was run over 15 minutes at a constant flow rate of 0.2 ml/min. In the case of mouse EGF an acetonitrile gradient of 25-50% was run over 15 minutes at 0.2 ml/min.

6.4(i) Epitope mapping of monoclonal antibodies and specific polyclonal antiserum using peptide synthesis.

Epitope mapping of 14 monoclonal antibodies and the specific polyclonal antiserum prepared by the candidate was undertaken using a solid-phase peptide synthesis method in collaboration with Dr. Mario Geysen (Department of Molecular Immunology, Commonwealth Serum Laboratories, Parkville, Victoria, Australia). The synthesis of homologous hEGF peptides, subsequent testing with each antibody by enzyme-linked immunosorbent assay (ELISA) and data processing utilizing computerized analysis of the results was carried out by Dr. Mario Geysen. Interpretation of the computerized analysis was then undertaken by the candidate. The procedure used for the peptide synthesis, ELISA and computer analysis of ELISA data was adapted from the method published by Geysen and colleagues (1987).

Forty-six 8-mer peptides, representing all possible overlapping octapeptides covering the total 53-amino acid sequence of hEGF, were synthesized concurrently onto solid support pins (see Fig 6.1) using conventional methods of solid-phase peptide chemistry (Meienhofer, 1973; Erickson and Merrifield, 1976). The peptides were of sufficient purity and quantity to allow subsequent reaction in an ELISA. The solid support pins were assembled into specially

molded polyethylene holders designed to hold 96 rods in the format and spacing of a microtitre plate (Geysen et al., 1987). The peptides, still attached to the support used for their synthesis, were tested for antigenicity by an ELISA using the polyclonal antiserum or monoclonal antibodies carried out in wells drilled in a teflon block in an 8 x 12 matrix to match the rod spacing. By this method, the sequential epitopes for the monoclonal antibodies and the polyclonal antiserum were examined in an accurate, rapid and systematic manner.

## RESULTS

### Generation of hEGF monoclonal antibodies

Four days prior to the fusion procedure, two immune mice were selected from the four immunized mice to provide immune donor spleen cells. To select the two mice with greatest immune response, the serum titre of anti-hEGF antibody was tested in each of the mice. Serum samples were obtained by retro-orbital bleeding of each mouse and the anti-hEGF activity tested in each serum by the ELISA and SPRIA screening methods. When the serum samples were tested for anti-hEGF activity using the ELISA method, approximately equivalent dilution curves were obtained see Fig. 6.2(a). However, when the same samples of antisera were tested for activity using the SPRIA method, the level of anti-hEGF activity in the sera of mice #1 and #4 appeared to be ten-fold greater than the level of activity measured in the serum of mice #2 and #3. The mice numbered #4 and #1 were subsequently chosen for use in the hybridization procedure on the basis of higher anti-hEGF activity as shown in the results of the SPRIA (see Fig. 6.2(b)).

The fusion procedure was carried out as described in

section 6.4(d). Fourteen days after the fusion procedure, ninety per cent of the microtiter wells plated contained hybridoma colonies. Supernatants from the wells containing hybridoma growth were removed on day sixteen and tested for hEGF antibody activity by means of the ELISA screening assay. Of the 260 supernatants tested, over fifty showed anti-hEGF activity. Hybridoma supernatants that were considered positive for hEGF antibody activity gave ELISA values that were at least ten times the background level of the assay. Due to practical constraints however, only fifteen of the colonies were chosen for further subcloning procedures., The parental cell line of fourteen of the fifteen chosen was P3-NS1-Ag4-1, the other being the variant P3-X63-Ag8.

#### Production of specific anti-hEGF polyclonal serum.

During the substantial time taken for the production of monoclonal antibodies, a specific polyclonal antiserum was raised in a rabbit for later use in radioimmunoassays required for the biological studies described in the following chapters. The polyclonal antibody is species specific, showing little affinity for mEGF as summarized in Table 6.2. The competitive radioimmunoassay subsequently developed with this polyclonal antiserum was found to be highly sensitive, requiring only 0.013 pmol of unlabelled hEGF to displace 50% of the labelled hEGF species (see Fig. 6.3).

#### Characterization of the monoclonal antibodies.

##### (1) Determination of antibody isotype.

Determination of the isotype of each monoclonal antibody was required to determine the strategy of purification. Utilizing the tissue culture supernatants for

each clone, the isotypes of monoclonal antibodies 1-14 inclusive were identified as IgG  $\gamma$  1, and IgG  $\gamma$  2A for monoclonal antibody 15 by double immunodiffusion analysis. Photographs of the four agarose gels used in this analysis are shown in Fig 6.4 (a)-(d). In Fig 6.4(a) lines of precipitate, indicating a positive reaction, can be seen for antibodies 1-14 inclusive when testing for IgG  $\gamma$  1, subclass. In Fig 6.4(b) only monoclonal antibody 15 was shown to be precipitated with the monospecific antisera raised against mouse IgG  $\gamma$  2A, the other antibodies indicating a negative reaction. No reaction was seen when testing for either IgG  $\gamma$  2B or IgM antibody [Fig 6.4(c) and (d)]. These results indicate that each monoclonal antibody is of the IgG class and therefore may be purified successfully from crude ascites fluid utilizing protein A-affinity chromatography, since protein-A has a high affinity for mouse IgG (Goding, 1978). Also, these results show that each antibody is exclusively of one particular subclass, supporting the notion that each antibody is truly monoclonal in nature.

(2) Purification of Ascitic fluid by Protein-A affinity chromatography.

Each monoclonal antibody was purified by protein-A affinity chromatography to allow further characterization of each antibody without the presence of interfering substances present in ascitic fluid such as fat, protein and the other immunoglobulins. The concentration of antibody present in each affinity-purified preparation was determined spectrophotometrically at 280 nm, as shown in Table 6.1.

(3) Reactivity of monoclonal antibodies with mouse and human EGF.

The reactivity of fourteen of the monoclonal

antibodies with hEGF and mouse EGF (mEGF) was studied using competitive displacement radioimmunoassays with  $^{125}\text{I}$ -hEGF labelled by the chloramine-T method. In Table 6.2 the amount of unlabelled hEGF or mEGF required to displace fifty percent of the radioactive ligand is given for each antibody.

The competitive assay was also performed using the purified specific polyclonal serum so that a comparison could be made between the monoclonal antibody binding characteristics with that of the polyclonal antibody. In the case of the polyclonal antisera and monoclonal antibody #1, 0.013 and 0.026 pmol hEGF respectively were required to displace approximately fifty percent of the tracer ligand. In contrast, the corresponding amount of unlabelled hEGF required with monoclonal antibody #2 and the remaining thirteen monoclonal antibodies was at least 2 ng (see Figure 6.3). Minimal cross-reactivity was seen between mEGF and the polyclonal antisera, indicating the the polyclonal antisera is highly species specific. In addition, at least 12.5 pmol of mEGF was required to displace fifty percent of labelled hEGF in all the competitive displacement radioimmunoassays performed with the monoclonals. These results are summarized in Table 6.2.

(4) Reactivity of monoclonal antibodies with 3 species of radiolabelled hEGF.

Radiolabelling procedures modify the native hEGF structure in varying degrees depending upon the nature of the reaction and conditions used in the procedure (Tack et al., 1980). The modifications of hEGF structure that occur following iodination by the iodogen or chloramine-T methods or labelling with tritium using the reductive methylation

technique were used to investigate indirectly the approximate location of possible epitopes and to determine the likely number of unique monoclonal antibodies amongst the 14 antibodies tested.

To compare the binding characteristics of the monoclonal antibodies, the relative binding affinities of each antibody and that of the specific polyclonal antisera with three different labelled hEGF species were studied using a fluid-phase radioimmunoassay. As mentioned above, the three labelled species were  $^3\text{H}$ -hEGF,  $^{125}\text{I}$ -hEGF labelled using the chloramine-T method, and  $^{125}\text{I}$ -hEGF labelled using the iodogen method. Without exception, all of the antibodies recognized the three forms of labelled hEGF, however, as expected, the binding affinities of the antibodies for each particular labelled species differed amongst antibodies. In Figure 6.5, the antibodies (including the specific polyclonal antisera) are ranked in order of relative binding affinity for each of the three labelled species. From this data, the monoclonal antibodies may be grouped into 5 or 6 distinct groups sharing approximate binding affinities for each labelled hEGF species. Therefore it is possible that at least five antibodies represent unique monoclonal antibodies amongst the fourteen tested. It is of interest to note that the order of antibodies for each labelled species is constant except for monoclonal antibody #1. This particular antibody has a relatively high affinity for  $^{125}\text{I}$ -labelled hEGF prepared by either method but a relatively low affinity for  $^3\text{H}$ -hEGF. This indicates that monoclonal antibody #1 binds to a region of the hEGF molecule that is modified during reductive methylation of hEGF. Since this labelling

technique is specific for the  $\alpha$  amino groups of  $\text{NH}_2$ -terminal residues and  $\epsilon$ -amino groups of lysyl residues of a protein, the epitope of monoclonal antibody #1 appears to involve either the  $\text{NH}_2$ -terminal residue or a lysine residue of hEGF.

(5) Production of S-carboxymethylated hEGF and mEGF.

The nature of the immunogenic epitopes for each monoclonal antibody was investigated indirectly by comparing the binding affinities of each antibody for hEGF and mEGF with the corresponding denatured S-carboxymethylated forms. The S-carboxymethylated forms of hEGF and mEGF were therefore prepared by the candidate for this investigation. Reverse-phase HPLC eluate profiles of analytical samples of native hEGF, reduced hEGF and S-carboxymethylated hEGF are given in Fig. 6.6. The corresponding profiles for mouse EGF are given in Fig. 6.7. The native, reduced and S-carboxymethylated forms of hEGF were found to elute at 38, 49 and 47% acetonitrile respectively. In the case of mEGF the corresponding percentages were 33, 37.5 and 35% respectively. Two peaks of S-carboxymethylated mEGF were detected in the profile eluting at 34 and 35% acetonitrile, possibly representing the alpha and beta forms of mEGF.

(6). Comparison of the reactivity of the monoclonal antibodies and polyclonal antiserum with the native and S-carboxymethylated forms of hEGF.

As mentioned above, the effect of S-carboxymethylation of hEGF and mEGF on antibody binding affinity was evaluated as an indirect method of assessing the nature of the antibody epitope. In Table 6.2 the amount of unlabelled native and S-carboxymethylated hEGF required to displace fifty percent of  $^{125}\text{I}$ -hEGF in competitive displacement radioimmunoassays is given for each antibody. Monoclonal

antibodies #1, 4, 6-14 inclusive showed similar reactivity with both the denatured and native forms of hEGF. In contrast, monoclonal antibodies #2, 3, 5, 15 and the polyclonal antiserum showed little reactivity with the denatured growth factor. Since a change in conformation did not appear to affect the binding of hEGF to antibodies #1, 4 and 6-14 inclusive, the hEGF epitope for each of these antibodies may be continuous or sequential in nature. However the epitope(s) of antibodies 2, 3, 5, 15 and the polyclonal antiserum are likely to be discontinuous or conformational since a change in the secondary structure of hEGF appeared to dramatically alter the antibody binding activity. Displacement RIA data demonstrating the immunological reactivities of antibodies 1 and 2 with both native and denatured forms of hEGF are illustrated in Fig. 6.8. Monoclonal antibody #2 is representative of the antibodies that are likely to bind to a conformational epitope whereas antibody 1 is representative of the antibodies that may recognize a sequential epitope.

In Table 6.2 the amount of unlabelled native and S-carboxymethylated mEGF required to displace fifty percent of  $^{125}\text{I}$ -hEGF in competitive displacement RIAs is given for each antibody. Monoclonal antibodies 1, 2 and 5 showed the greatest reactivity with mEGF, the amount required to displace 50% of the radioactive ligand being 12.5, 31.3 and 62.5 pmol respectively. These values are 500, 100 and 75 fold greater than the corresponding amount of hEGF required to displace fifty percent of the radioactive ligand, as summarized in Table 6.2. The remaining antibodies, including the polyclonal antiserum, showed minimal binding to mEGF. All monoclonal antibodies and the polyclonal



antiserum bound negligible amounts of the denatured form of mEGF.

(7) Use of peptide synthesis to identify hEGF monoclonal antibody and specific polyclonal antiserum epitopes.

All 46 possible octapeptides from the amino acid sequence of human EGF were synthesized as described previously (see Fig 6.1) for the purpose of detecting the hEGF binding site(s) for each antibody. The results obtained for the octapeptides when tested by ELISA (Geysen et al., 1987) with each of the monoclonal antibodies and polyclonal antiserum are illustrated in Fig. 6.9. In this figure, the results are shown as vertical lines proportional to the extinction obtained in the antibody-binding ELISA test, plotted above the number giving the location with the hEGF sequence of the NH<sub>2</sub>-terminal amino acid of each peptide. Each protein-A-affinity purified antibody was tested by ELISA at a concentration within the range of 3-60nM. The optimum concentration of each antibody chosen for the ELISA test was that which produced a reaction with the octapeptides with an acceptable level of non-specific binding. Examination of scans illustrated in Fig. 6.9 show that monoclonal antibodies #2-5 and 8-14 inclusive show either extremely weak or no reaction to any of the octapeptides, whereas monoclonal antibodies #1 and #13 show limited reaction with a series of octapeptides and monoclonal antibodies #6, 9 and 15 and particularly the polyclonal antiserum show very definite reaction to particular octopeptides.

In Figure 6.9 the antigenic profile for monoclonal antibody #1 shows that this antibody reacts weakly with a series of N-terminal octapeptides, the highest peaks of

reactivity occurring with peptides 7, 9, 11, 14 and 15. Therefore, from these results it would appear that this monoclonal binds to an epitope that involves the N-terminal residues of the hEGF molecule, particularly amino acid residues 12-22. Similarly, monoclonal antibody #13 (Fig. 6.9) appears also to bind to an epitope involving the N-terminal portion of the molecule, the strongest reactions being with octapeptides 7-12 inclusive.

The scan for monoclonal antibody #6 shows a strong reaction of this antibody with the first 3 octapeptides, with a lesser reaction with peptides 9, 11 and 14. It would appear that this antibody also binds to an epitope in an N-terminal region of the hEGF molecule. The antigenic profile for monoclonal antibodies #9 and #15 are difficult to interpret since strong reaction occurred with octapeptides spanning the entire hEGF amino acid sequence. However, the five highest peaks of activity for antibody #9 were measured with peptides 42, 39, 14, 9 and 11 and for antibody #15, peptides 22, 21, 43, 10 and 8.

The polyclonal antiserum showed very definite reaction with peptides 1, 2, 3, 22, 32 and 34 (see Fig. 6.9). This "triphasic" reactivity corresponds to amino acid residues 1-10, 21-29 and 31-41 of the hEGF sequence.

#### DISCUSSION

Monoclonal antibodies to hEGF were prepared primarily to provide an unlimited supply of specific antibody for routine use in EGF radioimmunoassays. Secondly, it was intended that a range of antibodies directed against different sites of the hEGF molecule be produced for examining the integrity of EGF in the studies of EGF intestinal survival and absorption described in Chapters 7 and 8 of this thesis.

However, since considerable time was taken to produce the range of monoclonal antibodies, a specific polyclonal antiserum was prepared and used in the studies discussed in Chapters 7 and 8. While the monoclonal antibodies were not produced and characterized in sufficient time for use in the biological studies, the hEGF binding sites for the antibodies were investigated for the purpose of future use in hEGF research.

The polyclonal antibody raised against hEGF proved to be highly species specific, consistent with the results of other research groups describing the specificity of other EGF polyclonal antibodies. In earlier studies comparing mEGF with urinary hEGF the antibodies were found to exhibit very limited cross-reactivity with heterologous EGF species (Gregory et al., 1977; Starkey and Orth, 1977). Furthermore, the specific polyclonal antisera raised against hEGF obtained by Dr. B. Kemp (see Chapter 4, section 4.3) also showed very little cross-reactivity with mEGF (see Chapter 5).

The primary aim in producing a range of monoclonal antibodies was to establish an unlimited supply of specific antibody for use in radioimmunoassays. With the notable exception of monoclonal antibody #1, the competitive radioimmunoassays developed with the monoclonal antibodies proved to be relatively insensitive, requiring 30-100 fold the amount of unlabelled hEGF to displace 50% of the tracer than that required in the assay using the polyclonal antiserum. In contrast, the radioimmunoassay developed using monoclonal antibody #1 was found to be comparable in sensitivity to that developed with the polyclonal antiserum. Therefore, further collection of polyclonal antiserum from

hEGF immune laboratory animals will not be required. In addition, the potentially unlimited supply of this antibody together with the advantage of being a consistent, standard reagent, make this particular antibody an invaluable tool for future hEGF research.

A competitive fluid-phase radioimmunoassay has already been published in the literature using a monoclonal antibody against hEGF (Ikuta et al., 1985). However, the reported sensitivity of this assay was 3-fold less sensitive than that reported in this study.

The second aim in producing a range of hEGF antibodies was to produce monoclonal antibodies of known antigenic specificity for use in biological studies. For example, a range of monoclonal antibodies may be of use as specific probes of hEGF structure to determine the nature of intracellular processing of EGF that is reported to occur following receptor binding and internalization (Matrisian et al., 1984; Planck et al., 1984; Wiley et al., 1985). Information is lacking about intermediate forms of EGF that might be formed during its intracellular processing and the potential activity of these intermediates. In addition, a range of monoclonal antibodies may also be of considerable use in determining the structure of EGF reaching peripheral tissues following oral administration in neonatal animals. There is evidence to suggest that whilst EGF reaches peripheral tissues in a form recognized by polyclonal antibodies, it has a diminished capacity to bind to specific receptors (Thornburg et al., 1984). Therefore the use of a range of monoclonal antibodies as specific probes may delineate the nature of EGF processing that appears to occur during its absorption from the neonatal intestine.

Given the potential use of this range of monoclonal antibodies, the hEGF binding site was examined for each antibody. As a first step in the characterisation, the reactivity of each antibody with both human and mouse EGF was compared using competitive displacement radioimmunoassays. The low degree of cross-reactivity of each antibody with mEGF indicated that the epitopes are not common to both mEGF and hEGF. Secondly, to investigate the nature of the immunogenic epitopes i.e. continuous or discontinuous, the binding affinities for native hEGF and mEGF were directly compared with that denatured forms of hEGF and mEGF respectively. A "continuous" epitope is a short linear sequence of amino acids homologous with the primary sequence of the protein antigen, and a "discontinuous" epitope is where spatially adjacent amino acids contributing to the interaction with antibody are linearly distant in the primary sequence of that protein (Tainer et al., 1985). The S-carboxymethylation procedure (Gurd, 1967) was chosen to produce a denatured but stable form of each EGF species for these studies. The relatively low affinity of monoclonal antibodies #2, 3, 5 and 15 and the polyclonal antiserum to S-carboxymethylated hEGF indicates that these antibodies bind to epitopes that are predominantly discontinuous in nature and are thus disrupted by denaturation of the peptide. Conversely monoclonal antibodies #1, 4 and 6-14 inclusive showed greater binding affinity to the denatured form, indicating that either the epitopes for each of these are purely sequential in nature or alternatively, the denaturation of the peptide did not disrupt the conformational epitope recognized by the antibodies.

More definitive studies investigating the amino acids involved in forming the epitope for each monoclonal was carried out using synthetic octapeptides homologous to the hEGF sequence. With the exception of antibodies #1, 6, 9, 13, 15 and the polyclonal antiserum it would appear that the remaining monoclonal antibodies recognize purely discontinuous epitopes, since negligible reactivity was detected with the series of octapeptides. Whilst monoclonal antibodies #1, 6, 9, 13 and 15 reacted with linear octapeptides homologous with the hEGF sequence, these continuous epitopes may be in some cases, part of a larger discontinuous epitope to which they contribute only a short stretch of residues. Indeed, it has been shown that antibodies directed to a discontinuous epitope may react weakly with subregions of the epitope made up of a few residues in linear sequence (Regenmortel, 1986).

The amino acid residues involved in the binding sites of antibodies #1, 6, 13 and the polyclonal antiserum were established using the synthetic octapeptides. Monoclonal antibodies #1, 6 and 13 were shown to possess binding sites associated with the N-terminus of the hEGF molecule while the polyclonal antiserum was shown to possess 3 distinct binding sites, 2 of which corresponded to the N- and C-termini of the molecule (see Fig. 6.10). Generally, it has been observed that the terminal residues of proteins are predominantly surface-oriented (Thornton et al., 1983), more flexible (Westhof et al., 1984) and therefore more antigenic (Van Regenmortel, 1986). Thus, the present findings are consistent with these previous observations.

The polyclonal antiserum was shown to bind to three distinct regions of the hEGF molecule corresponding to amino

acid residues 1-10, 21-29 and 31-41. The finding that this polyclonal antiserum binds to a region involving residues 21-29 is also consistent with previous studies describing predominant EGF antigenic determinants. In a study by Komoriya et al., (1984), a major EGF antigenic determinant for rabbit anti-EGF molecules was identified from residue 20 to 31 of the mouse EGF molecule by competition experiments utilizing synthetic EGF fragments. Moreover, in the same series of studies a major receptor-binding region was also located in this region of the molecule. Indeed, the accessibility of the residues in position 21-29 for antibody recognition is consistent with the conclusion by Komoriya and colleagues (1984) that these residues constitute or contain a major receptor-binding region for EGF (see Fig. 6.10 for antigenic sites of hEGF molecule).

To summarize, a specific polyclonal antiserum and a range of specific monoclonal antibodies have been produced. A highly sensitive, specific radioimmunoassay has been developed for the routine measurement of EGF using either polyclonal antiserum or monoclonal antibody #1. In addition, the fourteen monoclonal antibodies have been shown to be species specific, recognizing epitopes unique to hEGF. Nine out of fourteen antibodies have been shown to recognize discontinuous epitopes while antibodies #1, 6, 9, 13 and 15 react to varying degrees with synthetic peptides homologous to hEGF. Moreover, antibodies #1, 6 and 13 have been shown to bind to the N-terminus of the hEGF molecule, a predicted antigenic site.

Table 6.1 Characterization of Human EGF  
Monoclonal Antibodies

Antibody identification	Fusion partner	Isotype	Protein Conc. Stock Solution (uM)
Mc Ab 1	P3-NS1-Ag4-1	IgG $\gamma_1$	5.7
Mc Ab 2	"	"	2.3
Mc Ab 3	"	"	5.0
Mc Ab 4	"	"	7.6
Mc Ab 5	"	"	4.0
Mc Ab 6	"	"	5.9
Mc Ab 7	"	"	(no ascites produced)
Mc Ab 8	"	"	12.9
Mc Ab 9	"	"	13.1
Mc Ab 10	"	"	6.5
Mc Ab 11	"	"	6.3
Mc Ab 12	"	"	19.2
Mc Ab 13	"	"	6.6
Mc Ab 14	"	"	11.9
Mc Ab 15	P3-X63-Ag8	IgG $\gamma_{2A}$	36.8
Polyclonal Ab	Not applicable	Predominantly IgG	36.8



Table 6.2 Reactivity of monoclonal antibodies and polyclonal antiserum with hEGF, mEGF and the S-carboxymethylated forms of these growth factors.

Antibody identification		pmol unlabelled ligand required to displace 50% of <sup>125</sup> I-hEGF (chloramine T)			
		hEGF	S-carboxymeth hEGF	mEGF	S-carboxymeth mEGF
Mc Ab	1	0.026	0.016	12.5	>62.5
Mc Ab	2	0.33	>2.5	31.3	>62.5
Mc Ab	3	1.8	>2.5	>62.5	>62.5
Mc Ab	4	1.1	0.72	>62.5	>62.5
Mc Ab	5	0.83	>2.5	62.5	>62.5
Mc Ab	6	0.80	0.62	>62.5	>62.5
Mc Ab	8	0.83	0.63	>62.5	>62.5
Mc Ab	9	1.0	0.83	>62.5	>62.5
Mc Ab	10	1.13	0.75	>62.5	>62.5
Mc Ab	11	0.73	0.52	>62.5	>62.5
Mc Ab	12	1.17	0.75	>62.5	>62.5
Mc Ab	13	0.83	0.63	>62.5	>62.5
Mc Ab	14	0.93	0.70	>62.5	>62.5
Mc Ab	15	0.70	>2.5	>62.5	>62.5
Polyclonal Ab		0.013	>2.5	>62.5	>62.5

Fig. 6.1. The 53-amino acid sequence of hEGF was subdivided into octapeptide units, and each was synthesized on a separate polyethylene support in the orientation, and with a dipeptide spacer, as shown. Peptides are numbered according to the position of the NH-terminal amino acid within the hEGF sequence (PPA = polyethylene/polyacrylic acid).

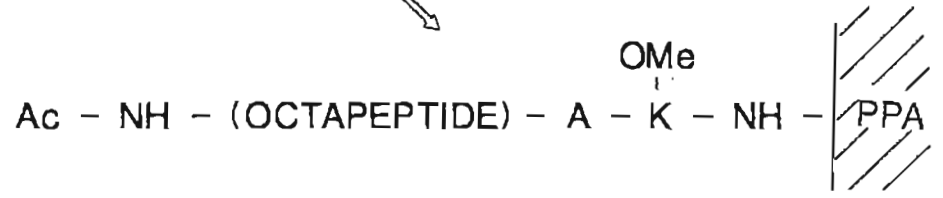
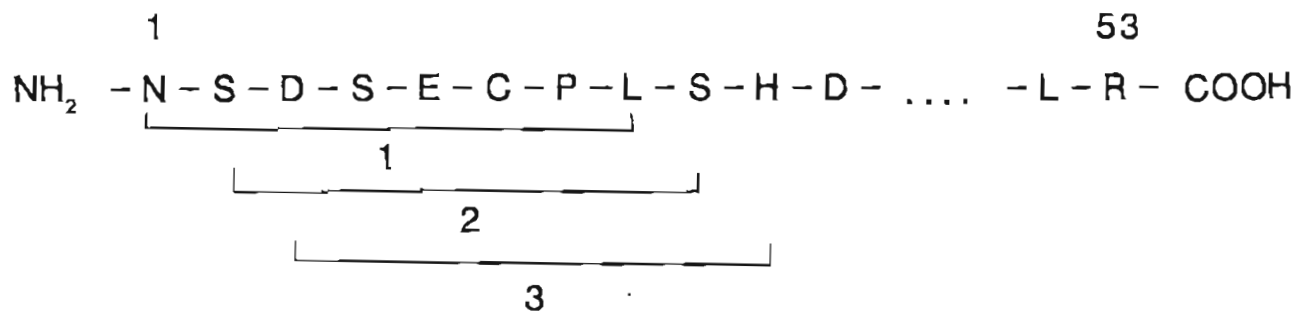


Fig. 6.2. Determination of the level of anti-hEGF activity in the serum of the four hEGF immune female BALB/C mice taken four days prior to the hybridization procedure:

(a) ELISA results of the antisera activity showing equivalent plots for all four mice. Absorbance units ( $A_{280}$ ) are plotted against the log of the dilution of antiserum. Solid lines indicate the test results and the dotted line represents an average of the negative controls for each antiserum tested.

(b) SPRIA results of the antisera activity showing marked differences between the levels of anti-hEGF activity in the sera taken from the four mice. The percentage of counts bound to counts added is plotted against the log of the dilution of antiserum. Non-specific binding of  $^{125}\text{I}$ -hEGF in the absence of antibody was subtracted from the counts bound in the presence of the various concentrations of antisera. Mice #1 and 4 were subsequently chosen for use in the hybridization procedure on the basis of the SPRIA results.

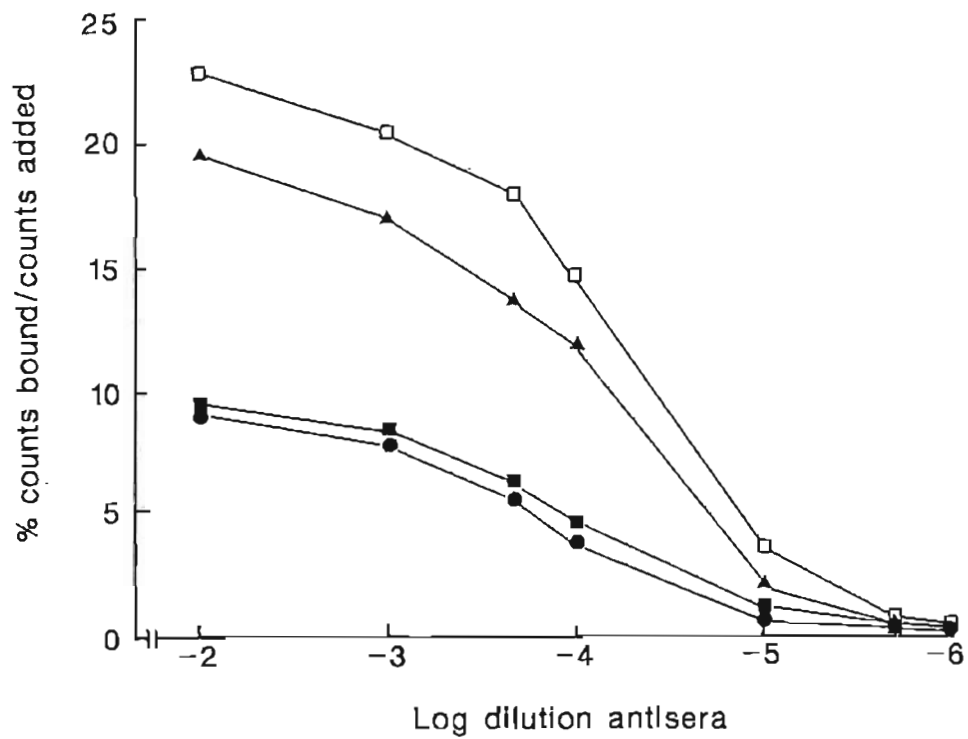
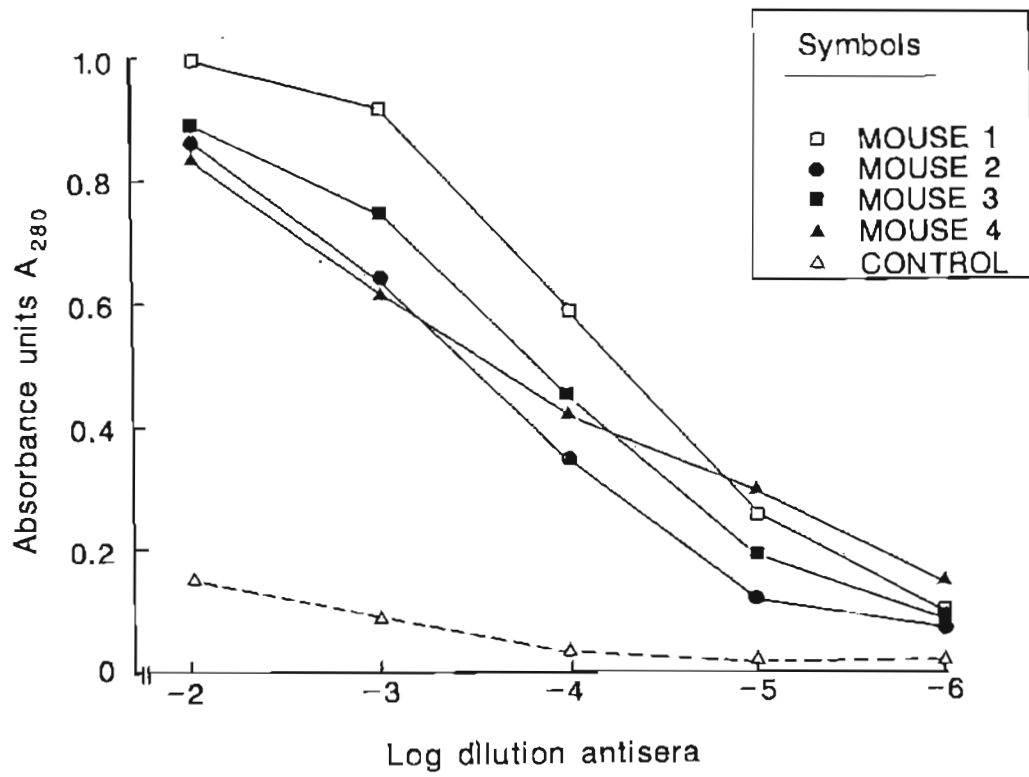


Fig. 6.3. Comparison of the competitive fluid-phase radioimmunoassay data obtained when using either specific polyclonal antiserum (●), purified monoclonal antibody 1 (■) or monoclonal antibody 2 (▲) as first antibody in the assay. The results of the assay using monoclonal antibody 2 is representative of the assays obtained with the remaining monoclonals. Labelled hEGF bound to antiserum or antibody is expressed as a % of the amount bound in the absence of unlabelled EGF (maximum). The % binding is plotted as a function of the ng unlabelled hEGF added to each tube drawn on a log scale. Values represent the mean  $\pm$  SEM of three replicates. The SEM falls within the area of the symbols where no bar is shown.

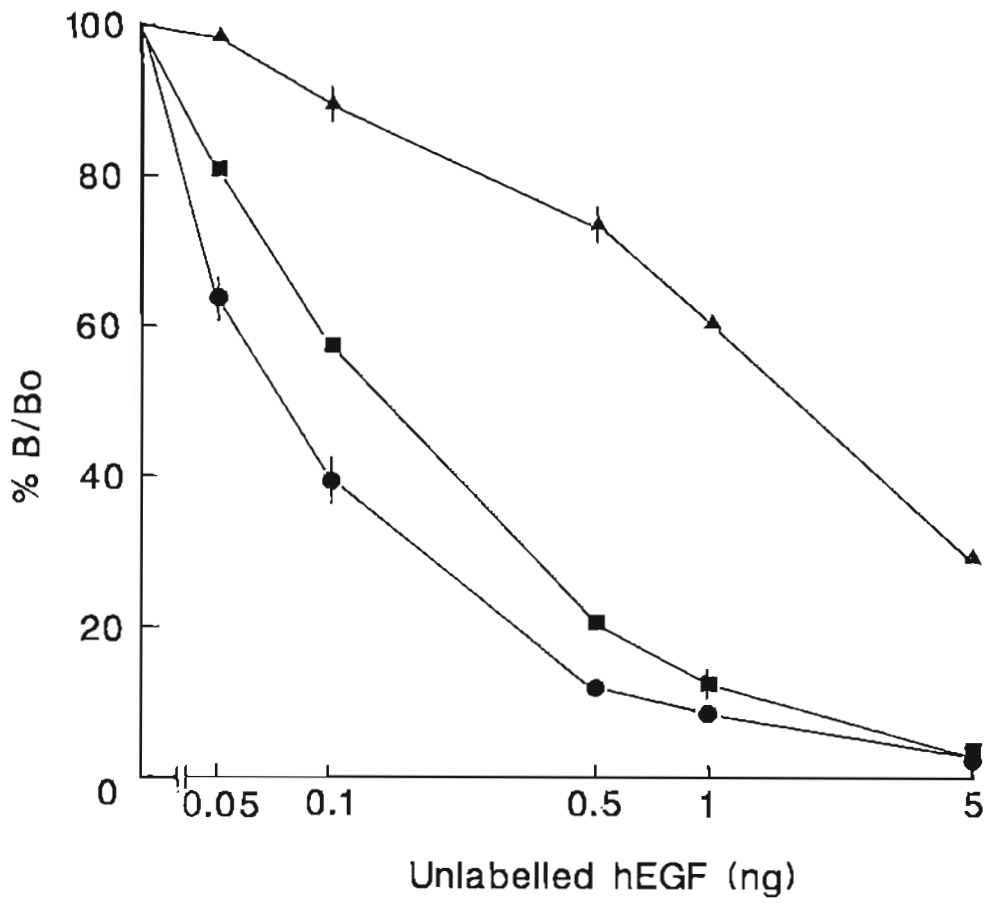
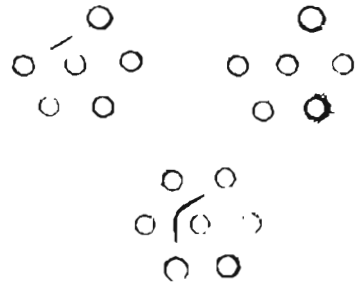


Fig. 6.4. Identification of the isotype of 15 monoclonal antibodies produced against hEGF using double immunodiffusion analysis. Centre wells of the rosettes of A, B, C and D contained monospecific antisera raised against mouse IgG  $\gamma_1$ , IgG  $\gamma_{2A}$ , IgG  $\gamma_{2B}$  and IgM respectively. The outer wells of each set of rosettes contained 10-fold concentrated samples of tissue culture supernatants from each of the 15 hybridoma colonies. The arrangement of test supernatants is illustrated diagrammatically (E). In addition to the hybridoma supernatants, immunoglobulin of the isotype being tested was added in one of the other wells to act as a positive control for each analysis. Results of the immunodiffusion analysis indicate that the isotype of antibodies #1 to 14 inclusive is IgG  $\gamma_1$  and IgG  $\gamma_{2A}$  in the case of monoclonal antibody #15.

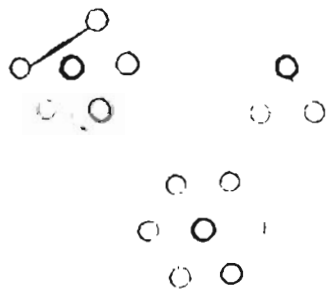




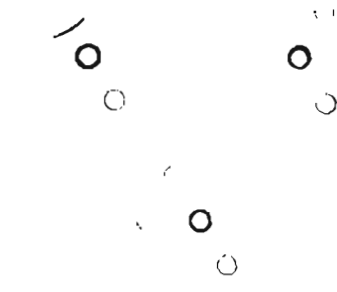
(A) IgG  $\gamma^1$



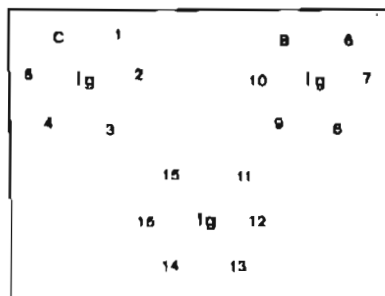
(B) IgG  $\gamma^{2A}$



(C) IgG  $\gamma^{2B}$



(D) IgM



(E)

Fig. 6.5. Reactivity of monoclonal antibodies and polyclonal antiserum with 3 species of radiolabelled hEGF. The monoclonal antibodies and polyclonal antiserum are ranked in order of binding affinities for 3 labelled species of hEGF, namely  $^{125}\text{I}$ -hEGF (iodogen method),  $^{125}\text{I}$ -hEGF (chloramine-T method) and  $^3\text{H}$ -hEGF (reductive methylation method). Note that the relative binding affinity of monoclonal antibody 1 for  $^3\text{H}$ -hEGF is much less than for both of the  $^{125}\text{I}$ -labelled hEGF species.

$^{125}\text{I}$ -hEGF (Iodogen method).

Polyclonal antiserum > 15 >> 1 > 6=13 > 10=11=14 > 4=8=9=12 > 2=3=5



$^{125}\text{I}$ -hEGF (Chloramine-T method).

Polyclonal antiserum > 15 > 1 > 6=12=13=14 > 4=8=9=10=11 > 2=3=5



$^3\text{H}$ -hEGF (Reductive methylation method).

Polyclonal antiserum > 15 > 6=13=14 > 4=8=9=10=11=12 > 1 > 2=3=5



Fig. 6.6. Reverse-phase HPLC elution profiles ( $A_{280}$ ) of analytical samples of native recombinant hEGF, reduced hEGF and S-carboxymethylated hEGF. The elution times for the native, reduced and S-carboxymethylated forms of hEGF were 6.8, 11 and 10.3 minutes respectively. The corresponding percent acetonitrile at which the three forms eluted were 38, 49 and 47% respectively. A butyl silica 2.1 mm x 3 cm reverse-phase column was utilized with mobile phases of 0.1% trifluoroacetic acid (pH 2.1, buffer A) and 0.1% trifluoroacetic acid / 100% acetonitrile (buffer B).

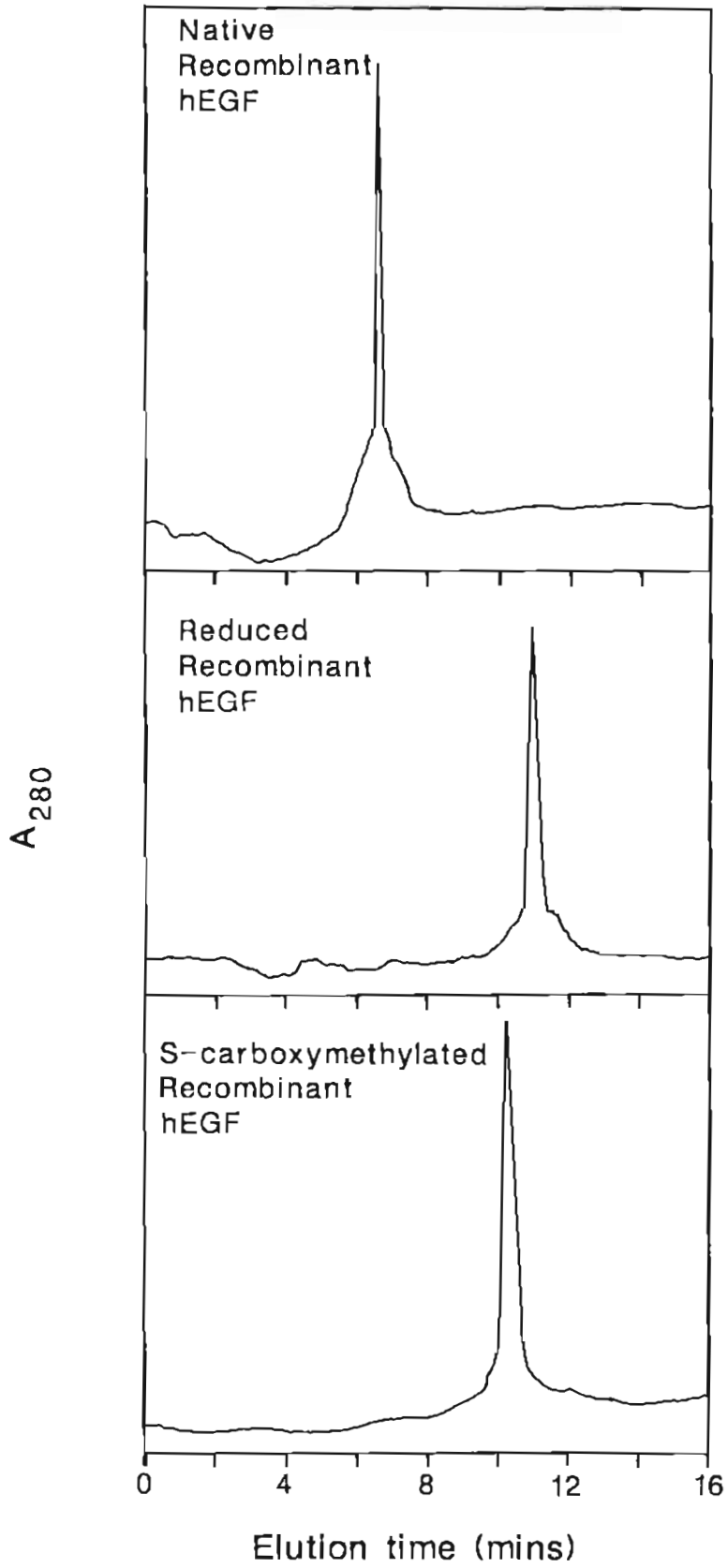


Fig. 6.7. Reverse-phase HPLC elution profiles ( $A_{280}$ ) of analytical samples of native mEGF, reduced mEGF and S-carboxymethylated mEGF. The elution times for the native, reduced and S-carboxymethylated forms (peak A and B) of mEGF were 4.8, 7.6, 5.6 and 6 minutes respectively. The corresponding percent acetonitrile at which the three forms eluted were 33, 37.5, 34 and 35% respectively. The conditions for chromatography were as described in Fig. 6.6.

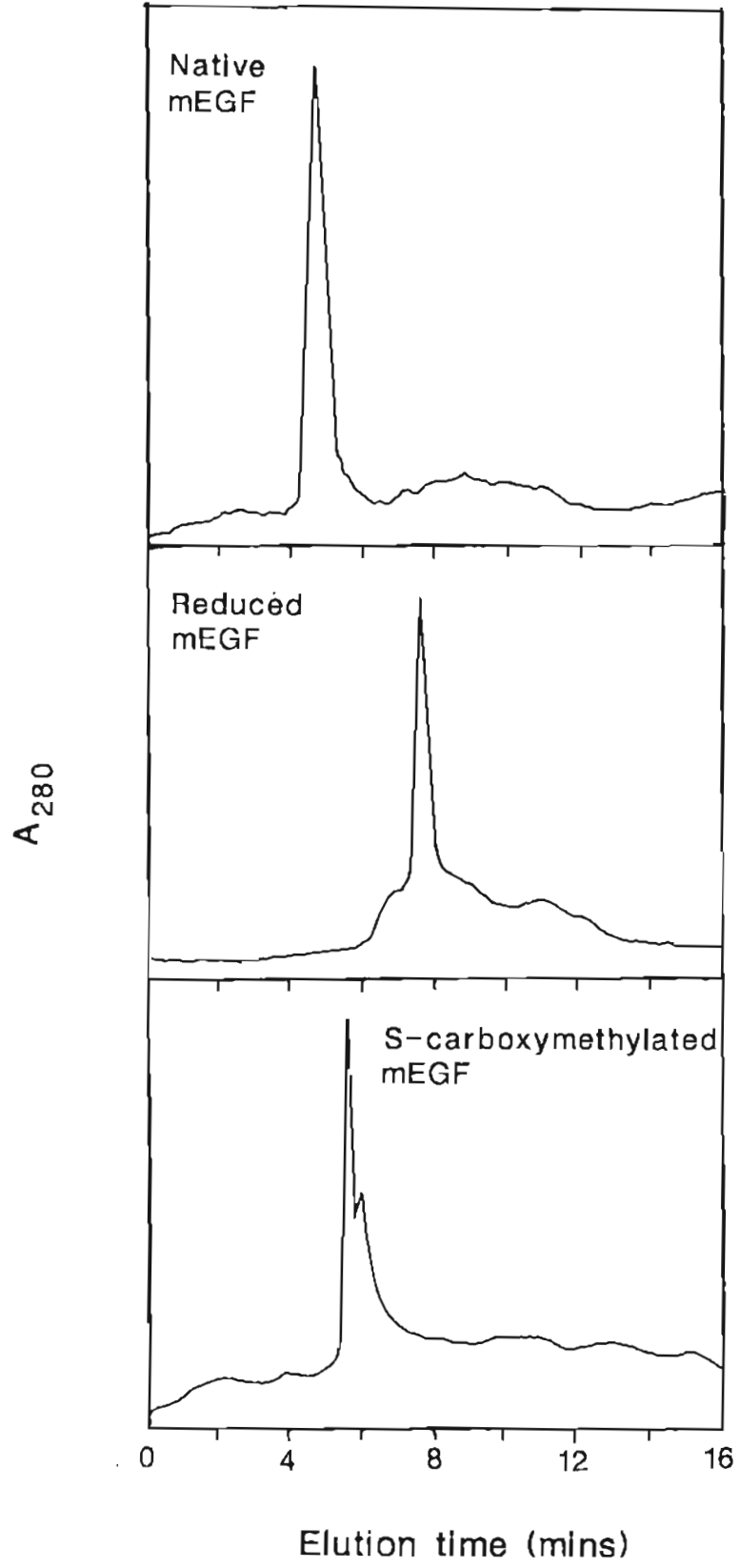
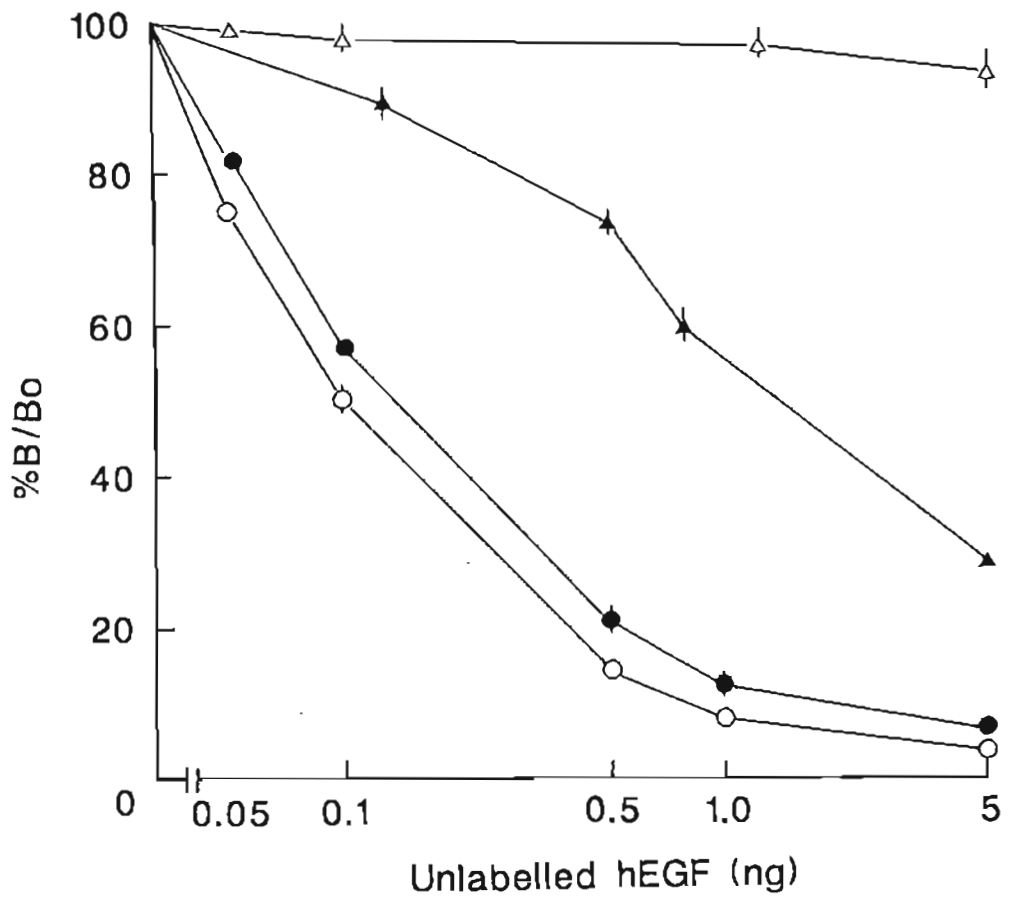


Fig. 6.8. Fluid-phase hEGF radioimmunoassay results showing the reactivity of monoclonal antibodies 1 and 2 with either hEGF or S-carboxymethylated hEGF. Monoclonal antibody 1 was incubated with  $^{125}\text{I}$ -labelled hEGF and the indicated amount of unlabelled hEGF ( ● ) or unlabelled S-carboxymethylated hEGF ( ○ ). Similarly, monoclonal antibody 2 was incubated with  $^{125}\text{I}$ -labelled hEGF and the indicated amount of unlabelled hEGF ( ▲ ) or unlabelled S-carboxymethylated hEGF ( △ ). Values are means  $\pm$ SEM, n=3. The SEM falls within the area of the symbols where no bar is shown.





**Fig. 6.9 Reactivity profiles (scans).** Results are shown as vertical lines proportional to the extinction at  $A_{420}$  nm obtained in the antibody-binding ELISA test, plotted above the number giving the location within the hEGF sequence of the  $\text{NH}_2$ -terminal amino acid of each peptide. Antibodies used to produce the scans shown were monoclonal antibodies #1-6 and 8-15 inclusive and the polyclonal antiserum. Significant peaks are greater than the level denoted by the "minimum significance" marker (-) calculated from the mean and standard deviation from each set of ELISA data for each antibody tested.

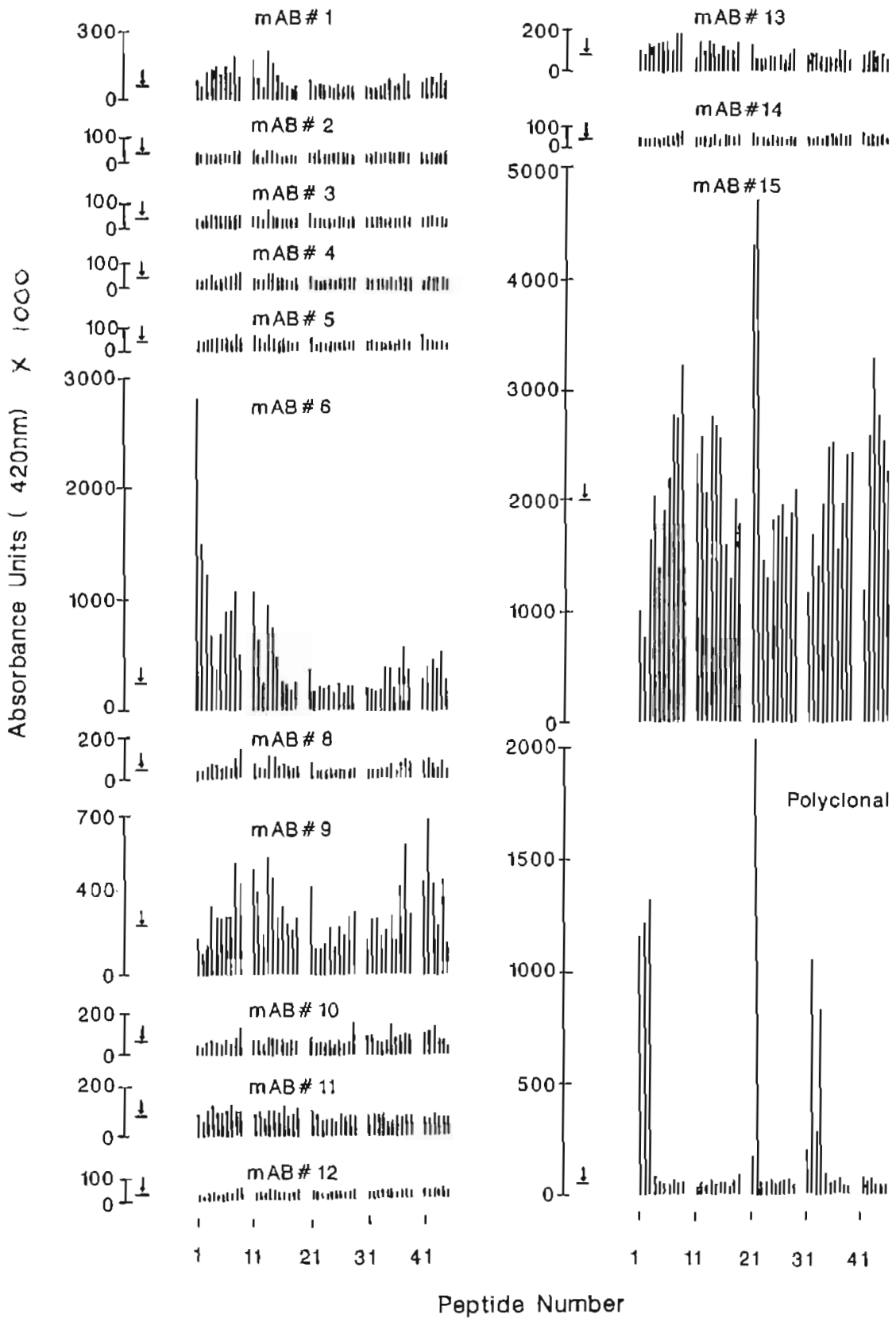
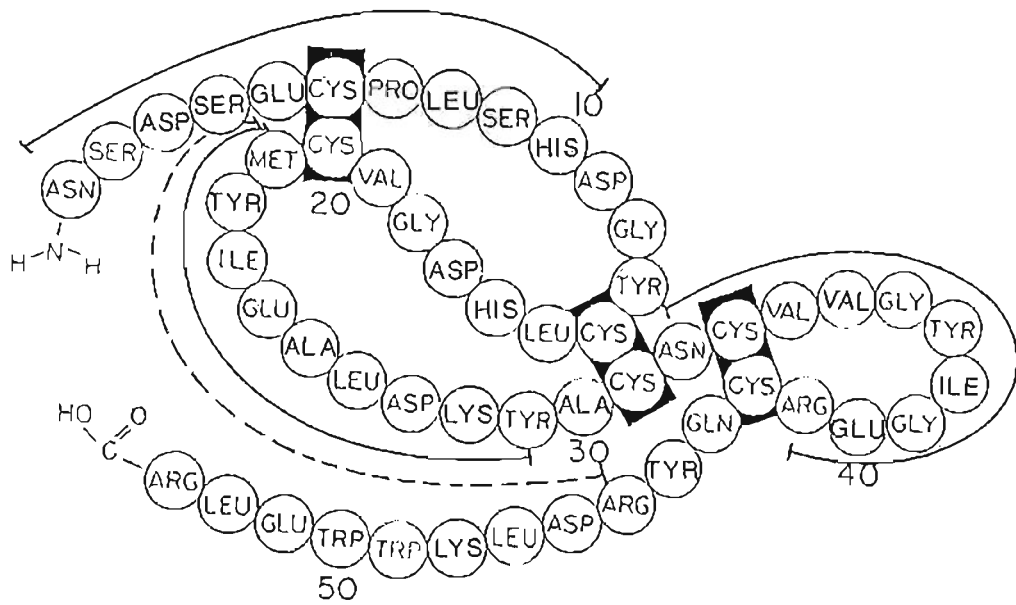


Fig. 6.10        Schematic diagram of human EGF showing the 3 main antigenic sites of the molecule. The receptor binding region (residues 21-30 inclusive) is denoted by the dotted lines. The 3 antigenic sites are denoted by the solid lines.



**CHAPTER SEVEN**

THE RELATIONSHIP BETWEEN DIETARY EGF INTAKE AND URINARY EGF  
EXCRETION IN THE PREMATURE INFANT: AN INDIRECT METHOD OF  
DETECTING EGF UPTAKE BY THE HUMAN NEONATAL INTESTINE

The work presented in this chapter has been recently accepted for publication:

Gale, S.M., Read, L.C., George-Nascimento, C., Wallace, J.C. and Ballard, F.J. (1988): Is dietary epidermal growth factor absorbed by premature human infants? *Biol. Neonate*. In press.

ABSTRACT

The analysis of urinary EGF provides an indirect yet ethically practical measure of EGF uptake from the gut in premature infants. Urinary EGF levels from infants receiving breast milk, a rich source of the growth factor, were compared with the levels excreted by infants receiving diets such as bovine-milk based formulae or total parenteral nutrition which contain low or negligible levels of EGF. Six to ten days following birth there was no significant difference in the urinary EGF output from infants fed breast milk, bovine milk or total parenteral nutrition. However, thirteen to seventeen days following birth, the urinary EGF output by breast-fed infants was higher than the level measured in counterparts fed either formulae or total parenteral nutrition. These latter results are consistent with the hypothesis that EGF crosses the gastrointestinal wall to enter the general circulation in the suckling infant.



INTRODUCTION

Injection of epidermal growth factor (EGF) to fetal rabbits or lambs leads to an increase in the rate of lung maturation that enhances the probability of survival upon premature delivery (Catterton et al., 1979; Sundell et al., 1980). Postnatal administration of EGF has also been shown to accelerate the functional maturation of the gastrointestinal tract of infant mice (Malo and Menard, 1982), rats (Moore et al., 1986; Oka et al., 1983) and rabbits (O'Loughlin et al., 1985). When these results are considered together with the findings that: (a) human milk contains approximately one hundred times the concentration of EGF present in bovine-milk based formulae (Read et al., 1984), and (b) premature human infants are at risk from complications arising from underdeveloped lungs and gastrointestinal tract (Baird, 1969), it seems plausible to propose that the EGF in breast milk may be beneficial to the suckling premature infant.

In this chapter, the hypothesis stated above is addressed by measuring the urinary excretion of EGF by premature infants fed either breast milk, bovine-milk based formulae or total parenteral nutrition (TPN). Since endogenous production of EGF has been reported to be relatively low in neonates (Hirata and Orth, 1979; Kasselberg et al., 1985), the analysis of urinary EGF provides an indirect yet ethically practical measure of EGF uptake from the gut in premature babies.

MATERIALS AND METHODSInfants

Premature human infants of gestational age 26-36 weeks were investigated while maintained in the Neonatal Intensive Care Units at either the Flinders Medical Centre or the Queen Victoria Hospital in South Australia. The study was approved by the Human Ethics Committees of both hospitals and did not involve changes to normal clinical practice. The three groups of infants were respectively fed TPN, formulae or breast milk exclusively over the analysis period except for the first 2-3 days after birth. The TPN diet was prepared and administered according to normal practices of the Intensive Care Unit; the formulae included S-26 (Wyeth), Nan (Nestle), De-Lact (Sharpe) or Prem-Enfalac (Mead & Johnson); breast milk was expressed by the infant's mother.

The groups were matched so far as possible for gestational age, body weight at birth and 5 minute Apgar scores (SEE TABLE 7.1). For the 9 TPN infants these parameters averaged 29.7 weeks, 1312 g and 8.1; for the 8 formulae-fed infants, 30.7 weeks, 1538 g and 8.6; and for the 18 breast milk-fed infants, 30.6 weeks, 1553 g and 8.3. All infants maintained satisfactory growth throughout the study while the incidence of respiratory distress was 8/9 in TPN infants, 6/8 in formulae-fed infants and 16/18 in breast milk-fed infants (see Table 7.1)..

Individual urine specimens were collected between 5 and 10 days after birth and between 13 and 17 days after birth to evaluate whether differences in EGF output were consistent. Urine was stored at -15° prior to analysis.

Children and adults.

Twenty four hour urine collections were obtained from 11 male and 16 female subjects of ages 5-31 years. All subjects chosen were healthy at the time of the urine collection. This part of the study was approved by the Humans Ethics Committee of the University of Adelaide, South Australia.

### Materials

Polyvinyl chloride, round-bottom 96-well microtiter plates were purchased from Dynatech, Alexandria, VA, U.S.A.; affinity purified sheep anti-rabbit IgG antiserum from Silenus Laboratories (Hawthorn, Vic., Australia), and Spectropor dialysis membrane tubing of molecular weight cut-off 3,500 from Spectrum Medical Industries Inc., Los Angeles, CA, U.S.A. A polyclonal antiserum to hEGF was prepared by the candidate as previously described (See Chapter Four).

### Preparation of urine samples prior to radioimmunoassay.

All urine samples were dialysed and concentrated ten-fold by lyophilisation prior to the measurement of EGF. Concentration of infant samples was considered necessary due to the low levels of EGF in neonatal urine; dialysis was performed prior to lyophilisation to remove excess salts. Urine samples from subjects of ages 5-31 years were treated in the same manner. The recovery of EGF added to urine averaged 94%. All urine samples were suitably diluted before radioimmunoassay to ensure that the EGF concentration was in the linear range of the assay. (see below).

### Solid-phase radioimmunoassay

Human EGF was iodinated using the Iodogen method as described in Chapter Four, section 4.2. Each well of the

polyvinyl chloride 96-well plates was coated overnight at room temperature with 75  $\mu$ l (60 ng protein) of sheep anti-rabbit IgG antiserum diluted in PBS (25mM  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 150mM NaCl, 10 mM EDTA, adjusted to pH 7.4 with NaOH). After three washes with PBS containing 5% bovine serum albumin, 50  $\mu$ l of PBS, containing sufficient anti-EGF antiserum to bind approximately 50% of the tracer was added per well and the plates incubated at room temperature for one hour, followed by washing as before.  $^{125}\text{I}$ -hEGF (50  $\mu$ l, 10,000 cpm) in PBS containing 0.25% bovine serum albumin was added to each well together with 50  $\mu$ l of a urine specimen or an EGF standard. After a further one hour incubation at room temperature the wells were washed as before, cut apart and the radioactivity determined in each well .

A standard curve from a typical EGF solid-phase radioimmunoassay is represented on a semi-logarithmic plot (Figure 7.1). Fifty per cent of labelled EGF was displaced when 0.45 ng of EGF was added to the assay. The urine specimens were diluted before radioimmunoassay to an EGF concentration of 0.05-1.0 ng per 50  $\mu$ l to be within the most precise region of the assay. Displacement curves parallel to the standard curve were obtained when different dilutions of the urine samples were assayed (Figure 7.1).

#### Creatinine Determination.

Urinary creatinine was determined using an automated adaptation of the Jaffé reaction (Techicon Auto-Analyser Method N11b).

#### Statistics.

Comparison between means have been made using the Student's t-test. Values are reported in Table 7.2 and the Figures as means  $\pm$  SEM.

RESULTSInfluence of dietary EGF on urinary EGF excretion.

The excretion of EGF by premature infants born between 28 and 33 weeks of gestation was measured over two postnatal periods; from 5-10 and from 13-17 days after birth. During the first collection period, the concentration of EGF in urine from infants receiving TPN was  $0.34 \pm 0.07$  pmol per ml, significantly ( $P < 0.01$ ) less than observed in urine from breast-fed infants (Figure 7.2a). However, since the creatinine concentrations were also lower in infants receiving TPN than the other two dietary groups, expression of the EGF excretion data per  $\mu$ mole of creatinine removed all the differences between the groups (Figure 7.2).

Urine collected during the period between 13 and 17 days after birth from infants receiving TPN had a mean EGF concentration of 0.33 pmol per ml, significantly lower than the values in urine from either formulae-fed ( $p < 0.05$ ) or breast-fed infants ( $p < 0.02$ ) (Figure 7.2b). Moreover, the concentration of 1.41 pmol per ml of urine from breast-fed infants was higher ( $p < 0.05$ ) than the combined average ( $0.54 \pm 0.10$  pmol/ml) measured in the urine samples from infants fed formulae or TPN. Unlike the situation in the first collection period, expression of the EGF content per  $\mu$ mole of creatinine in urine collected between 13 and 17 days after birth did not remove statistical significance between the breast-fed and the other groups. Thus the value obtained in urine from breast-fed infants,  $1.04 \pm 0.20$  pmol EGF per  $\mu$ mole creatinine, was higher ( $p < 0.05$ ) than both the values in infants given TPN ( $0.49 \pm 0.1$  pmol/ $\mu$ mole), (Figure 7.2b) and the combined group of babies not fed breast milk ( $0.54 \pm 0.07$  pmol/ $\mu$ mole). However, no statistical

significance at the 5% level was reached in a comparison between breast-fed and formulae-fed infants.

#### Influence of Age on Urinary EGF Excretion.

The relationship between gestational age and urinary EGF excretion was studied in premature infants aged 5-10 days following birth between 26 and 37 weeks of gestation. Since the level of dietary EGF intake did not appear to affect the urinary EGF output of infants aged 5-10 days [Figure 7.2(a)], data from all infants during this postnatal period was included irrespective of the type of dietary intake. The urinary EGF output (pmole EGF/ $\mu$ mole creatinine) was found to increase proportionately with gestational age (Figure 7.3). Statistical analysis of this data showed that the correlation between the two variables was significant ( $n=39$ ,  $r=0.758$ ,  $p<0.02$ ).

The urinary EGF excretion by premature infants was compared with that measured in several age groups, and was found to increase three-fold from birth to a maximum in subjects aged 5-8 years after which a gradual decline occurred (Table 7.2).

#### DISCUSSION

If milk-derived EGF is to act directly to promote growth and differentiation of infant tissues other than the gastrointestinal tract, dietary EGF must first enter the general circulation. There is little information available on the intestinal uptake of protein hormones in humans or other species (Udall et al., 1981). However, since endogenous EGF production is very low in neonates (Hirata and Orth, 1979; Kasselberg et al., 1985) and it has been demonstrated that radioactive EGF passes very rapidly into urine following intravenous administration (Gregory et al.,

1977), it was considered at the outset of this study that urinary EGF should provide a useful estimate of EGF uptake across the gastrointestinal wall of premature infants. The approach taken in this study was to compare the urinary output of EGF by infants receiving an EGF-rich diet, breast milk, with two different EGF-poor diets, bovine-milk based formulae and TPN. It is demonstrated that the excretion of EGF by premature infants receiving breast milk is higher than by the other groups. This observation was made from data obtained from infants of age 13-17 days and applied whether urinary EGF excretion was expressed as a concentration or normalised for creatinine excretion. However, between 5 and 10 days after birth, urinary EGF excretion expressed per  $\mu$ mole of creatinine was not influenced by dietary EGF intake, perhaps because the infants may have had only had 3-7 days of total enteral nutrition before the urinary samples were taken.

The discovery that dietary EGF intake influences the urinary EGF output does not prove that the higher EGF levels are a direct consequence of a greater EGF absorption rate across the intestine. An alternative possibility would be an indirect effect of a component in breast milk to enhance the endogenous production of EGF, which would then lead to an increased rate of excretion. Some endogenous EGF production certainly occurs because it is shown here that premature infants receiving a diet with negligible EGF content excreted significant amounts of urinary EGF. Moreover, an increase in the rate of EGF excretion occurred between the first and second postnatal weeks even in babies who were not receiving dietary EGF. Further evidence of increased endogenous EGF production with age during early

development is given by the demonstration that a positive correlation occurs between gestational age and urinary EGF excretion. This finding agrees with previous reports (Evans et al., 1986; Mattila et al., 1985).

In this study it was found that the amount of urinary EGF excreted by children aged 5-8 years is three-fold greater than that excreted by the premature neonate, in agreement with a previous study that demonstrated an increase up to the beginning of the third year of life (Mattila et al., 1985). Thereafter EGF excretion decreased gradually with increasing age. The change in the rate of EGF production with age has also been studied by immunohistochemical analysis of human tissue sections in order to identify the site or sites of EGF production (Kasselberg et al., 1985). In that investigation EGF was detected in the adult kidney but not in kidney tissue from the foetus or the newborn infant. These results are pertinent because the kidney has been suggested as an important endogenous source of urinary EGF due to the presence of precursor EGF messenger RNA in the distal tubules of the mouse kidney (Rall et al., 1985). In addition, measurements of urinary EGF in unilaterally nephrectomised rats were consistent with endogenous production of EGF by the kidney (Olsen et al., 1984).

It is plausible that urinary EGF originates from a larger EGF precursor molecule located in the cell membrane of the kidney. The sequences of mouse submandibular gland EGF mRNA (Scott et al., 1983) and human renal EGF mRNA (Bell et al., 1986) predict that EGF is synthesized as an unusually large precursor, consisting of 1217 amino acid residues in the mouse and 1207 residues in man, more than 20



times larger than EGF itself. The structure of the predicted precursor is similar in both species and suggests that it may be a membrane bound protein (Pfeffer and Ullrich, 1985) consisting of an amino-terminal hydrophilic extracellular domain of about 1032 residues in man, a hydrophobic transmembrane region of about 22 residues, and a carboxy-terminal hydrophilic intracellular domain of about 153 residues; the EGF sequence lies just outside the plasma membrane near the carboxyl terminus of the extracellular domain (Scott et al., 1983; Bell et al., 1986). In addition, there is considerable homology between the precursor and the low-density lipoprotein receptor, a known membrane bound protein (Pfeffer and Ullrich, 1985). It has been suggested that the precursor molecule is synthesized in renal tubular cells (Bell et al., 1986; DiAugustine and Brown, 1986) and is inserted into the plasma membrane with the extracellular domain containing EGF projecting into the tubular lumen. Whether the whole precursor is released into urine and is subsequently cleaved by enzymes present in urine into high molecular weight intermediate forms and the 6000 Da EGF species itself or is cleaved in situ, leaving the intracytoplasmic and transmembrane regions behind in the tubular epithelial cell, is an interesting subject for further research. However, urinary EGF in humans may also originate from other endogenous sources, since immunoreactive EGF has been detected in many human tissues (Kasselberg et al., 1985). This suggests that EGF production occurs in these tissues which may in turn also contribute to urinary EGF.

Regardless of the actual source of urinary EGF in humans, the present study indicates that babies fed breast

milk excrete more urinary EGF after the second postnatal week, thus being consistent with the hypothesis that EGF crosses the gastro-intestinal wall to enter the circulation of the premature infant. A role for EGF in early human development is suggested by experiments with mice, rats, rabbits and lambs, in which EGF given to fetal animals accelerated developmental processes in the lung (Catterton et al., 1979; Sundell et al., 1980) and gastrointestinal tract (Calvert et al., 1982; Dembinski et al., 1982; Oka et al., 1983, Goodlad et al., 1985). From these results it seems likely to be the poorly developing infant that would benefit most from the trophic actions of EGF. Moreover, this concept is supported by studies demonstrating that the rat intestine is more responsive to EGF following gut resection (Read et al., 1986), starvation (Dembinski et al., 1982) or atrophy associated with removal of enteral nutrition (Goodlad et al., 1985), while the onset of respiratory distress syndrome in the prematurely-delivered lamb can be prevented by injection of EGF into the fetus (Sundell et al., 1980). Thus, although it is clear from extensive clinical experience that both full-term and premature infants develop at normal rates when fed an EGF-deficient diet, it is plausible that infants born prematurely with inadequate gut or lung function may gain an advantage from the presence of EGF in breast milk, particularly the higher concentrations in milk from their own mothers (Read et al., 1985).

**Table 7.1 Summary of the Clinical data of the 3 dietary groups of premature infants investigated in the study.**

Clinical Data	Breast milk	Formulae	TPN
Sex (M:F)	14:4	6:2	4:5
Gestational age (weeks) (mean $\pm$ SEM)	30.6 $\pm$ 0.3	30.7 $\pm$ 0.4	29.7 $\pm$ 0.4
Birth weight (g) (mean $\pm$ SEM)	1553 $\pm$ 70	1538 $\pm$ 90	1312 $\pm$ 79
5 minute Apgar Score (mean)	8.3	8.6	8.1
Incidence of Respiratory Distress	16/18	6/8	8/9

Table 7.2 Changes in EGF excretion with age.

Age	EGF excretion	
	pmol/ $\mu$ mol creatinine	nmol/kg/day
Premature infants, 5-10 days (40)	0.60 $\pm$ 0.05	-
Premature infants, 13-17 days (26)	0.71 $\pm$ 0.06	-
5-8 years (5)	2.14 $\pm$ 0.37	0.36 $\pm$ 0.08
10-12 years (10)	1.49 $\pm$ 0.16	0.28 $\pm$ 0.02
14-17 years (6)	1.53 $\pm$ 0.25	0.30 $\pm$ 0.05
24-31 years (6)	1.20 $\pm$ 0.11	0.24 $\pm$ 0.01

Values represent means  $\pm$  SEM for the number of subjects given in parentheses.

**Figure 7.1.** EGF solid-phase radioimmunoassay. Labelled EGF bound to antiserum as a percentage of the amount bound in the absence of unlabelled EGF (control) is plotted as a function of (●) unlabelled EGF (ng); (▲) pooled adult urine ( $\mu$ l) or (■) pooled neonatal urine, diluted as required. Non-specific finding has not been subtracted.

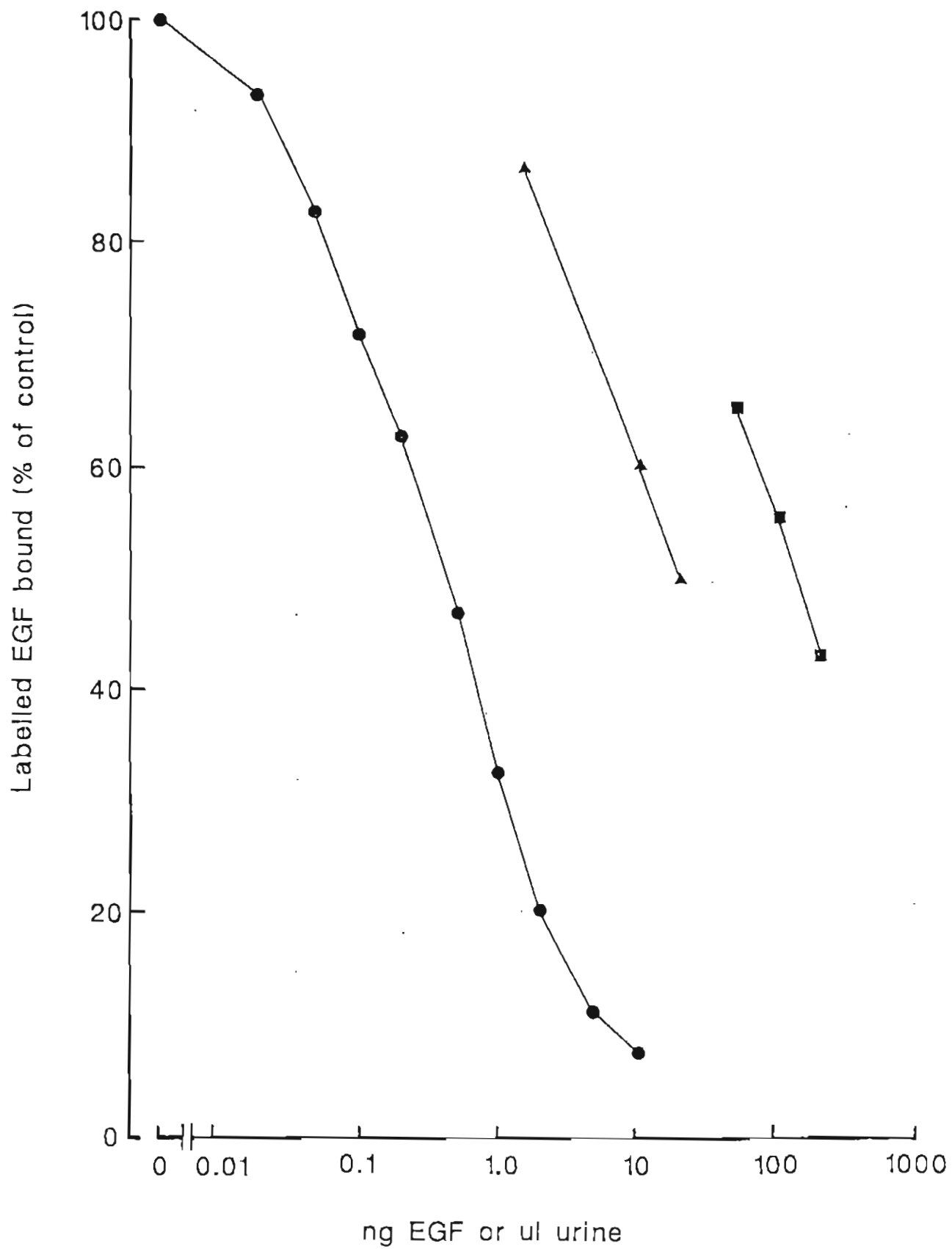
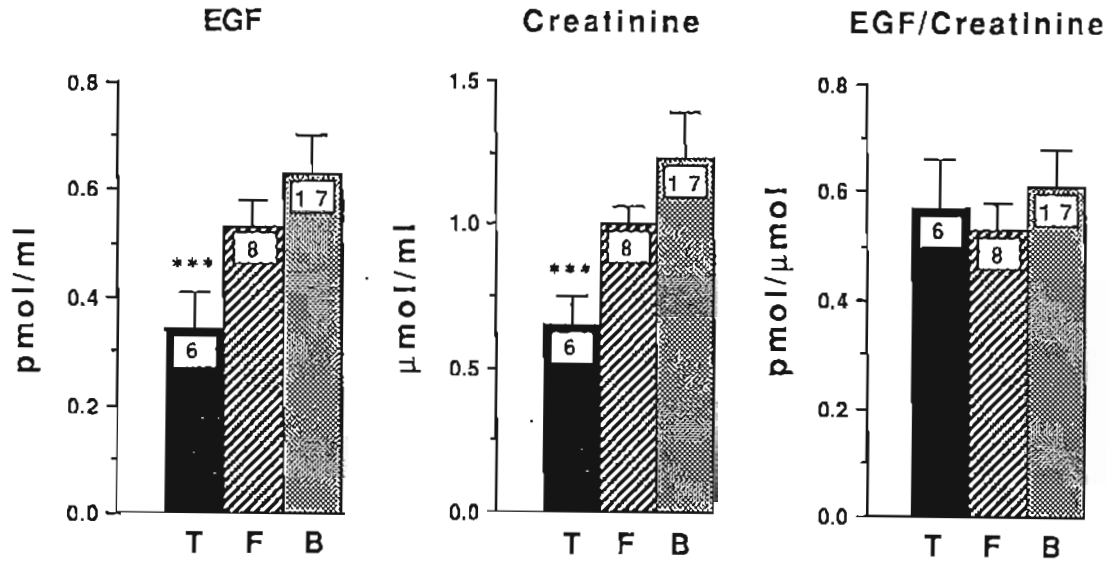


Figure 7.2. The effect of dietary source on urinary EGF excretion by premature infants (a) 5-10 days and (b) 13-17 days after birth. The urinary output of both EGF and creatinine was measured and these data are shown as urinary concentrations. Urinary excretions of EGF are also expressed as values normalized with respect to urinary creatinine concentration. The infants received either total parenteral nutrition (T), formula (F) or breast milk (B). Each value is the mean  $\pm$  SEM for the number of subjects as shown. Asterisks denote the level of statistical significance between the data from breast-fed infants and the corresponding data from infants receiving formula or total parenteral nutrition, where \* $p$ <0.05, \*\* $p$ <0.02 and \*\*\* $p$ <0.01.

a) 6-10 days



b) 13-16 days

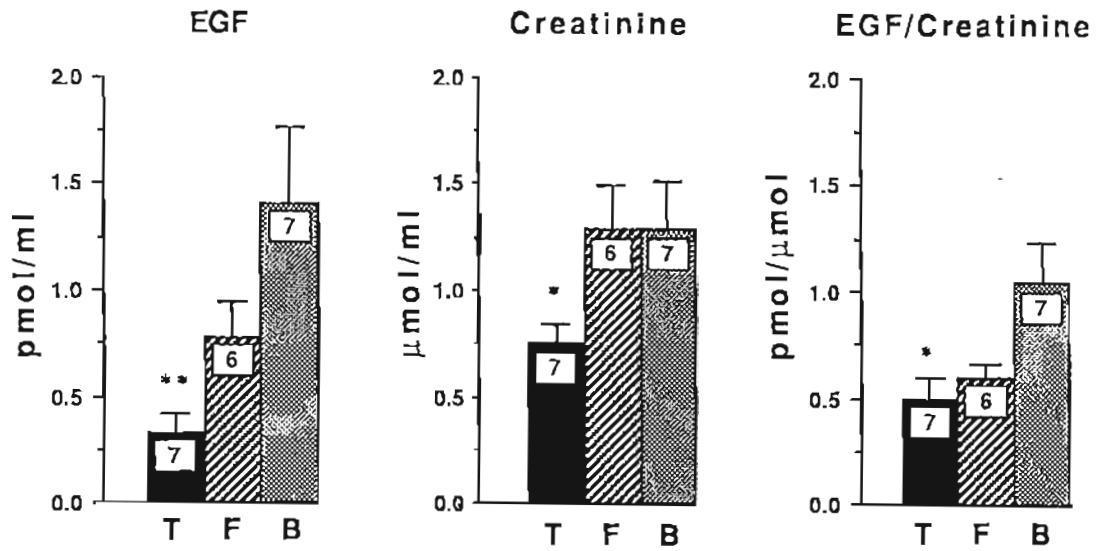
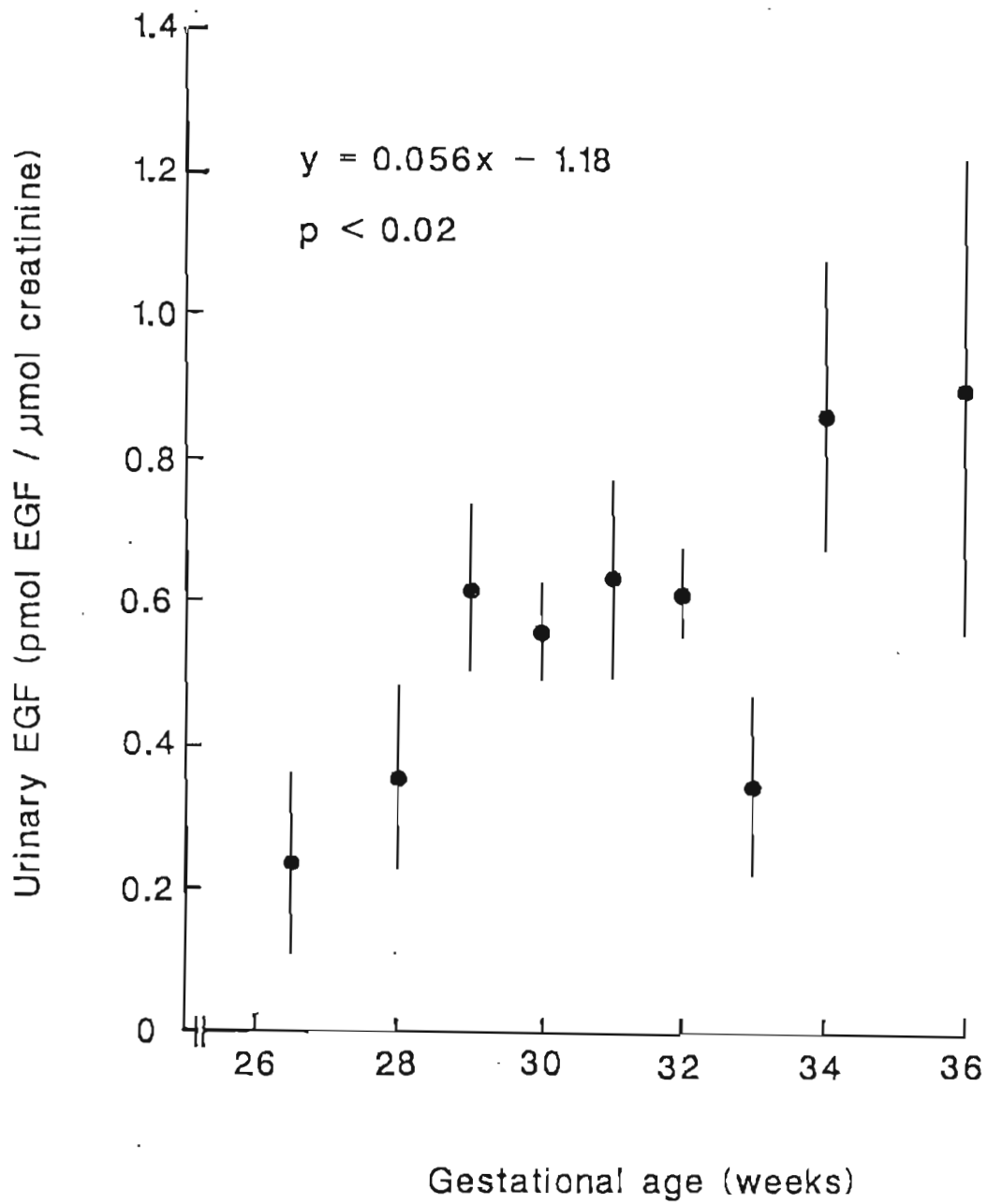




Figure 7.3. . . . EGF excretion (pmole EGF/ $\mu$ mole creatinine) in urine collected between 5 and 10 days after delivery has been plotted as a function of gestational age. Each plotted value is the mean  $\pm$  SEM for between 3 and 10 urine samples.



CHAPTER EIGHT

A QUANTITATIVE ASSESSMENT OF THE INTESTINAL ABSORPTION OF  
IMMUNOLOGICALLY - INTACT EGF  
USING THE AUTOPERFUSED LAMB INTESTINE METHOD.

Some of the work presented in this chapter has already been accepted for publication:

Read, L.C., Gale, S.M. and George-Nascimento, C. (1986):  
In: The effects of human milk on the recipient infant.  
Second International Symposium on Human Lactation, Konstanz,  
G.D.R., Sept., 1986, edited by A.S. Goldman, Plenum Press,  
U.S.A. In Press.

ABSTRACT

The potential for milk-derived epidermal growth factor (EGF) to survive gastrointestinal digestion and to be subsequently absorbed biologically intact by the neonatal intestine was examined. To assess the survival of gastrically-administered EGF, chronic re-entrant catheters were surgically implanted in 7-10 day old lambs for complete collection of small intestinal contents. Using this technique, it was established that after intragastric infusion of  $^{125}\text{I}$ -labelled EGF, a minimum of 15-33% of the radioactivity infused reached the lamb small intestine as immunologically active EGF. Analysis of intestinal absorption of EGF was achieved by adaptation of the in situ auto-perfused intestine to the newborn lamb. This technique enables the quantitative analysis of absorption of luminal proteins into both blood and lymph under near-physiological conditions. Gamma globulin (IgG) and EGF were infused simultaneously into the lumen of an intestinal segment of anaesthetized lambs and the absorption into both blood and lymph determined. IgG was not detected in either efferent blood or lymph of the autoperfused intestinal segment while EGF, a relatively small molecular weight moiety was absorbed into blood, but not lymph, at rates that showed no dependency on age of the lamb, but varied considerably between animals. The results of the study indicate that the amount of EGF absorbed by the neonatal intestine is unlikely to influence the growth and maturation of peripheral neonatal tissues, however, the findings support the hypothesis that luminal EGF has a trophic effect on the gastrointestinal tract itself.

## INTRODUCTION

In the preceding chapter, it was shown that during the second postnatal week of the life urinary EGF output is higher in breast-fed infants than in counterparts receiving diets deficient in EGF (see Chapter 7). These results are consistent with the hypothesis that EGF resists digestion and crosses the gastrointestinal wall intact to enter the general circulation in the suckling human infant. Furthermore, there is other direct evidence in experimental animals that EGF remains intact in the neonatal gastrointestinal tract and may be absorbed in significant amounts (Thornburg et al., 1984; Mulvihill et al., 1986; Koldovsky et al., 1986; Thornburg et al., 1987). While these previous studies support the gastrointestinal survival and absorption of EGF in the human infant and in experimental animals, they have provided only qualitative information on the fate of orally-derived EGF. Quantitative analysis of the survival of orally-administered EGF in the neonatal intestine and subsequent intestinal absorption is necessary in order to assess the physiological role of milk-derived EGF in the suckling neonate. If EGF is to have direct effects on the growth and maturation of infant tissues such as the lung, it must first resist digestion to reach the site of absorption and secondly, cross the intestinal barrier to enter the general circulation in a biologically active form. In the studies described hereafter in this chapter, the lamb has been used as an experimental model to assess both the intestinal survival and absorption of EGF in the newborn in a quantitative manner under conditions close to the physiological.

To investigate the gastrointestinal survival of EGF,

chronic indwelling re-entrant catheters were implanted in the small intestine to allow complete collection of intestinal contents from suckling, unanaesthetized lambs. It was thereby possible to examine the in vivo gastrointestinal metabolism of orally-administered EGF in newborn lambs under near-physiological conditions.

Quantitation of the intestinal absorption of EGF is necessary in order to evaluate the influence of milk-derived EGF in the development of peripheral tissues, such as the lung. It is important, not only to measure the concentrations of EGF crossing the intestinal barrier, but also to determine the route of uptake. EGF absorbed into portal blood will pass directly to the liver, a known site of EGF clearance (Burwen et al., 1984), whereas EGF in lymph would enter the bloodstream without an initial passage through the hepatic circulation. The concentrations of orally-derived EGF that finally reach a peripheral target organ are therefore likely to depend on the route of intestinal absorption.

Various methods have been described for the determination of the intestinal absorption of luminal proteins, both in vivo and in vitro. However, in vitro preparations, such as intestinal sacs or strips (Crane et al., 1958; Field et al., 1971) or the isolated perfused intestine (Shichiri et al., 1973; Windmueller et al., 1977; Windmueller et al., 1984) do not allow determination of rates of uptake into both blood and lymph nor do they provide a model close to the in vivo situation. On the other hand, the relatively non-invasive methods for use in the whole animal, such as portal blood sampling following placement of proteins into the small intestine (Warshaw et

al., 1971), or measurement of the disappearance from the lumen, are difficult to quantitate, especially for proteins such as EGF, that are cleared rapidly from the circulation (for review, see Csaazy, 1984).

To evaluate the intestinal absorption of EGF in the suckling infant in a quantitative manner under near physiological conditions, the in situ autoperfused intestine was adapted to the newborn lamb. This method, previously described by Windmueller and Spaeth (Windmueller et al., 1977; Windmueller et al., 1984) in the adult rat, allows determination of the rates of transmission of a luminal compound into both blood and lymph, while maintaining normal blood, lymph and neural supplies.

In this chapter, the intestinal absorption of EGF, a relatively small protein (MW 6000 Da), is compared with that of a large molecular weight moiety, human gamma globulin (hIgG, MW 150,000 Da). Human IgG was included for comparison because there is considerable data on the intestinal transmission of IgG in neonatal animals (Comline et al., 1951; Balfour and Comline, 1959; Asplund et al., 1962; Leece, 1973; Healy, 1977; Patt, 1977; Cabello and Levieux, 1981; Grongnet et al., 1986), information that can be used to assess the validity of the auto-perfused intestine as a technique for studies on the absorption of proteins in the newborn.

#### MATERIALS AND METHODS.

##### MATERIALS

Rabbit anti-hIgG and sheep anti-rabbit IgG antisera were purchased from Silenis Laboratories (Hawthorn, Victoria, Australia).  $^{22}\text{Na}$  Cl was from the Radiochemical Centre, Amersham, Bucks, U.K.



Cannula tubing was purchased from Dural Plastics and Engineering, Dural, N.S.W., Australia or Dow Corning Corp. (Michigan, USA), i.v. catheters (Surflo) from Terumo Corporation, Tokyo, Japan, and anaesthetics from Bomac Lab., Asquith, N.S.W., Australia (Sodium Pentothal) or I.C.I. Australia Operations Ltd., Villawood, Vic., Australia (Fluothane). Sterile 430 ml bags for collection of donor sheep blood (CPDA modified blood-Pack Units) were purchased from Travenol Laboratories, N.S.W., Australia. Polyvinyl chloride, round-bottom 96-well microtiter plates were purchased from Dynatech, Alexandria, VA, USA.

#### ANIMALS.

Merino or Dorset/Merino cross lambs of 1-18 days of age were obtained from the Waite Agricultural Research Institute Experimental Station.

#### Radio-labelled compounds.

EGF and hIgG were iodinated to a specific activity of 95 Ci/g using the Chloramine-T method of iodination as described in Chapter Four, section 4.2. In the case of the experiments undertaken to determine the intestinal processing of EGF, EGF was iodinated to a specific activity of 95 Ci/g using the iodogen method also as described in Chapter Four, section 4.2.

#### Insertion of chronic gastric and re-entrant small intestinal catheters to determine the survival of ingested EGF.

Chronic gastric and re-entrant intestinal catheters were inserted into anaesthetized lambs of 5-10 days of age, using surgical techniques similar to those described by Brown et al (Brown et al., 1968).

The gastric cannula was an open-ended length of vinyl tubing (3.0 mm ID, 4.0 mm OD) fitted with a cuff so that

when it was secured into the gastric wall by a purse-string suture it would not dislodge. The catheter was then exteriorized and sutured to the skin on the back of the animal to prevent disruption. The re-entrant intestinal catheter was comprised of 2 lengths of cuffed vinyl tubing (3.0 mm ID, 4.0 mm OD) connected by a joining piece of silastic tubing (4.0 mm ID, 6.0 mm OD). It was placed near the midpoint of the small intestine following resection of a small length of bowel to prevent any volvulus of the adjacent intestine. The catheter ends were secured in the intestinal lumen by ties behind the cuffs. The two sections of the re-entrant catheter were then exteriorized, sutured to the skin of the animal in the same manner as the gastric catheter, and joined using the connector piece of tubing. A surgical net tube was placed around the trunk of the animal to prevent dislodgement of the catheters by the animal or by the dam. Lambs were returned to their mothers after they regained consciousness and generally they resumed suckling within several hours. Lambs were given a recovery period of at least 24 hours prior to experimentation, during which time they were left undisturbed with their mothers, and allowed to suckle naturally. The lambs were not fasted before any experiment. To determine the intestinal processing of EGF,  $^{125}\text{I}$  labelled EGF (95 Ci/g,  $1-2 \times 10^6$  cpm, 5-10 ng EGF) was infused as a bolus (EGF infusate) into the gastric catheter, the re-entrant catheter was disconnected and the entire intestinal contents were collected over the next 3 hours into tubes kept on ice (see Fig. 8.1). Also aliquots of gastric contents were taken periodically throughout the experimental period. Lambs were confined in a box but required no sedation during the 3 hour

experimental period. At the end of the collection period, gastric and intestinal samples were centrifuged at 4°C for 30 mins at 40,000 x g and the fat-free infranatant recovered. For determination of the percentage of immunoreactive EGF, aliquots (100 µl) of infranatant or EGF infusate were incubated at 4°C with an excess of polyclonal antiserum to EGF. Buffers and precipitation of bound radioactivity were as described previously in Chapter Four, section 4.5. The percentage of immunoreactive EGF (referred to subsequently as "intact EGF") in gastric and intestinal samples was calculated from the ratio of the proportion of radioactivity in the samples that bound to the antiserum to the proportion of radioactivity in the original infusate that bound to the specific antiserum.

#### Auto-perfused lamb intestine.

The auto-perfused intestine, described in the adult rat by Windmueller and Spaeth (Windmueller and Spaeth, 1977 and 1984) was adapted to the newborn lamb. All animals were maintained with their respective dams to suckle normally from birth. Lambs were taken from their mothers a few minutes prior to the commencement of the procedure so that the animals were well hydrated and fed prior to experimentation. The wool was clipped from the lamb's neck to expose the jugular vein, which was then punctured using a 14-gauge i.v. catheter. A polyethylene cannula (1.0 mm ID, 1.5 mm OD) containing sodium pentothal (25 mg/ml in 0.9% NaCl) was passed through the i.v. catheter and advanced approximately 15 cm into the jugular vein. Pentothal was infused slowly until light anaesthesia was achieved (approximately 20 mg/Kg body wt.), at which time the trachea was intubated with a cuffed endotracheal tube for subsequent

maintenance of anaesthesia using Fluothane (0.5-2.0%), delivered through a CIG Midget MK 2, with an O<sub>2</sub> flow of 2 litres/min. The jugular vein cannula was retained and used subsequently for infusion of donor blood.

The lamb was laid in a supine position on a heating pad and the carotid artery was cannulated and connected to a pressure transducer (Model 13119,P23AA, Statham, Puerto Rico) for continuous measurement of arterial pressure and heart rate, recorded using a Beckman Type R Dynograph. After the wool was clipped from the abdomen, the small intestine was exteriorized through a midline incision onto a piece of plastic film stapled to the edges of the abdominal wound. The intestine was moistened frequently with warm Krebs's-Ringer solution and wrapped in the plastic film whenever possible to prevent dehydration and to help maintain temperature. A 20 cm segment of small intestine was chosen such that its blood supply drained into a single branch of the superior mesenteric vein (Fig. 4.2). Suitable segments were available throughout the small intestine of the lamb. For the experiments described in this paper, a segment within the proximal small intestine was cannulated. Two silk ligatures (3-0) were secured around each end, forming a closed luminal segment, with a silastic infusion cannula (1.0 mm ID, 2.2 mm OD) inserted into the proximal end of the segment lumen (Fig. 8.2). Ligatures (4-0 silk) were also tied around the veins adjacent to the luminal ties, to ensure that all venous blood arising from the segment would subsequently drain into the venous catheter.

Next, the intestinal lymph duct was cannulated at a site that allowed complete collection of the lymph drainage from the chosen segment. Any minor lymph ducts that

emanated from the segment, but did not drain into the lymph catheter, were occluded using 4-0 silk ties. Complete collection of lymph from the segment was routinely verified at the completion of each experiment by injecting a small volume of a dye, 0.5% pontamine sky blue, into the lymph nodes of the segment. Cannulation of the lymph duct was achieved using an i.v. catheter (20 or 22 gauge), subsequently connected to a 20 cm length of silastic tubing (1.0 mm ID, 2.2 mm OD), or alternatively, the duct was cannulated directly using bevelled polyethylene tubing (0.58 mm ID, 1.0 mm OD, approximately 20 cm long). With either method, the catheter was secured by two 4-0 silk ties around the lymph duct. To prevent clotting, heparin (5000 I.U./ml) was flushed through the lymph catheter prior to cannulation and also added to the collection tubes (final concentration of 10 I.U. heparin/ml lymph). Collection tubes were kept on ice.

As the final step, the segmental vein was cannulated. Taking care not to occlude any arteries, two 3-0 silk ties were passed under the vein approximately 1.0 cm apart, one as close as possible to the superior mesenteric vein. Heparin (500 I.U./Kg body wt.) was then infused into the circulation through the jugular vein catheter. After the heparin had circulated (1-2 min.), the ligature nearest to the superior mesenteric vein was tied to occlude the blood flow and the vein was cannulated as rapidly as possible, using a 20 gauge i.v. catheter. The catheter was secured in place with a second tie and connected to a silastic cannula (1.0 mm ID, 2.2 mm OD) of approximately 20 cm length for continuous collection of blood into 5 ml plastic tubes kept on ice. Blood and lymph flow was monitored by timing the

collection of these fluids into volume calibrated tubes.

Coincident with the venous cannulation, replacement sheep blood was transfused into the lamb's jugular vein at a rate equal to the rate of blood collection from the intestinal vein (generally 5-6 ml/min), plus a small amount typically 0.3-0.5 ml/min, to compensate for any additional loss of blood associated with the surgery. Continuous monitoring of the lamb's blood pressure permitted fine adjustment of the transfusion rate. On the day of the experiment, donor blood was obtained from an adult sheep maintained with a chronic jugular catheter and was collected using 20 ml syringes into sterile bags containing heparin (20 I.U./ml blood). During the perfusion, the blood bag was kept on ice and gently mixed by rocking to prevent settling of red blood cells (Windmueller and Spaeth, 1977 and 1984). Donor blood was pumped with a peristaltic pump (Gilson MiniPuls 11, Gilson Medical Electronics, France) through a gauze filter, a water-jacketed 39°C heat exchanger and a bubble trap before reaching the jugular vein (Windmueller and Spaeth, 1977 and 1984). To maintain the temperature of the intestine at 39°C, the segment was positioned in a small water-jacketed bath constructed from coiled plastic tubing, and was wrapped entirely in plastic film and kept moist with warm Krebs-Ringer solution. Rectal and intestinal temperature were monitored continuously during the experiment.

Following an equilibration period of 15-30 min. during which time blood and lymph were collected as control samples, a 1.0 ml volume of Krebs-Ringer solution pH 7.4 containing EGF (50 µg, 8.3 nmol) and hIgG (5 mg, 33 nmol) was infused into the luminal catheter of the segment. The

intestinal segment was not emptied of digesta before infusing the protein mixture into the segment. This ensured that the proteins delivered to the intestinal lumen would be present in luminal fluid approximating the physiological state. Timed collections from the intestinal lymph and venous catheters were then continued for 30-60 minutes. Blood samples were also taken from the carotid arterial catheter several times during the experiment. At the end of the experimental period, the intestinal segment was rapidly excised and the luminal contents flushed out with 10 ml of ice-cold Krebs-Ringer solution containing 0.1% bovine serum albumin. The intestinal segment was blotted dry and weighed to obtain the wet tissue weight. A portion was then homogenized in ice-cold 10ml Krebs-Ringer solution using an Ultra Turrax homogenizer (Janke and Kunkel KG, GDR), while the remainder of the segment was dried overnight at 70°C to obtain the wet weight:dry weight ratio. The lamb was sacrificed under anaesthetic by intravenous administration of a saturated KCl solution.

Samples of blood, lymph, luminal contents and homogenized intestine were centrifuged at 1500g for 30 min. and the supernatants removed and stored frozen at -20°C for later analysis by radioimmunoassay. Blood and lymph collected before the luminal infusion of protein were also centrifuged to obtain control plasma and lymph.

The initial preparative surgery was generally completed within 60 minutes. With the additional 15-30 min equilibration period plus the 30-60 minutes experimental time following luminal infusion, the entire procedure lasted about 2.5 hours, although it was found that the experiment was not compromised by longer periods of surgery, as lambs

remained stable for many hours under Fluorothane anaesthesia.

#### EGF RADIOIMMUNOASSAYS

The concentration of EGF present in the plasma and lymph samples generated from the perfusion experiments was determined using two different radioimmunoassay protocols. The concentration of EGF in plasma was determined by fluid-phase radioimmunoassay as described in Chapter Four, section 4.5, method 2. Some plasma samples were concentrated four-fold before assay by freeze-drying and reconstituting in 25% of the original volume. In all radioimmunoassays, tubes containing EGF standards also contained 50  $\mu$ l of control plasma, treated in the same manner as the samples, in order to detect any non-specific interference in the assay.

Since control lymph samples were found to cause significant interference in the fluid-phase radioimmunoassay, a solid-phase radioimmunoassay was used to measure EGF activity in lymph samples. The procedure for this assay has previously been described (see Chapter 7).

#### IgG RADIOIMMUNOASSAYS

Two different double-antibody fluid-phase radioimmunoassays were used to determine IgG activity in the lymph and plasma samples. The protocol for each assay was similar to that described in Chapter Four, section 4.5, method 2. In the case of the plasma samples, sheep anti-human IgG serum and donkey anti-sheep serum were used in the first and second phase of the assay respectively. However, since significant non-specific interference occurred with this assay when lymph was present, another fluid-phase radioimmunoassay was developed using a specific antiserum against human IgG raised in a rabbit rather than a sheep.



Even using this alternative assay protocol, no more than 5 $\mu$ l of each lymph sample could be added to the assay without significant non-specific interference occurring.

Rates of intestinal absorption of EGF or IgG were calculated as the product of the concentration in blood or lymph (pmol/ml, determined by RIA) and the flow rate of blood or lymph (ml/min), and are expressed as pmol EGF or IgG absorbed/min/g dry weight intestine.

## RESULTS

### Integrity of EGF in the small intestine of the lamb following intragastric administration.

Determination of the immunoreactivity of  $^{125}\text{I}$ -labelled EGF in gastric samples taken periodically during the experimental period indicated that EGF was not degraded significantly in the stomach of 7-13 day-old lambs; 90-100% of the radioactivity present in the gastric samples was immunoreactive (results not illustrated). The percentage of radioactivity representing immunologically intact EGF in individual samples taken from the small intestine after intra-gastric infusion of  $^{125}\text{I}$ -labelled EGF was approximately 70-90% (see Fig. 8.3). The amount of radioactivity (cpm) in samples taken from the small intestinal catheter remained essentially constant during the 3 hour experimental period. Table 8.1 shows the calculations of the estimated minimum percentage of intact EGF reaching the small intestine after intra-gastric infusion in two lambs aged 11 and 13 days. The data obtained from the experiment in the 7 day-old lamb was incomplete due to poor intestinal flow during the procedure and therefore is not included in this analysis.

The amount of radioactivity infused intra-gastrically

was of the order of  $1-2 \times 10^6$  cpm, which represents 5-10 ng EGF. The residual radioactivity, expressed in cpm, in the stomach of the 11 day-old lamb was estimated by counting an aliquot of gastric contents at the end of the experiment, assuming a gastric volume of 50 mls. Since an obstruction in the gastric catheter prevented sampling of stomach contents from the 13 day-old lamb the residual gastric radioactivity could not be determined for this experiment. The total cpm recovered from the small intestine during the experimental period was calculated by the summation of the total immunoreactive and non-immunoreactive cpm in the samples of the small intestinal (S.I.) contents collected. The total amount of radioactivity recovered in the small intestinal contents collected during the experimental 3 hour period expressed as a percentage of the difference between the amount of radioactivity infused and the amount retained in the stomach, was 17 and 49% for the 11 day and the 13 day-old lamb respectively. The percentage of radioactivity representing intact EGF in the small intestinal samples was equivalent to the average percentage of intact EGF in intestinal samples over the entire timecourse, as shown in Fig.8.3. The product of the percentage of radioactivity recovered and the percentage of radioactivity representing intact EGF in the intestinal contents gives an estimated minimal percentage of the total amount of infusate reaching the small intestine as intact EGF. This estimation is likely to be conservative as the recovery of the radioactivity in the small intestinal samples could not include any intact EGF remaining in the intestinal contents proximal to the catheter.

Absorption of EGF by the autoperfused lamb intestine.

(1) Perfusion characteristics.

Prior to cannulation of the intestinal vein, blood pressure and heart rate generally remained stable at the rates given in Table 8.2, although the depth of anaesthesia caused some fluctuation. The initial blood pressure was maintained following cannulation of the segmental vein by adjustment of the rate of transfusion of donor blood. Blood flow through the intestinal catheter was dependent both on the size of the intestinal segment and on the length of the venous catheter. Consequently the length of the intestinal segment was standardized to 20 cm and the venous cannula length adjusted to allow a venous blood flow of approximately 5 ml/min in all experiments (Table 8.2). Lymph flow varied considerably between experiments, ranging from 0.014-0.252 ml/min (Table 8.2), variability that presumably reflected differences between lambs in feeding patterns prior to surgery as well as in the position of the lymph catheter which drained not only the segment but also regions distal to the segment. Figure 8.4 gives the physiological data on a time-course basis for a representative experiment. Heart rate, peak systolic and diastolic pressure and venous blood flow remained steady during the entire perfusion while lymph flow was generally more variable during the course of the perfusion.

Initial experiments were carried out to verify that the cannulation procedure resulted in complete collection of blood from the intestinal segment.  $^{125}\text{I}$ -labelled EGF or  $^{22}\text{NaCl}$  was infused into the luminal cannula and blood samples were taken over the next 60 minutes from the carotid arterial catheter as well as the intestinal venous catheter. The absence of radioactivity in the carotid arterial samples

demonstrated that none of the segment's venous drainage escaped the intestinal vein catheter. Conversely,  $^{22}\text{NaCl}$  injected into the lumen of the intestine adjacent to the segment could be detected in the carotid arterial samples, but not in the intestinal venous samples, establishing that the venous catheter only collected blood derived from the chosen intestinal segment (results not illustrated). Quantitative lymph collection was established visually at the end of each experiment, as described in the Methods section.

(2). Radioimmunoassays.

(a) EGF Radioimmunoassays

The EGF concentration in venous blood collected during the perfusions contained approximately 0.15 ng per ml. Since the minimum sensitivity of the assay was of the order of 0.01 ng EGF, it was necessary to assay 200 $\mu\text{l}$  of plasma to accurately measure the EGF concentration. A standard curve from a typical EGF fluid-phase is represented on a semi-logarithmic plot in Fig.8.5 to demonstrate that the addition of 50 $\mu\text{l}$  of four-fold concentrated plasma, collected from a lamb prior to luminal infusion of EGF (control plasma), did not cause interference in the assay.

In contrast to plasma, lamb lymph was found to cause interference in the fluid-phase RIA. Consequently, lymph samples were assayed for EGF by means of a solid-phase RIA, in which up to 50 $\mu\text{l}$  of lymph caused no interference (Fig 8.6). Higher concentrations of lymph could not be assayed due to significant interference, resulting in a non-parallel standard curve. (results not shown). The sensitivity of the solid-phase RIA was similar to that of the fluid-phase RIA, with fifty per cent of labelled EGF displaced by 0.1 ng

(0.016 pmol) of EGF.

(b) IgG Radioimmunoassays.

In Fig.8.7, a standard curve from a typical IgG fluid-phase radioimmunoassay is represented on a semi-logarithmic plot, demonstrating that the addition of 50 $\mu$ l of control plasma caused no interference. Fifty per cent of tracer IgG was displaced by the addition of 4ng (0.026 pmol) of unlabelled hIgG. Using this fluid-phase method, non-parallel standard curves were generated by the addition of as little as 2 $\mu$ l control lymph (results not shown). Accordingly, lymph samples were assayed for IgG by another fluid-phase radioimmunoassay using specific antiserum for hIgG raised in the rabbit, in which up to 5 $\mu$ l of control lymph caused minimal interference (Fig. 8.8).

(3) Intestinal absorption of EGF and IgG.

The time-courses for absorption of EGF and IgG are illustrated for a 4 day-old lamb in Fig. 8.9. In all of the lambs studied, immunologically-intact EGF was absorbed exclusively into blood, whilst IgG was not detected in either blood nor lymph arising from the auto-perfused segment of intestine. It should be noted however, that while the sensitivity of the EGF and IgG radioimmunoassays were approximately equivalent on a molar basis, the maximum volume of each sample of blood or lymph that could be assayed without interference was much less in the case of IgG than EGF. Thus, EGF assays routinely included 250 $\mu$ l of plasma or 50 $\mu$ l of lymph, without interference whereas IgG radioimmunoassays tolerated no more than 50 $\mu$ l of plasma or 5 $\mu$ l of lymph. Therefore, the failure to detect IgG in either blood or lymph may be due to the relatively low amounts of each sample assayed compared to the amounts

assayed for EGF determination.

In view of the fact that cannulation of the venous drainage of an intestinal segment is likely to modify blood flow through that segment, the possibility was considered that the exclusive transmission of EGF into blood may have been an artifact associated with high rates of blood flow following cannulation. To test this possibility,  $^{125}\text{I}$ -labelled EGF was infused into the lumen of a segment in which the lymph, but not the venous drainage, was cannulated. As EGF was still undetected in lymph under these conditions, it was concluded that venous cannulation did not modify the distribution of absorbed EGF between lymph and blood.

In all of the lambs studied, the absorption of EGF into blood generally plateaued within 5-10 minutes of infusion into the lumen, and then remained fairly stable for the rest of the perfusion. For each lamb, the rates of absorption of EGF into blood and lymph were averaged over the entire experimental period and are shown in Fig. 8.10 as the mean  $\pm$ SEM of animals in each age group. EGF was absorbed exclusively into blood and the rates of uptake showed no apparent dependency on age during the first 4-5 days of life. However, there appeared to be an increased rate of uptake between day 4-5 and day 16-18 although it is difficult to tell if this trend is real due to the large variability in EGF uptake between animals. The rate of IgG uptake was assumed to be negligible as no IgG was detected in either venous blood nor lymph at any age.

From the data in Fig. 8.10, it was calculated that over an experimental period of 30-60 minutes, less than 0.1% of the luminally-infused EGF was absorbed as immunologically

intact protein. Measurement of the quantities of EGF and IgG in the luminal contents and intestinal homogenate at the completion of the perfusion indicated that 50-70% of the infused protein could be recovered from these sources (results not shown), suggesting that 30-50% of luminal EGF and IgG had been degraded, either in the lumen or in the intestinal tissue.

#### DISCUSSION

The potential for milk-derived EGF to stimulate growth and differentiation of neonatal tissues depends upon the metabolism of EGF in the immature neonatal intestine. If EGF is to promote growth or maturation of infant tissues it must survive gastrointestinal digestion to reach the small intestine biologically intact. Furthermore, any direct actions on tissues other than the gastrointestinal mucosa would require transport from the gut lumen to the general circulation. In this study, the metabolism of dietary EGF was examined using the lamb as a model of the suckling human infant. The gastrointestinal metabolism of EGF in the lamb during suckling is likely to be similar to that in other mammals, including the human, because during this time the only source of nutrition is maternal milk. The lamb is a monogastric animal during the first 2-3 weeks of suckling, but later ingests solid vegetable matter, stimulating the development of a rumen, thereby altering its whole metabolic status. Since the human is a monogastric species, the age of lamb studied was restricted to the first 18 days of postnatal life.

The intestinal survival of gastrically-administered EGF was examined in studies of lambs aged 7-13 days. With the techniques employed in this study, it was demonstrated that

a minimum of 15-33% of intra-gastrically administered EGF reached the small intestine immunologically intact in suckling lambs. The fate of the remaining EGF is uncertain. One possibility is that 67-85% of the EGF was degraded during passage through the gastrointestinal tract, and the resulting  $^{125}\text{I}$ -labelled fragments absorbed at sites proximal to the intestinal catheter. However, if EGF was unstable in the lamb gastrointestinal tract, then the proportion of immunologically-intact radioactivity, or alternatively the total amount of radioactivity reaching the intestinal catheter would be expected to decrease with time. In view of the finding that both the total amount of radioactivity and the percentage representing immunoreactive EGF remained essentially constant for the 3 hour observation period, it appears unlikely that EGF is degraded rapidly in the lamb gastrointestinal tract. Rather, it is possible that at least some of the unaccounted  $^{125}\text{I}$ -hEGF remained intact, either in the stomach or in the proximal intestine, thereby preventing its quantitation. Considering that the unaccounted EGF was assumed to be totally degraded for the purposes of calculating the intestinal survival, it is likely that the estimated 15-33% represents a considerable underestimation. Accordingly, the percentage of immunologically intact EGF in intestinal samples (70-90%) may in fact be a close reflection of the gastrointestinal survival of EGF.

To determine whether radioactivity bound to either fragments or intact EGF is absorbed proximal to the small intestinal catheter, the simultaneous infusion of a non-absorbable marker such as polyethylene glycol with EGF should be done in future experiments, in order to assess the



luminal dilution of  $^{125}\text{I}$  (Westrom et al., 1984). Further studies are also required to determine the degree to which immunological activity reflects biological activity. Attempts were made to assess the biological activity of the intestinal samples by measuring receptor binding activity (results not given), but since the samples caused unacceptable interference in the receptor assay this was not possible using the current radioreceptor assay technique.

Although further studies will be needed to verify the structural integrity of ingested EGF, it appears likely that a large proportion of orally-derived EGF survives digestion in the neonatal gastrointestinal tract, a conclusion supported by results obtained by Thornburg et al in studies with suckling rats (Koldovsky et al., 1984; Thornburg et al., 1984). After  $^{125}\text{I}$ -labelled EGF was administered per os to sucklings 30-55% of the radioactivity detected in the gastric or intestinal wall and lumina was capable of binding to cell-surface EGF receptors. Other evidence for the intestinal survival of EGF has been obtained from studies involving oral administration. Cohen and Taylor (1974) reported that oral EGF could induce precocious eyelid opening and incisor eruption in newborn mice.

Once having estimated that a significant proportion of orally administered EGF may survive intestinal digestion to reach the small intestine in a biologically active form, the intestinal absorption of EGF was quantitated using the autoperfused intestine model (Windmueller and Spaeth, 1977 and 1984). This technique allows the simultaneous determination of the rates of absorption of proteins, or indeed any other compounds, into both blood and lymph under near physiological conditions. Although the animal must be

maintained under anaesthetic, other conditions closely resemble the physiological situation. Thus, surgical intervention is relatively minor compared to many other techniques, while heart rate, blood pressure, temperature, blood and lymph flow and the rates of absorption of luminal proteins remain stable for long periods.

In this study, IgG was not detected in the blood or lymph draining the intestinal segment. However, since such relatively low amounts of each sample of lymph or blood could be added to the IgG assays it is possible that undetectable amounts of IgG were present in either blood or lymph samples. Indeed, evidence for the absorption of IgG in neonatal animals has been reported by other investigators. IgG has been measured in the peripheral blood of lambs during the first 24-48 hours of life, a time when the lamb intestine has been reported to be relatively permeable to large milk proteins. After this time "intestinal closure" is reported to occur, and intestinal permeability to large proteins is found to be very much reduced (Comline et al., 1951; Asplund et al., 1962; Leece and Morgan, 1962; Leece, 1973; Cabello and Levieux, 1981; Grongnet et al., 1986).

It has also been reported that IgG is absorbed into efferent lymph but not into venous blood (Comline et al., 1951; Pierce and Johnson, 1960). In a study by Comline et al (1951) cannulae were introduced into the duodenum, thoracic duct and jugular vein of anaesthetized newborn calves. After duodenal administration of colostrum the lymph and blood were collected and analysed for the presence of colostral IgG. They reported that unchanged globulins of the colostrum did not enter the portal circulation but were

carried in the lymph to peripheral blood. Therefore, it is plausible that IgG may have been present in the lymph samples from animals aged 1-2 days but was undetectable in the low amounts of lymph sample assayed (5 $\mu$ l).

EGF was absorbed into blood, but not lymph, at rates that showed no dependency on the age of the lamb during the first 4-5 days of postnatal life, but varied considerably between animals. An increase in EGF uptake is suggested from day 4-5 to day 16-18, although due to the large variability in absorption rates between animals it is not possible to determine if this trend is accurate. However, in preliminary studies in which older animals were included, the 17 day-old lamb also showed an increased rate of uptake than younger animals but the inferred trend did not continue with increasing age. While the recent study by Murray et al., (1987) examines the absorption characteristics of single amino acids in the neonatal bovine intestine using the autoperfused intestinal method, little information is available from the literature on the absorption of small proteins in the neonatal intestine. No previous studies have examined the intestinal absorption of proteins in newborn animals using a technique, such as the autoperfused intestine, that can quantitate absorption simultaneously into blood and lymph. Most previous studies have either used techniques such as in vitro tissue segments or perfusions, or have involved peripheral blood sampling following gastrointestinal administration of proteins. None of these techniques provide adequate, quantitative information, particularly for proteins such as EGF that are cleared rapidly from the general circulation (Elder et al., 1978a).

The limited information available from other studies appears to confirm our finding that a small protein such as EGF would be transmitted into blood, rather than lymph. Warshaw et al (1971) used intact adult rats with chronic intestinal lymph and portal vein catheters to show that horse-radish peroxidase, a protein of molecular weight 40,000 D, is absorbed into both lymph and portal blood. While they were unable to quantitate the absorption into blood, it appeared that a substantial proportion of the protein entered the circulation via the venous route. Similarly, Balfour and Comline (1959) observed uptake of orally administered albumin (40,000 Da) into both the blood and lymph of calves. Absorption of insulin, a protein of similar size to EGF, into portal blood has also been demonstrated using an in vitro, vascularly-perfused, adult rabbit intestine (Shichiri et al., 1973).

The large variability observed between individuals in rates of uptake of EGF is also consistent with other studies on the intestinal absorption of proteins. Cabello and Levieux (1981) reported marked differences between lambs of the same postnatal age in the rates of appearance of colostral IgG into the circulation, while in the above-mentioned study of Warshaw et al., (1971), horseradish peroxidase infused directly into the small intestine, was absorbed at highly variable rates in adult rats. Many factors are thought to influence the intestinal absorption of macromolecules including the total luminal protein concentration, the mucosal surface area, mucosal mucus barrier and the unstirred water layer which is dependent upon the degree of peristalsis of the intestinal segment (Bjarnason et al., 1984). In addition, Murray et al., (1987)

observed that fluctuations in venous blood flow affected the absorption rate of single amino acids across the intestinal wall. Therefore, it is not surprising that such a variability in EGF uptake exists between individual animals as seen in this study.

Small proteins such as EGF or insulin are known to be absorbed to some extent in animals of all ages, including the adult rat (Danforth and Moore, 1959; Pierce et al., 1964; Thornburg et al., 1984), and the present study has demonstrated for the first time that in the lamb the rate of absorption of EGF is not dependent on the animal's age, at least until 18 days of age. Whether the permeability characteristics of the lamb intestine change 18 days postnatally has yet to be determined. Possibly the major factor limiting intestinal absorption of small proteins in adult animals is not the permeability of the intestinal mucosa, but rather the efficiency of luminal degradation in the gastrointestinal tract. The newborn lamb has an immature gastrointestinal system that permits survival of intact protein. Indeed, the preliminary findings in this study suggest that 70-90% of  $^{125}\text{I}$ -labelled EGF, administered intragastrically to lambs, reaches the small intestine intact.

The concentration of EGF in venous blood was determined by means of a radioimmunoassay using a polyclonal antiserum. The level of immunoreactive EGF in venous blood may represent an overestimation of the actual concentration of the biologically active growth factor, since the ability of EGF to bind to cell surface receptors is lost with relatively small changes in EGF structure (Thornburg et al., 1984), whilst immunoreactivity with a polyclonal antiserum

is less affected. Thornburg (1984) found that EGF detected in the intestinal contents of a rat following oral administration showed minimal loss of immunoreactivity even though considerable degradation had occurred as shown by loss of receptor binding activity. It was not possible to analyse the plasma samples arising from the present study by receptor assay techniques since the sensitivity of the radioreceptor assay (1-2 ng) was too low to detect EGF in the samples, and the addition of concentrated plasma samples to the assay caused significant interference. The use of a receptor assay in the analysis of the integrity of absorbed EGF would necessitate the infusion of a labelled species of EGF rather than native unlabelled EGF. Since modifications to EGF through radioactive or enzyme labelling procedures may affect the intestinal absorption or produce artifacts in the results, unlabelled EGF was chosen as the infusate for the present study.

The concentrations of EGF in venous blood reported here differ somewhat from those obtained in preliminary experiments (Read et al., 1986). The mean concentration of EGF in venous blood averaged from each experiment in the present study was approximately 0.12 ng/ml  $\pm$ SEM (0.02nM), whereas preliminary results indicated a venous concentration of 1.98 ng/ml. This ten-fold discrepancy is probably due to the different assays used in each study. The EGF fluid phase radioimmunoassay employed to analyse the plasma samples from the preliminary studies was a relatively insensitive assay. Fifty per cent of labelled EGF was displaced when 0.5 ng of EGF was added to the assay. A fluid-phase radioimmunoassay with a sensitivity 5 fold greater was subsequently developed for use in the present

study. Re-assay of the preliminary experimental samples using the more sensitive assay indicated that the preliminary data quoted was an overestimate of the EGF concentration in venous blood samples. However, since long-term storage of EGF present in the biological samples may have been detrimental to the integrity and therefore immunoreactivity of EGF, accurate determination of EGF concentration in these samples was not considered possible. Consequently, the preliminary data was not included in the present study. However, the route of absorption and the overall pattern of EGF absorption with respect to age was similar for both studies.

From the perfusion experiments illustrated in Figure 8.10, the concentration of immunoreactive EGF in venous blood averaged 0.02nM. The amount of EGF infused (50 $\mu$ g) represented a saturating concentration since the rate of absorption into blood plateaued in 5-15 minutes, and over 50% of the infused dose was recovered intact in luminal contents at the end of the experiment. Thus, it would appear that a portal vein concentration of 0.02 nM reflects the maximum rate of absorption of EGF across the lamb small intestine. If these results can be extrapolated to the human infant, it is unlikely that a portal vein concentration of 0.02 nM EGF could ever be achieved following suckling, given that the concentration of EGF in human breast milk is 1000-fold lower than the concentration in the infusate used in the studies. After dilution with luminal contents the concentration of EGF in the segment after infusion was generally 3 $\mu$ g/ml, whereas the concentration of EGF in the lumen following breast feeding would be approximately 9-18 ng/ml, given a concentration in

breast milk is 30-60 ng/ml (Read et al, 1984), and assuming that a minimum of 30% of ingested EGF reaches the small intestine intact.

Even if a portal concentration of 0.02 nM could be attained under physiological conditions in a suckling infant, mitogenic responses would presumably be small (Read et al, 1984), given the known sensitivities of cultured cells from various tissue origins. Furthermore, the actual concentration of EGF reaching most potential target organs, such as the lung, would be considerably less than 0.02 nM. EGF is shown here to be exclusively absorbed into the portal circulation and therefore must pass through the liver, a known site of EGF clearance (Burwen et al., 1984). Burwen demonstrated in the rat that 95% of EGF in the portal circulation was taken up by hepatocytes, transported to lysosomes and degraded. Given that: (1) a concentration 0.02 nM EGF in portal blood is likely to represent the maximum achievable concentration; (2) that it represents immunoreactive but not necessarily biologically active EGF; (3) that any EGF absorbed in portal blood initially passes through the liver before finally entering the general circulation, and finally (4) the maximal concentration of 0.02 nM is likely to elicit a minimal mitogenic response, it is unlikely that dietary EGF would significantly influence the growth and maturation of peripheral neonatal tissues, with the possible exception of the liver.

Some evidence from other studies indicates that a proportion of orally-derived EGF reaches the peripheral tissues. Thornburg et al (1984) detected immunoreactive EGF in the lung of newborn suckling rats following oral administration of labelled EGF. Nevertheless, the amount



of intact EGF was not quantitated in these studies and therefore the levels may have been consistent with results of this study. In other studies, the reduced amount of EGF in milk in sialoadenectomized mothers was shown to cause delayed eyelid opening in newborn mice (Okamoto and Oka, 1985). One interpretation of these findings is that while orally-derived EGF may cross the gastrointestinal barrier in physiologically active amounts, it is not possible to attribute these effects directly to milk EGF.

As mentioned previously, the rate of absorption of EGF from the gut lumen into the portal circulation does not appear to be related to the age of the lamb. The lack of age dependency of intestinal absorption of EGF suggests that a similar, low rate of uptake of EGF may occur in the intestine of the adult. Nevertheless, since it has been shown that oral administration of EGF does not appear to elicit any biological response in the adult (Oka et al., 1983), there must be developmental changes occurring to account for this. Possibly a lowered intestinal survival, rather than changes in intestinal absorption capacity. In the neonatal animal, the immature luminal digestive systems together with the presence of protease inhibitors from breast milk (Laskowski and Laskowski, 1951) would favour the survival of dietary EGF. These conclusions are supported by the work of Mosinger et al., 1959 and Warshaw et al., 1971. Mosinger et al demonstrated that insulin administration per os to infant but not adult rats caused hypoglycaemia. However, when insulin was introduced directly into loops of small intestine in adult rats blood glucose levels dropped (Warshaw et al., 1971).

From the data presented here on the survival of

gastrically-administered EGF in the neonatal small intestine, it is plausible that EGF may exert a trophic effect on the gut by topical action. Since human breast milk contains an EGF concentration of 30-60 ng/ml (Read et al., 1984), and assuming that a minimum of 30% of ingested EGF reaches the small intestine intact, a minimum concentration of 10 ng/ml may be present in the neonatal gut lumen following breast feeding. This concentration of EGF would produce substantial mitogenic responses in cultured cells (Burwen et al., 1984). The interaction of EGF with specific receptors on the surface of epithelial intestinal cells has been demonstrated by several researchers (Chabot et al., 1982; Gallo-Payet and Hugon, 1985). Indeed, functional EGF receptors have been extensively characterized from rat intestinal epithelial cells (Blay and Brown, 1985a and 1985b), and recently it has shown that human mammary secretions stimulate the proliferation of these intestinal cells in culture (Corps and Brown, 1987). In addition, it has been shown that EGF is necessary for the normal growth and maturation of the fetal rabbit gastrointestinal tract (Mulvihill et al., 1986). Therefore, it appears likely that luminal EGF derived from milk is able to exert direct topical mitogenic effects on the neonatal gut mucosa.

It is also conceivable that luminal EGF could induce other mitogenic responses indirectly by stimulating the release of gastrointestinal peptides such as gastrin and enteroglucagon, which in turn could enhance the growth and maturation of various neonatal tissues in addition to the gut mucosa (Gleeson et al., 1971; Johnson, L.R., 1976; Johnson, L.R., 1977). Lucas et al., (1981) demonstrated that 6-day-old breast-fed and bottle-fed infants differ

markedly not only in their basal circulating concentrations of several gut peptides (gastric inhibitory peptide and neurotensin) but also in their dynamic hormonal responses to feeding, notably with respect to the release of insulin, motilin, neurotensin, enteroglucagon and pancreatic polypeptide. While further studies are needed to assess the possible influence of nutrition on physiological development, it would appear likely that several gut peptides are involved in the development of the neonatal gastrointestinal tract in human neonates.

While the above studies suggest that luminal EGF can exert direct trophic effects on the gastrointestinal tract, other recent preliminary studies have provided evidence that EGF must reach the basolateral surface of enterocytes to exert effects (Scheving et al., 1987). If so, the observed in vivo effects of EGF on the growth of the intestinal mucosa suggest that EGF is absorbed intact from the gut lumen, at least into the interstitial spaces, or it is transported intact to the basolateral membrane receptors. Nevertheless, the present study indicates that little EGF absorbed in this manner is able to reach the general circulation either via the blood or lymph.

In summary, the results of the present study suggest that a large proportion of milk-derived EGF survives intestinal digestion in the suckling neonate, of which only a small fraction is subsequently absorbed. While it is unlikely that dietary EGF influences the growth and development of peripheral neonatal tissues, trophic effects on the gut mucosa by luminal dietary EGF are implicated.

Table 8.1. Estimation of the minimum percentage of immunologically intact  $^{125}\text{I}$ -EGF reaching the small intestine after intra-gastric infusion.

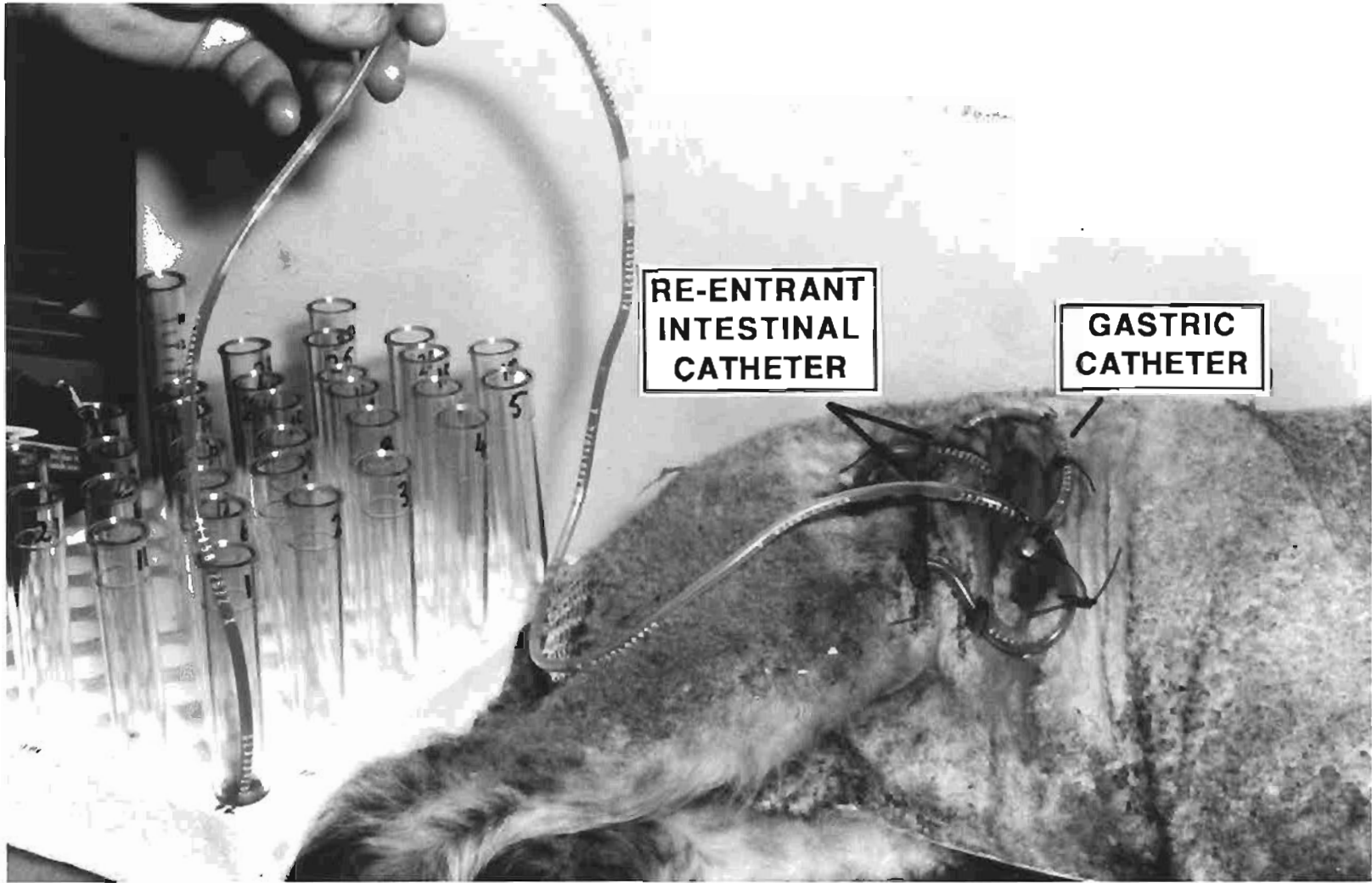
	A	B	C	D	E	F
Lamb	Infused cpm	Residual cpm in stomach	Total cpm recovered in S.I.	% recovery of $^{125}\text{I}$ in S.I. samples	% intact EGF in S.I. samples	Est. min. % intact EGF in S.I.
11 day	$1.2 \times 10^6$	$2.4 \times 10^5$	$1.6 \times 10^5$	17	88	15
13 day	$2.0 \times 10^6$	n.d.	$9.8 \times 10^5$	49	67	33

Table 8.2. Physiological parameters measured during auto-perfusion of the lamb intestine.

Blood flow	5.27 ± 0.5	ml/min/gdw
Lymph flow	0.071 ± 0.018	ml/min/gdw
Blood pressure: peak systolic	65.3 ± 3.3	mm Hg
peak diastolic	43.7 ± 3.4	mm Hg
mean arterial	50.9 ± 3.3	mm Hg
Heart rate	200 ± 15	beats/min
Rectal temperature	39.1 ± 0.24	°C
Intestinal segment temperature	38.6 ± 0.22	°C

Values represent mean ± SEM for 14 lambs, varying in age between 1-18 days, and represent the average for the entire experimental period (30-60 min). Blood and lymph flow represent the flow through the intestinal venous and lymph catheters, respectively; gdw = g dry weight intestinal segment. Mean arterial pressure was calculated by: Peak systolic pressure + 1/3 (peak systolic pressure - peak diastolic pressure).

Figure 8.1. Photograph showing the exterior portions of the gastric and re-entrant intestinal catheters in the lamb. In the photograph the re-entrant intestinal catheter has been disconnected for the purposes of collecting the entire intestinal contents following intra-gastric administration of  $^{125}\text{I}$ -labelled EGF.



RE-ENTRANT  
INTESTINAL  
CATHETER

GASTRIC  
CATHETER

Figure 8.2. Schematic diagram showing the auto-perfused lamb intestine, allowing for in situ perfusion of a segment of small intestine in the newborn lamb.



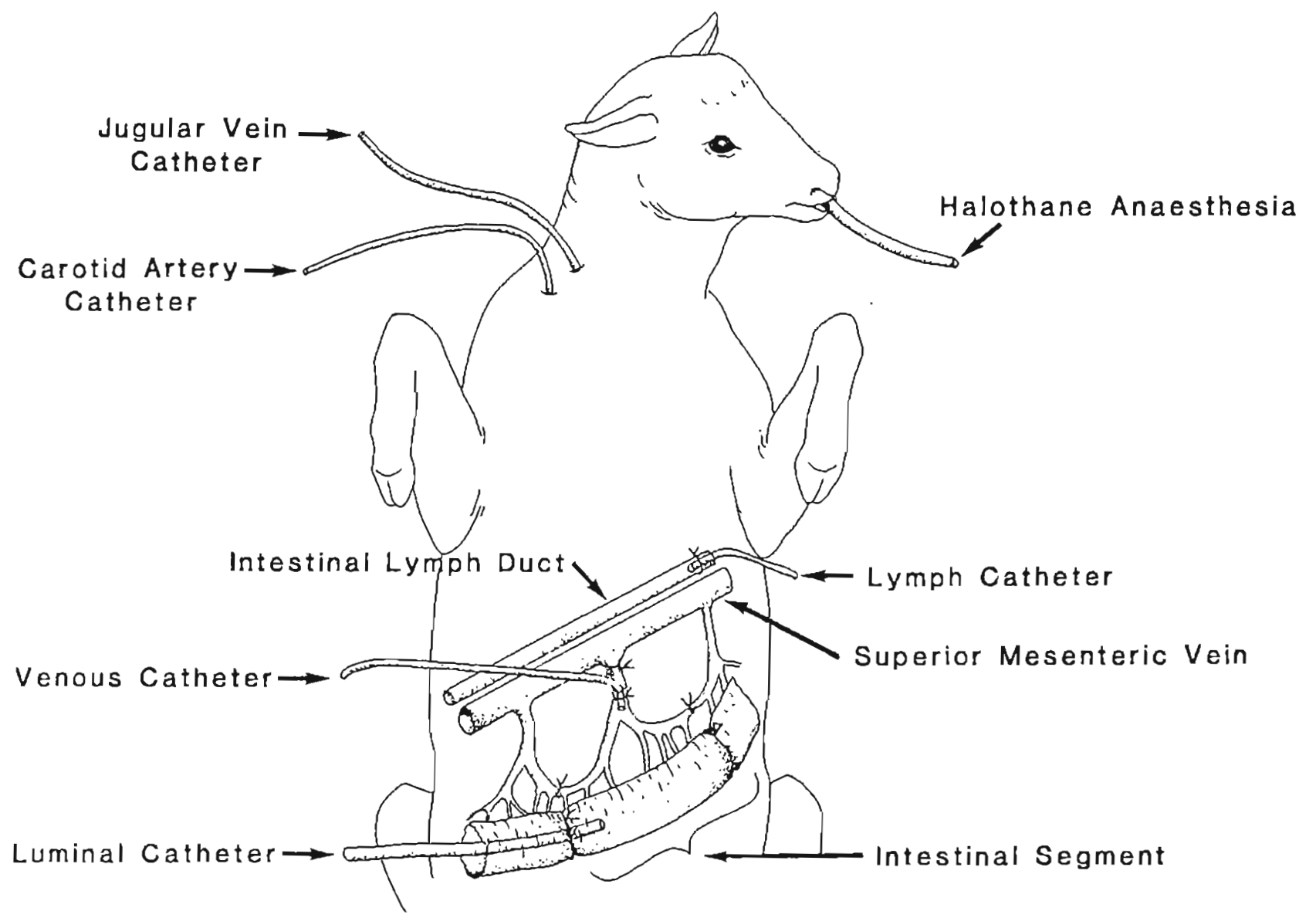


Figure 8.3. Time-course of the percentage of immunologically intact  $^{125}\text{I}$ -labelled EGF reaching the small intestine following intragastric infusion. Data are given for 3 individual lambs of the ages indicated.

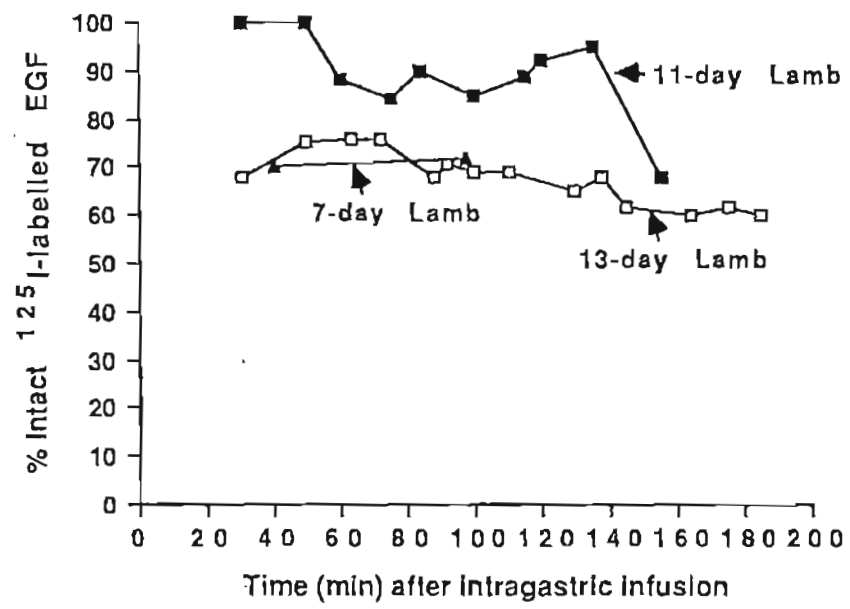


Figure 8.4. Time-course of physiological parameters during a typical auto-perfusion experiment, showing heart rate, peak systolic pressure (PSP), peak diastolic pressure (PDP), venous blood flow and lymph flow from the perfused segment.

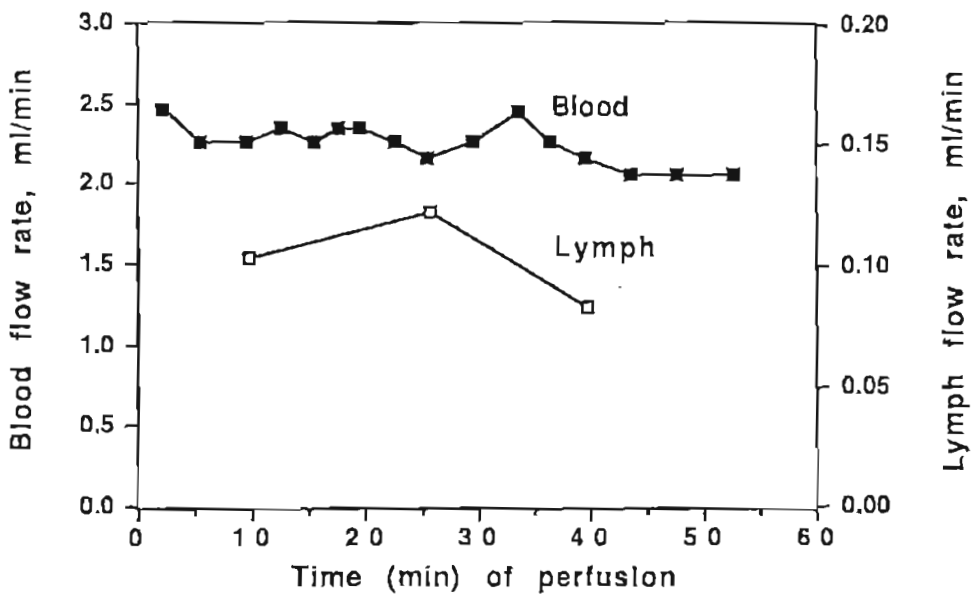
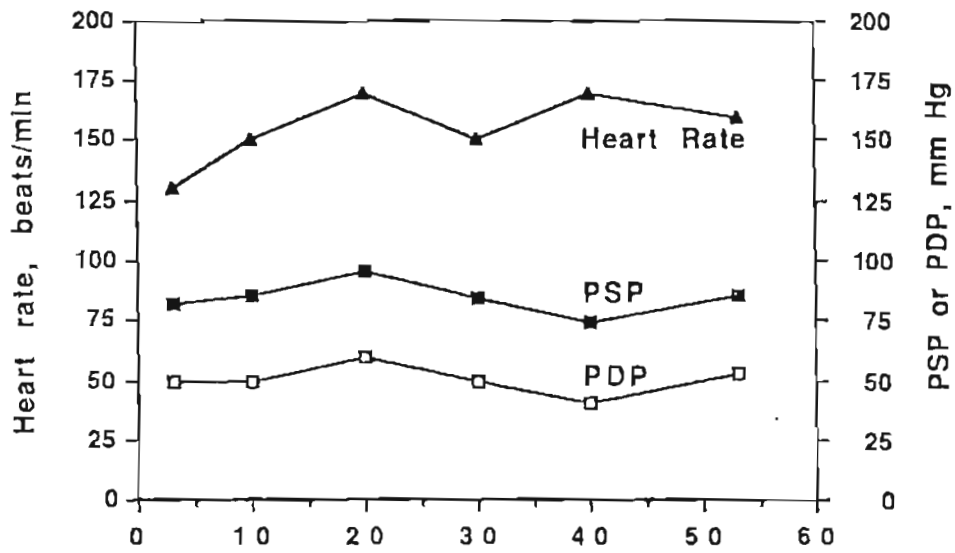
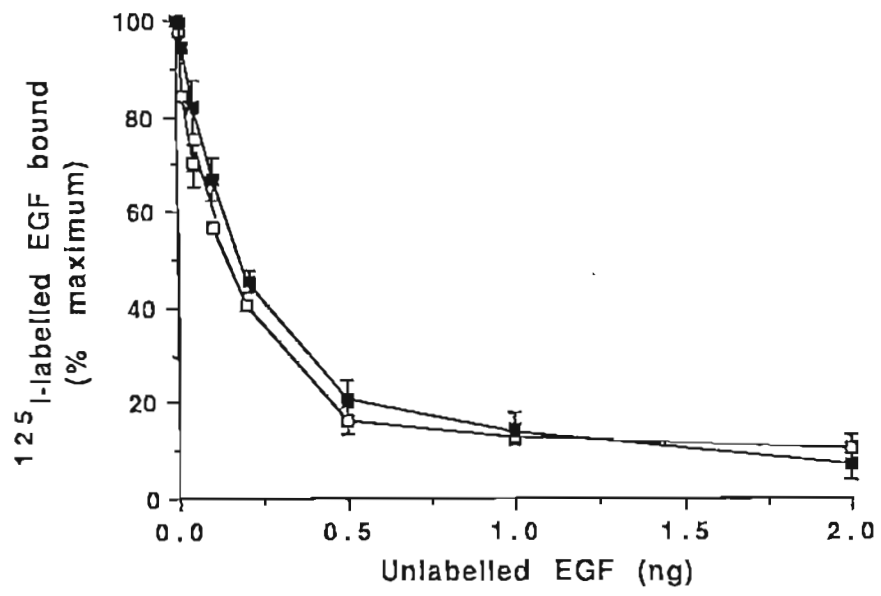
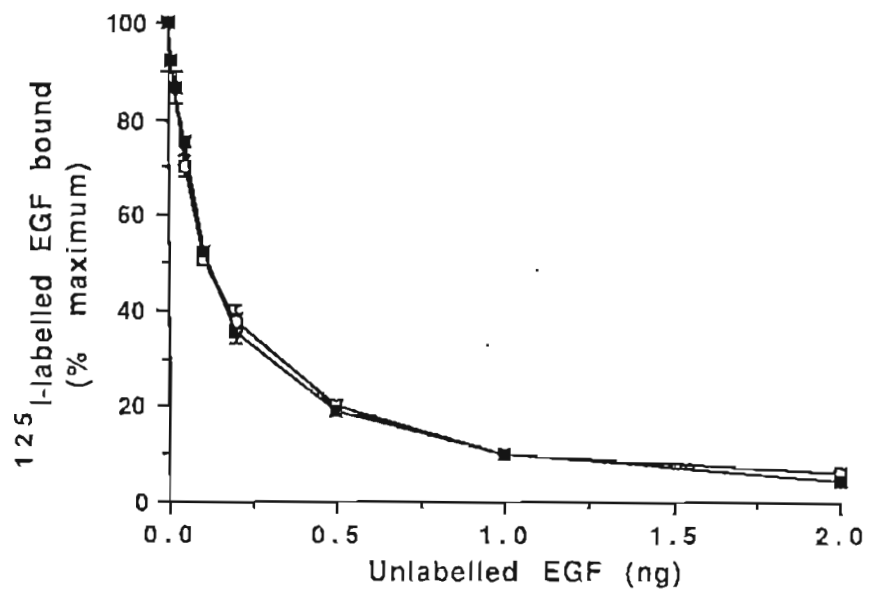


Figure 8.5. EGF fluid-phase radioimmunoassay. Labelled EGF bound to antiserum is expressed as a % of the amount bound in the absence of unlabelled EGF (maximum). The % binding is plotted as a function of the ng unlabelled EGF added to each tube, in the absence (open squares) and presence (closed squares) of 50 $\mu$ l of four-fold concentrated control plasma. Values represent the mean  $\pm$ SEM of three replicates.

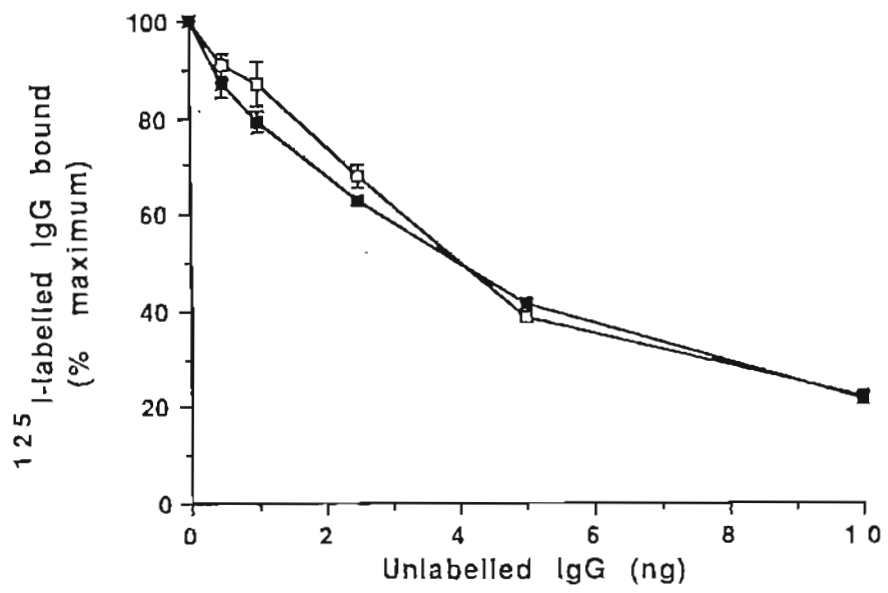


**Figure 8.6. EGF solid-phase radioimmunoassay.** Labelled EGF bound to antiserum is expressed as a % of the amount bound in the absence of unlabelled EGF (maximum). The % binding is plotted as a function of the ng unlabelled EGF added to each well, in the absence (open squares) and presence (closed squares) of 50 $\mu$ l of control lymph. Values represent the mean  $\pm$ SEM of three replicates.

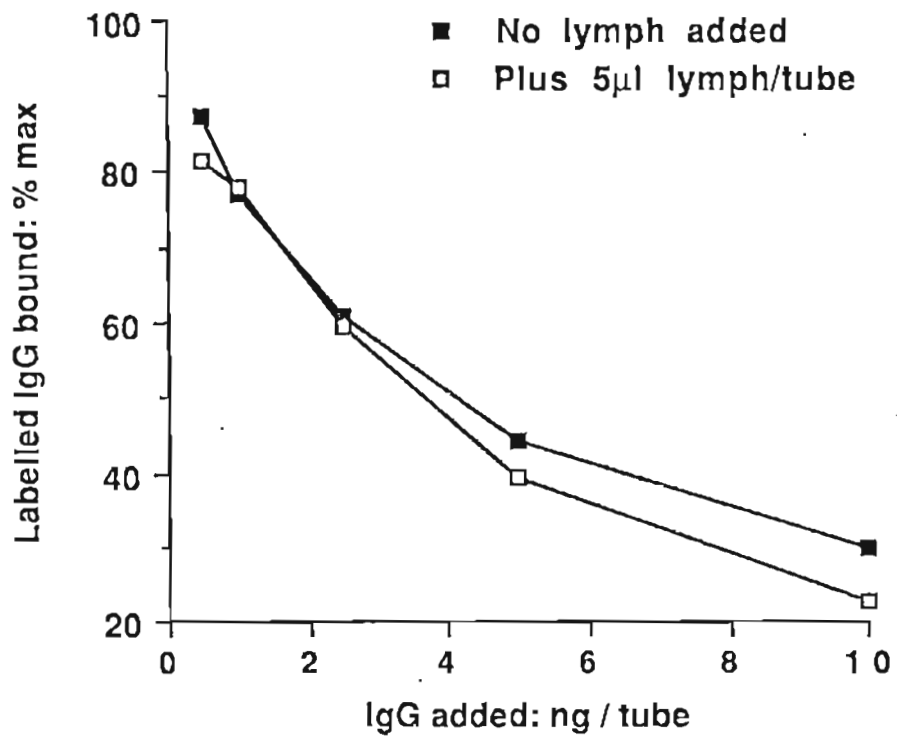




**Figure 8.7. IgG fluid-phase radioimmunoassay.** Labelled IgG bound to antiserum is expressed as a % of the amount bound in the absence of unlabelled IgG (maximum). The % binding is plotted as a function of the ng unlabelled IgG added to each tube, in the absence (open squares) and presence (closed squares) of 50 $\mu$ l control plasma. Values represent the mean  $\pm$ SEM of three replicates.



**Figure 8.8.** IgG fluid-phase radioimmunoassay for IgG detection in lymph. Labelled IgG bound to antiserum is expressed as a % of the amount bound in the absence of unlabelled IgG (maximum). The % binding is plotted as a function of the ng unlabelled IgG added to each tube, in the absence (closed squares) or presence (open squares) of 5 $\mu$ l of control lymph. Values represent the mean of three replicates.



**Figure 8.9. Auto-perfused lamb intestinal segment.**

Representative experiment (4 day-old lamb) showing time-course of intestinal blood flow (at top) and the rates of EGF (open squares) and IgG (closed squares) absorption (at bottom).

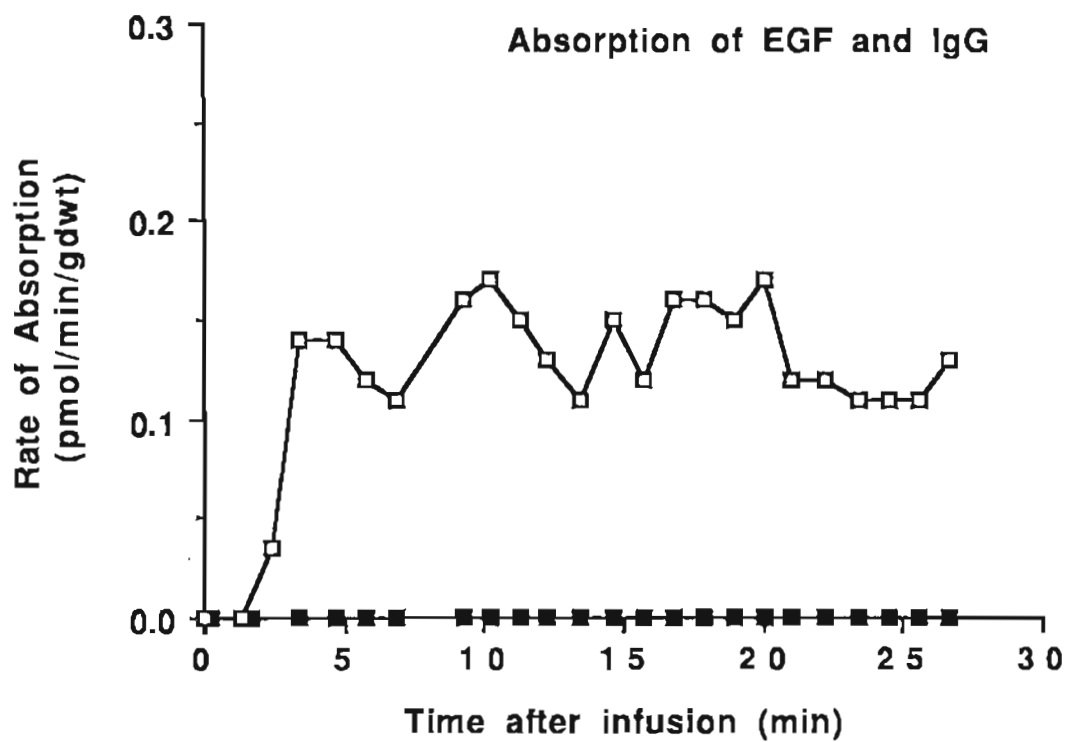
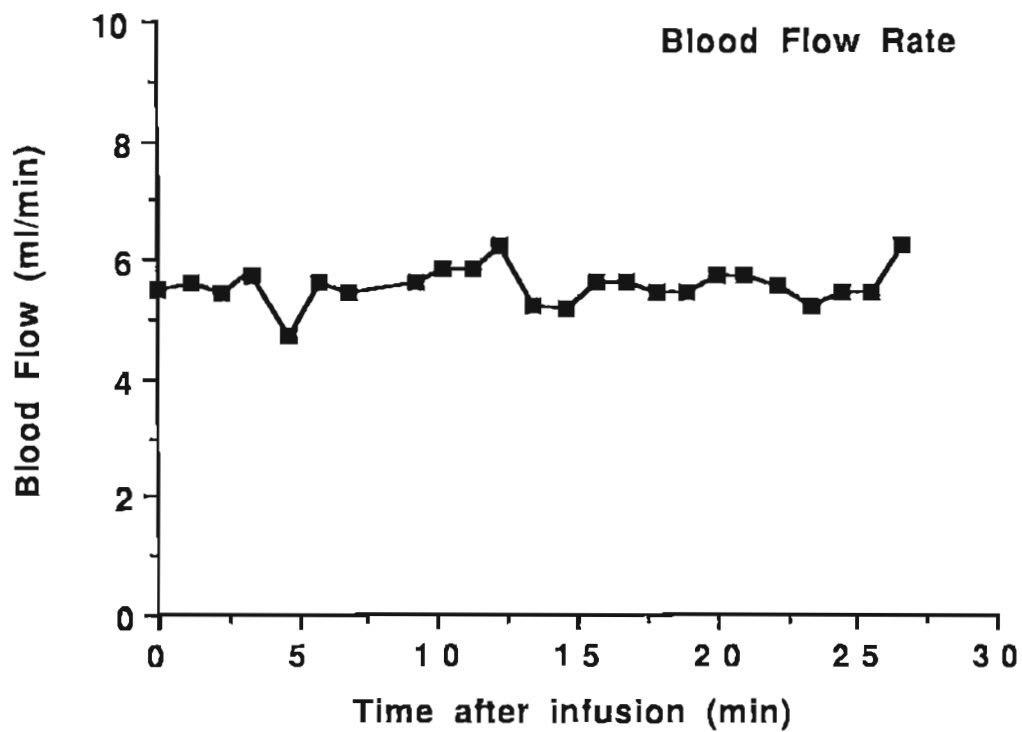
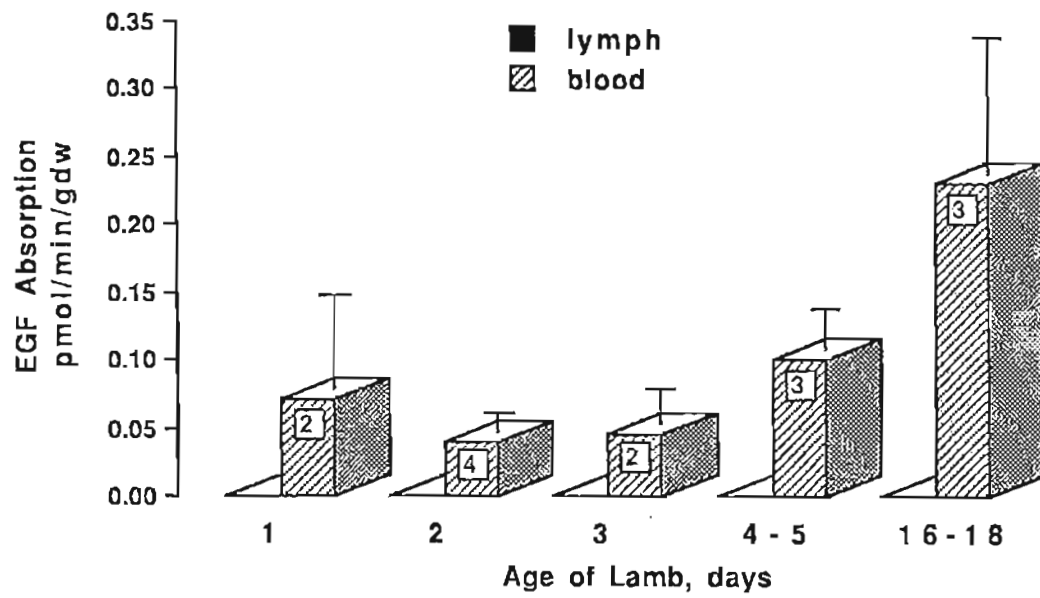


Figure 8.10. Average rates of intestinal absorption of EGF into blood and lymph in lambs of 1-18 days of age. Each value represents the mean  $\pm$ SEM for the number of animals in each age group. The numbers of animals representing each age group are given inside the columns.





CHAPTER NINE

CONCLUSIONS.

The physiological roles of milk growth factors are uncertain, although it would appear likely that the functions of growth factors in milk relate either to the development of the suckling infant and/or the maintenance of the maternal breast during lactation. In the case of EGF, a role in the growth and development of the human infant may be inferred firstly because human breast milk contains relatively high concentrations of EGF, secondly, the suckling human infant has a relatively low endogenous production of this growth factor, and thirdly, EGF has been shown to be a potent growth-promoting agent for crucial neonatal tissues such as the gut and the lung.

The extent of a direct mitogenic effect of milk-derived EGF in the suckling neonate depends upon four major factors. Firstly, if milk-derived EGF is to act directly to promote growth and differentiation of infant tissues, it must initially survive gastrointestinal digestion. Secondly, if milk-derived EGF is to reach neonatal tissues other than the luminal gut mucosa it must be absorbed across the intestinal wall into either the portal circulation or lymphatic system in biologically active amounts. Thirdly, if EGF is absorbed into the portal circulation, the amount reaching the general circulation will depend upon hepatic clearance of EGF, and finally, the rate of EGF clearance from the general circulation will influence the concentration of milk-derived EGF in peripheral blood reaching tissues such as the lung. Thus, the effectiveness of milk-derived EGF as a growth-promoting agent in neonates cannot be adequately assessed unless quantitative information on these four factors is known. As outlined in Chapter Two, both the intestinal survival and absorption were examined in this thesis in a

quantitative manner since only qualitative studies have been reported previously for this topic.

Studies investigating the biological roles of milk growth factors have been hindered in the past by the lack of adequate supplies of purified growth factors, a problem that has recently been overcome by the use of recombinant techniques allowing the production of potentially unlimited supplies of purified growth factors for research purposes. Recombinant human EGF has now been synthesized in *E coli* bacterial cells (Smith et al., 1982) and in yeast cells (Urdea et al., 1983; Brake et al., 1984), cells that have been transformed with plasmids containing the chemically synthesized gene for human EGF. The use of yeast-derived hEGF in biological studies was considered appropriate in view of the findings that recombinant hEGF was indistinguishable from the growth factor purified from human milk and urine, on the basis of immunological, receptor binding and biological potency. The in vivo studies described in this thesis involved the infusion of recombinant hEGF into lambs. A potential problem with this approach could arise if hEGF was not as active in other species. However, given the equipotency of mouse and human EGF in both receptor binding and biological potency in human fibroblasts, it appears that hEGF would be equally active in any heterologous species. Moreover, the use of a different species for in vivo studies has distinct advantages, namely that antibodies prepared against hEGF are unlikely to react with heterologous EGF. It was determined that the antibodies prepared against recombinant hEGF, both polyclonal and monoclonal, did not bind mouse EGF. While reactivity to sheep EGF cannot be ruled out, past studies

have shown that other antisera to EGF are highly species specific (Dailey et al., 1978). In support of this, it was shown in the present studies that sheep plasma showed no activity in hEGF radioimmunoassays. Human EGF could therefore be quantitated without interference from endogenous EGF in the lamb.

Studies investigating the survival of gastrically-administered EGF in the neonatal lamb intestine showed that a minimum of 15-33% of administered EGF reached the small intestine immunologically intact, as assessed by the capacity to bind to a specific polyclonal antiserum. It would have also been informative to assess receptor binding activity in view of the known correlation between receptor binding and biological response to EGF (Heath and Merrifield, 1986). Thus, receptor binding is more likely to reflect biologically intact EGF than is immunological activity which may include inactive fragments. Receptor binding activity was not assessed because the samples of digesta caused unacceptable interference in the radioreceptor assay. In future studies, it may be possible to overcome these problems through the use of formaldehyde-fixed cells or placental membranes as a source of EGF receptors. However, these assays may still be subject to interference by some biological samples. An alternative approach would be the use of a range of monoclonal antibodies directed against different sites of the EGF molecule. In this regard, three of the monoclonal antibodies produced in this thesis were found to be directed to the N-terminal portion of the hEGF molecule while another two antibodies recognized amino acid residues thought to be involved with the formation of the receptor binding region

(Komoriya et al., 1984). Whilst the biological potency of EGF surviving digestion in the neonatal lamb intestine was not tested, the results of the present work are supported by previous studies. Thornberg and colleagues (1987) found that while 56% of the radioactivity reaching the rodent small intestine represented immunologically intact EGF, 44% of the radioactivity was able to bind to specific EGF receptors.

If these results can be extrapolated to the suckling human infant, the concentration of immunologically intact EGF in the gut lumen following breast feeding would be 9-18 ng/ml, assuming a minimum of 30% of ingested EGF reaches the small intestine intact and the concentration of EGF in breast milk is 30-60 ng/ml (Read et al., 1984). Since an EGF concentration of 9-18 ng/ml is sufficient to promote mitogenic responses in cultured cells (Burwen et al., 1984), milk-derived EGF may well exert trophic effects on the gut mucosa following breast feeding in human infants. Moreover, a role for milk-derived EGF in the neonatal human gastrointestinal tract is consistent with some clinical findings. While adequate gastrointestinal development is apparent in most infants fed artificial diets low in EGF content, the incidence of various gastrointestinal diseases is greater amongst formula-fed than amongst breast-fed infants (Myers et al., 1984). Therefore, whilst exogenous EGF may not represent an essential requirement for gastrointestinal development, there are indications for a role in maintenance and repair of the neonatal gut. Trophic effects of milk-derived EGF could probably occur through a topical action, in view of the in vitro studies demonstrating that enterocytes are EGF responsive (Beaulieu and Calvert, 1981;

Al-Nafussi and Wright, 1982) and possess specific EGF receptors on the cell surface (Blay and Brown, 1985a and 1985b).

It would be of interest in future studies to determine whether dietary EGF reaches more distal sections of the gut to determine whether any trophic effects of milk EGF are confined to the upper gastrointestinal tract or are more generalized, including the ileum and the colon. Such studies could be achieved by extending the current studies to include the placement of re-entrant catheters at various sites along the intestine distal to the mid-small intestine.

Given that a substantial proportion of milk-derived EGF would appear to reach the small intestine intact and the available evidence strongly suggests a topical action to stimulate mucosal growth, the question arises as to the extent of absorption of luminal EGF. In studies of intestinal absorption of EGF in lambs, significant absorption of intestinal EGF was observed, the EGF entering the portal rather than the lymphatic circulation. Nonetheless, the rate of absorption was found to be quite low. Following the introduction of 50  $\mu\text{g}$  EGF into the lumen of a segment of the small intestine, the concentration of EGF measured in the intestinal vein was 0.02 nM. Given that the concentration of EGF in breast milk is at least 1000-fold lower than the concentration in the infusate used in these studies, it is unlikely that a portal vein concentration of 0.02 nM could ever be attained following suckling. Moreover, even if a portal concentration of 0.02 nM could be attained under physiological conditions in the suckling infant it would elicit only a minimal mitogenic response (Read et al., 1984), given the known sensitivities

of cultured cells from various tissue origins. Furthermore, the actual concentration of EGF reaching potential peripheral organs would be considerably less than 0.02 nM since EGF is absorbed exclusively into the portal circulation and therefore must pass through the liver, a known site of EGF clearance (Burwen et al., 1984), before reaching the general circulation. Therefore, it is unlikely that milk-derived EGF would directly influence the growth and maturation of peripheral neonatal tissues other than the liver. It should also be noted that a radio-immunoassay, rather than a radioreceptor assay was used to measure concentrations of EGF in venous blood. For reasons discussed above, the radioimmunoassay is likely to overestimate rather than underestimate the concentration of intact EGF. Again, additional studies using the range of monoclonal antibodies directed at different regions of the EGF molecule would assist in elucidation of the structural integrity of absorbed EGF.

While the rates of EGF absorption in the auto-perfused lamb intestine suggest that milk-derived EGF contributes little to the circulating levels in the newborn, the possibility remains that the gut of the human infant is more permeable to EGF than is the lamb intestine. This may be especially so in the premature infant in which gastrointestinal development is very immature. Indeed, the results of the human study (Chapter 7) were found to be consistent with the hypothesis that EGF crosses the gastrointestinal wall to enter the general circulation. Premature infants receiving higher levels of EGF in their diet showed greater urinary EGF excretion after the second postnatal week. While this finding could also be explained



by an indirect effect of some breast milk component on rates of endogenous EGF synthesis, they are nonetheless consistent with the absorption of intact EGF across the neonatal intestine.

To address the question of species differences in intestinal permeability, it would be pertinent to extend the auto-perfused intestine studies to include another species such as the piglet, given that any one experimental animal model is not likely to reflect entirely the physiological situation in the human. For example, while EGF has been detected in sheep tissues and biological responses to EGF have been observed in sheep (Thorburn et al., 1981a; Waters et al., 1982), in this species, the concentration of EGF in milk appears to be very much less than that measured in human milk. As an alternative species, the piglet would appear the model of choice, in view of the apparent similarity in gastrointestinal structure and function to that in the human (Dodds, 1982). Whilst the piglet is a more difficult animal to work with, both in terms of handling and intestinal anatomy, auto-perfusions in the intestine are feasible in this species, as determined recently by Dr. Leanna Read (unpublished results). Moreover, the concentration of EGF in pig milk is greater than that measured in sheep milk (Read et al., 1984), more closely resembling the human situation.

The intestinal absorption of dietary proteins has been shown to be directly related to the maturity of the intestine (Henning, 1981). While the rate of intestinal absorption of EGF was not found to be dependent upon the age of the lamb, at least until 18 days of age, it is possible that greater absorption of EGF and other proteins may occur

in prematurely-delivered animals. Indeed the intestine of the premature human infant is known to be particularly undeveloped compared with term infants and thus may be more permeable to dietary EGF. Future studies investigating intestinal EGF absorption in premature animals would provide the required evidence for this hypothesis.

To conclude, the studies using the lamb as an experimental animal model of the human infant provide strong evidence that a substantial proportion of milk-derived EGF would reach the small intestine intact. Whilst some intestinal absorption does occur, the amount is low, and this combined with the fact that EGF is transferred exclusively into portal blood and therefore passes through a known EGF clearance organ, the liver, suggests that a very small proportion of milk-derived EGF would reach peripheral tissues. Therefore, the direct growth-promoting actions of milk-derived EGF in the suckling human infant are likely to be confined to the gastrointestinal tract and possibly the liver. Any actions in other tissues are more likely to be mediated by secondary responses to the actions of EGF in the gut and the liver.

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