IgA NEPHROPATHY AND LIVER DISEASE

A thesis presented to the University of Adelaide
for the degree of
DOCTOR OF PHILOSOPHY

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SUMMARY

IgA nephropathy, the most common renal biopsy diagnosis in Australia, is characterised pathologically by diffuse mesangial deposits of IgA and C_3. The finding of IgA and C_3 in extramesangial sites such as the skin, together with the recurrence of disease in renal grafts, has lent support to the concept of an immune complex aetiology for IgA nephropathy. A morphologically similar condition often is found secondary to alcoholic liver disease.

The liver occupies a central role in the portal clearance of antigens, circulating immune complexes and IgA polymers, so that mesangial IgA deposition secondary to alcoholic liver disease may reflect defects in this role. This hypothesis would be supported by the identification of A_2 subclass, secretory component binding and IgA polymers in the mesangial deposits, since these features are characteristic of enteric IgA.

The data presented here are derived from the examination of material from patients with alcoholic liver disease and from rats with cirrhosis induced by carbon tetrachloride.

i) The introductory chapter describes the immunochemistry of immunoglobulin A, the clinicopathological features of primary IgA nephropathy, mesangial IgA deposition secondary to alcoholic liver disease, and the development and manipulation of an animal model.

ii) Mesangial IgA deposition in alcoholic liver disease: an autopsy study

This autopsy study showed a significant association between alcoholic liver disease and mesangial IgA deposition, and provided renal tissue for further study. Choroid plexus, skin and liver, was also available for study by immunofluorescence. While extrarenal tissues from subjects with alcoholic liver disease showed a greater incidence of IgA staining than that seen in controls, there was no statistical correlation between the presence of mesangial or extramesangial deposits.

iii) Immunochemical characterisation of mesangial IgA deposits

Mesangial IgA subclasses were sought in patients with deposits secondary to alcoholic liver disease, compared with those with primary IgA nephropathy and other conditions having mesangial IgA deposition. A_2 was not more common in patients with alcoholic liver disease, and secretory component could not be demonstrated in any IgA deposits. The capacity to bind purified secretory component, however, was almost exclusive to the mesangial deposits in alcoholic liver disease, suggesting that these differed from those in the primary deposits by being polymeric.

iv) The immune complex mediation of mesangial IgA deposits in alcoholic liver disease

The mesangial IgA deposits were eluted by acid citrate buffer from glomerular preparations of nine kidneys from patients with ALD. This technique was shown to reduce

both IgA and C_3 staining intensity in frozen sections, indicating immune complex dissociation. The eluates were tested for antibody specificity to food antigens, auto-antigens and bacterial flora. Two eluates showed anti-bovine serum albumin activity in a liquid phase radioimmune assay, seven demonstrated anti-Mallory body activity in an immunofluorescent system, and five had anti-bacterial activity in an enzyme linked immuno-sorbent assay. In addition, serum from patients with alcoholic liver disease was tested in a solid phase Cl_q assay and a high incidence of circulating immune complexes was demonstrated. These data support an IC origin for the mesangial deposits and have identified some of the antigenic stimuli involved.

v) <u>A rat model of mesangial IgA deposition in CCl_4 induced cirrhosis</u>

An animal model of mesangial IgA deposition in rats was developed using subcutaneous CCl_4 injections. Body weight, serum urea, creatinine and immunoglobulin levels were monitored and the rats killed after three months. Those treated with CCl_4 developed mesangial deposits of IgA, IgG and C_3. Renal failure did not occur but there was cirrhosis and progressive hyperglobulinaemia was confirmed. In cirrhotic rats, IgA and C_3 could be eluted from kidney sections by treatment with citrate buffer. This finding indicated that, as in the human, the renal deposits were immune complexes.

vi) Therapeutic manipulation of the model

Several therapeutic agents used in humans have been shown to affect the parameters thought to be important in the pathogenesis of mesangial IgA deposition. Phenytoin lowers serum IgA levels; neomycin alters gut flora; d-penicillamine lowers circulating immune complex levels; dapsone is effective in dermatitis herpetiformis (a disease characterised by IgA and C_3 deposits); and prostaglandins have been shown to protect animals against immune complex nephritis. Although serum IgA levels could be lowered by penicillamine, only 15-(S)-15-methyl prostaglandin E_1 reduced mesangial IgA deposition. At the doses used, 15-(S)-15-methyl prostaglandin E_1 also protected the liver against damage caused by carbon tetrachloride.

The conclusions to be drawn from each segment of this study are discussed in the relevant chapters, and in a final discussion chapter.

DECLARATION

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 and belief, the thesis contains no material previously published or written by another person, except when due reference is made in the text of the thesis.

SIGNED:

JANE LOMAX-SMITH

CANDIDATE: ......Jane Lomax-Smith......

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

DATE: ..16/7/86..... **SIGNATURE:**

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ABBREVIATIONS

ALD	alcoholic liver disease
BSA	bovine serum albumin
B-cell	bursa-equivalent derived lymphocyte
C	complement component
CCl_4	carbon tetrachloride
CIC	circulating immune complex
Con A	concanavalin A
cpm	counts per minute
DDD	dense deposit disease (a form of glomerulonephritis)
DIF	direct immunofluorescence
DH	dermatitis herpetiformis
DNP	dinitrophenyl
ELISA	enzyme linked immunosorbant assay
EM	electron microscope
Fab	antigen-binding fragment of immunoglobulin molecule
Fc	constant fragment of immunoglobulin molecule
FITC	fluoroscein isothiocyanate
g	relative centrifugal force
g	gram
GN	glomerulonephritis
H&E	haematoxylin and eosin
HBV	hepatitis B virus
HLA	human leucocyte antigen
HSA	human serum albumin
HSP	Henoch Schönlein purpura
^{125}I	iodine-125
IC	immune complex
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IF	immunofluorescence
IIF	indirect immunofluorescence
ip	intraperitoneal
iv	intravenous
J chain	joining chain
kg	kilogram
MCGN	mesangiocapillary glomerulonephritis
mCi	millicurrie
µg	microgram
mg	milligram
ml	millilitre
MW	molecular weight
NHS	normal human serum
NZB/W	New Zealand Black/White (mouse)
nM	nanometre
NMS	normal mouse serum
OKT	Ortho-Kung T cell
PBL	peripheral blood
PB(S)	phosphate buffer (saline)
PG	prostaglandin
pIgA	polymeric IgA

PIGN	postinfectious glomerulonephritis
PWM	pokeweed mitogen
RIA	radioimmune assay
RID	radial immune diffusion
S	Svedberg unit of sedimentation coefficient
sIgA	secretory IgA
SC	secretory component
sc	subcutaneous
SLE	systemic lupus erythematosus
T cell	thymus-derived lymphocyte
TNP	trinitrophenyl
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

DILUTIONS

In this thesis, dilutions of fluids are described as "one in" abbreviated to "1:" with a colon. This avoids the use of dilution ratios such as one to 1279. Ratios, when discussed, are introduced as such in the text.

CORRIGENDA

Chapter 1
p.9,paragraph 3, "in the perfused rat liver" to read "from the rat circulation"
p.23,paragraph 1, "difference in SRBC haemolysis" to read "difference in haemolysin titres".

Chapter 4
p.148,paragraph 3, "S typhimurium" to read "S minnesota"
p.150 ,Table 4.8, "S enteroides" to read "S enteritidis"
p.326 Bacteroides to be listed amongst Gram-negative organisms

Some of the people who made the preparation of this thesis such an enjoyable experience. Left to right: Hans Schoppe, Dale Caville, Lorraine Zabrowarny, Gordon Howarth, Dianne Broughton, Sonia Priadko, Alma Bennett and Anna Langen-Zueff.

CHAPTER 1

IgA nephropathy and liver disease: an introduction

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CONTENTS

1 THE IMMUNOCHEMISTRY OF IMMUNOGLOBULIN A (IgA)

1.1 A mucosal antibody

1.2 The transport of IgA across epithelia

1.3 The role of IgA in mucosal protection

1.4 Hepatobiliary transport of IgA

2 PRIMARY IgA NEPHROPATHY

2.1 Clinical presentation and epidemiology

2.2 The proposed aetiology of primary IgA nephropathy

3 MESANGIAL IgA DEPOSITION SECONDARY TO ALCOHOLIC LIVER DISEASE

3.1 The association

3.2 The proposed aetiology of secondary mesangial IgA deposition

3.3 Characterisation and specificity of the mesangial IgA deposits

3.4 The investigation and use of human tissues in this thesis

3.5 The investigation and use of an animal model of mesangial IgA deposition in this thesis

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1 THE IMMUNOCHEMISTRY OF IgA

1.1 A mucosal antibody

IgA is distributed widely within the animal kingdom being found in birds, rodents and humans. In all species studied, it represents a relatively small proportion of circulating globulins, comprising 2-3 percent (%) of human serum protein while IgG accounts for 10%. In contrast, however, IgA represents a comparatively large proportion of exocrine secretions such as saliva or colostrum (Tomasi and Zigelbaum 1963). Brandtzaeg et al (1970) showed that the ratio of the concentration of IgA:IgG in pure secretions such as colostrum was 400-500 times higher than the ratio in serum. This suggests that such glands actively or selectively secrete IgA into exocrine fluids.

The basic monomeric unit of IgA has a molecular weight (MW) of 162,000 daltons and a sedimentation coefficient of 6.9 Svedberg units (S) (Tomasi et al 1965). In addition, there are a variety of polymeric forms having sedimentation coefficients up to 18 S. Density gradient ultracentrifugation has shown that in man, 90% of serum IgA is monomeric whilst that found in saliva or colostrum is almost entirely polymeric (Tomasi et al 1965). This would suggest that IgA polymers have a greater propensity for secretion or transport into exocrine fluids.

In common with all immunoglobulin classes, IgA consists of either kappa or lambda light chains and class specific heavy chains. The specific alpha heavy chains themselves exist in two forms, corresponding to two IgA subclasses recognised by

characteristic differences in antigenic determinants (Vaerman and Heremans 1966). The two subclasses, termed IgA_1 and IgA_2, exhibit differences in both amino acid sequence and stereoscopic configuration. The IgA_2 subclass is resistant to certain IgA specific bacterial hydrolases probably because of a fortuitous deletion of the target amino acid sequence (Plaut et al 1974). In addition, IgA_2, except for rare allotypes, lacks the disulphide bonds which link other immunoglobulin heavy and light chains (Grey et al 1968).

The percentage of IgA comprising A_2 subclass is up to two times higher in secretions - such as saliva, intestinal fluid, milk, tears, bronchial fluid or bile - than in serum (Grey et al 1968; Delacroix et al 1982a). The cells producing IgA subclasses can be identified in the circulation and in tissues using immunofluorescent microscopy. Approximately 20% of adult IgA-bearing, circulating and nodal plasma cells contain cytoplasmic A_2 (Conley et al 1980a) compared with 35% of mucosal or glandular IgA-bearing plasma cells (André et al 1978a). A_2 subclass, although more common in secretions, does not form the major proportion of polymeric IgA. IgA_2 comprises 35% of biliary polymers compared with 25% of biliary monomers (Delacroix et al 1982a).

Serum IgA is predominantly produced by the bone marrow (Kutteh et al 1982a). Circulating IgA-bearing plasma cells secrete up to 60% polymers (Kutteh et al 1982a) and after pokeweed mitogen (PWM) stimulation exhibit approximately equal subclass expression suggesting that they may represent migrating mucosal-type

plasma cells (Kutteh et al 1980).

In common with IgM, polymers of IgA contain an additional polypeptide chain first described in rabbit and human colostrum by Halpern and Koshland (1970). This "joining" polypeptide, the J chain, functions as a structural bridge in the polymerisation of monomers (reviewed by Koshland 1975). Its MW is approximately 15,000 daltons and it is bound to the alpha chain by disulphide bonds. Splitting these disulphide bonds in polymers, results in the release of J chain which may be identified by its characteristic fast mobility in gel electrophoresis in urea at alkaline pH.

1.2 The transport of IgA across epithelia

The marked polymeric IgA concentration gradient observed between serum and exocrine fluids, implies that there is an active and specific transport mechanism across such epithelia. Like IgA, IgM is a polymeric J-chain containing immunoglobulin, and there is an IgM:IgG ratio some 50 times lower in serum than in secretions (Brandtzaeg et al 1970). Even so, the concentration of IgM in secretions is only one-twentieth that of IgA (Brandtzaeg et al 1970). In reptiles and amphibians, IgM performs a similar mucosal protective role to that of IgA in phylogenetically more advanced animals (Portis and Coe 1975). Furthermore, in humans with IgA deficiency there may be partial compensation by enhanced mucosal synthesis of IgM (Crabbe and Heremans 1966; Ammann and Hong 1971) so that J-chain containing polymeric immunoglobulins may share some functional roles. In secretions, these immunoglobulins have been found to be associated with an

additional immunochemically distinct subunit (Tomasi et al 1965). South et al (1966) suggested that this protein, which they called "transport piece" and found both free and associated with secreted IgA, was in some way instrumental in transepithelial transport. Now termed "secretory component" (SC), this protein of MW 85,000 daltons, has been shown *in vitro* to bind non-covalently to J-chain containing polymeric immunoglobulins but not to their corresponding monomers (Mach 1970; Rádl et al 1971). Normal human saliva and colostrum contain almost as much free as bound SC (Brandtzaeg 1971). IgM is less firmly bound than IgA, to SC, with coupling relying upon an excess of SC (Brandtzaeg 1974a). Rádl et al (1971) have further suggested that IgM-SC binding may be limited to larger polymers (25-30 S), although Brandtzaeg (1974a) has found even 19 S pentamers to bind free SC.

Brandtzaeg (1974b) has demonstrated immunohistochemically the production by glandular epithelial cells of SC which becomes a membrane-bound receptor for the J-chain containing polymers. His work suggests that polymer is bound non-covalently to the membrane-associated SC receptor, absorbed into the cell by pinocytosis and subsequently secreted onto the external or luminal surface. The IgA-SC complexes, termed secretory IgA (sIgA), separate from the mucosal surface and are stable in exocrine secretions with 90% of the dimers retaining their union with SC (Mestecky et al 1970).

1.3 The role of sIgA in mucosal protection

The high concentration of IgA in enteric secretions is thought to confer a unique functional role in mucosal protection. This appears to be dependent upon specific immunisation allowing IgA to fix substances in the mucosal coat with subsequent antigenic breakdown (Walker and Isselbacher 1974). Dietary macromolecules (Udall et al 1981) and endotoxins (Ravin et al 1960) have been shown to be capable of crossing the intestinal barrier. Infants, in particular, absorb food antigens which can be demonstrated in the circulation after feeding and be shown to decrease in concentration as the child grows (Udall et al 1981).

Both *in vitro* studies, using inverted gut sacs in the rat (Cornell et al 1971; Walker et al 1972), and *in vivo* studies of mesenteric lymph fistulae (Warshaw et al 1971), have shown that horseradish peroxidase, an exogenous macromolecule, is taken-up by pinocytosis into the enterocyte, whence it is transported via the lamina propria to the efferent lymphatics. Oral, and to a lesser extent, parenteral immunisation reduces subsequent *in vitro* mucosal absorption of antigens by inverted gut sacs (Walker et al 1972; Walker et al 1973). Pre-immunisation of rats reduces the *in vivo* absorption into the circulation of radiolabelled bovine serum albumin (BSA) (Pang et al 1981). Furthermore, IgA obtained from immunised animals and infused into inverted gut sacs, can be shown to prevent the passage of antigen to the systemic surface (André et al 1974). Failure of this system is exemplified by the increased incidence of atopy, particularly in relation to food antigens in infants with IgA deficiency. In such patients, there is an increased incidence

of milk protein precipitins (Buckley and Dees 1969) and, following milk feeds, a high incidence of circulating immune complexes (CIC) (Cunningham-Rundles et al 1979). Sloper et al (1981) examined children with partial villous atrophy and found that those with eczema had significantly fewer IgA plasma cells in their jejunal biopsies than those without, suggesting a less efficient immunological barrier. In this context, patients with primary IgA deficiency may develop chronic glomerulonephritis (GN) with immune deposits, CIC, and raised titres of serum antibodies to BSA (Kwitko et al 1979).

Normal intestinal IgA function is required for mucosal immunity. Antibacterial activity at mucosal sites must progress largely independent of complement and leucocyte function. It has been suggested that at luminal sites, bacterial colonisation is dependent upon surface adherence which can be prevented by sIgA (Gibbons 1974). Williams and Gibbons (1972) have shown that surface adherence of streptococcal species to isolated epithelial cells, can be inhibited by prior incubation of the bacteria with purified sIgA. Inhibition of adherence correlates precisely with species specific agglutination. Thus, it would appear that mucosal IgA functions in at least two ways: first, it prevents intestinal surface adherence by bacteria, thus reducing both colonisation and the quantity of free antigen available for mucosal transport; and second, it ensures that antigens, bacterial or otherwise, escaping this control are complexed to facilitate clearance by the liver from the portal blood.

1.4 Hepatobiliary transport of IgA

Patients with cirrhosis have hyperglobulinaemia, predominantly of the IgA class (Jori et al 1977). The increased serum IgA represents a seven to nine fold rise in dimers but only a two to four fold rise in monomers (André and André 1976). Although there is evidence that sIgA is transported across the enterocytes, the liver has been shown to play an important role in enteric IgA transport.

The majority of investigations concerning IgA in association with the liver have been carried out in the rat which has far more IgA in bile than in serum (Lemaître-Coelho et al 1977). A key role for the liver in IgA metabolism was suggested by the 15 fold elevation in serum IgA level which follows within 24 hours of bile duct ligation in the rat (Lemaître-Coelho et al 1978). This rise comprised sIgA and was associated with free SC.

The role of the liver in biliary IgA transport has been investigated in a series of experiments in both mice and rats using intravenously injected monoclonal IgA from a mouse myeloma (Jackson et al 1977a, 1977b, 1978). MOPC 315 myeloma IgA was shown to be rapidly cleared from the circulation of mice after intravenous injection (Jackson et al 1977b). There was rapid specific clearance of IgA into bile and intestinal fluids in association with SC (Jackson et al 1977a). These phenomena occurred against a concentration gradient and furthermore, in the perfused rat liver, 93% of the injected myeloma IgA was removed in only four hours (Jackson et al 1978). In the rat, ultrastructural autoradiographic studies of the liver after

intravenous injection of ^{125}I labelled polymeric IgA (pIgA) have shown membrane attachment, followed by transport across hepatocytes to bile ducts (Birbeck et al 1979). In monolayer cultures, similar studies show that pIgA uptake by hepatocytes was associated with membrane-bound vesicles (Jones et al 1982) and could be blocked by prior incubation with antiserum to SC (Orlans et al 1979). Studies of hepatocytes in cell culture have demonstrated production of both free and membrane-bound SC (Socken et al 1979). Using isolated rat liver perfusion systems, the transhepatic transport of dimeric IgA can be blocked by prior infusion with anti-SC antibody or sIgA (Fisher et al 1979). It would seem, therefore, that hepatobiliary transport of IgA in the rat is similar to that described via the enterocyte, and is SC dependent.

The mechanisms of hepatobiliary transport of IgA in man are somewhat controversial. Hopf et al (1978) have demonstrated the capacity of isolated human hepatocytes to bind IgA polymers, but not monomers, despite an apparent lack of membrane SC. Nagura et al (1981) reported immunohistochemical evidence of SC-mediated IgA transport by both intrahepatic and extrahepatic biliary epithelium but failed to demonstrate SC in or on hepatocytes. This confirmed earlier fluorescence work performed by Kater et al (1979) who, while looking for IgA deposits in normal livers and those from patients with alcoholic liver disease (ALD), observed SC staining only in the bile ducts. Notwithstanding the singular report by Hsu and Hsu (1980) of SC on 50% of isolated human hepatocytes, it would appear that there are fundamental differences between man and rat in

the hepatobiliary transport of IgA. First, unlike the situation in the rat where there is considerably more IgA in bile than in serum (Lemaître-Coelho et al 1977), humans, although demonstrating selective IgA transport into bile, never show a higher biliary than serum IgA concentration (Delacroix et al 1982b, reviewed by Vaerman et al 1978). Second, the rapid rate of clearance into bile of perfused IgA demonstrated in the rat (Jackson et al 1978) does not seem to occur in the normal human. Dooley et al (1982) showed that, in man, only 1% of an injected trace dose of radiolabelled dimeric myeloma IgA was cleared into bile in eight hours compared with around 30% in rats treated in parallel. Again in man, Delacroix et al (1982b) injected radio-labelled IgA monomers and dimers obtained from patients with alcoholic cirrhosis into normal patients and showed preferential transport of polymers, but with a maximum clearance of only 2.8% in 24 hours. Furthermore, when these authors compared the percentage of labelled IgA in serum and bile, results suggested that only about half of the biliary IgA was derived from the serum. Thus, it would seem that IgA polymers are secreted selectively into bile in both humans and rats. In humans, however, less biliary IgA is derived from the serum, implying local production by the liver - perhaps by peribiliary plasma cells.

Since protein fragments, macromolecules and other potentially antigenic substances are capable of crossing the gastro-intestinal barrier in normal animals, it has been postulated that hepatic secretion of IgA may act as a mechanism for the clearance of absorbed antigen in the form of complexes. Experi-

mentally, complexes may be formed *in vivo* by injection of a myeloma monoclonal antibody together with its specific antigen, haptenised to human serum albumin (HSA) or BSA. Mouse myeloma monoclonal antibodies of different classes with anti-dinitrophenyl (DNP) activity have been injected into mice (Russell et al 1981) and rats (Harmatz et al 1982). Only the IgA-myeloma complexes could be shown to be cleared rapidly into bile. Brown et al (1982) have further shown this IgA specific secretion to be independent of the mononuclear phagocytic system, whose blockade did not affect IgA-complex clearance. The same mouse myeloma, named MOPC 315, but with anti-trinitrophenyl (TNP) activity has further been used in rat liver perfusion experiments by Socken et al (1981) to demonstrate that this mechanism is SC dependent and may be blocked by prior incubation of the MOPC 315 with human SC. It would appear that the preferentially transported complexes have an antigen:antibody ratio of 1:2 (MW<460,000) and that larger complexes (MW≃970,000), although capable of binding SC, are cleared less effectively (Socken et al 1981). This may reflect the filtering effect of the venous sinusoidal pore size which, however, would still represent less of a hindrance to IgA diffusion than the blood-mucosa barrier in salivary or mammary organs. Russell et al (1982), using similar *in vitro* formed complexes, suggested that this may explain the lack of IC transport into the other external secretions that they examined.

The preferential biliary secretion of pIgA in humans, although less efficient than in rats, would serve two functions in mucosal immunity. IgA would be returned to the gastro-intestinal tract, re-inforcing intestinal immunity, and IgA antibody-antigen

complexes would be cleared from the circulation. This latter effect would protect the body from subsequent IC deposition and complement activation.

2 PRIMARY IgA NEPHROPATHY

2.1 Clinical presentation and epidemiology

Berger and Hinglais (1968) described a group of patients in whom mesangial IgA staining was the predominant immunofluorescence (IF) finding in renal biopsies. Reviewing 300 biopsies, these authors recorded IgA as the most prominent IF finding in eight cases of systemic lupus erythematosus (SLE) and 14 with Henoch-Schönlein purpura (HSP).

However, in a further 55 cases, IgA deposits were associated with isolated haematuria in young men who had no accompanying systemic syndrome. Since IgG was also present, the pathological features in this latter group were summarised by the name "nephritis with IgA-IgG deposits". Over the next decade the condition became eponymously linked with the name of Berger, and patients with the syndrome were described in London (Davis et al 1973), in the USA (Lowance et al 1973; McCoy et al 1974), in Japan (Shirai et al 1978), in Singapore (Sinniah et al 1976) and in Australia (Woodroffe et al 1975). Thus, a syndrome originally considered to be uniquely French became recognised throughout the world. Patients with Berger's disease may present with a wide variety of clinical manifestations (Clarkson et al 1977). Classically, they have recurrent macroscopic haematuria which is often synpharyngitic, but presentation may be at routine

examination with the detection of microscopic haematuria, proteinuria, or hypertension.

Renal biopsy usually shows diffuse mesangial proliferation with an increase in cells and matrix, and with segmental lesions that may range from proliferation to hyalinosis with sclerosis. The pathognomonic IF finding is of diffuse mesangial IgA and C_3 deposits which may be accompanied by IgG and IgM and, rarely $C1_q$ and C_4. Electron microscopy (EM) confirms the presence of mesangial and paramesangial electron dense deposits. Occasionally, these extend along the capillary basement membrane which is often "etched" on its epithelial surface (Spargo et al 1980). Now, the condition is known generally as "IgA nephropathy" and this term is used throughout this thesis to designate cases of isolated GN without clinical associations other than those described above.

Primary IgA nephropathy must be differentiated from other conditions associated with mesangial IgA deposits. One such example is SLE, an auto-immune disorder which affects predominantly females and is characterised by the LE-cell phenomenon (Hargraves et al 1948) and antinuclear antibodies in high titre (Zvaifler 1981). SLE is a widespread disease with diverse clinical features which may include involvement of the skin, joints, eyes and nervous system. Renal involvement is common and a wide range of morphological changes may be found in the GN of SLE. Although the light microscopic (LM) appearances in some patients are strikingly similar to those of IgA nephropathy, features of SLE such as wire loops, haematoxyphil bodies or a membranous

nephropathy facilitate differentiation. EM of glomeruli in SLE generally demonstrates extramesangial deposits which occasionally have a characteristic organised structure. The IF pattern in SLE differs from that in IgA nephropathy by showing, in addition to IgA, the presence of a plethora of other immunoglobulins and complement components, especially Cl_q which, as noted above, is seen rarely in IgA nephropathy.

Another condition with which mesangial IgA deposits may be associated is HSP. This disorder occurs most often in children particularly following an upper respiratory tract infection, and is recognised chiefly by its presentation as a systemic vasculitis involving skin, gut and joints. The renal morphology of HSP is often similar to that of IgA nephropathy but crescents are more frequent, IF often shows abundant fibrin, and EM may demonstrate capillary wall deposits.

Reports of the prevalence of IgA nephropathy in biopsy populations throughout the world have indicated a notable geographical variation. Studies in Singapore (Sinniah et al 1976), Australia (Clarkson et al 1977) and Japan (Shirai et al 1978) have shown prevalences of 20-40% in immunopathologically positive biopsy populations. These figures approach the 40% reported in France (Berger and Hinglais 1968) but exceed the 16% recorded in the USA (Lowance et al 1973) and 10% in the UK (Davis et al 1973). In comparing these figures, it should be borne in mind that the criteria used in selection of patients for renal biopsy vary considerably in different countries and centres. Thus apparent variations in the prevalence of IgA nephropathy merely could

reflect different policies, with more frequent biopsy of patients having abnormal urinary findings, as in some centres in Australia, or the more ready discovery of such abnormalities during conscription and routine screening, as in Singapore. Reports of associations between IgA nephropathy and the presence of human leucocyte antigen (HLA) BW 35 (Berthoux et al 1978; Noël et al 1978), DRW4 (Fauchet et al 1980) or B12 (Richman et al 1979) have been noted although disputed by some workers (Clarkson et al 1977; Mustonen et al 1981). Such associations may explain, in part, the recorded geographical variations in the incidence of IgA nephropathy but are highly contradictory with workers in the same country conceding a higher frequency of DR4 but suggesting a correlation between a benign (Hiki et al 1982) or aggressive course (Kashiwabara et al 1982).

2.2 The aetiology of IgA nephropathy

i An increase in circulating IgA

It has been suggested that IgA deposition in the kidney is related to excessive levels of circulating IgA (Egido et al 1980). In support of this, the majority of studies have reported an increase in circulating IgA levels in some (Zimmerman and Burkholder 1975; Finlayson et al 1975; Nomoto et al 1979; Woodroffe et al 1980; Lesavre et al 1982) or most (Lopez-Trascasa et al 1980) instances of IgA nephropathy. Nomoto et al (1979) have described a marked increase in IgA-producing peripheral blood lymphocytes (PBL) in each of 12 patients with primary IgA nephropathy and two-thirds of close family members (Sakai et al 1979a). The same authors subsequently

reported a decrease in IgA-specific suppressor T-cell activity (Sakai et al 1979b) and an increase in helper T cells with Fc receptors for IgA (T-α-cells) in such patients (Sakai et al 1982). The significance of this latter finding is uncertain as it may reflect merely a normal response to an increase in serum IgA, since an increase of a given immunoglobulin class is known to cause expansion of the T-cell population with the corresponding Fc receptor (Hoover et al 1981). In the mouse such Fcα receptor expression is induced by polymers rather than monomers (Yodoi et al 1983). Adachi et al (1983) have further shown that PBL from patients with IgA nephropathy are refractory to such induction despite a tendency to elevated native serum IgA levels. They suggest that this may be a result of maximal Fcα T-cell expression in such patients induced by a proportional elevation in serum pIgA. This would be supported by the observations of Egido et al (1982a) who noted that, in patients with IgA nephropathy, PWM stimulated PBL preferentially produced polymeric IgA. T-cell subpopulations can be identified by IF studies using monoclonal antibodies. In the Ortho Kung T-cell series (OKT), OKT4+ represents helper T cells and OKT8+, suppressor T cells (Reinherz et al 1980a, b). Chatenoud and Bach (1981) have reported an increased OKT4:8 T-cell subset ratio in five of nine patients with IgA nephropathy which suggests a defect in suppression; however, Egido et al (1983) have shown that the helper OKT4 cells are disproportionately elevated. These abnormalities in IgA production could be genetically determined and may thus be manifestations of the tissue typing studies described earlier.

ii The presence of CIC

There is a large body of opinion which supports the theory that IgA nephropathy is mediated by the mesangial deposition of IC from the circulation. The original work of Dixon et al (1961) in the rabbit showing the formation of BSA-anti-BSA complexes and their deposition in glomeruli produced a morphological model of IC disease. The features of human mesangial IgA disease with granular IF staining and electron dense deposits are similar to those seen in this animal model and suggest an IC aetiology. Support for this mechanism has come from the demonstration of CIC in 44% of patients with IgA deposition in the kidneys (Woodroffe et al 1980). Unfortunately, conventional IC assay systems are not suited to the measurement specifically of IgA class complexes. Most commonly used assays preferentially detect complexed IgG (McDougal et al 1982). The solid phase Cl_q ($SpCl_q$) assay, measures the binding of complexes containing Cl_q fixing immunoglobulin, by a radiolabelled antibody to human IgG. IgA-containing complexes would only register in such an assay if there were included IgG. Similarly, radiolabelled antibody to IgG is the endpoint used to quantitate binding by C_3 products in the bovine conglutinin and Raji, C_3 binding, B-cell assay. Modifications designed to detect IgA in CIC have had limited success. Woodroffe et al (1980) modified the $SpCl_q$ assay by the use of α heavy chain-specific antisera and found mixed immunoglobulin classes in more than half of the sera tested. In a similar group of patients, Lesavre et al (1982) used IgA class-specific Raji cell and bovine conglutinin assays and showed IgA containing IC in 66% and 39% of patients respectively. IgA class IC have been demonstrated also in HSP

by Levinsky and Barratt (1979) using a latex bead agglutination test dependent upon rabbit IgM antiserum to human IgA. This assay had a high background reactivity which could be avoided in a modified assay by polyethylene glycol precipitation of probable IgA polymers (Kauffman et al 1981). These modifications are time-consuming and not practised in routine laboratories.

The recognition of recurrent IgA deposition in kidneys transplanted into patients with IgA nephropathy (Berger et al 1975), and the finding in some patients of IgA and C_3 deposits in extrarenal sites, such as the skin (Baart de la Faille-Kuyper et al 1976; Thompson et al 1980) and vessels from striated muscle (Tomino et al 1981a) have provided further support for a circulating origin for the deposits. Further support for an IC aetiology in primary IgA nephropathy was afforded by an animal model of IC-mediated mesangial IgA deposition developed by Rifai et al (1979), which employed the injection of IgA myeloma protein complexed in vitro or in vivo with its specific antigen, DNP. Isaacs et al (1981) extended this approach to the induction in mice of active immunity to neutral dextran (MW 10,000-70,000). Intraperitoneal immunisation with dextran was followed by intravenous injection of antigen and after 10 weeks all mice showed granular IgA deposition in the mesangium.

The similarity of the human disease to such animal IgA-IC models, together with the high incidence of CIC in patients, the presence of extramesangial IgA deposits, and the recurrence of deposits in grafted kidneys, all support a CIC origin for IgA nephropathy.

iii The mesangial deposition of circulating IgA polymers

An alternative theory to explain the immune deposits occurring in IgA nephropathy was proposed by Sancho et al (1981) who suggested that aggregates form as a result of elevated concentrations of polymeric IgA in the circulation. In support of this theory, these authors have reported, in patients with IgA nephropathy, an increase in circulating polymers identified by ultracentrifugation, and detected J chain and SC affinity in the heavier fractions. Furthermore, they showed that the mesangial deposits contained J chain and were capable of binding free SC (Egido et al 1980; Lopez-Trascasa et al 1980). They have suggested that human IgA nephropathy may be analogous to the animal model of Rifai et al (1979), where IgA complexes only induced GN when formed from polymers. Tomino et al (1982a) were able to elute IgA from human renal biopsy tissue and found it to be polymeric by ultracentrifugation and absorption with antiserum to J chain. Other groups have not been able to demonstrate elevated levels of circulating IgA polymers in patients with IgA nephropathy (Woodroffe et al 1980; Lesavre et al 1982), and the matter remains unresolved. If IgA deposition merely were related to elevated serum levels of polymers one might expect to see more cases of mesangial IgA deposition amongst patients with IgA myelomas. Such reports are rare (Dosa et al 1980).

3 MESANGIAL IgA DEPOSITION SECONDARY TO ALD

3.1 The association

In a series of 10 cirrhotics undergoing portacaval shunt procedures, Callard et al (1975) found nine to have clinically unsuspected GN with mesangial IgA deposits. Subsequently, 26 of 34 patients with ALD and overt GN were shown to have varying degrees of segmental sclerosis and mesangial proliferation with IgA and C_3 deposition (Nochy et al 1976). In France, autopsy studies of cirrhotic patients have shown mesangial IgA deposits in approximately two-thirds of 100 cirrhotic patients, of whom 90% were alcoholics (Berger et al 1978). It has been established, therefore, that there is a significant association between alcohol-induced cirrhosis and mesangial IgA deposition, and this constitutes the most common form of secondary IgA nephropathy. There are several possible explanations for the occurrence of IgA deposition in this group of patients, and the mechanism may be multifactorial in each patient. Deposition of IgA in the mesangium could result from abnormal handling of gut-derived IgA, either alone or complexed, or an abnormal affinity of the mesangium for material normally present within the circulation.

3.2 Proposed aetiology of mesangial IgA deposition in alcoholic cirrhosis

i Defective mucosal barrier

Alcohol is known to increase gastro-intestinal absorption of macromolecules (Worthington et al 1978), and this increase in

mucosal permeability could produce hyperimmunisation. Patients with IgA nephropathy secondary to ALD have been shown to have abnormally high titres of serum anti-BSA antibody as well as antibody activity towards intestinal bacteria (Woodroffe et al 1980). Increased jejunal absorption could be the result of superficial mucosal erosions or may be related to defective intraluminal fixation of antigenic substances by IgA. The jejunal production of IgA can be assessed experimentally using intraluminal balloons to isolate bowel segments from bile. Studies of small intestinal secretion by this technique in decompensated alcoholic cirrhosis have shown a reduction in sIgA production but no alteration in passive transudation of monomers (Pelletier et al 1982). There is no evidence of a decreased number of intestinal mucosal plasma cells in alcoholics but a defect in SC-dependent active transport, described in other situations (Strober et al 1976), could occur. Such a defect could reduce intraluminal binding of antigens, with consequent absorption, complexing and entry into the circulation as CIC.

ii <u>Defective hepatic clearance</u>

The liver normally is responsible for clearing gut-derived antigens from portal blood. Reduction in this function, brought about by intrahepatic portacaval shunting in cirrhosis, is likely to impose a greater antigen load on the systemic circulation, possibly resulting in increased antibody production. This has been shown experimentally in the rat by Triger et al (1973) who demonstrated a greater humoral immune response to sheep red blood cells (SRBC) injected into the inferior vena cava than into the portal vein. Delayed hypersensitivity was also examined in

these animals by measuring the increase in ear thickness produced by SRBC challenge. Only those rats immunised repeatedly via the inferior vena cava responded. In the same animal model, carbon tetrachloride (CCl_4) abolished the difference in SRBC haemolysis between inferior vena cava and portal vein immunisation (Triger and Wright 1973).

Systemic loading of antigens may not be the sole mechanism whereby patients with liver destruction and portacaval shunts develop raised antibody titres and CIC. Vaerman et al (1981) have shown in rats that portacaval shunting, but not transposition, results in an 18-fold increase in serum IgA levels. The fact that this increase takes some three weeks to develop supports the postulate that the change is a result of reduced liver mass secondary to reduced blood flow and not to direct systemic loading of antigens. This implies that increased serum IgA levels occur only when hepatocyte SC-mediated clearance is reduced. Thus, shunting may be of importance only in conjunction with a reduced liver cell mass.

The liver is thought to clear polymeric IgA, and IgA class IC, by SC-dependent secretion through bile duct cells and, perhaps, the hepatocyte. In ALD, with extensive derangement of liver architecture, it is to be expected that these cellular mechanisms also may be deranged. Evidence for this derangement, however, currently is restricted to the finding of altered IF for IgA within the liver. Hopf et al (1978) examining isolated hepatocytes found half of normal cells to bear surface IgA compared with all of seven hepatocyte preparations from patients with

alcohol-induced cirrhosis. Kater et al (1979) demonstrated continuous surface hepatocyte IF staining reactions for IgA in alcoholics. This finding appears to be highly specific for ALD and has been demonstrated also in deparaffinised trypsinised sections (Swerdlow et al 1982a). This reaction may represent a specific antibody reaction or defective IgA handling with non-specific SC binding.

iii <u>Deposition of CIC</u>

There is much evidence to support an IC theory for the aetiology of all forms of IgA nephropathy. However, the evidence is more compelling in patients with IgA nephropathy secondary to alcoholic cirrhosis since cryoglobulins (Jori et al 1977) and detectable IC (Penner et al 1978; Gluud and Jans 1982) are usually present in addition to the typical glomerular morphological appearances associated with IC deposition. CIC have been detected frequently in patients with chronic or severe liver failure of any aetiology, and they have been implicated as mediators of continued liver damage (Canalese et al 1981).

Penner et al (1978), using a Raji cell assay to study serum from patients with ALD, found IgG-IC in none of the patients with steatosis, in 80% of patients with alcoholic hepatitis and 50% of those with cirrhosis. Using an IgA endpoint, seven of these 13 patients with ALD and IgG-IC also had IgA-IC. Using a complement consumption test, Gluud and Jans (1982) found CIC in 40% of patients with steatosis compared with 60% of those with alcoholic hepatitis or cirrhosis. Woodroffe et al (1980)

considered that CIC are almost always present in those patients with alcoholic cirrhosis who develop IgA nephropathy.

IgA-containing complexes are thought to arise as a result of a failure in normal IC clearance. This has been demonstrated in patients with alcoholic cirrhosis as a loss in the normal gradient in CIC concentration between portal and hepatic veins (Kaufman et al 1982). To be of IgA class such CIC might be expected to contain antigens which, having gained access to the portal system via a defective gastro-intestinal barrier, might then be associated with mucosal antibodies.

iv Defective control of IgA production by PBL

Hyperglobulinaemia in chronic liver disease, whilst due largely to hyperimmunisation and defective clearance, may in part be related to reduced suppressor-cell activity. In patients with ALD there is an increase in IgA production, but this is in the context of a generalised hyperglobulinaemia. Wands et al (1981) have demonstrated an increased *in vitro* IgG production by PBL from patients with ALD, despite normal responses to stimulation by PWM or suppression by concanavalin A (Con A). In contrast, this group showed that Con A suppressor-cell activity was reduced in patients with chronic active hepatitis, implying that alterations in ALD are not merely non-specific effects of liver damage. Holdstock et al (1982a) confirmed the normal PWM response in a heterogenous group of patients with chronic liver disease. Furthermore, they described a significant increase in IgG production by normal lymphocytes after exposure to serum from cirrhotic as compared with normal patients. This work suggested

that T-cell dependent control of B-cell function was not abnormal in chronic liver disease, but that the B-cell populations were being stimulated by non-specific serum factors capable of stimulating normal cells.

3.3 Characterisation and specificity of the mesangial deposits

The main sites of IgA production in the body are mucosal surfaces where active SC-dependent secretion of J-chain containing A_1 or A_2 polymers occurs. It is germane to the finding of IgA in the mesangium to question its origin, and the finding of a predominance of A_2 subclass, J chain or SC in mesangial deposits would suggest a mucosal origin. Thus far, subclass studies have been contradictory and dependent upon the specificity of reagents (Tomino et al 1981b). André et al (1980), using rabbit antisera, found A_2 in all forms of mesangial IgA deposition, but Conley et al (1980b), using monoclonal mouse antibodies, found it in none. J chain is a normal constituent of IgM, so that its presence is not specific for IgA dimers (Conley et al 1980b). SC has not been found in IgA deposits (McCoy1974; Dobrin et al 1975; Bené et al 1982).

Another approach to the characterisation of IgA deposits is the study of their ability to bind free SC. This is a property of polymeric IgA (Brandtzaeg 1974b). Such binding has been demonstrated both in primary IgA nephropathy and in mesangial IgA nephritis associated with alcoholic cirrhosis (Egido et al 1980; Sancho et al 1981; Bené et al 1982) suggesting that the deposits in both diseases contain polymers.

3.4 The investigation and use of human tissues in this thesis

In this thesis the kidneys of patients coming to autopsy with ALD were examined by IF and compared with those of normal controls and patients with other liver diseases (Chapter 2). The incidence of mesangial IgA deposition in patients with alcoholic cirrhosis coming to autopsy in a general hospital was determined. Evidence for CIC was sought by $SpC1_q$ assay of serum and by IF of extrarenal tissue (choroid plexus and the skin). In addition, the liver was studied by indirect immunofluorescence (IIF) to investigate the difference between the IgA distribution in patients with and without mesangial IgA deposits.

The immunochemical nature of mesangial IgA deposits in alcoholic cirrhosis was compared with those in primary IgA nephropathy, SLE and HSP, as well as IF negative material (Chapter 3). IIF was performed using sheep or rabbit antisera to J chain, SC and IgA subclasses. Mouse monoclonal antibodies to A_1 and A_2 were used in an avidin-biotin IF system (Warnke and Levy 1980). The presence of polymers was sought by the capacity of the deposits to bind free SC.

The IgA in mesangial deposits can be eluted by incubation with citrate buffer (Sancho et al 1981; Tomino et al 1982a). The deposits were examined *in situ* and *in vitro* after acid elution (Chapter 4). To identify antibody specificity against food proteins, eluates were tested in a radioimmune assay (RIA) for anti-BSA activity and kidney sections were incubated with

fluorescein isothiocyanate (FITC) labelled BSA and HSA. The re-activity of eluted IgA to bacterial antigens was examined by IIF after incubation on a slide preparation of E coli and in an enzyme linked immunosorbant assay (ELISA). Auto-antibody activity against mesangium and Mallory's hyaline was tested in an IF system and against aggregated serum albumin by gel precipitation.

3.5 The investigation and use of an animal model of mesangial IgA deposition in this thesis

The recognition of a high incidence of mesangial IgA deposition in patients with alcoholic cirrhosis has stimulated the development of animal models of cirrhosis. Some of these models have resulted in mesangial IgA deposits but progressive renal failure has rarely occurred. Sakaguchi et al (1964) showed that cirrhosis produced in rats by CCl_4 and ethionine caused mesangial sclerosis with electron dense deposits, but IF studies were not performed. Subsequently, Gormly et al (1981) examined the kidneys of rats with cirrhosis induced by inhalation of CCl_4 and found diffuse deposits of IgA, IgM, IgG and C_3 in the mesangium.

Experimental hepatosplenic schistosomiasis is accompanied by GN and CIC (Digeon et al 1979). Partial ligation of the portal vein of mice imitates the portacaval shunting of cirrhosis (Cheever and Warren 1963), and has been used to enhance the renal consequences of hepatosplenic schistosomiasis. Used alone on a group of uninfected control animals in a study of schisto-

somal GN by van Marck et al (1977), it was found to produce mesangial deposits of IgM, IgA, IgG and C_3 in 71% of animals.

Other models of mesangial IgA deposition independent of liver damage have been developed by injection of mice with preformed MOPC 315-DNP/BSA complexes (Rifai et al 1979) or by active immunisation (Isaacs et al 1981; Emancipator et al 1983) leading to glomerular IC deposition. In the study presented in this thesis, the model of Gormly et al (1981) was modified by the administration of subcutaneous (sc) CCl_4. This avoided respiratory damage to the rat and reduced operator hazards. This model, established in a pilot study using two strains of rats (Chapter 5), formed the basis of the experimental portion of the following study. It was found to be reproducible and allowed immunological, serological and morphological definition of a form of mesangial IgA deposition secondary to liver disease. The reproducibility of the model enabled its manipulation by various therapeutic agents (Chapter 6).

Proposed aetiological factors considered in the mechanisms of mesangial IgA deposition secondary to liver disease are the absorption of gut floral antigens, raised serum IgA levels, CIC and alternate pathway activation by IgA complexes or polymers. There are clinical precedents suggesting that these factors may be affected by the drugs which were used to manipulate the animal model. These drugs were prostaglandin E_1 (PGE_1), d-penicillamine, phenytoin, neomycin and dapsone.

Neomycin is an orally active antibiotic used pre-operatively for bowel sterilisation (Sande and Mandell 1980). In rats, it might be expected to reduce the absorption of floral antigens, and has been shown to reverse hyperglobulinaemia in rats with portacaval shunts (Keraan et al 1974).

The observation of increased serum IgA concentrations in patients with IgA nephropathy (Woodroffe et al 1980) has led to speculation about the possible therapeutic value of reducing these concentrations to normal. Phenytoin significantly lowers serum IgA levels in epileptic patients (Sorrell and Forbes 1975) as well as in patients with mesangial IgA deposition (Clarkson et al 1977). However, there is controversy over the therapeutic efficacy of this manoeuvre. Clarkson et al (1977) showed no clinical or pathological effect upon patients treated for one year, whilst Lopez-Trascasa et al (1980) reported a reduction in serum levels of IgA polymers and some clinical improvement.

Dermatitis herpetiformis (DH) is a vesicular rash of flexor surfaces, commonly found in association with gluten enteropathy (coeliac disease). DH is characterised immunopathologically by IgA and C_3 deposition at the dermo-epidermal junction. The condition has been shown to respond to oral dapsone (Fry et al 1980). The IgA deposits in DH have been shown to bind SC suggesting that they are dimeric (Leonard et al 1982). Secondary IgA nephropathy has been described in association with DH and coeliac disease (Katz et al 1979). Follow-up biopsies in dapsone-treated DH patients have shown a disappearance of C_3 but no decrease in epidermal IgA deposition (Van der Meer et al

1979). Nonetheless, the clinical efficacy of dapsone in this condition encouraged its trial in the model of IgA nephropathy in the rat.

D-penicillamine has been shown to be of value in the treatment of human rheumatoid arthritis and experimentally induced arthritis in rabbits (Hunneyball et al 1978). In addition it has been shown to lower the levels of CIC in primary biliary cirrhosis (Epstein et al 1979). In the rat model of mesangial IgA deposition, where CIC might be expected to be of aetiological significance, d-penicillamine may affect the development of the deposits.

Prostaglandins (PG) are complex compounds with diverse and incompletely understood effects, particularly upon immune-mediated inflammation. In the New Zealand Black/White (NZB/W) mouse, which develops a spontaneous nephritis morphologically similar to SLE in man, PGE_1 has been shown to increase survival and reduce IC deposition in the glomerulus (Zurier et al 1978). The rat model of mesangial IgA nephropathy is thought to be IC-mediated and for this reason the effect of 15-(s)-15-methyl PGE_1, a stable derivative of PGE_1, was tested in the CCl_4 model of mesangial IgA deposition.

CHAPTER 2

Mesangial IgA deposition in ALD: an autopsy study

INTRODUCTION

The association between IgA nephropathy and alcoholic cirrhosis, demonstrated in France (Callard et al 1975; Nochy et al 1976; Berger et al 1978) now has been recognised in Spain (Sancho et al 1981) and Japan (Nakamoto et al 1981; Endo et al 1983). In this study, material was obtained from consecutive autopsies performed on patients with ALD between October 1978 and May 1981 at The Royal Adelaide Hospital (RAH). In addition, material was obtained from autopsies performed on patients with other serious liver diseases. Control tissues were obtained from patients dying in hospital without evidence of ALD or renal disease, and from autopsies performed at the State Coroner's Office upon people with violent or sudden deaths. The kidneys were examined by IF to determine and compare the prevalence of mesangial IgA deposits amongst these groups of patients in South Australia.

Immunoglobulin deposits outside the kidney substantiate a CIC aetiology for a specific form of GN, and this study offered the opportunity to examine tissues from extrarenal sites by IF. Most importantly, the autopsy study provided material for the immunochemical study of mesangial IgA deposits (Chapter 3) and whole kidneys for elution studies (Chapter 4). In some instances, laboratory stored serum was available from the Division of Clinical Chemistry, The Institute of Medical and Veterinary Science (IMVS). This serum had been inadequately stored for CIC assay but was screened for hepatitis B antigens and antibody, as a precaution before further manipulation of stored tissue.

MATERIALS

Forty-three consecutive patients coming to autopsy at the RAH between October 1978 and May 1981 with a history of alcohol abuse, were included in this study. Each patient had been assessed by an admitting clinic as being a chronic alcohol abuser. For comparison, material was obtained from six patients with other forms of chronic liver disease. Control tissue was obtained from 17 Coronial autopsies (State Coroner's Office, Divett Place, Adelaide) performed upon subjects whose death was violent or sudden, and from eight routine Hospital (RAH) autopsies performed upon patients with no clinical evidence of preceding liver or kidney disease. Twenty-nine of 74 autopsies were performed by the author; the remaining 45 being performed by pathologists of the Division of Tissue Pathology, IMVS.

The hospital case notes were reviewed, and blood pressure, serum creatinine, urinalysis results and the cause of death of each patient were recorded. Where possible, these clinical parameters were taken between a year and one month prior to the terminal event to avoid agonal abnormalities.

No such data was available from the Coroner's Office so that for all of the normal control patients, only age, sex and cause of death were recorded.

Kidney

Tissue blocks were taken for IF and LM, and at least one kidney was frozen at $-20^{o}C$ after stripping the capsule, and discarding pelvic fat and most of the medullary tissue.

Liver

Material was taken for IF and LM study.

Choroid plexus

Choroid plexus for IF was extracted from the fourth ventricle in the cerebellomedullary cistern with blunt forceps. The technique allowed the preservation of the brain structure for later neuropathological investigation.

Skin

A skin ellipse from the left forearm was taken for IF.

Serum

Nineteen of the 58 patients who died within the RAH had premortem blood taken and serum stored within the Division of Clinical Chemistry, IMVS. The serum was screened in the Division of Virology for hepatitis B antigens and antibody.

METHODS

A Light microscopy

Material for LM was fixed in 10% neutral buffered formalin, processed to paraffin wax and sections of liver cut at 5 µm and kidney at 2 µm. Sections of kidney were stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and periodic acid-methenamine silver (after Jones 1957) (PASM). Sections of liver were stained with H&E, adaptations of Fouchet's bile stain with Sirius red as a collagen stain, Gordon and Sweet's reticulin stain, and where appropriate, Perl's iron stain. The processing and staining methods are described in Appendix I.

The LM slides were examined using a Leitz Orthomat Microscope. Renal sections were examined and the glomerular size assessed together with the presence of an increase in glomerular mesangial cells, segmental lesions, and vascular or glomerular hyaline. Glomerulomegaly was judged subjectively as being present or absent. Mesangial proliferation was recognised as the presence of three or more mesangial nuclei per glomerular segment, and recorded as diffuse if two or more areas were affected in at least 80% of the glomeruli, or focal if less than 80% of glomeruli were affected. Sclerotic lesions were those with collapse or expanded areas of PAS-positive matrix, with or without hyalinosis.

Liver sections were examined and evaluated for the presence of steatosis, Mallory bodies, an increase in stainable iron,

alcoholic hepatitis, increased fibrosis, or cirrhosis. The changes were assessed using the criteria laid down by Christofferson and Poulson (1979).

B Immunofluorescence

1 Material for IF was embedded in Tissue Tek II OCT compound (Lab-Tek Products, Mile Laboratories, IL) and snap frozen in isopentane and liquid nitrogen.

2 Unfixed frozen sections were cut at 2 µm and placed on slides coated with glycerine and formaldehyde (Appendix II).

3 The sections were washed for 10 minutes in a bath of phosphate buffered saline (PBS) (Appendix IX), with an electric stirrer.

4 Direct IF (DIF) was performed in a moist chamber by incubation of sections with FITC-conjugated monospecific antisera to human IgA, IgG, C_3 (Wellcome, Beckenham, UK), IgM (Hyland-Travenol Laboratories, Costa Mesa, CA), fibrinogen and $C1_q$ (Hoechst, Marburg, W Germany) at a dilution of 1:10 in 1% BSA (Commonwealth Serum Laboratories) in PBS (BSA/PBS), for 30 minutes.

5 Indirect IF (IIF) was performed by incubation of sections with monospecific rabbit antisera to human IgA, IgG, IgM, C_3 and fibrinogen (Hoechst) at a dilution of 1:8 in BSA/PBS for 30 minutes, followed by 1:10 FITC-conjugated

goat anti-rabbit IgG (Dakopatts, Copenhagen, Denmark) for 45 minutes.

6 Between antibody layers in the IIF, and finally, all sections were washed twice for 10 minutes in fresh PBS in an electrically stirred bath.

7 Sections were mounted in glycine buffered glycerol pH 8.6 (Appendix II).

8 Sections were examined using a Leitz Ortholux microscope fitted with Pleom epi-illumination, an HB200 mercury lamp as a light source, dichroic mirrors on position 3, and BG38 and K510 filters.

Initial studies were performed using DIF and IIF in parallel, but the latter system was more time consuming and only showed less background staining in the liver. For other tissues therefore only DIF was used.

The choroid plexus and skin specimens were examined by Miss A E Thompson, Immunohistologist, Division of Tissue Pathology, IMVS.

C <u>Hepatitis serology</u>

Hepatitis B virus (HBV) status was tested by RIA using Ausria for hepatitis B surface antigen (HBsAg), Ausab for hepatitis B surface antibody (anti-HBs), and Corab for hepatitis B core antibody (anti-HBc) (Abbott Laboratories, N Chicago, IL). The

tests were performed by Mr Peter Lindschau in the Division of Virology, IMVS.

D Statistics

The Mann-Whitney U test was used to compare the ranked ages of the patients in the various categories (Siegel 1956). A Yates modified chi square test was used to compare the relationship between variables.

RESULTS

A Light microscopy of liver tissue, to confirm ALD and define the type of liver damage.

B Review of clinical status: cause of death, and hepatitis serology.

C Examination of renal tissue:
  i) Direct immunofluorescence
  ii) Light microscopy

D Indirect immunofluorescence of liver tissue.

E Direct immunofluorescence of skin.

F Direct immunofluorescence of choroid plexus.

A The LM of liver tissue

The liver sections from the patients in the study were examined and the results are shown in Table 2.1. The livers of 34 of the 43 alcohol abusers were cirrhotic (Figure 2.1) with regenerative nodules and evidence of alcohol-associated changes, such as Mallory bodies (Figure 2.2) and iron deposition (Figure 2.3). There were no histological changes in these patients to suggest another aetiology for the cirrhosis. Sixteen of these patients had acute hepatitis with perihepatocytic neutrophil infiltration of the type seen in ALD. The remaining nine alcoholic patients had varying degrees of liver disease compatible with an alcoholic aetiology including steatosis, perivenular sclerosis, Mallory bodies, giant mitochondria, or an increase in stainable iron. Three of these patients without cirrhosis showed alcoholic hepatitis. Fibrous linkage of portal tracts was seen in four instances.

The control patients occasionally showed steatosis, centrilobular congestion, or a moderate portal infiltration, but all had normal architecture (Figure 2.4). None showed Mallory bodies or excess stainable iron (Table 2.2).

B Review of patients' clinical status and cause of death

The case notes of the 43 patients known clinically to be chronic alcohol abusers were reviewed. The data obtained from review of the case notes are shown in Table 2.3. Three patients had biopsy proven mesangial IgA deposition. Of the 34 patients with alcoholic cirrhosis, 19 died as a direct result of hepatic failure. The terminal events recorded were gastro-intestinal

Table 2.1 The histological features in the livers of 43 patients with ALD

Autopsy number	Steatosis	Cirrhosis	Alcoholic hepatitis	Mallory bodies	Other features
50721.78	+	+	-	-	-
50816.78	+	+	-	-	Hepatoma
50871.78	-	+	-	+	-
50903.78	+	+	-	+	-
50939.78	-	+	-	+	-
50912.78	-	+	-	-	-
50975.78	-	+	-	+	-
50010.79	-	+	-	+	-
50078.79	-	+	+	+	-
50132.79	+	+	+	+	-
50141.79	+	+	-	-	-
50257.79	+	+	-	+	-
50329.79	+	+	+	+	-
50358.79	+	+	+	+	-
50364.79	-	+	-	+	-
50432.79	+	+	+	+	-
50439.79	+	+	+	+	-
50442.79	-	+	+	+	-
50443.79	+	+	-	-	-
50502.79	-	+	-	-	-
50504.79	+	+	+	+	-
50510.79	+	+	-	+	-
50528.79	+	+	+	+	-
50538.79	+	+	-	+	-
50561.79	-	+	+	+	-
50629.79	+	+	+	+	-
50730.79	+	+	+	+	-
50203.80	+	+	+	+	-
50228.80	-	+	-	-	-
50317.80	-	+	+	+	-
50331.80	+	+	+	+	-
50359.80	+	+	-	-	-
50916.80	+	+	-	+	-
50512.81	+	+	+	+	-
50012.79	-	-*	+	-	PVS/GM
50055.79	+	-*	+	-	-
50355.79	+	-	-	-	GM
50486.79	+	-	-	+	-
50493.79	+	-	+	+	-
50698.79	+	-	+	-	GM
50873.79	+	-*	+	+	PVS
50264.80	-	-*	-	-	PVS
50271.80	+	-	-	-	GM

PVS = perivenular sclerosis
GM = giant mitochondria
C = cirrhosis
* = fibrous linkage portal tracts

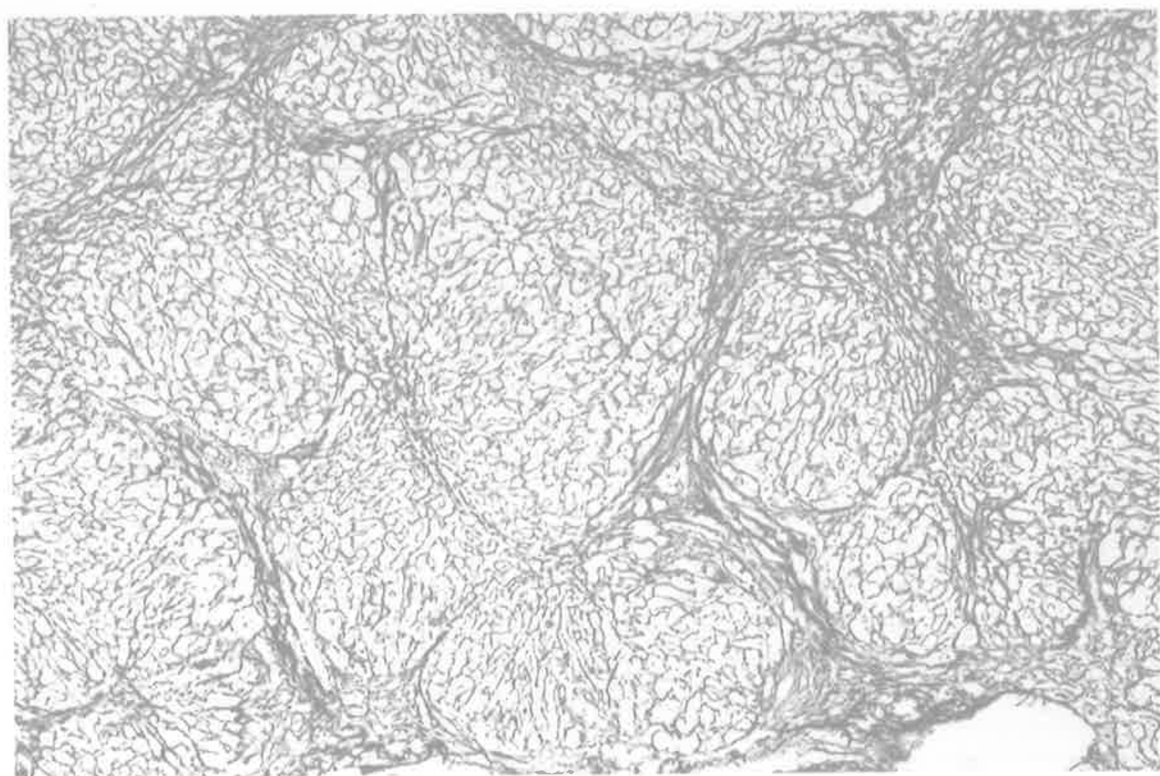


FIGURE 2.1
Liver of a patient with a history of alcohol abuse (50629.79), showing cirrhosis (Reticulin stain, X55).

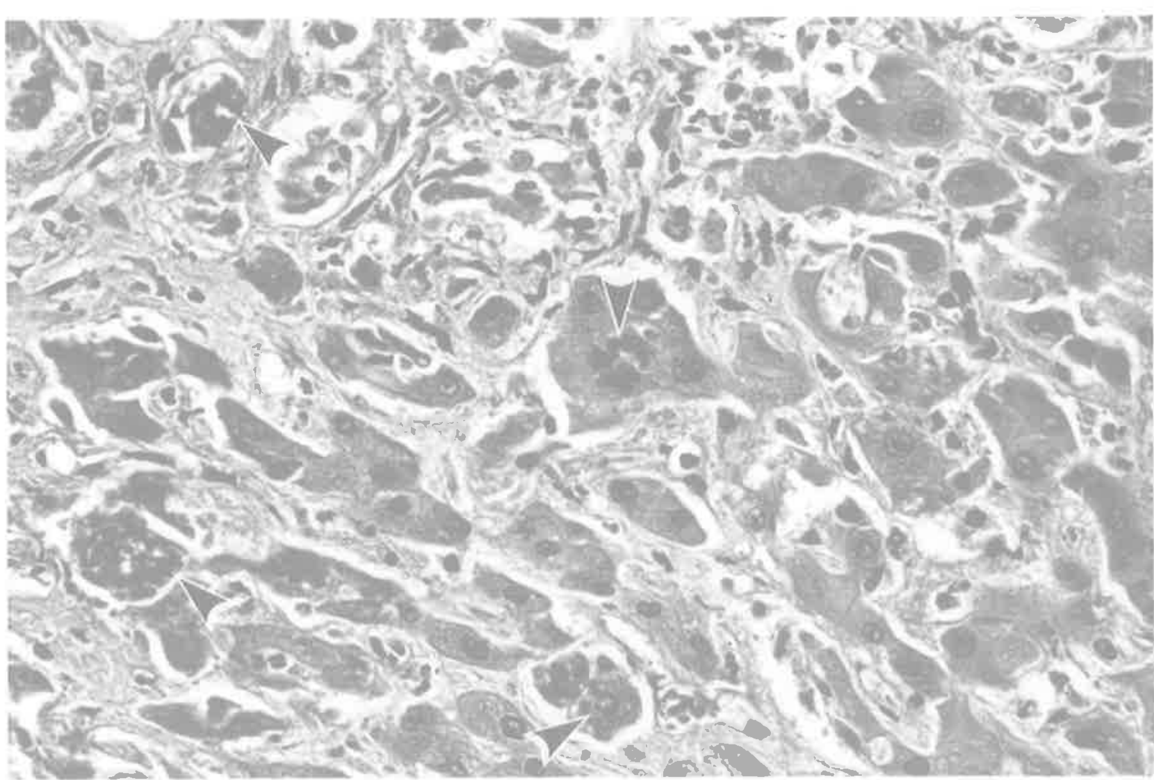


FIGURE 2.2
Liver of a patient (50132.79) with alcoholic cirrhosis. There is alcoholic hepatitis with perihepatocyte neutrophils and irregular cytoplasmic hyaline masses representing Mallory bodies (arrows) (H&E, X340).

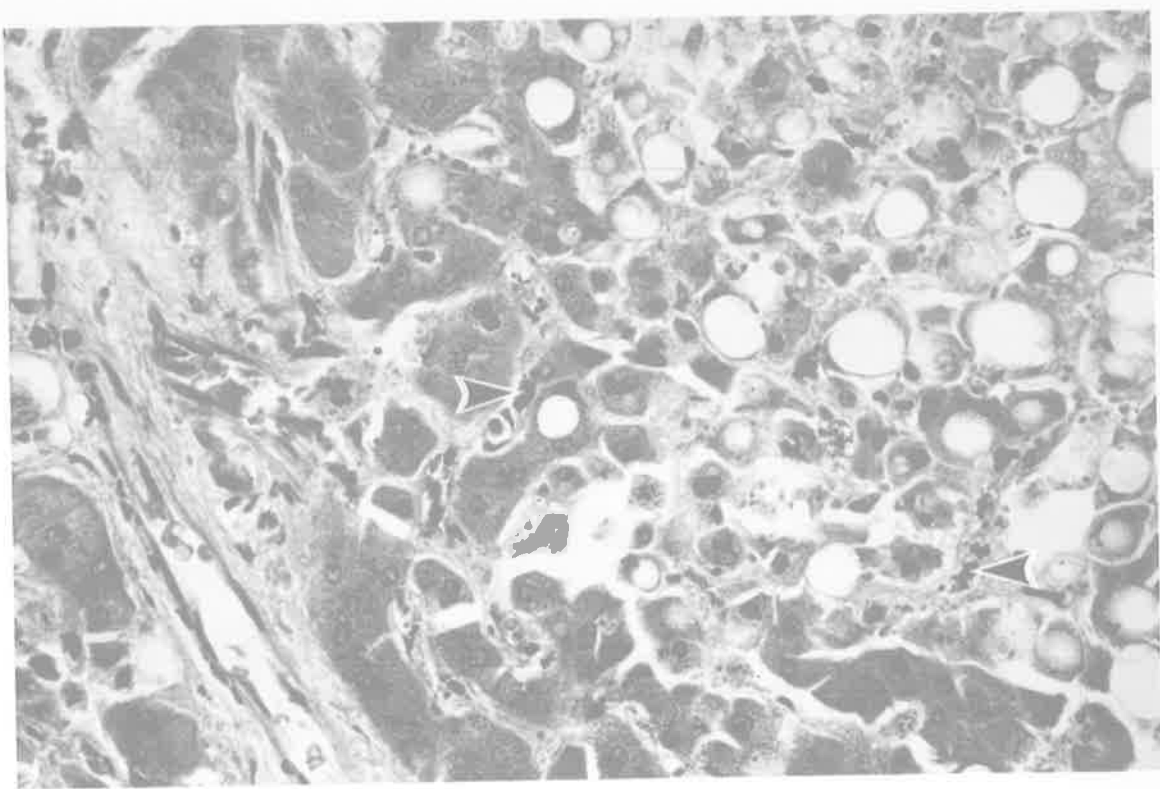


FIGURE 2.3
Liver from a patient with alcoholic cirrhosis (50358.79) showing iron granules in hepatocytes and Kupffer cells (arrows). There is distension of liver cells by lipid vacuoles (Perl's stain, X340).

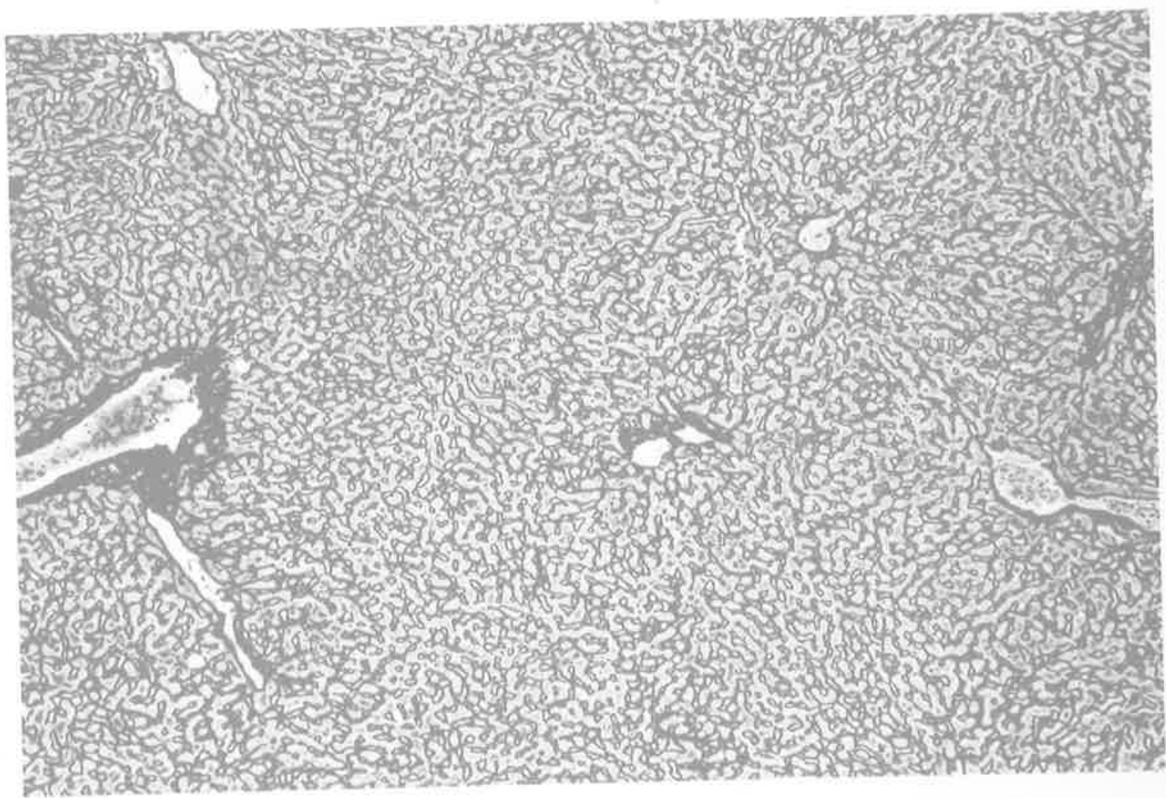


FIGURE 2.4
Liver of a control patient who died suddenly after major trauma (50810.80). The architecture is normal (Reticulin stain, X70).

Table 2.2 Summary of the hepatic light microscopic features of patients in the autopsy series excluding the six patients with other specific liver conditions

Patient category (number)		Light microscopic features in the liver				
		Steatosis	Mallory bodies	Increase stainable iron	Alcoholic hepatitis	Perivenular sclerosis
Cirrhotic ALD	(34)	22 (65%)	26 (76%)	24 (70%)	16 (47%)	NA
Non-cirrhotic ALD	(9)	7 (89%)	3 (44%)	1 (11%)	5 (56%)	3 (33%)
Controls	(25)	8 (32%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

NA = not assessed

Table 2.3 Clinical status and cause of death of patients with ALD and other serious liver diseases

				Clinical status			Death	
Patient category	Autopsy number	Sex	Age	Diastolic pressure (mm)	Serum Creatinine mmol/L	Abnormal urinalysis	Cause	Relation to alcohol
Cirrhotic ALD	50721.78	M	57	90	0.10	NT	Heart failure + GIH	A
	*50816.78	M	60	100	0.40	+	Myocardial infarct	I
	50871.78	F	64	85	0.08	0	Lymphoma	I
	50903.78	M	56	80	0.08	0	Heart failure + GIH	A
	50939.78	M	69	70	0.11	+	Road traffic accident	R
	50912.78	F	60	80	0.07	0	Cerebrovascular accident	I
	50975.78	M	61	110	0.08	+	Heart failure + GIH	A
	50010.79	M	55	90	1.60	+	Heart failure + GIH	A
	50078.79	F	68	70	0.13	0	Septicaemia	R
	50132.79	M	58	90	0.26	0	Heart failure + GIH	A
	50141.79	M	51	80	0.11	+	Heart failure + GIH	A
	50257.79	M	59	85	0.05	+	Encephalopathy	A
	50329.79	M	65	90	0.16	0	Heart failure + GIH	A
	50358.79	F	42	100	NT	+	Encephalopathy	A
	50364.79	M	76	100	0.16	0	Heart failure + septicaemia	R
	50432.79	M	49	50	0.35	+	Heart failure + septicaemia	R
	50439.79	F	42	85	0.15	0	Encephalopathy	A
	*50442.79	M	57	95	1.60	+	Hepatorenal failure	A
	50443.79	M	73	90	0.09	0	Myocardial infarct	I
	50502.79	M	68	80	0.08	0	GIH	A
	50504.79	M	68	NT	NT	0	Inhalation	R
	50510.79	M	54	80	NT	+	Pancreatitis	R
	50528.79	F	60	80	0.10	0	Septicaemia	R
	50538.79	M	58	80	0.07	0	Heart failure + GIH	A
	50561.79	M	53	70	0.07	0	Myocardial infarct	I
	50629.79	M	32	70	0.07	0	Heart failure + GIH	A
	50730.79	M	48	70	0.40	0	Heart failure + GIH	A
	50203.80	M	60	95	0.07	+	Cerebrovascular accident	I
	50228.80	F	53	80	0.07	0	Heart failure + GIH	A
	50317.80	M	62	70	0.09	+	Heart failure + GIH	A
	50331.80	M	69	70	0.34	0	Heart failure + GIH	A
	50359.80	M	74	80	0.07	0	Septicaemia	R
	50916.80	F	38	65	NT	0	Septicaemia	R
	50512.81	M	58	80	0.05	0	Heart failure + GIH	A
Non-cirrhotic ALD	*50012.79	M	56	95	0.23	+	Hepatorenal failure + CCF	A
	50055.79	M	48	100	NT	+	Encephalopathy	A
	50355.79	M	64	100	0.15	+	Myocardial infarct	I
	50486.79	M	54	80	0.12	0	Cardiomyopathy	A
	50493.79	M	52	105	0.10	+	Septicaemia	R
	50698.79	M	48	80	0.09	0	Encephalopathy	A
	50873.79	M	59	NT	NT	+	Heart failure + GIH	A
	50264.80	M	69	100	NT	+	CCF	I
	50271.80	M	49	80	0.09	0	Encephalopathy	A
Other serious liver conditions	50843.78	M	57	95	0.11	+	Metastatic carcinoma, pancreas	0
	50885.78	M	77	90	0.10	0	Hepatoma	0
	50234.79	F	75	100	0.08	NT	Secondary biliary cirrhosis	0
	50436.79	F	65	70	0.10	0	Primary biliary cirrhosis	0
	50892.79	F	90	90	0.15	0	Hepatoma	0
	50137.80	M	56	80	0.11	+	Haemochromatosis	0

NT = not tested prior to the terminal event
GIH = gastro-intestinal haemorrhage
CCF = congestive cardiac failure

A = directly attributable to alcohol abuse
R = related to alcohol abuse
I = incidental to alcohol abuse

haemorrhage (GIH) (15), encephalopathy (3) and hepatorenal failure in a patient known to have mesangial IgA nephritis. Nine had causes of death which were alcohol related, including acute pancreatitis (1), inhalation of vomit (1), road traffic accident (RTA) (1), and septicaemia (6). There were six incidental causes of death. These were malignant lymphoma (1), cerebrovascular accident (CVA) (2), and myocardial infarction (MI) (3). One of the latter patients was known to have mesangial IgA nephritis, and also had a hepatocellular carcinoma (50816.78). Of the nine patients with non-cirrhotic ALD, the causes of death could be attributed directly to alcohol in six, including encephalopathy (3), massive GIH (1), hepatorenal failure (1), and alcoholic cardiomyopathy (1). The patient with hepatorenal failure also had congestive cardiac failure (CCF), and was known to have mesangial IgA nephritis. One patient died from septicaemia, one from MI and one in CCF. There was no relationship between the type of ALD and the cause of death.

The non-alcoholic serious liver diseases taken for comparison were primary biliary cirrhosis (1), haemochromatosis (1), hepatocellular carcinoma (2) (one without cirrhosis), and carcinoma of the head of the pancreas (2). One of the patients with carcinoma of the head of the pancreas had secondary biliary cirrhosis, and the other massive liver metastases.

Seventeen Coronial autopsies were included in the study as controls. The causes of death (Table 2.4) were RTA (11), gunshot injury (1), suicidal overdose (1), drowning (1), subarachnoid haemorrhage from an aneurysm (1), inhalation of vomit (1),

Table 2.4 Age, sex and cause of death of control patients from the Coroner's Office and The Royal Adelaide Hospital

Autopsy	Sex	Age	Cause of death
50261.79	F	66	Tamponade
50500.79	M	46	Septicaemia
50515.79	M	74	Septicaemia
50690.79	F	68	Myocardial infarct
50696.79	M	58	Congestive cardiac failure
50702.79	F	70	Tamponade
+ 50781.79	M	22	Road traffic accident
50915.79	M	71	Septicaemia
50116.80	M	58	Carcinoma of lung
+ 50222.80	M	37	Drowning
+ 50224.80	M	17	Overdose
+ 50810.80	M	29	Gunshot
+ 50815.80	M	61	Road traffic accident
+ 50915.81	F	42	Subarachnoid haemorrhage
+ 722.81	M	34	Road traffic accident
+ 787.81	M	17	Road traffic accident
+ 788.81	M	18	Road traffic accident
+ 1262.81	M	56	Road traffic accident
+ 1263.81	M	15	Road traffic accident
+ 3699.81	M	22	Road traffic accident
+ 3715.81	M	18	Road traffic accident
+ 3716.81	F	36	Road traffic accident
+ 4674.81	M	33	Road traffic accident
+ 4782.81	M	69	Inhalation
+ 4783.81	M	29	Jump from high building

+ = Coronial autopsy

and jumping from a high building (1). The control group also included eight hospital patients who died from septicaemia (3), CCF (1), MI (1), cardiac tamponade (2), and carcinoma of the lung (1).

The mean age of patients dying with ALD was 58.1 years compared with 43.0 years for controls (p=0.0026), and 70.0 years for those with other serious liver diseases (p=0.063). Of the patients with ALD, 82% were male compared with 80% of controls, and 50% of those with other chronic liver diseases.

Clinical status

Review of the case notes of the 49 patients with ALD and other severe liver diseases disclosed blood pressure recordings and urinalysis results in 47 prior to the terminal admission, but less than 12 months before death. Forty-two pre-terminal creatinine measurements were available. Diastolic pressures of 95 mm of mercury and above were regarded as indicative of hypertension, and creatinine levels of greater than 0.12 mmol/L as indicative of renal impairment. Using these criteria in all categories of liver disease investigated, 13 of 47 patients had hypertension, and 14 of 42 had renal impairment. Of the 47 patients with recorded urinalysis results, 20 had both haematuria and proteinuria, which was recorded as abnormal urinalysis in Table 2.3. In five patients, this abnormal urinalysis was without hypertension or raised serum creatinine levels. None of the 19 serum samples from controls or patients, had HBsAg, but five had anti-HBs or anti-HBc activity. Isolated, anti-HBs antibody was found in two of three control sera tested, and

anti-HBc antibody in one of two sera from patients with other serious liver diseases. Two of 11 sera from patients with cirrhotic ALD showed both antibodies (50010.79, 50502.79). Three sera from patients with non-cirrhotic ALD were negative in all three tests (Appendix III). These findings confirmed that none of the patients were carriers or had active hepatitis B infection.

C Results of examination of renal tissue

i DIF of autopsy kidneys

The renal IF findings are shown in Table 2.5. Seventeen of the 34 patients (50%) who died with alcoholic cirrhosis had mesangial IgA deposition (Figure 2.5-2.6). Of the nine patients with non-cirrhotic ALD, three (33%) showed mesangial IgA deposition. Only one (17%) of the six patients with other liver diseases had mesangial IgA deposits. One (4%) of 25 controls had mesangial IgA, but the remainder showed no significant abnormality by IF. Thirteen of the 22 patients with mesangial IgA, also had capillary wall staining. Of the 22 patients with mesangial IgA, 10 (45%) had IgG, 15 (68%) IgM, 10 (45%) Cl_q and nine (41%) C_3 staining. C_3 staining, in the absence of mesangial IgA, was present in five patients with ALD, one with another serious liver disease, and in two controls. This was an isolated finding except in two cases with ALD (50871.78, 50141.79) where there were other immunoglobulins and Cl_q.

ii LM examination of the autopsy kidneys

Of the 43 patients with ALD, of whom 34 had alcoholic cirrhosis, 29 showed glomerulomegaly and 13 showed diffuse mesangial hyper-

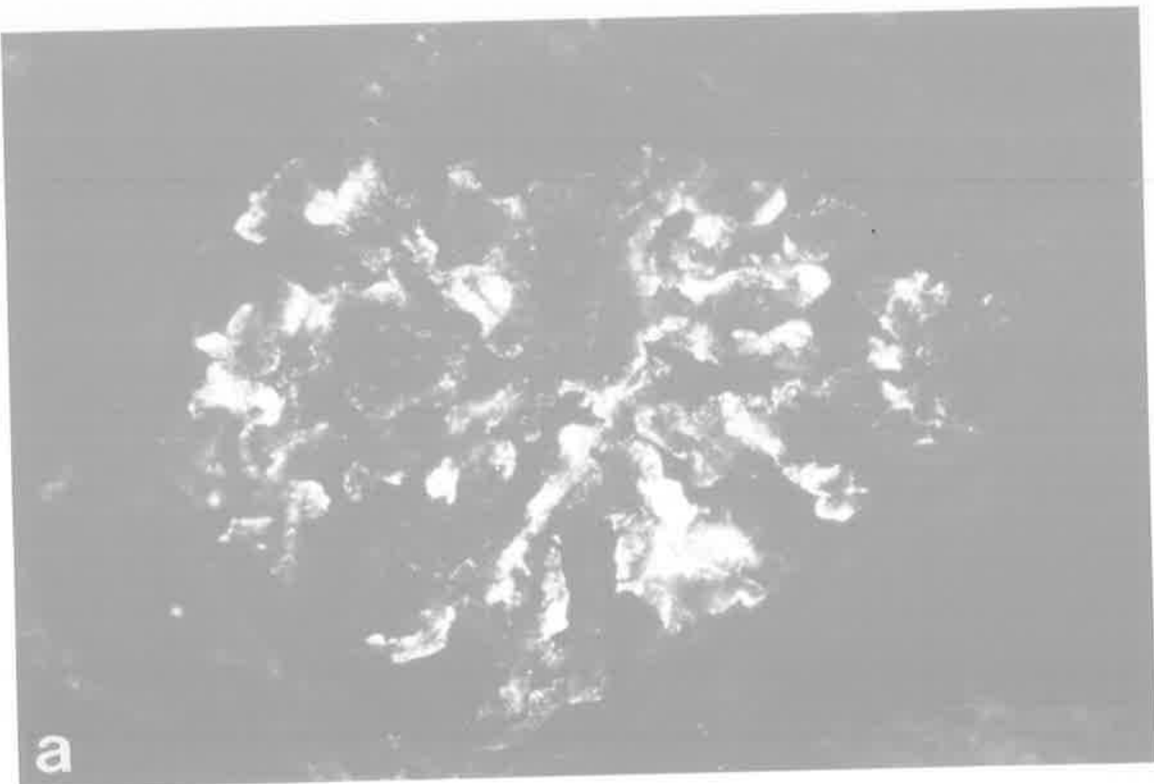
Table 2.5 Renal IF performed on 74 autopsy kidneys from controls and patients with ALD or other serious liver diseases

Patient category	Autopsy number	Renal IF					
		IgA	IgG	IgM	Clq	C_3	Fibrinogen
Cirrhotic ALD	50721.78	3	0	2	1	2	0
	*50816.78	2	2	3	1	1	0
	50871.78	0	2	1	2	2	0
	50903.78	2	0	2	0	0	0
	50912.78	0	0	3	0	0	0
	50939.78	2	0	2	0	0	0
	50975.78	4	0	2	2	2	3
	50010.79	3	0	0	1	0	0
	50078.79	0	0	0	0	0	0
	50132.79	2	2	3	3	0	0
	50141.79	0	0	1	1	1	0
	50257.79	0	0	1	2	0	0
	50329.79	2	2	2	2	0	0
	50358.79	3	0	0	0	2	0
	50364.79	0	0	0	0	0	0
	50432.79	0	0	0	0	0	0
	50439.79	2	0	2	0	0	0
	*50442.79	4	0	2	1	2	0
	50443.79	0	0	0	0	0	0
	50502.79	0	0	0	0	0	0
	50504.79	0	0	0	0	0	0
	50510.79	0	0	0	0	2	0
	50528.79	0	0	0	0	0	0
	50538.79	0	3	0	2	0	0
	50561.79	0	0	0	0	0	0
	50629.79	0	0	0	0	0	0
	50730.79	3	2	1	2	0	0
	50203.80	3	2	3	0	2	2
	50228.80	3	2	2	2	2	2
	50317.80	0	0	3	0	0	0
	50331.80	2	2	3	2	2	3
	50359.80	0	0	2	0	0	0
	50916.80	3	0	2	0	2	0
	50512.81	2	0	0	0	0	0
Non-cirrhotic ALD	*50012.79	2	0	0	0	0	0
	50055.79	0	0	1	0	0	2
	50355.79	0	0	0	0	0	0
	50486.79	0	0	2	0	0	0
	50493.79	2	2	0	0	0	0
	50698.79	1	1	0	0	0	0
	50873.79	0	0	0	0	2	0
	50264.80	0	0	4	0	4	0
	50271.80	0	0	1	1	0	0
Other serious liver conditions	50843.78	2	0	0	0	0	0
	50885.78	0	0	0	0	0	0
	50234.79	0	0	2	0	0	0
	50436.79	0	0	0	0	3	0
	50892.79	0	0	0	0	0	0
	50137.80	0	0	0	0	0	0
Controls	50261.79	0	0	0	0	0	0
	50500.79	0	0	0	0	2	0
	50515.79	0	0	0	0	0	0
	50690.79	0	2	0	0	0	0
	50696.79	0	1	0	0	0	0
	50702.79	0	0	2	0	0	0
	+50781.79	0	0	0	0	2	0
	50915.79	0	0	0	0	0	0
	50116.80	0	0	0	0	0	0
	+50222.80	0	0	3	3	0	0
	+50224.80	0	0	0	0	0	0
	+50810.80	0	0	0	0	0	0
	+50815.80	0	0	0	0	0	0
	+50915.81	0	0	0	0	0	0
	+ 722.81	2	1	2	0	0	0
	+ 787.81	0	0	0	0	0	0
	+ 788.81	0	0	0	0	0	0
	+ 1262.81	0	0	0	0	0	0
	+ 1263.81	0	0	0	0	0	0
	+ 3699.81	0	0	0	0	0	0
	+ 3715.81	0	0	0	0	0	0
	+ 3716.81	0	0	0	0	0	0
	+ 4674.81	0	0	0	0	0	0
	+ 4782.81	0	0	0	0	0	0
	+ 4783.81	0	0	0	0	0	0

Staining graded subjectively from 0-4+

* = known to have mesangial IgA deposits
\+ = Coronial autopsy

Autopies with five digit codes were performed by IMVS staff at either the RAH or State Coroner's Office Mortuary



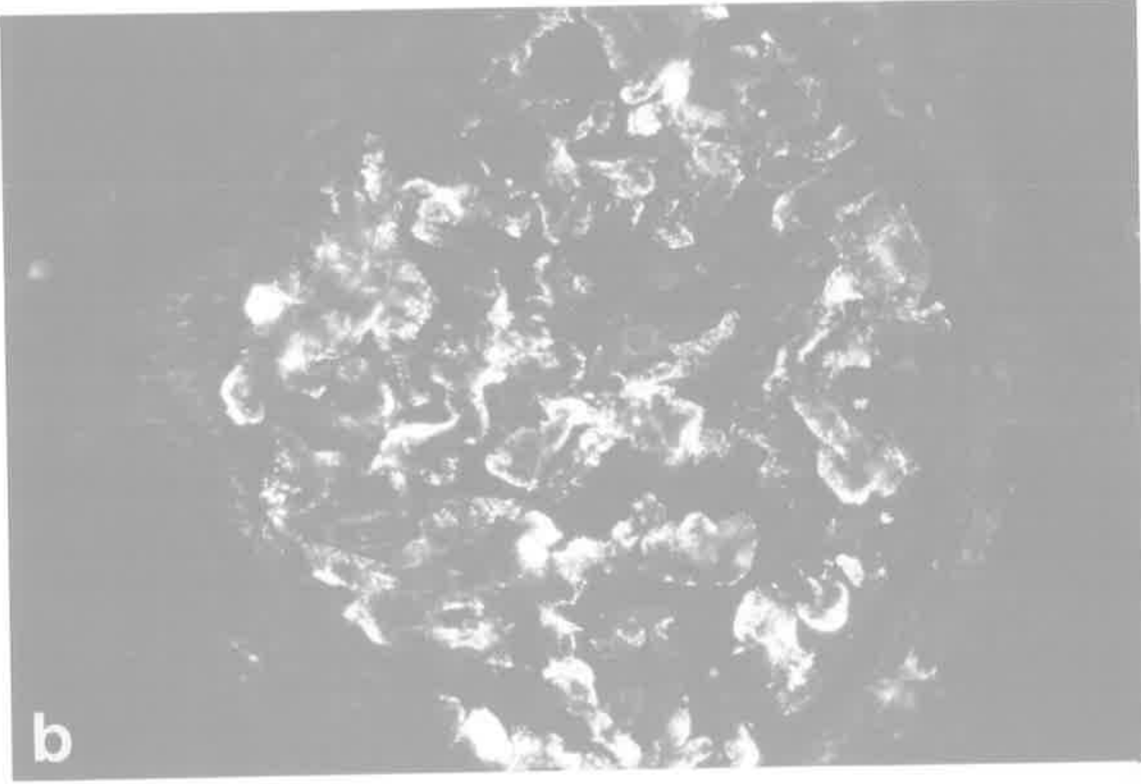
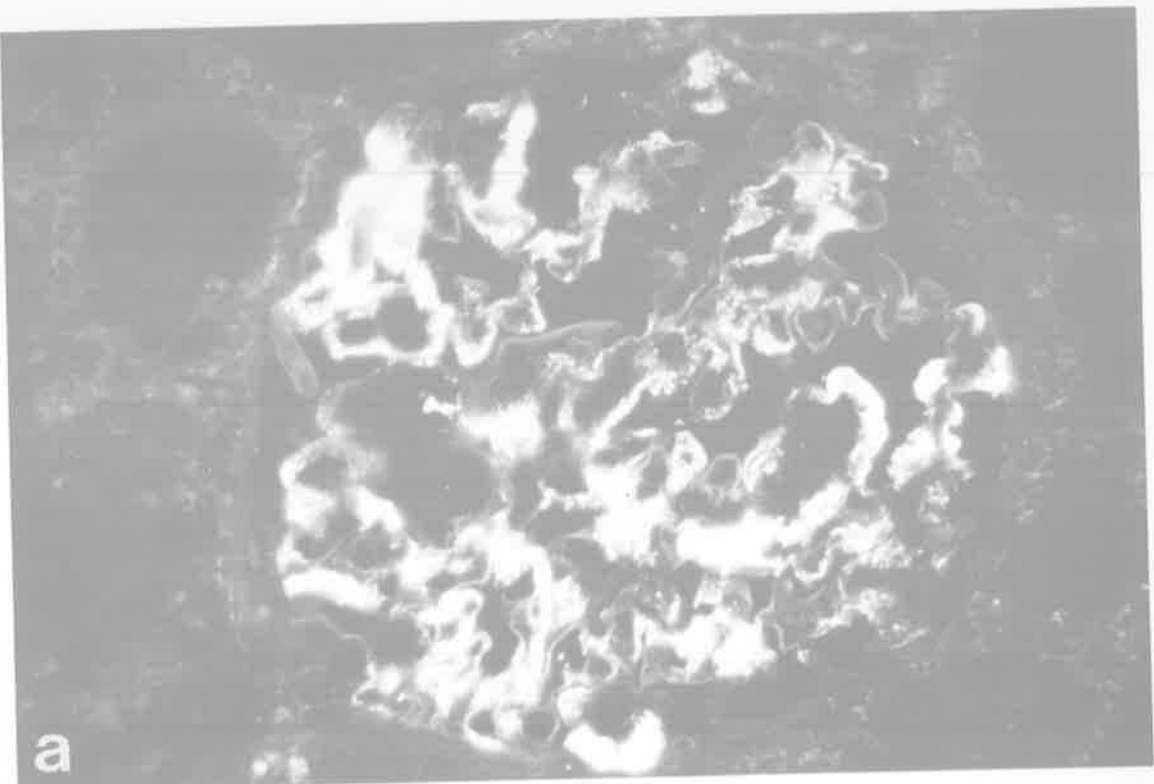


FIGURE 2.5
Glomeruli from a patient with ALD (50228.80).
a Mesangial granular diffuse staining for IgA (graded 3+).
b Mesangial granular diffuse staining for IgG (graded 2+).
There is also capillary wall staining.
(DIF, X1000)



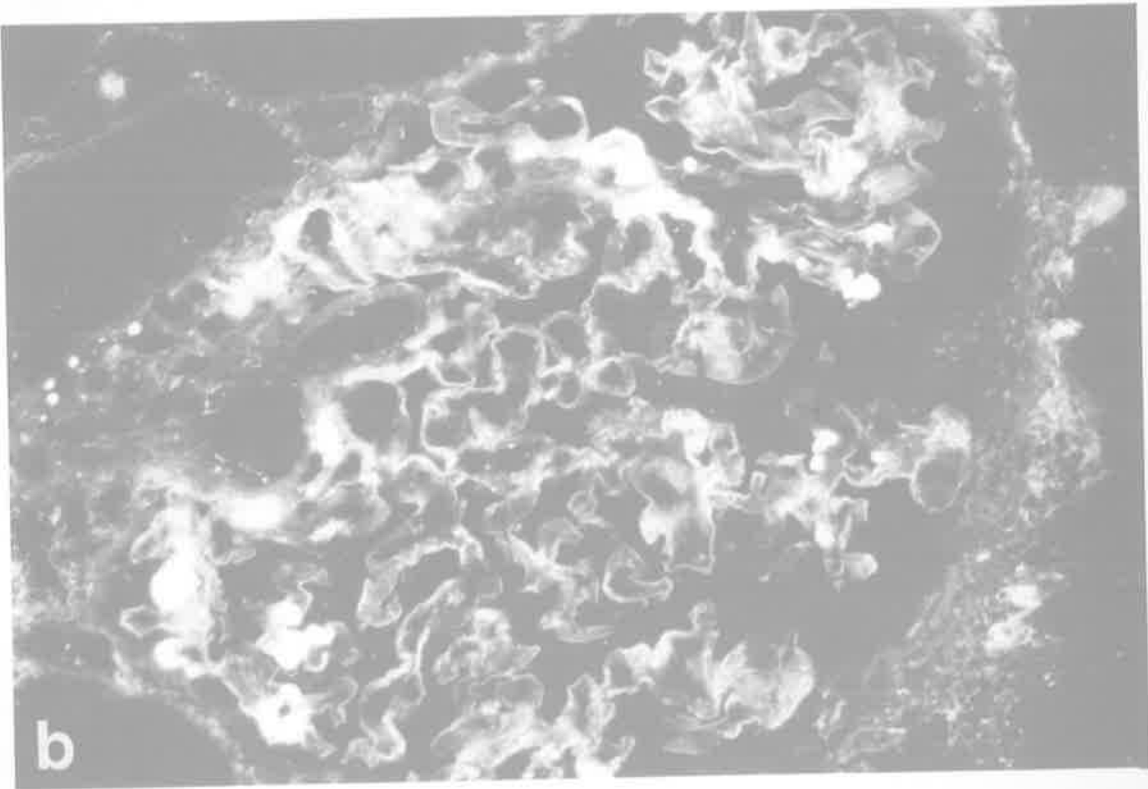


FIGURE 2.6
Glomeruli from a patient with ALD (50228.80).
a Mesangial granular diffuse staining for IgM (graded 2+).
b Mesangial granular diffuse staining for C_3 (graded 2+).
There is also linear basement membrane staining (graded 2+).
(DIF, X1000).

cellularity (Table 2.6, Figure 2.7). Glomerular obsolescence was rare, as was vascular abnormality. Glomerular hyaline nodules were present in 11 cases. The kidneys of control patients showed no significant abnormalities. Mesangial IgA deposition showed no significant correlation with glomerulomegaly ($p>0.1$) but was more common in patients with diffuse mesangial hypercellularity ($p<0.001$), vascular hyaline ($p<0.02$) and glomerular hyaline ($p<0.02$).

D Liver IIF

Liver tissue from 41 patients with ALD, three with other liver diseases, and 10 controls, was available for examination. The results are shown in Table 2.7. Two patterns of fluorescence were recognised. One showed continuous perihepatocyte staining which formed a mosaic-like appearance (Figure 2.8), and the other exhibited spider-like contours often between groups of hepatocytes (Figure 2.9) in a distribution suggestive of bile canaliculi. The mosaic-like pattern for IgG was seen in all disease groups and occurred in 23 of 41 patients with ALD compared with two of ten controls ($p=0.045$). IgA in this distribution was seen in 16 of 41 patients with ALD and in one control case. This difference was not significant ($p>0.05$). Mosaic-like continuous contours of IgM were present in four of 41 patients with ALD but in none of the controls.

Some livers showed no hepatocyte staining with any reagents, but plasma cells could often be recognised in the fibrous septae.

Table 2.6 Glomerular morphology in patients with ALD. All patients were cirrhotic unless asterisked (*). Glomerular obsolescence was graded 3-15%=1+, 16-40%=2+. Hypercellularity was diffuse (D) or focal (F)

Mesangial IgA deposition	Autopsy No.	Glomerular morphology					
		Glomerulomegaly	Mesangial hypercellularity	Sclerosis + hyalinosis	Vascular hyaline	Hyaline nodules	Obsolescence
Present	50721.78	+	-	+	-	+	-
	50816.78	+	D	-	+	+	-
	50903.78	-	-	+	+	+	-
	50939.78	+	-	-	-	-	-
	50975.78	+	D	+	-	-	-
	50010.79	+	D	-	-	-	-
	50012.79*	+	D	-	-	-	-
	50132.79	-	F	-	-	-	-
	50329.79	+	-	-	-	-	1+
	50358.79	-	D	-	-	+	-
	50439.79	+	D	-	+	-	-
	50442.79	+	D	+	+	+	-
	50698.79*	+	D	-	+	+	-
	50730.79	+	D	-	-	-	-
	50203.80	+	D	-	+	+	1+
	50228.80	+	-	-	-	-	-
	50331.80	-	-	+	-	+	1+
	50493.80*	+	-	-	+	+	-
	50916.80	+	-	-	+	-	-
	50512.81	+	D	-	-	-	-
Absent	50871.78	-	-	-	-	-	-
	50912.78	+	-	-	-	+	-
	50055.79*	-	-	-	-	-	-
	50078.79	+	-	-	+	-	-
	50141.79	-	-	-	-	-	-
	50257.79	-	-	-	-	-	-
	50355.79*	+	-	-	-	-	-
	50364.79	+	-	+	-	-	-
	50432.79	-	-	+	+	-	-
	50443.79	+	-	-	-	-	-
	50486.79*	+	-	-	-	-	-
	50202.79	-	F	-	-	-	-
	50504.79	-	-	+	-	-	-
	50510.79	+	D	+	-	-	-
	50528.78	+	-	-	-	+	-
	50538.79	+	-	-	-	-	-
	50561.79	+	-	-	-	-	-
	50629.79	+	-	-	-	-	-
	50873.79*	-	-	-	-	-	-
	50264.80*	-	-	+	-	-	2+
	50271.80*	+	-	-	-	-	-
	50317.80	+	D	-	-	-	-
	50359.80	-	-	-	-	-	-

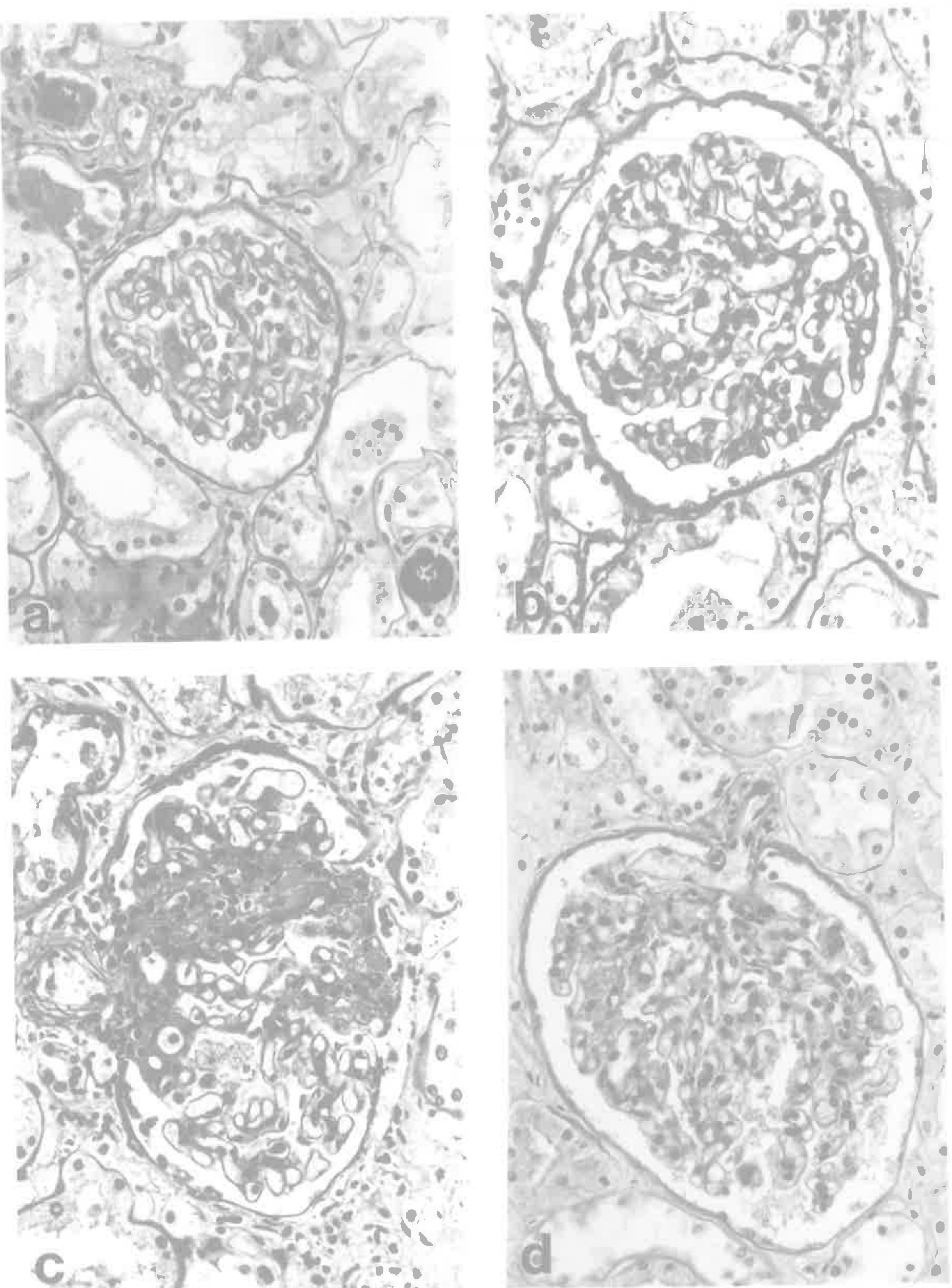


FIGURE 2.7
Glomeruli from patients with ALD showing a range of changes (PAS, X280).

a Normal size and cellularity (50132.79).

b Glomerulomegaly and normal cellularity (50493.79).

c Glomerulomegaly with hypercellularity (50010.79).

d Arteriolar hyaline (arrow) associated with an enlarged glomerulus (50493.80).

Table 2.7 Results of hepatic IIF in patients and controls

Patient category	Autopsy number	IIF of liver				
		IgA	IgG	IgM	C_3	Fibrinogen
Cirrhotic ALD with mesangial IgA deposits	50903.78	-	-	-	-	-
	50975.78	S	M	M	-	M
	50010.79	-	-	M	-	-
	50132.79	-	-	-	-	-
	50329.79	-	-	-	-	-
	50358.79	-	-	-	-	M
	50439.79	-	-	-	-	-
	50442.79	M	M	-	M	M
	50730.79	-	-	-	-	-
	50939.79	-	-	-	M	-
	50203.80	-	M	-	S	-
	50228.80	-	S	-	-	-
	50331.80	M	M	S	-	-
	50916.80	M	M	S	-	-
	50512.81	-	-	-	-	-
Cirrhotic ALD without mesangial IgA	50871.78	-	-	-	-	-
	50912.78	-	S	-	-	S
	50078.79	M	M	-	M	M
	50141.79	-	-	-	-	-
	50257.79	M	M	S	-	M
	50364.79	M	M	M	-	-
	50432.79	-	-	-	-	-
	50443.79	M	M	-	M	M
	50502.79	M	M	-	-	-
	50504.79	M	M	-	-	-
	50510.79	-	M	-	-	-
	50528.79	-	M	-	-	-
	50538.79	-	-	-	-	-
	50561.79	M	M	-	-	-
	50629.79	M	M	S	-	-
	50317.80	S	M	S	-	-
	50359.80	-	M	S	S	S
Non-cirrhotic ALD with mesangial IgA	50012.79	M	M	-	-	-
	50698.79	-	M	-	-	-
	50493.80	M	M	-	-	-
Non-cirrhotic ALD without mesangial IgA	50055.79	M	M	M	M	-
	50355.79	-	-	-	-	-
	50486.79	-	-	-	-	-
	50873.79	M	M	S	S	S
	50264.80	-	-	-	-	-
	50271.80	M	M	-	-	-
Other liver diseases	50885.78	-	-	-	-	S
	50234.79	-	-	-	-	-
	50436.79	-	S	S	S	-
Controls	3699.81	-	-	-	-	-
	3715.81	-	-	-	-	-
	50261.79	S	S	-	-	M
	50810.80	-	S	-	-	-
	722.81	-	S	-	-	-
	787.81	-	M	-	-	-
	788.81	M	M	-	-	-
	1263.81	-	S	-	-	-
	4782.81	-	-	-	-	-
	4783.81	-	-	-	-	-

m = mosaic continuous contours

s = spider outlines

See text

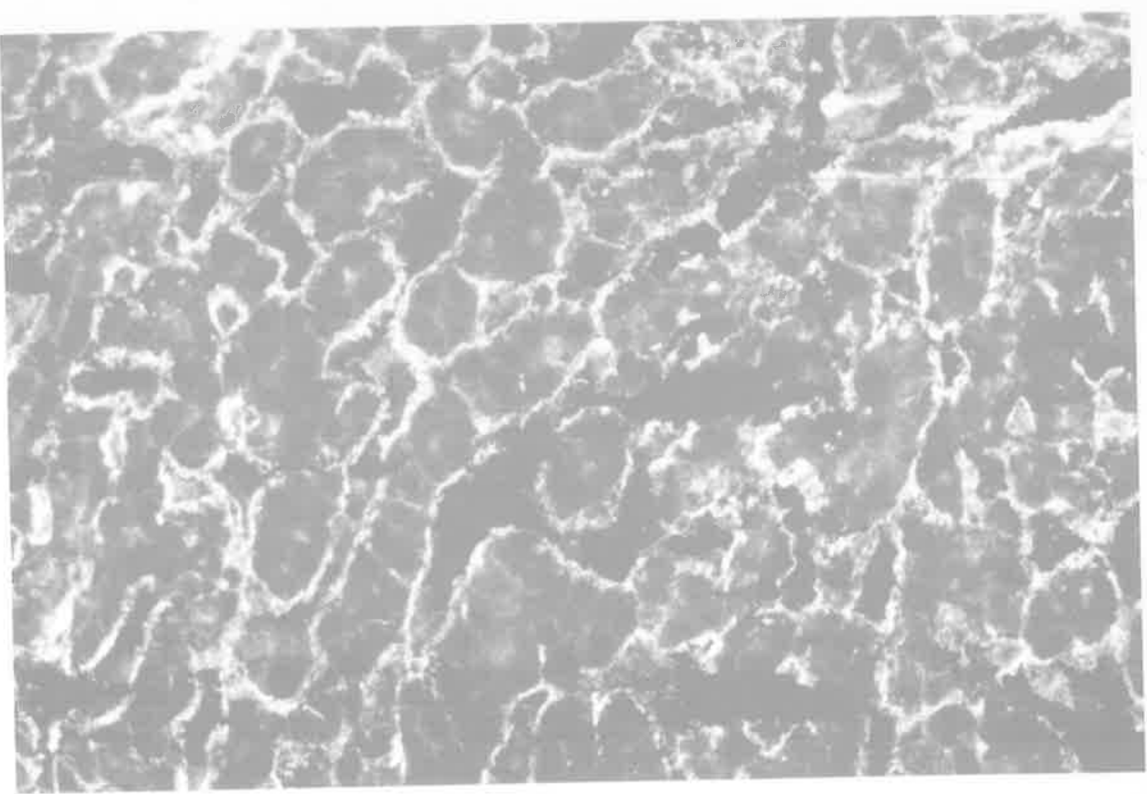


FIGURE 2.8
Liver from a patient with alcoholic cirrhosis (50442.79) and mesangial IgA deposition. The section shows a "mosaic" pattern of stain ing for IgA (IIF, X725).

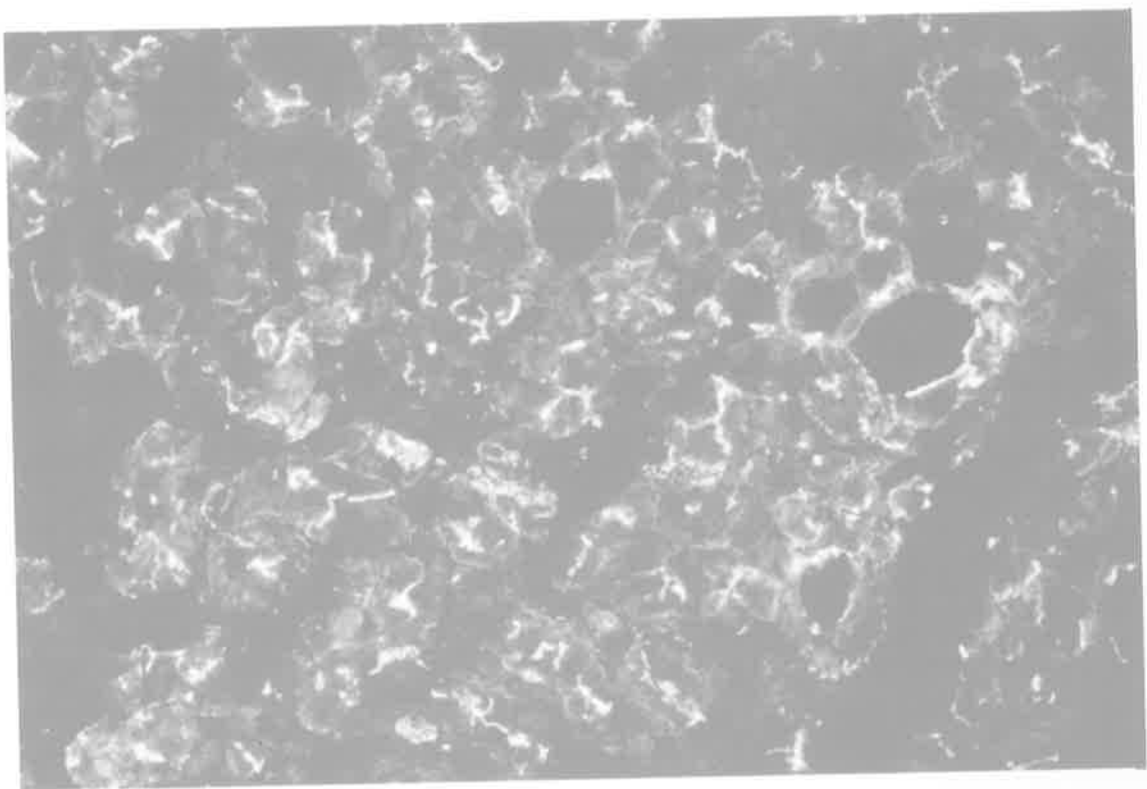


FIGURE 2.9
Liver from a patient with non-cirrhotic ALD and no mesangial IgA deposition (50873.79). The section shows intrahepatocyte "spider contours" after staining for IgM. The pattern suggests bile canalicular walls (IIF, X550).

E Skin DIF

Skin IF was performed on 30 patients with ALD and 17 controls. The results are shown in Table 2.8. IgA was seen in 21 of 30 patients with ALD, one of two patients with other liver diseases, and eight of 17 controls. The deposits usually were located in small vessels superficially in the papillae (Figure 2.10), or around the skin appendages. Infrequent intracellular deposits were seen in the deep dermis, presumably in plasma cells. Although cutaneous vascular deposits were more frequently seen in patients with ALD than in controls the difference was not significant.

F Choroid plexus DIF

Choroid plexus tissue was available for examination in 24 patients within the series, and the results are shown in Table 2.8. There was IgA in the walls of small vessels in three of the six choroid plexi available from patients with ALD, and in five of 16 from controls (Figure 2.11-2.12). There was no significant correlation between ALD and IgA deposition in choroid plexus vessels.

G Examination of the correlation between clinical or pathological features and mesangial IgA deposits

The mode of death in patients with cirrhotic ALD was compared in those with or without mesangial IgA deposition. Mesangial IgA deposition appeared to be more common amongst those patients with alcoholic cirrhosis who died in hepatic failure (13 of 21) than in those who died from alcohol-related or incidental causes (four of 13), but the difference was not significant ($p=0.5$). Of the patients with severe liver disease of alcoholic and non-

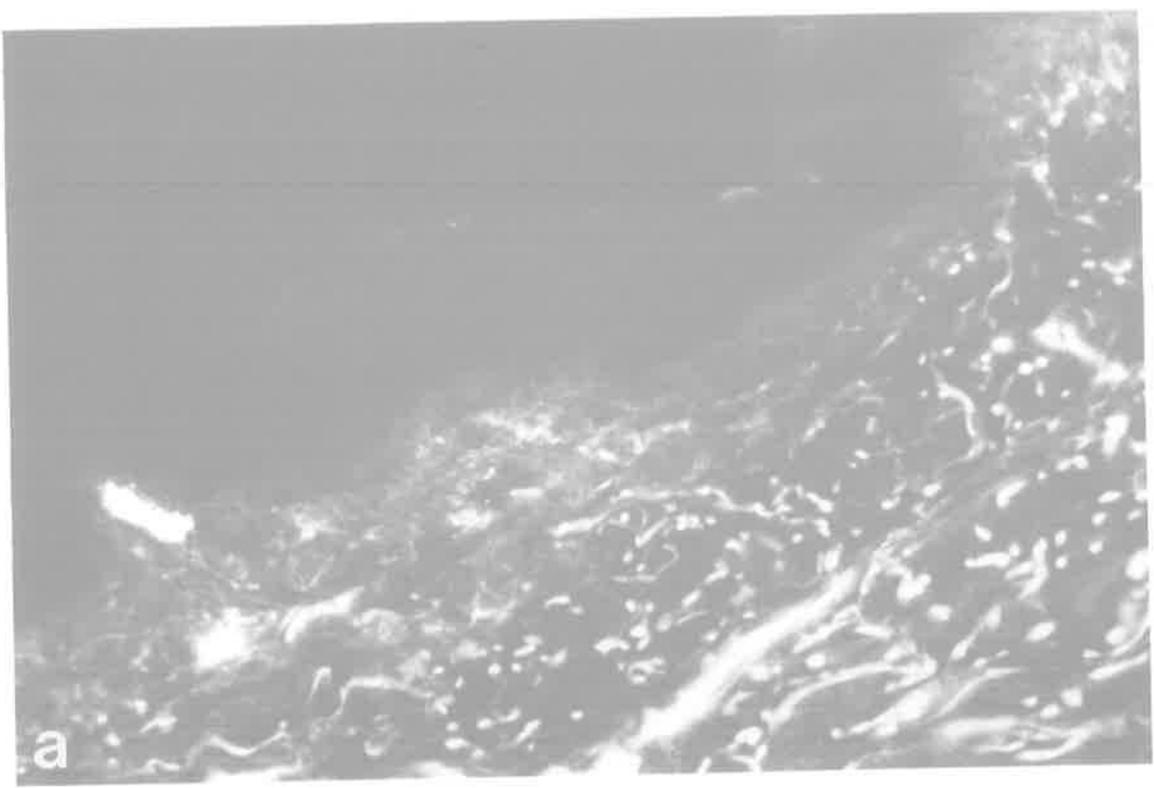
Table 2.8 Results of immunofluorescence performed on skin and choroid plexus in autopsy study

Patient Catergory	Autopsy Number	Cutaneous immunofluorescence						Choroid plexus immunofluorescence					
		IgA	IgG	IgM	Clq	C_3	Fibrinogen	IgA	IgG	IgM	Clq	C_3	Fibrinogen
Cirrhotic ALD with mesangial IgA deposits 12 (17)	50903.78	2	0	2	0	1	NT	-	-	-	-	-	-
	50939.78	0	0	0	2	0	2	-	-	-	-	-	-
	50975.78	4	0	0	0	3	1	-	-	-	-	-	-
	50010.79	2	0	0	0	0	1	-	-	-	-	-	-
	50132.79	1	0	1	0	0	1	-	-	-	-	-	-
	50329.79	4	0	4	1	1	3	-	-	-	-	-	-
	50358.79	4	1	4	0	0	3	-	-	-	-	-	-
	50439.79	4	0	4	3	0	3	-	-	-	-	-	-
	50442.79	0	0	0	0	0	3	-	-	-	-	-	-
	50730.79	2	0	3	0	NT	1	-	-	-	-	-	-
	50203.80	3	2	4	3	0	3	-	-	-	-	-	-
	50228.80	0	0	4	0	0	NT	0	0	4	0	0	NT
Non-cirrhotic ALD with mesangial IgA deposits 2 (3)	50012.79	3	0	3	0	0	3	-	-	-	-	-	-
	50698.79	0	0	2	0	0	0	0	0	1	0	0	1
Cirrhotic ALD without mesangial deposits 13 (17)	50912.78	2	0	1	0	0	3	-	-	-	-	-	-
	50078.79	3	1	1	0	1	3	-	-	-	-	-	-
	50141.79	0	0	0	0	0	3	-	-	-	-	-	-
	50257.79	0	0	3	0	0	3	-	-	-	-	-	-
	50364.79	3	0	1	0	0	3	-	-	-	-	-	-
	50432.79	1	0	2	0	0	0	-	-	-	-	-	-
	50502.79	1	0	0	0	0	1	-	-	-	-	-	-
	50504.79	0	0	3	0	0	3	2	0	1	0	0	2
	50510.79	4	2	3	2	0	4	1	0	1	0	0	1
	50528.79	1	0	2	0	0	2	-	-	-	-	-	-
	50538.79	4	0	0	0	0	2	-	-	-	-	-	-
	50561.79	3	1	0	0	0	3	2	0	4	3	1	3
	50629.79	0	0	1	1	NT	1	0	0	1	0	0	1
Non-cirrhotic without mesangial deposits 3 (6)	50055.79	2	0	4	0	0	4	-	-	-	-	-	-
	50486.79	2	0	4	3	1	4	-	-	-	-	-	-
	50873.79	0	0	0	0	NT	2	-	-	-	-	-	-
Other serious liver diseases 3 (6)	50234.79	3	0	1	0	0	3	-	-	-	-	-	-
	50436.79	0	0	0	0	0	0	0	0	2	0	0	0
	50137.80	-	-	-	-	-	-	2	0	3	0	0	1
Controls 21 (25)	50261.79	0	0	1	0	0	1	-	-	-	-	-	-
	50500.79	0	0	2	0	0	2	-	-	-	-	-	-
	50515.79	0	0	0	0	0	2	0	0	0	0	0	0
	50690.79	-	-	-	-	-	-	2	0	4	2	0	NT
	50696.79	4	2	1	0	NT	2	0	0	0	0	0	0
	50702.79	1	0	1	1	NT	0	0	0	2	0	0	2
	50781.79	0	0	2	0	0	1	0	0	0	0	0	1
	50915.79	3	0	4	3	NT	3	-	-	-	-	-	-
	50116.80	0	0	0	0	NT	0	1	0	0	0	1	0
	50222.80	0	0	1	0	0	1	0	0	0	0	0	NT
	50224.80	1	0	4	1	0	3	3	0	0	0	0	0
	50810.80	2	0	2	0	2	1	3	0	2	0	0	2
	50915.81	2	0	3	1	0	0	-	-	-	-	-	-
	722.81	1	0	0	1	0	0	0	0	0	0	0	NT
	787.81	0	0	4	2	0	0	3	0	2	1	0	0
	788.81	4	0	1	0	0	1	0	0	0	0	0	0
	1262.81	0	0	2	0	0	1	-	-	-	-	-	-
	1263.81	0	0	1	1	0	0	0	0	0	4	4	0
	4674.81	-	-	-	-	-	-	0	0	0	0	0	0
	4782.81	-	-	-	-	-	-	0	0	0	0	0	1
	4783.81	-	-	-	-	-	-	0	0	0	0	0	0

Staining intensity graded subjectively from 0-4

NT = not tested
 - = tissue not available

Number beneath patient category refers to number in whom extra mesangial tissue was tested, with the total in that category in parenthesis



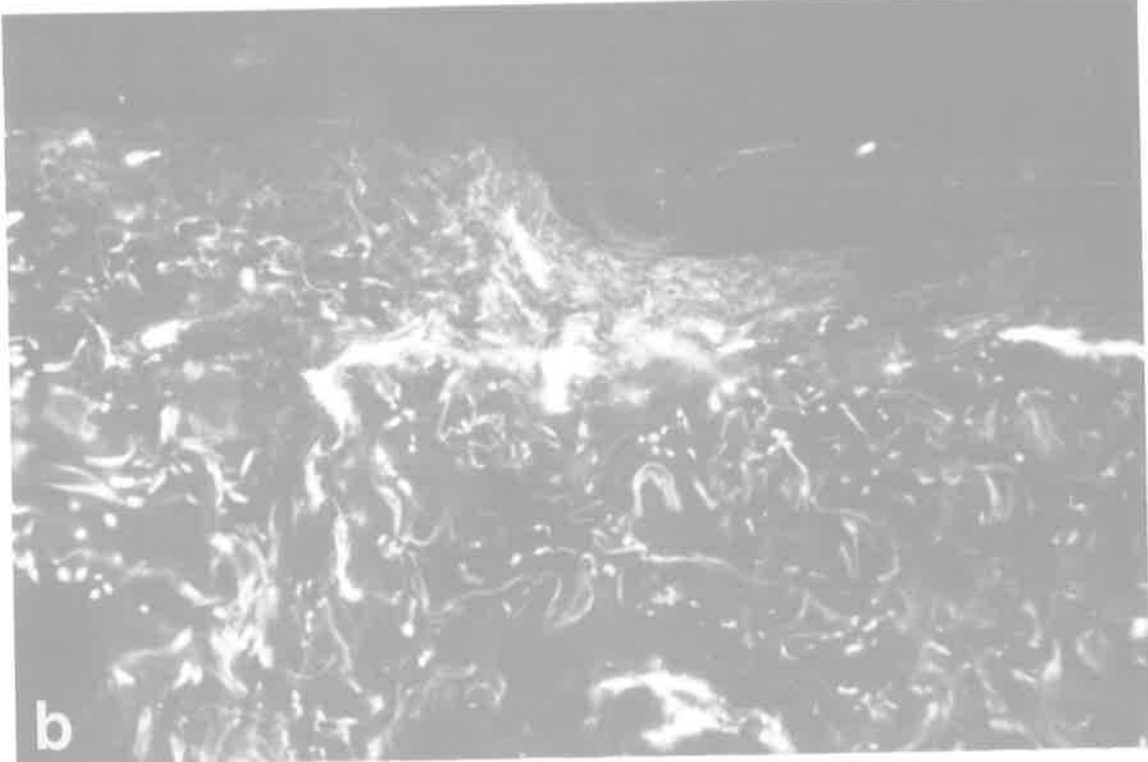


FIGURE 2.10
Normal forearm skin from a patient with ALD and mesangial IgA deposition (50329.79). The sections show a faint outline of the epidermis, with autofluorescent collagen in the dermis (DIF, X650).

a Bright granular IgA is seen in the wall of a papillary dermal capillary (mid-left) and in the subpapillary horizontal plexus.

b Similar bright granular IgM staining is seen in a papillary dermal capillary (centre) and horizontal vessel (right).

FIGURE 2.11
Choroid plexus from a patient with ALD (50561.79) stained for IgA, showing the villous structure covered with columnar epithelial and goblet cells. Note faint linear outline of epithelial basement membrane and granular 2+ reaction in capillary wall (DIF, X3200).

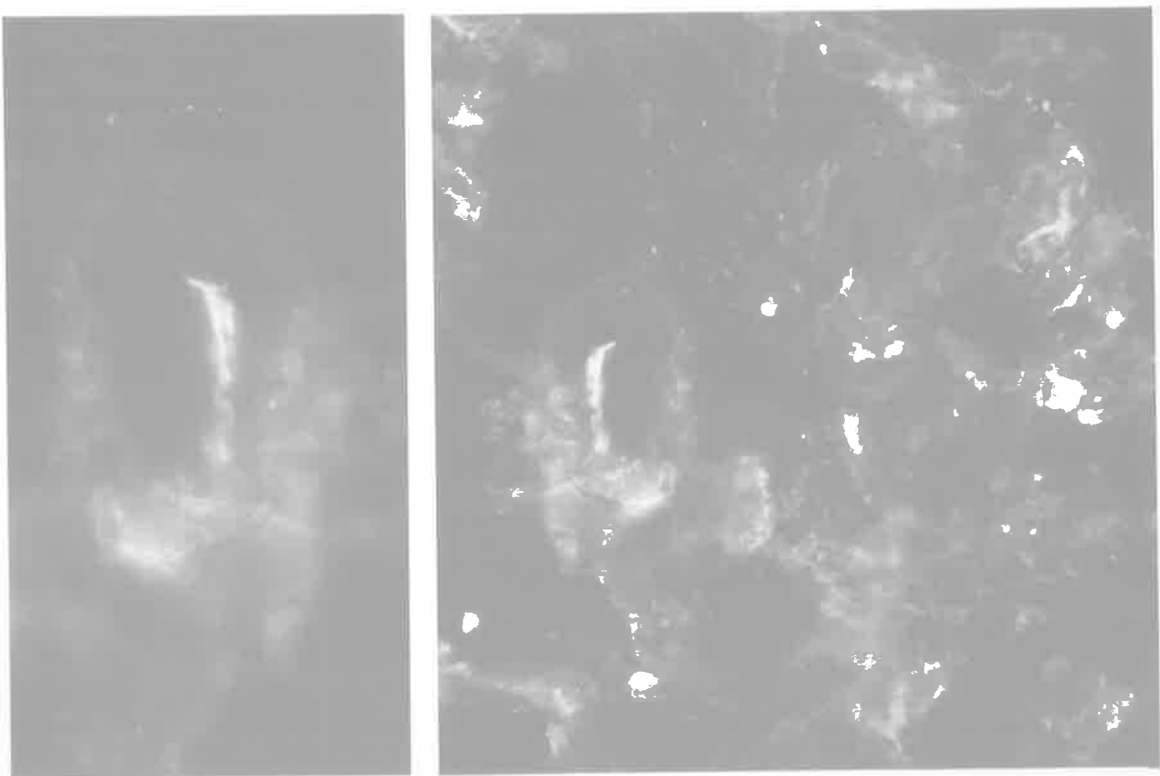


FIGURE 2.12
Choroid plexus from the same patient as Figure 2.11, stained for IgA, and showing more clearly the staining on two sides of a capillary wall. There is a granular 2+ reaction (DIF, X2500, inset X3200).

alcoholic origin, hypertension was found in 13 of 47, renal failure in 14 of 42 and abnormal urinalysis in 20 of 47 (Table 2.9). None of these clinical parameters was significantly more common in patients with or without mesangial IgA deposition.

The correlation between cutaneous and mesangial IgA deposition in the autopsy series is shown in Table 2.10. Cutaneous IgA was seen in 11 of 15 cases associated with mesangial deposits, and in 19 of 34 without mesangial deposits. The association was not significant (p=0.1).

The correlation between choroid plexus and mesangial IgA deposition is shown in Table 2.11. DIF was performed on choroid plexus from only two patients in the series with mesangial IgA deposition and IgA was found in neither. Choroid plexus IgA was found, however, in the absence of mesangial IgA in three of four patients with cirrhotic ALD, one of two with other liver diseases, and five of 16 controls. During the period when the autopsy study was being performed, material from three patients with myeloma (kappa light chain (1), IgG (2)), one with membranous nephropathy, and one with primary IgA nephropathy, were available for study. Only the latter subject showed choroid plexus IgA. Thus, only one of three patients with mesangial IgA deposition showed IgA deposits in the choroid plexus, compared with nine of 26 patients without mesangial IgA. There was no correlation between choroid plexus and mesangial IgA deposits (p=0.5).

Hepatic staining for IgA was apparently more common in patients with ALD than in control patients but in view of the low numbers

Table 2.9 The relationship between the presence of mesangial IgA deposits and clinical status in patients with liver disease. Number of patients with data available in parenthesis

	Hypertension	Renal failure	Abnormal urinalysis
Patients with mesangial IgA deposition	8 (21)	9 (19)	11 (20)
Patients without mesangial IgA deposition	5 (26)	5 (23)	9 (27)
TOTAL	13 (47)	14 (42)	20 (47)

Table 2.10 Correlation between cutaneous and mesangial IgA deposition in the autopsy series

Patient group	IgA in skin of autopsy patients	
	With mesangial deposits	Without mesangial deposits
Cirrhotic ALD	9 (12)	9 (13)
Non-cirrhotic ALD	1 (2)	2 (3)
Other liver disease	NT	1 (2)
Controls	1 (1)	7 (16)
TOTAL	11 (15)	19 (34)

Table 2.11 The correlation between the deposition of IgA in the choroid plexus and the mesangium of autopsy patients

Patient category	IgA in choroid plexus	
	With mesangial IgA	Without mesangial IgA
Cirrhotic ALD	0 (1)	3 (4)
Non-cirrhotic ALD	0 (1)	NT
Other liver disease	NT	1 (2)
Controls	NT	5 (16)
Membranous nephropathy	-	0 (1)
Primary IgA nephropathy	1 (1)	-
Myeloma	-	0 (3)
TOTAL	1 (3)	9 (26)

in the latter group the difference was not significant. There was no correlation between hepatic and mesangial IgA deposition.

There was, however, a striking association between alcoholic cirrhosis and mesangial IgA deposits (Table 2.12). They co-existed in 50% of the cases in this study. Mesangial deposits were seen in 33% of non-cirrhotic alcoholics and only 4% of a control population. The correlation between ALD and mesangial IgA deposits was significant (p=0.01), but there was no difference between the prevalence in cirrhotic and non-cirrhotic patients with ALD (p=0.5).

The presence of mesangial IgA deposition showed a significant correlation with ALD, but extrarenal IgA deposits correlated neither with ALD (Table 2.13) nor with mesangial deposits.

Table 2.12 The incidence of mesangial IgA deposition in patients with liver disease

Patient category (total number)		Mesangial IgA deposits
Cirrhotic ALD	(34)	17 (50%)
Non-cirrhotic ALD	(9)	3 (33%)
Other liver disease	(6)	1 (17%)
Controls	(25)	1 (4%)

Table 2.13 The prevalence of mesangial and extramesangial IgA deposits in patients with ALD

Patient category	IgA deposits			
	Mesangium	Liver (mosaic pattern)	Skin	Choroid plexus
ALD	20 (43)	16 (41)	21 (31)	3 (6)
Control	1 (25)	1 (10)	8 (17)	4 (16)
	($p<0.01$)	($p>0.1$)	($p>0.1$)	($p>0.5$)

DISCUSSION

In this autopsy study, 50% of patients with alcoholic cirrhosis and 33% of those with non-cirrhotic ALD were found to have mesangial IgA deposition. This is in contrast to 4% of normal and hospital controls, and 17% of patients with a variety of other serious liver conditions.

This study has shown that in Australia, as in France, a large percentage of patients with alcoholic cirrhosis have mesangial IgA deposition. Apart from confirming the work of Callard et al (1975) and Berger et al (1978) in cirrhotic patients, this study examined a small number of patients with non-cirrhotic ALD and demonstrated an incidence of mesangial IgA nephritis significantly above that of the control group. The incidence of IgA nephropathy in the general population is difficult to assess since the disease is of relatively low mortality and may be asymptomatic.

One of the major problems in any study of disease populations, is the selection of a suitable control group. Here a heterogenous group of sudden Coronial deaths was chosen. Clearly, many patients dying in hospital have malignancies and infections which could be associated with IC nephritis. Similarly, it is possible that those subjects who have died from violent or traffic-associated deaths were also alcohol abusers. Since evidence relating to alcohol consumption was not always available in Coronial cases, the absence of alcohol-associated changes within the liver was accepted as the only criterion of normality.

Despite these reservations, the control incidence of mesangial IgA deposition in this study (4%) has been confirmed by a series of consecutive autopsies performed on patients with traumatic deaths in Singapore (Sinniah 1983). The sex ratio of the control autopsies approximated that of the patients with ALD, but the ages could not be matched and were statistically dissimilar. The patients with other liver diseases were significantly different from both controls and patients with ALD, being older and more often female.

The light microscopical appearances of the kidneys in secondary IgA nephropathy are similar to those in primary IgA nephropathy, with mesangial proliferation and hyaline deposition. Diffuse mesangial hypercellularity is, however, less common in primary IgA nephropathy than in patients with ALD (personal unpublished observations).

A correlation between extrarenal IgA deposits and ALD could not be established. Extrarenal IgA deposits did not occur significantly more often in patients with mesangial deposits than in those without. These results are in contrast to the clear distinction found by Tomino et al (1981a). This group examined the arteries of muscle biopsies from the loin obtained at the time of renal biopsy, and showed that there was IgA in five of 15 patients with primary IgA nephropathy, but none of 11 patients with IgA negative renal biopsies. Miss A E Thompson, however, examined multiple levels, and because of this, may have found more cutaneous staining in controls. The data presented here agree with some previous reports by showing that skin biopsy is

not useful in the diagnosis of mesangial IgA deposition (Thompson et al 1980; Moatamed et al 1981). Further there was no correlation between dermal papillary IgA deposition and ALD. Swerdlow et al (1982b) have suggested such a correlation on the basis of synchronous liver and skin biopsies but, unlike the study reported here and previously (Thompson et al 1980), showed no deposits in the dermal vessels of control patients.

The finding here of a mosaic-like pattern of IF in 16 of 41 (39%) livers from patients with ALD compared with one of 10 controls is similar to the findings of Kater et al (1979) and Swerdlow et al (1982a), although the association was not significant. The lack of a significant difference between the incidences is a reflection of the small numbers of Coronial liver samples obtained for IF. Kater et al (1979) using frozen sections of liver biopsy material and DIF, found a continuous pattern of IgA in 50 of 58 patients with ALD and only 16 of 246 control patients. Swerdlow et al (1982a) found a similar pattern by DIF in 50 of 64 ALD biopsies and three of 72 non-ALD biopsies, but used deparaffinised and trypsin-digested sections. The lower incidence of IgA-staining in this study compared with those of Kater et al (1979) and Swerdlow et al (1982a), who both examined biopsy material, may reflect the dissociation of non-bound immunoglobulin in autopsy tissues. Chandy et al (1983) have also examined autopsy liver tissue from patients with liver disease and noted a fainter, more diffuse pattern of IgA staining, which they explained as an effect of postmortem autolysis. The cause for the relative prominence of IgG staining in this study is unclear. Swerdlow et al (1982a) believed that IgG and IgM

were non-specific findings, being unrelated to the presence of ALD.

The spider-like outlines seen in this study appeared to correspond to intrahepatic bile canaliculi. This pattern was seen more often, however, with reagents for IgM and IgG than IgA. Chandy et al (1983), also working with autopsy livers, recently described IgA in the walls of bile canaliculi with appearances similar to those here described as spider-like.

The clinical parameters studied in patients with ALD showed no correlation with mesangial IgA deposition. Hypertension, abnormal urinalysis or elevated serum creatinine were not more common in patients with mesangial IgA deposits. This confirms the occult nature of mesangial IgA deposition found by Callard et al (1975) when they examined the kidneys of cirrhotic patients fit enough to undergo portacaval shunting procedures. None of their 10 patients, of whom nine had mesangial IgA deposition, had proteinuria, hypertension or elevated serum creatinine levels. Conversely the patients with ALD studied by Nochy et al (1976) were selected on the basis of overt GN, all had proteinuria, and half had elevated serum creatinine levels. The patients studied here were decompensating patients who died in hospital with a multitude of clinical problems. Blood pressure, urinalysis and renal function were often abnormal in such patients, with and without mesangial IgA deposition.

Hepatitis screening showed evidence of previous HBV infection in two of 14 patients with ALD and two of three controls but

active infection or carrier status was not seen.

The autopsy study formed the basis of this thesis by confirming the high incidence of mesangial IgA deposition in ALD. Renal material obtained from these autopsies was used in a series of experiments to characterise the immunochemical nature of the mesangial IgA deposits in ALD (Chapter 3) and provided whole kidneys for elution studies (Chapter 4). These subsequent studies were designed to elucidate the immunopathogenesis of mesangial IgA deposition secondary to ALD.

CHAPTER 3

The immunochemical characterisation of mesangial IgA deposits in patients with alcoholic cirrhosis

INTRODUCTION

The finding of mesangial IgA deposits secondary to alcoholic cirrhosis allows speculation about the origin of the immunoglobulin. This may, be resolved by examining the immunochemical characteristics of such deposits. Woodroffe et al (1982), have suggested that in this group of patients the mesangial deposits and the documented increases in circulating IgA and IgA-class IC might be accounted for, either by a defective mucosal barrier with increased absorption of floral or dietary antigens and systemic hyperimmunisation, or by defective hepatic clearance of antigens, IgA polymers or complexes.

IgA is present in the circulation mainly as monomeric A_1 subclass units, but at mucosal sites it is secreted actively as A_1 or A_2 dimers (Delacroix et al 1982a), polymerised by J chain, and transported by binding to SC. The bowel is the main site of IgA secretion in the body. Thus the presence of J chain, subclass A_2 or SC in the mesangium, would support a mucosal origin of the IgA. SC rarely has been identified in any form of mesangial IgA nephritis (McCoy et al 1974; Dobrin et al 1975) and the reported presence of J chain (Conley et al 1980b) must be qualified because this can be found with IgM which often accompanies the IgA deposits (Spargo et al 1980). The IgA subclass studies so far reported have been contradictory (Conley et al 1980b; André et al 1980; Tomino et al 1981b).

It has also been suggested that primary IgA nephropathy is mediated not by IC, but the deposition of IgA polymers from the

circulation (Egido et al 1980; Lopez-Trascasa 1980). Tomino et al (1982a) have shown eluted mesangial IgA to be of polymeric size.

The capacity to bind to free SC (Rádl et al 1971; Brandtzaeg 1974a) remains an important method of distinguishing polymeric J-chain containing immunoglobulins from monomers.

Egido et al (1980) showed SC binding capacity in 16 of 20 biopsies with primary IgA nephropathy compared with six of seven with HSP and none of three with SLE. Subsequently Bené et al (1982) have shown SC binding capacity in all of a series of 15 patients with IgA nephropathy. Patients with ALD represent a group of patients where a mucosal origin for the mesangial IgA deposits may be predicated (Woodroffe et al 1982). In support of this, Sancho et al (1981) found SC binding capacity in all of six acid eluates from autopsy kidneys and in all of three frozen sections from patients with mesangial IgA deposition and ALD.

The present study examines the immunochemical characteristics of the IgA mesangial deposits in patients with ALD and compares them with the deposits of primary IgA nephropathy, SLE and HSP. A_1 and A_2 subclasses were sought by conventional IIF using sheep antisera and by an avidin-biotin system using monoclonal anti-body reagents. J chain and SC were sought by IIF. The ability of mesangial IgA deposits to bind purified SC was examined.

MATERIALS

Postmortem tissue from 16 patients with alcoholic cirrhosis (Chapter 2) shown to have mesangial deposits of IgA and C_3 were compared with renal biopsies from patients with primary IgA nephropathy (11), HSP (7), and SLE (5). The renal biopsies were arbitrarily coded 1-23. Patients' autopsy code numbers were shortened by removing the final two digits which designated the year of death. IF negative renal tissue from autopsies (2) and biopsies (7) were examined as controls. The frozen tissue was collected, stored and sectioned as previously described (Chapter 2). Purified human SC was a gift from Dr J LaBrooy, Department of Medicine, RAH.

METHODS

A . Direct immunofluorescence

DIF for IgA, and IgM was performed as described in Chapter 2.

B Indirect immunofluorescence

i Using antisera against J chain and SC

Sections received a preliminary five minute wash in PBS, and were then incubated in a moist chamber with either 1:5 rabbit anti-human J chain (Nordic Immunologic Laboratories, Tilberg, Netherlands) or 1:4 rabbit anti-human SC (Hoechst) which had been absorbed with 1:10 normal human serum (NHS) for 45 minutes. Incubation of sections with 1:4 normal rabbit serum was used as a control.

- The sections were washed in PBS for 20 minutes and stained for 30 minutes with 1:5 FITC-conjugated goat anti-rabbit IgG (Dakopatts) which had previously been absorbed with 1:10 NHS for 30 minutes.

- Sections were washed for 20 minutes in PBS and mounted in glycine buffered glycerol at pH 8.6.

The specificity of the rabbit antisera was examined by staining sections from normal human small bowel biopsies obtained by Crosby Capsule and treated according to the method of Savilahti (1972).

ii <u>Using antisera against IgA subclasses</u>

- Sections received a preliminary five minute wash in PBS, and were incubated in a moist chamber for 30 minutes with 1:4 dilution of antisera raised in sheep to A_1 and A_2 (Nordic). Incubation of sections with 1:4 normal sheep serum was used as a control.

- Sections were washed in PBS for 20 minutes, and stained with FITC-conjugated rabbit anti-sheep IgG (Wellcome) which had been absorbed with 1:10 NHS for 30 minutes. Sections were washed in PBS for 20 minutes and mounted in glycine buffered glycerol at pH 8.6.

- The specificity of these reagents was examined by staining sections from normal human small bowel biopsies and an IgA_1 myeloma bone marrow smear.

iii Using murine monoclonal antibody

Monoclonal antibodies were obtained from a mouse myeloma cell line, P3-X63-Ag8 653 hybridised with spleen cells from immunised BALB/C mice (IgA_1) or AJ mice (IgA_2) (Becton Dickinson, Sunnyvale, CA). The monoclonal antibodies were tested initially by IIF using FITC-conjugated rabbit anti-mouse IgG (Cappel, Cochranville, PA) and, later, in an avidin-biotin system (Warnke and Levy 1980). Subsequently, only the avidin-biotin system was used because in nine weakly positive cases, by the monoclonal avidin-biotin system, the FITC-conjugated rabbit anti-mouse system showed a reduction in the staining intensity in four to A_1 and six to A_2. Overall, in six instances these reductions amounted to a change from a positive to negative staining reaction. The avidin-biotin system was clearly more sensitive.

. Sections were washed for two minutes in PBS and were incubated in a moist chamber with the monoclonal antibody (20 μg/ml) for 30 minutes. Staining was controlled by incubation with 1:10 normal mouse serum (NMS), and by use of PBS instead of monoclonal antibody.

. Sections were washed for 10 minutes in PBS, and were incubated in a moist chamber with biotinylated goat anti-mouse IgG (Tago, Burlingame, CA) (500 μg/ml).

. Sections were washed for 10 minutes in PBS and were stained with FITC-conjugated avidin (Becton Dickinson) (50 μg/ml).

. Sections were washed for 10 minutes in PBS and mounted in glycine buffered glycerol pH 8.6. The specificity of the murine antibody was examined by staining sections of normal human small bowel biopsies and a human A_1 myeloma bone marrow smear.

C SC binding

. The binding capacity of the SC preparation was tested by incubation at 200 μg/ml with a jejunal biopsy, for 30 minutes in a moist chamber, followed by IIF staining for human SC as outlined above. A control slide pre-incubated with PBS was used to demonstrate the distribution of native SC in the section.

. The SC binding capacity of the mesangial IgA deposits in renal sections was tested by incubation of adjacent sections with either SC or PBS followed by IIF for human SC as described above.

D Statistics

The significance of differences in J-chain content or SC binding between the disease groups and in relation to IgM were calculated using a chi-square test with a Yates correction.
A ranked Mann-Whitney U test was applied to the SC binding capacities of deposits in primary IgA nephropathy compared with those in alcoholic cirrhosis. A Spearman's rank test was applied as a measure of covariance to the relationship between IgM staining and J-chain staining or SC binding capacity (Siegel 1956).

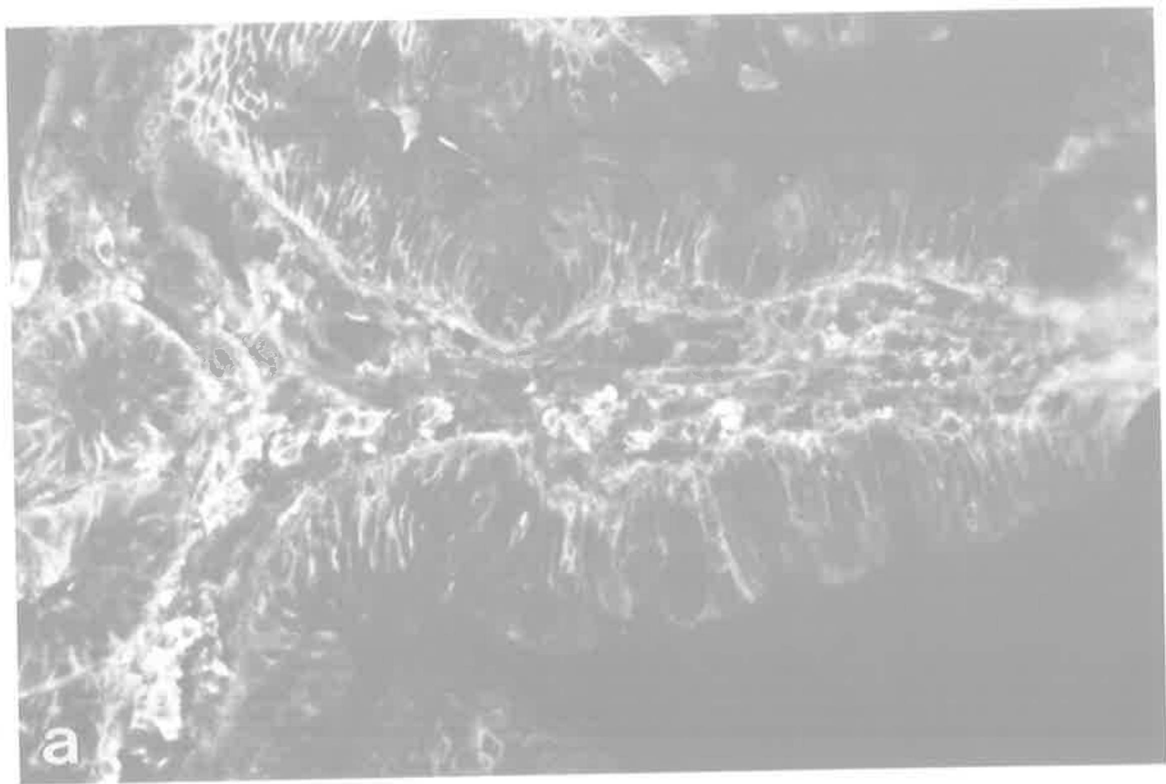
RESULTS

Antiserum to human J-chain stained the cytoplasm of lamina propria plasma cells but not enterocytes. Antiserum to human SC stained both lamina propria plasma cell cytoplasm and enterocytes, the latter exhibiting a "picket fence" distribution along lateral walls and basal surfaces. After absorption with NHS, the antiserum to human SC stained only enterocytes (Figure 3.1).

Sheep antisera to A_1 and A_2 subclasses were tested on normal human jejunum and showed similar bright 3+ staining of lamina propria plasma cell cytoplasm. Neither antiserum stained enterocytes. Staining of an IgA_1 myeloma bone marrow smear however, gave a 3+ reaction for A_1 and 1+ reaction for A_2 (Figure 3.2).

Murine monoclonal antibodies tested on normal small intestine showed a 2+ reaction for large numbers of lamina propria plasma cells stained for subclass A_1 and 4+ reaction for lesser numbers of plasma cells stained for A_2 (Figure 3.3). A_2 subclass staining was weakly present on the borders of some enterocytes suggesting a reaction with bound IgA. The IgA_1 myeloma bone marrow smear stained with monoclonal antibody to A_1 showed a bright cytoplasmic 4+ reaction, while for A_2 there was faint nuclear but no cytoplasmic staining (Figure 3.4). Faint nuclear staining was noted also with the NMS and PBS controls.

The results of the renal IF studies are shown in Table 3.1 and Figure 3.5 Using sheep antisera, 10 of the 11 patients with



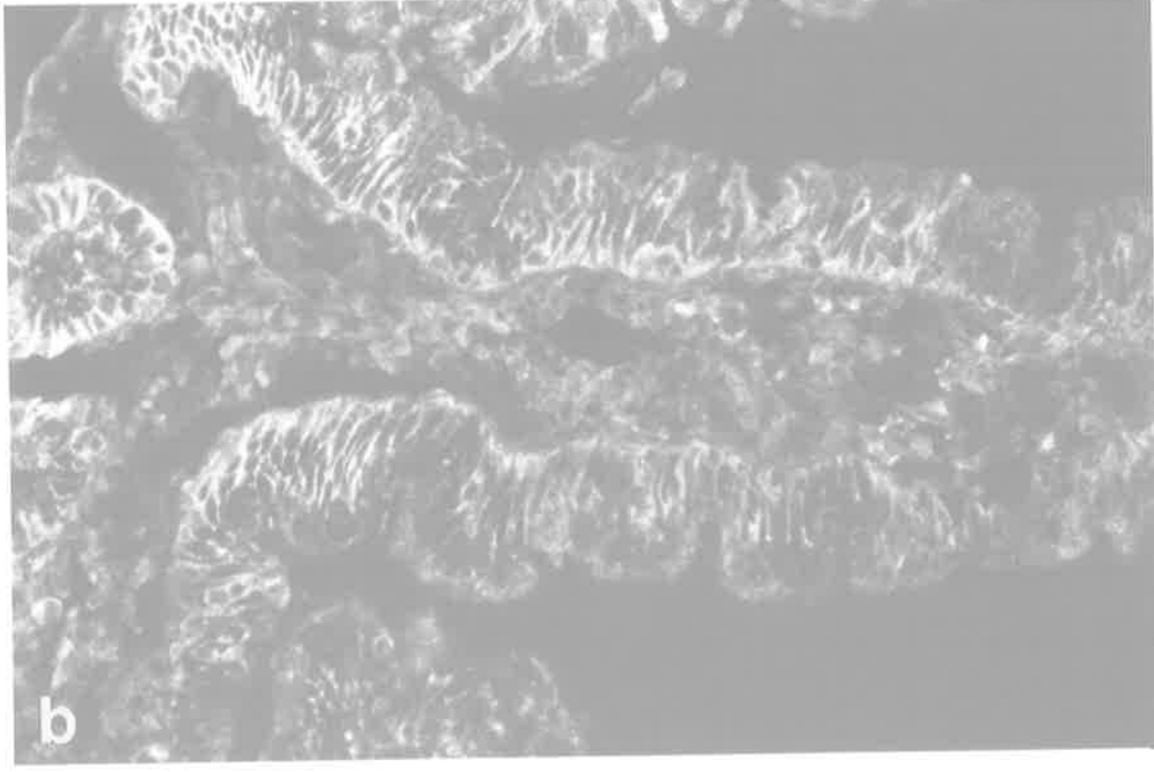
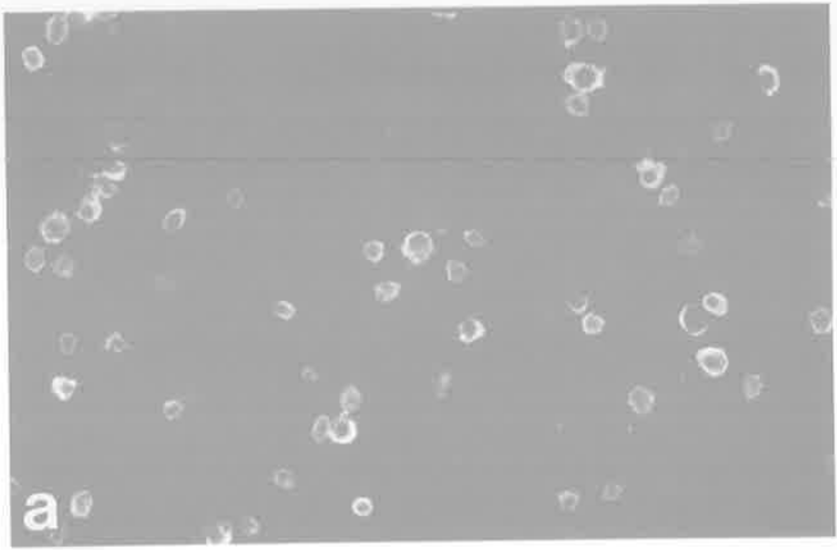


FIGURE 3.1
A normal human jejunal biopsy, stained with rabbit anti-human SC (a) unabsorbed, (b) absorbed with NHS, and FITC goat anti-rabbit IgG. Both photographs show a "picket-fence" distribution of staining for SC. Plasma cell staining (a) presumed to be a cross-reaction with IgA is lost after absorbing the antiserum with NHS (IIF, X1000).



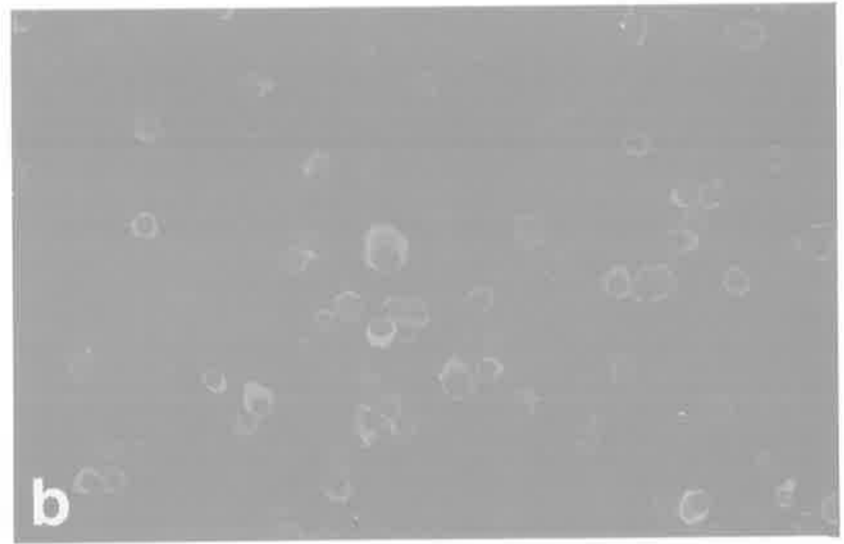
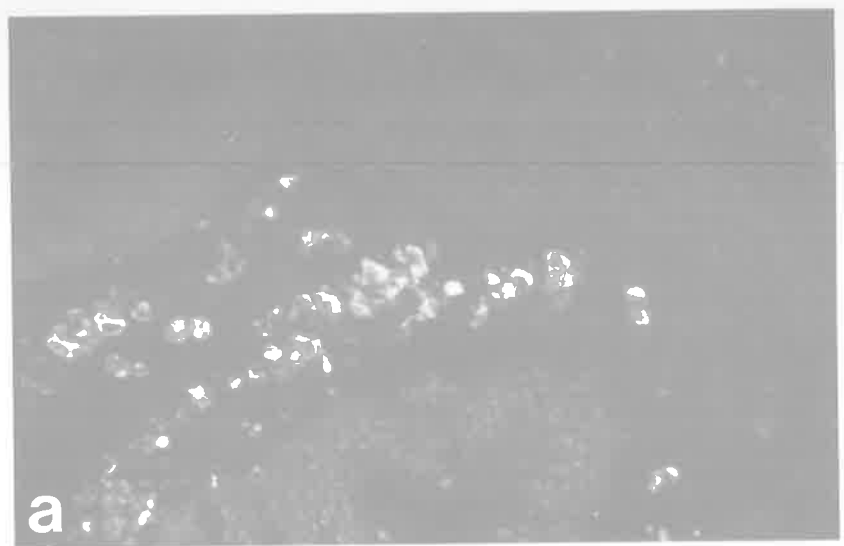
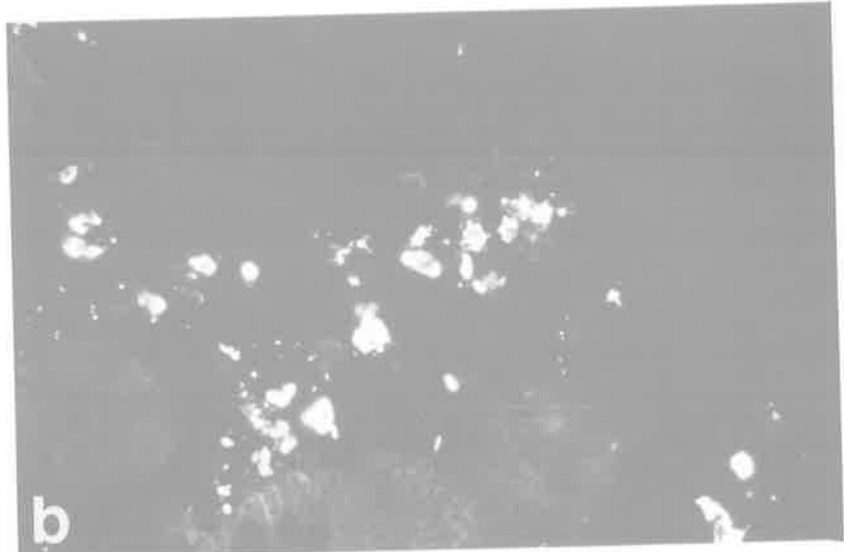


FIGURE 3.2
Bone marrow smears from a patient with an IgA_1 myeloma. Stained with sheep antisera to IgA subclasses and FITC rabbit anti-sheep IgG. (a) The staining for A_1 is graded 3+. (b) A_2 staining is graded 1+. (c) A control section was stained with FITC anti-sheep IgG after incubation with NSS and was negative (IIF a and b, X300; c, X150).





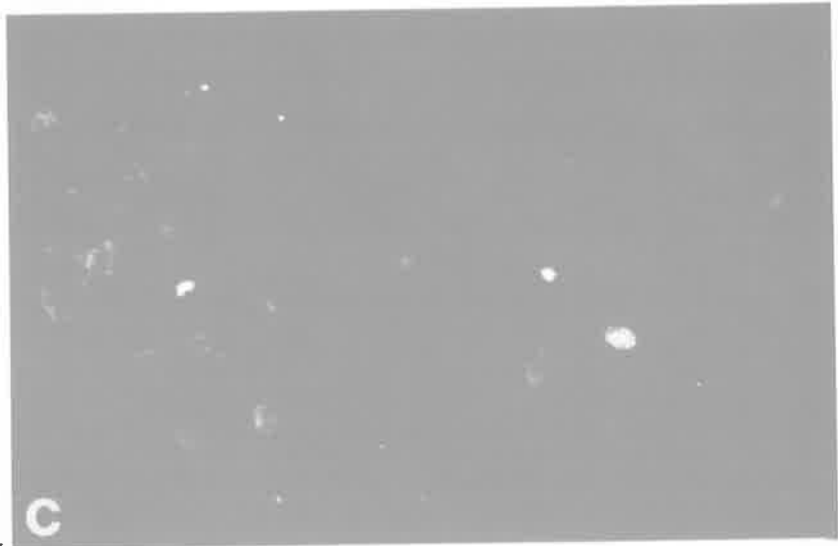
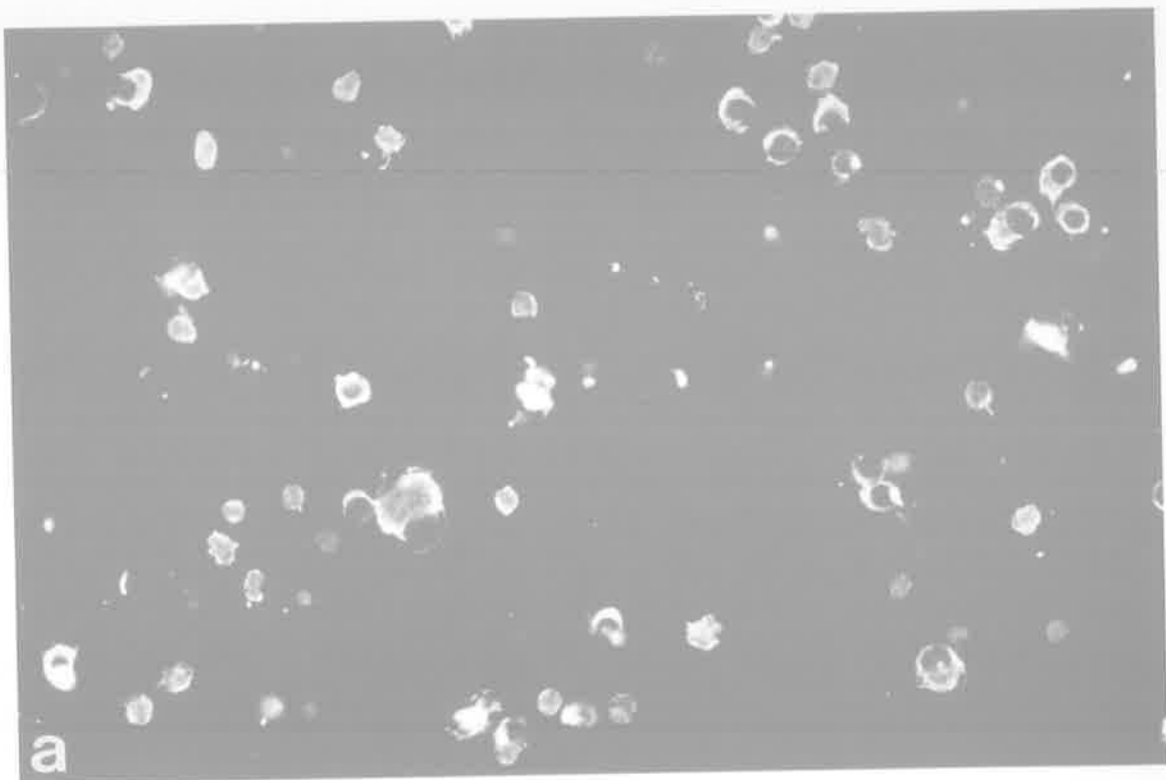


FIGURE 3.3
Avidin-biotin linked IIF of sections of a normal human jejunal biopsy using (a) monoclonal antibody to IgA_1, (b) monoclonal antibody to IgA_2 and (c) NMS. There are more plasma cells staining for IgA_1 than IgA_2 but the latter show a greater intensity of staining. Faint nuclear staining is seen. Staining for A_2 seen on the walls of enterocytes may represent IgA-SC complexes (X300).



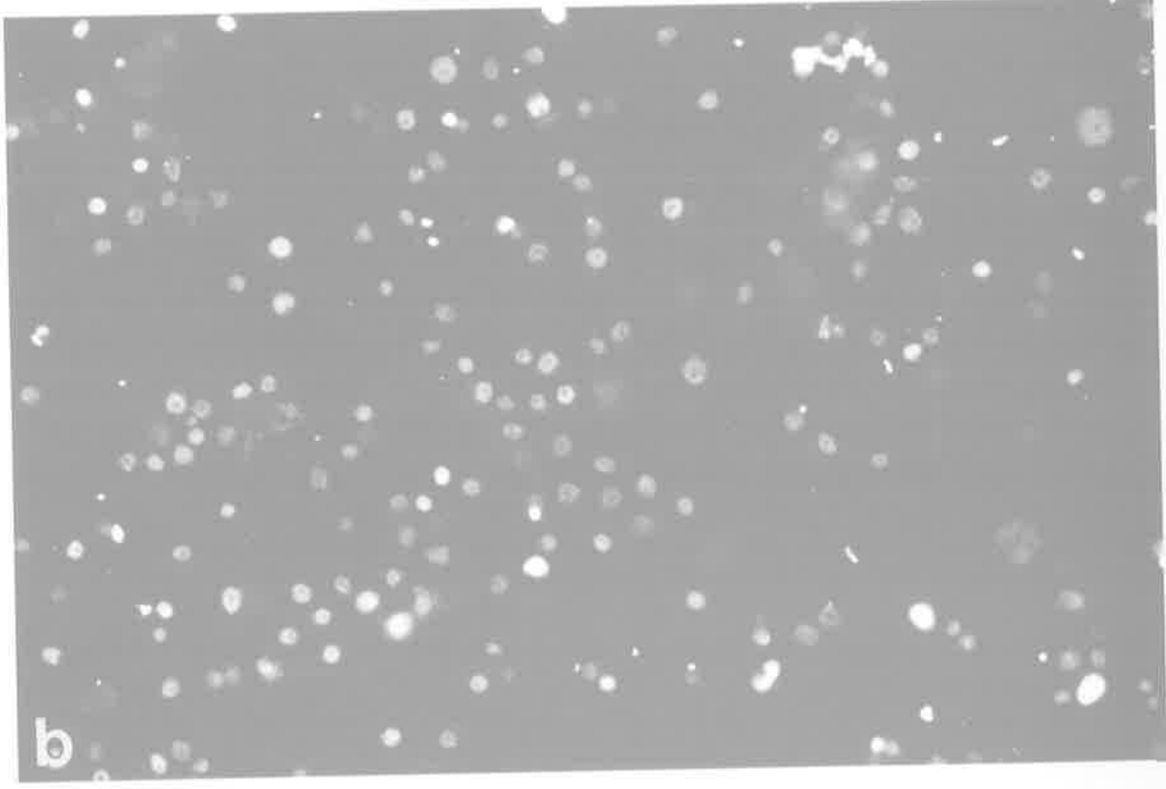


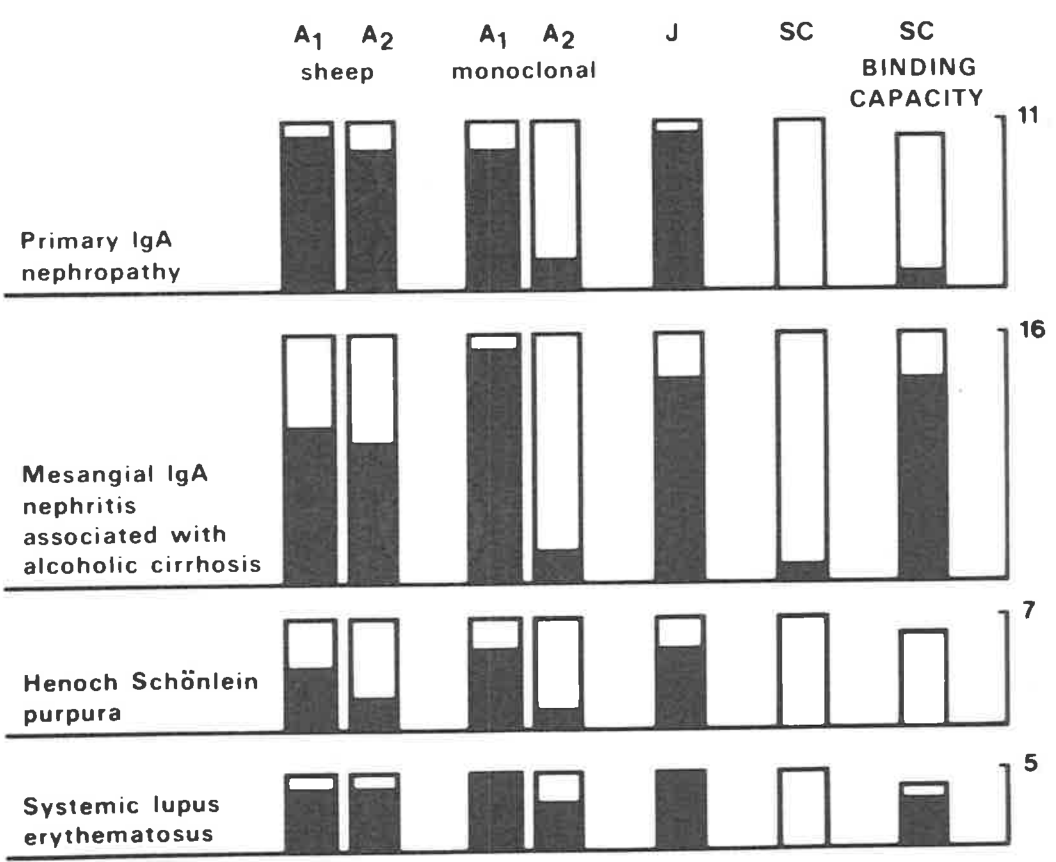
FIGURE 3.4

Avidin-biotin linked IIF of a bone marrow smear from a patient with an IgA_1 myeloma. The smears are stained with (a) monoclonal antibody to IgA_1, (b) monoclonal antibody to IgA_2. There is a cytoplasmic 4+ reaction in (a) but no cytoplasmic fluorescence in (b). Note once again the faint nuclear staining (X900).

Table 3.1 The mesangial immunofluorescence and SC binding capacity of patients with various diseases showing mesangial IgA deposits

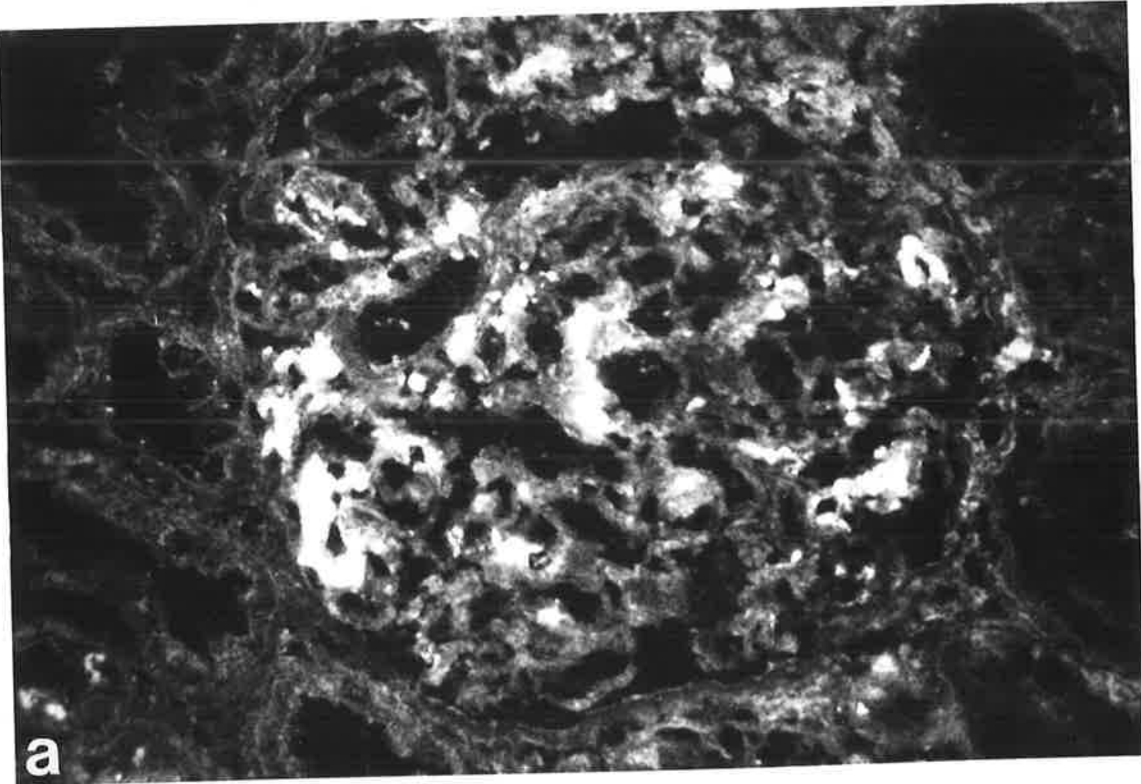
Patient category	Patient no. (#)	Mesangial immunofluorescence								SC binding capacity
		IgA	A subclasses				IgM	J	SC	
			Antiserum A_1	Antiserum A_2	Antibody A_1	Antibody A_2				
Mesangial IgA nephritis associated with alcoholic cirrhosis	50010	4	0	0	1	0	0	3	0	0
	50132	2	1	1	3	0	4	1	0	4
	50203	3	0	0	2	0	4	1	0	4
	50228	3	3	0	3	0	3	1	0	4
	50329	3	0	1	2	1	3	2	0	3
	50331	3	1	1	2	0	4	2	0	4
	50358	2	3	3	4	0	0	3	0	1
	50439	1	0	0	0	0	3	0	0	0
	50442	4	4	4	4	0	3	3	1	3
	50512	3	1	1	3	0	0	2	0	3
	50721	3	1	1	2	0	3	1	0	4
	50730	3	1	1	3	1	1	3	0	3
	50816	3	3	3	3	0	1	3	0	3
	50939	3	0	0	2	0	3	0	0	1
	50903	2	1	0	1	0	3	0	0	1
	50975	4	2	2	3	0	3	2	0	0
Primary IgA nephropathy	1	3	3	2	3	0	2	3	0	0
	2	3	3	3	3	0	0	2	0	0
	3	3	3	2	3	0	2	2	0	2
	4	4	3	1	3	0	2	3	0	0
	5	3	2	1	3	3	0	1	0	0
	6	3	0	0	0	0	2	1	0	0
	7	3	1	0	1	0	0	1	0	0
	8	4	2	1	3	1	0	3	0	0
	9	4	3	4	4	0	1	3	0	0
	10	3	1	1	4	0	1	0	0	NT
	11	4	3	1	3	0	3	3	0	0
Henoch-Schönlein purpura	12	2	0	0	2	0	0	1	0	0
	13	2	4	4	4	4	0	3	0	NT
	14	3	2	1	3	0	0	2	0	0
	15	3	0	0	3	0	1	3	0	0
	16	3	1	0	0	0	0	0	0	0
	17	2	0	0	0	0	0	0	0	0
	18	2	1	0	1	0	0	0	0	0
Systemic lupus erythematosus	19	4	4	3	3	2	2	2	0	2
	20	4	4	3	3	2	4	2	0	4
	21	3	2	1	2	0	3	2	0	NT
	22	3	1	0	2	1	3	3	0	1
	23	2	0	1	1	0	4	3	0	0
Controls (9)		0	0	0	0	0	0	0	0	0

Figure 3.5 Histogram illustrating mesangial immunofluorescence and SC binding capacity in patients with mesangial IgA deposition. Open bars represent the number of patients tested, closed bars those with fluorescence or SC binding capacity



primary IgA nephropathy showed mesangial A_1 and nine showed A_2 (Figure 3.6). Monoclonal antibodies showed A_1 in the same 10 patients but A_2 in only two. Of the 16 patients with alcoholic cirrhosis, the sheep antisera showed nine to have both A_1 and A_2 whereas with monoclonal antibodies 15 had A_1 and two had A_2. Of the seven patients with HSP, four showed staining for A_1 and two for A_2 when tested with the sheep antisera whereas with monoclonal antibody five had A_1 but only one (#13) had A_2 (Figure 3.7). Of the five patients with SLE, sheep antisera showed A_1 in four and A_2 in four; with monoclonal antibodies A_1 was shown in five and A_2 in three. Overall, monoclonal antibodies against A_1 were positive in almost all cases (35 of 39) compared with 29 of 39 by sheep antiserum. Monoclonal antibody for A_2, however, was positive only in eight, compared with 25 of 39 patients with sheep antiserum.

J-chain staining was present in 32 of the 39 patients. Twenty-six of the 39 kidneys had mesangial IgM; seven of the 11 patients with primary IgA nephropathy, 13 of the 16 patients with alcoholic cirrhosis, one of the seven with HSP, and all of those with SLE. Four of these 26 had no J-chain staining. Of the 13 patients without IgM, 10 showed staining for J chain. There was no correlation between staining for J chain and IgM. J-chain staining was not found more frequently in patients with A_2 subclass ($p>0.5$). SC staining of the mesangium was lost after pre-incubation of the antiserum with NHS (Figure 3.8) in all but one case (50442), in which only a trace remained.



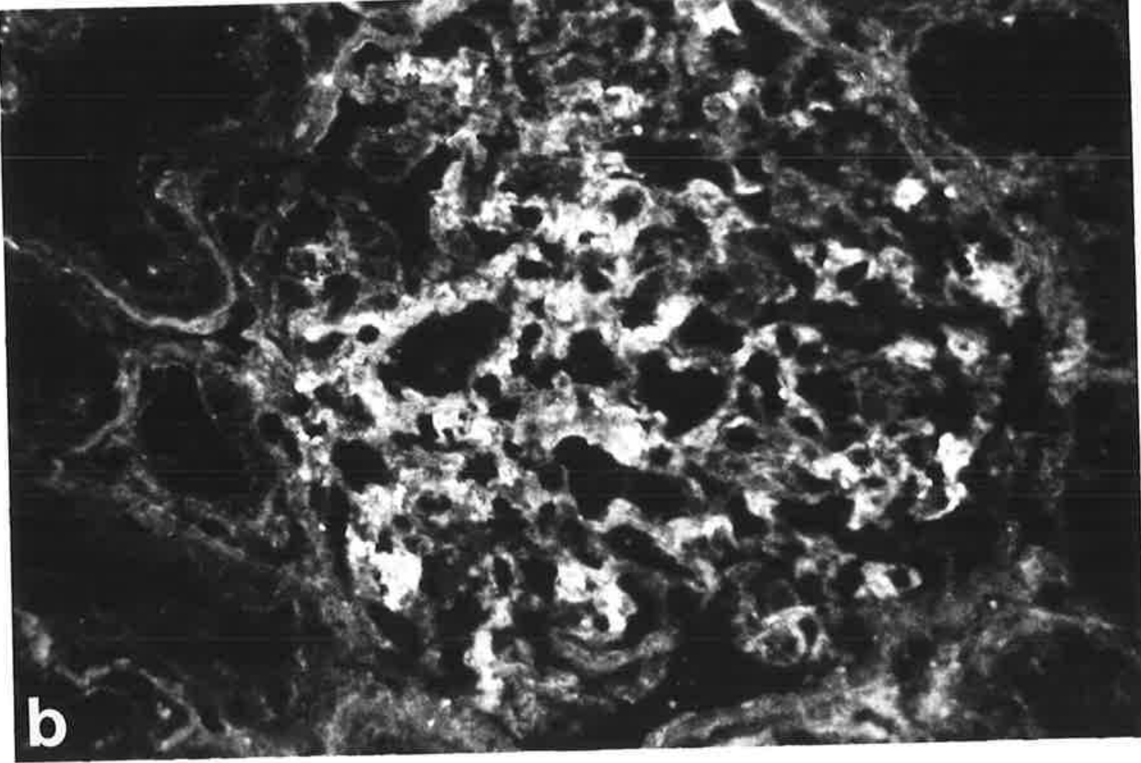


FIGURE 3.6
Glomerulus from a patient with primary IgA nephropathy (#3) stained with sheep antisera to IgA subclasses and FITC rabbit anti-sheep IgG. (a) Granular mesangial staining for A_1 graded 3+ and (b) A_2 graded 2+ (IIF, X900).

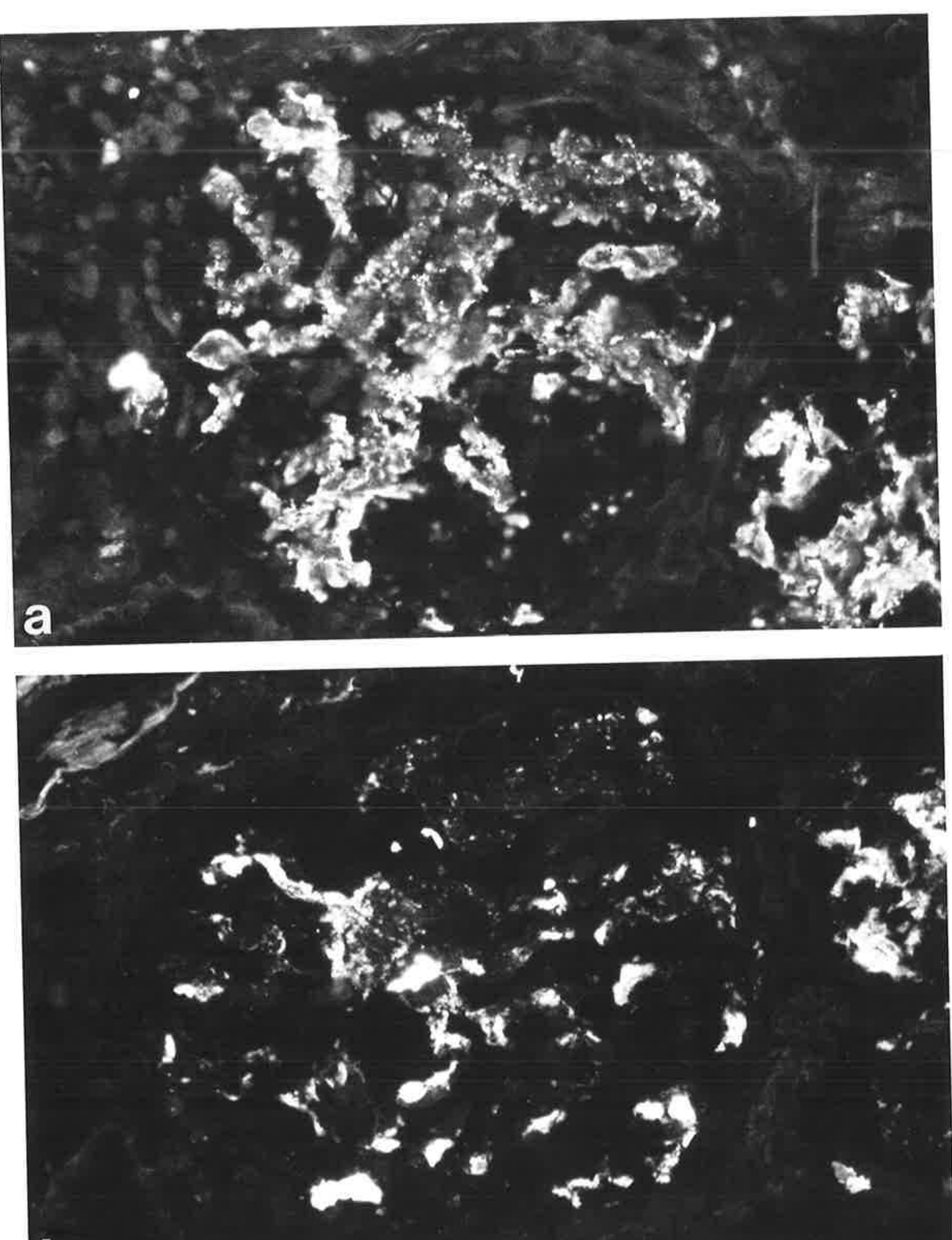


FIGURE 3.7
Glomerulus from a patient with HSP (#13) stained with monoclonal antibody to IgA subclasses, biotinylated goat anti-mouse IgG and FITC-conjugated avidin. (a) Mesangial staining for IgA_1 is graded 4+ and (b) mesangial staining for IgA_2 is graded 3+ in this field (IIF, X950).

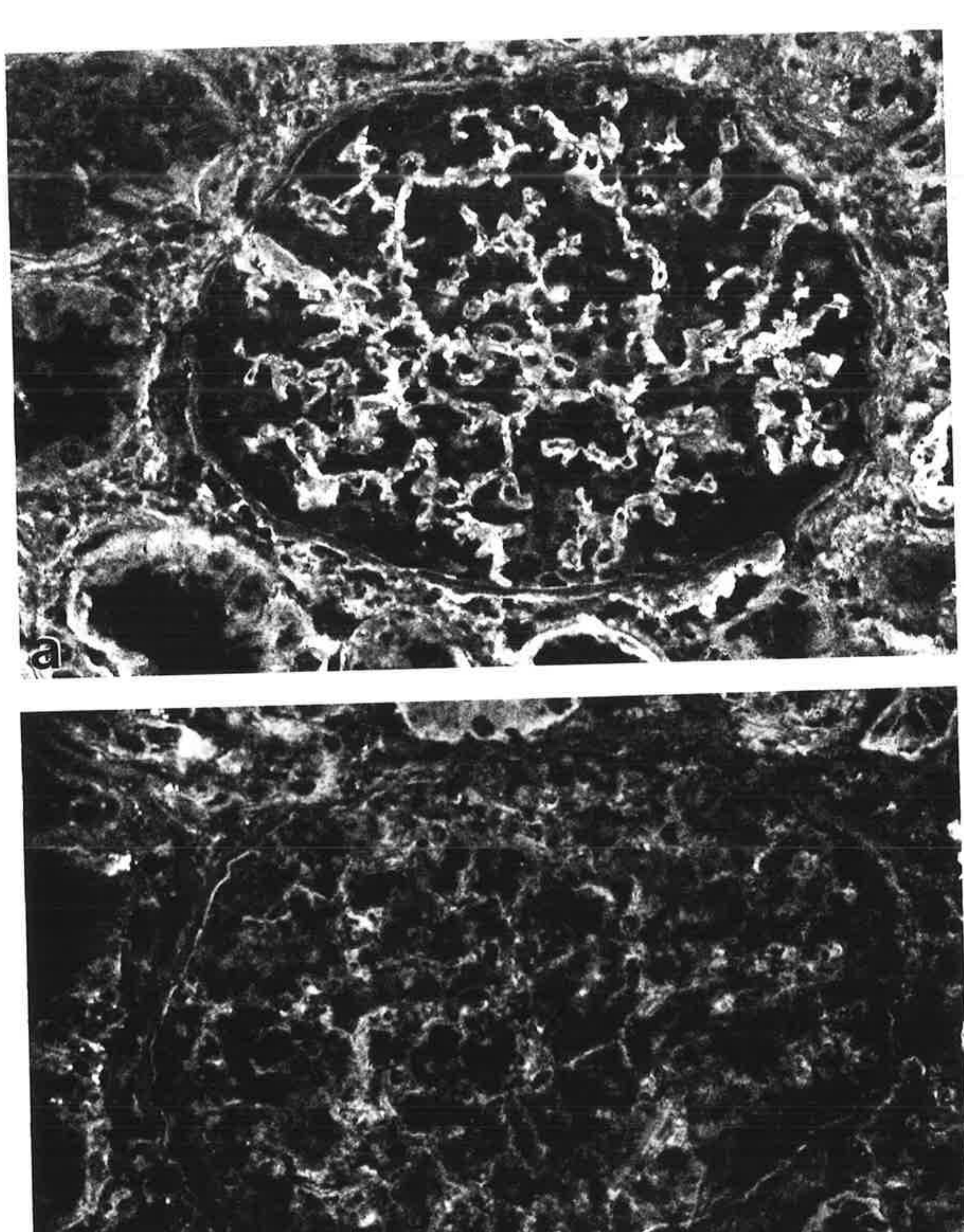


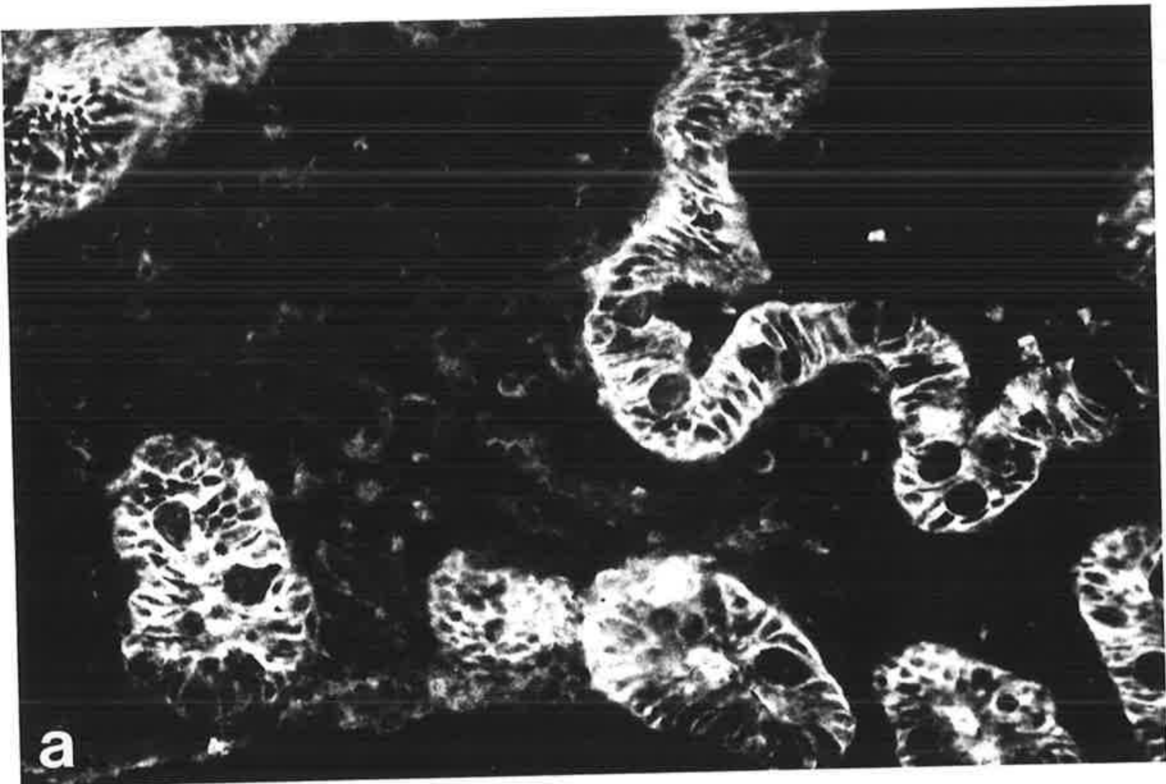
FIGURE 3.8
IIF of the same glomerulus from a patient with HSP (#18) stained with rabbit anti-human SC and FITC goat anti-rabbit IgG. Mesangial staining for SC was graded 2+ with unabsorbed anti-human SC (a), but was negative (b) using antiserum absorbed with NHS (X1250).

In the SC binding tests, a control jejunal section incubated with PBS showed localisation of SC to the basal and lateral walls of enterocytes by IIF and FITC anti-human SC. Sections incubated with SC and similarly stained also showed localisation of native SC on enterocytes, but in addition, bright staining of the cytoplasm of mucosal plasma cells was evident (Figure 3.9).

The SC binding capacity of the mesangial IgA deposits is shown in Table 3.1. In only one patient (50442) did the PBS control section show mesangial staining for SC using absorbed antiserum to human SC. This atypical case showed enhancement of staining after incubation with SC. Twelve of the remaining 15 kidneys from patients with alcoholic cirrhosis and mesangial IgA deposits showed SC binding (Figure 3.10).

The nine IF negative control kidneys and all of six HSP kidneys failed to bind SC, whereas biopsies from three of four patients with SLE and one of the 10 patients with primary IgA nephropathy, bound free SC.

As a measure of covariance, a Spearman's rank test showed a poor but significant correlation (rho=0.571) between the intensity of IgM staining and SC binding. There was an overall correlation between SC binding and IgM deposits ($p<0.05$) but no difference in the frequency of IgM in the patients with alcoholic cirrhosis compared with primary IgA nephropathy ($p>0.5$), despite a significant difference in SC binding ($p<0.01$). A Mann-Whitney U test showed the difference in SC binding amongst these groups to be highly significant ($p=0.0014$).



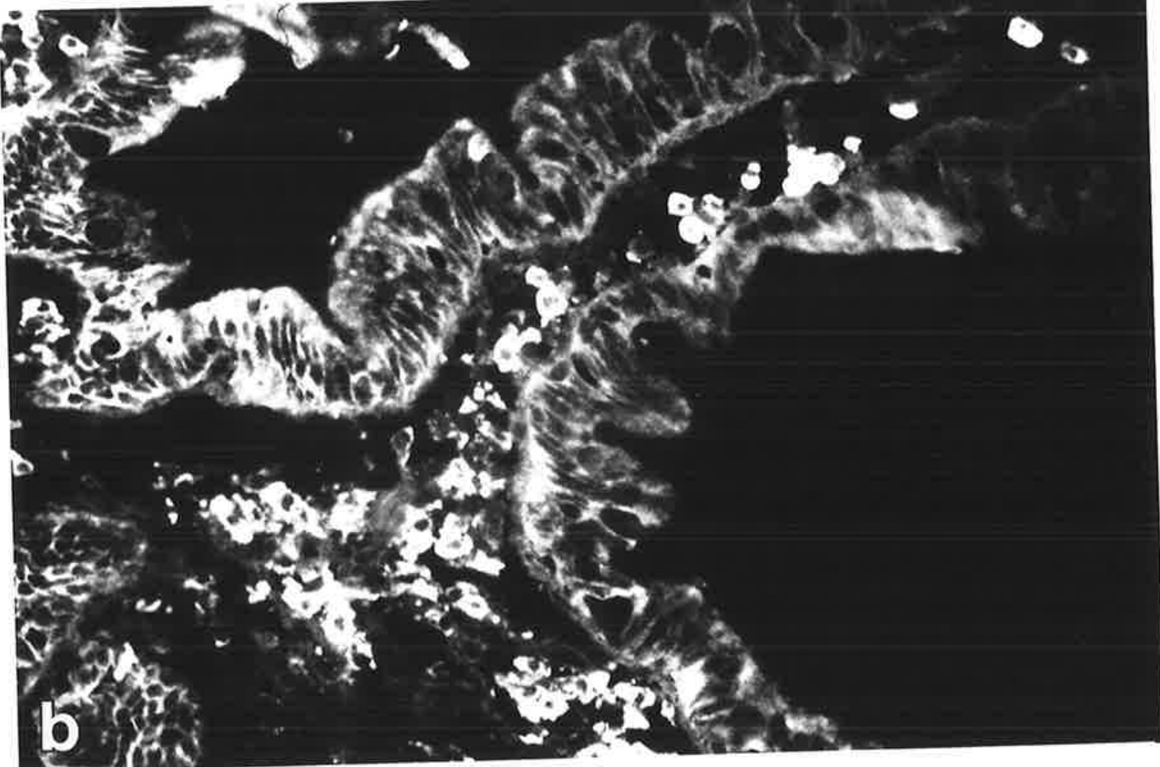
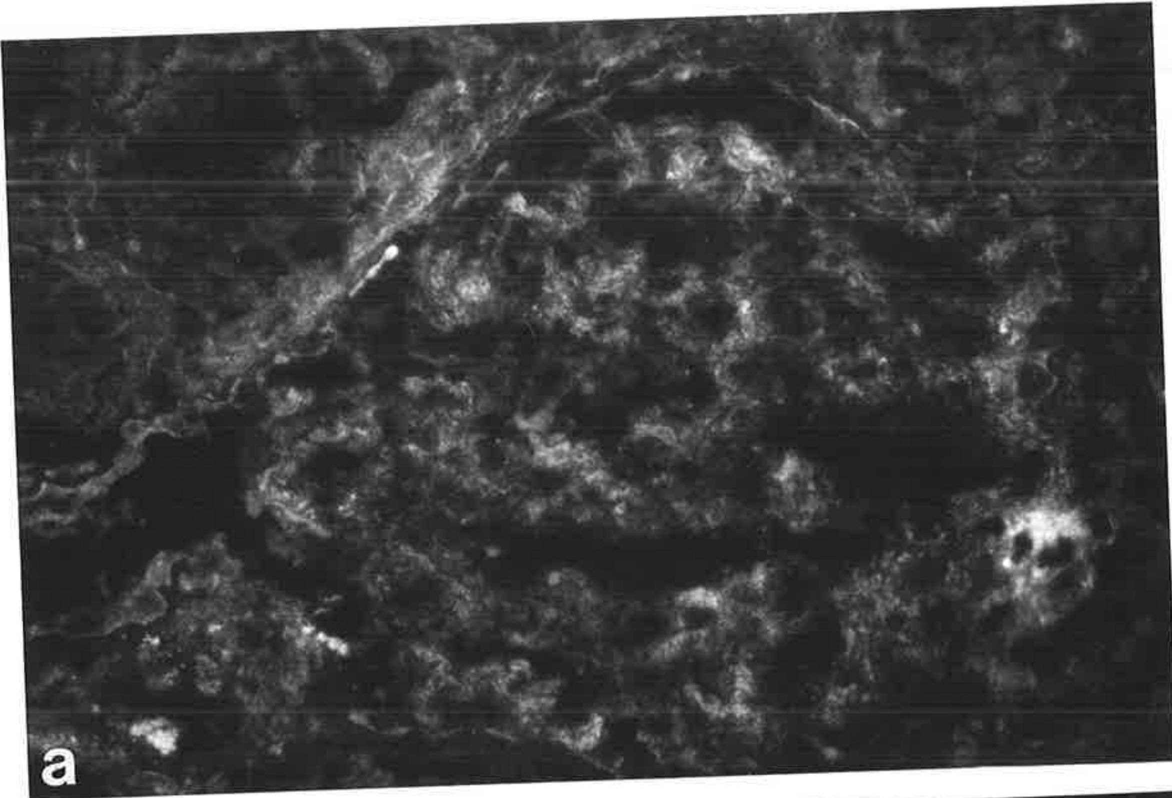


FIGURE 3.9

The binding of free SC to normal human jejunum stained with absorbed antiserum to human SC (IIF, X700).

a After incubation with PBS, native SC is seen on enterocytes. The lamina propria plasma cells are not stained.

b After incubation with free SC, there is in addition to enterocyte staining, prominent staining of lamina propria plasma cells.



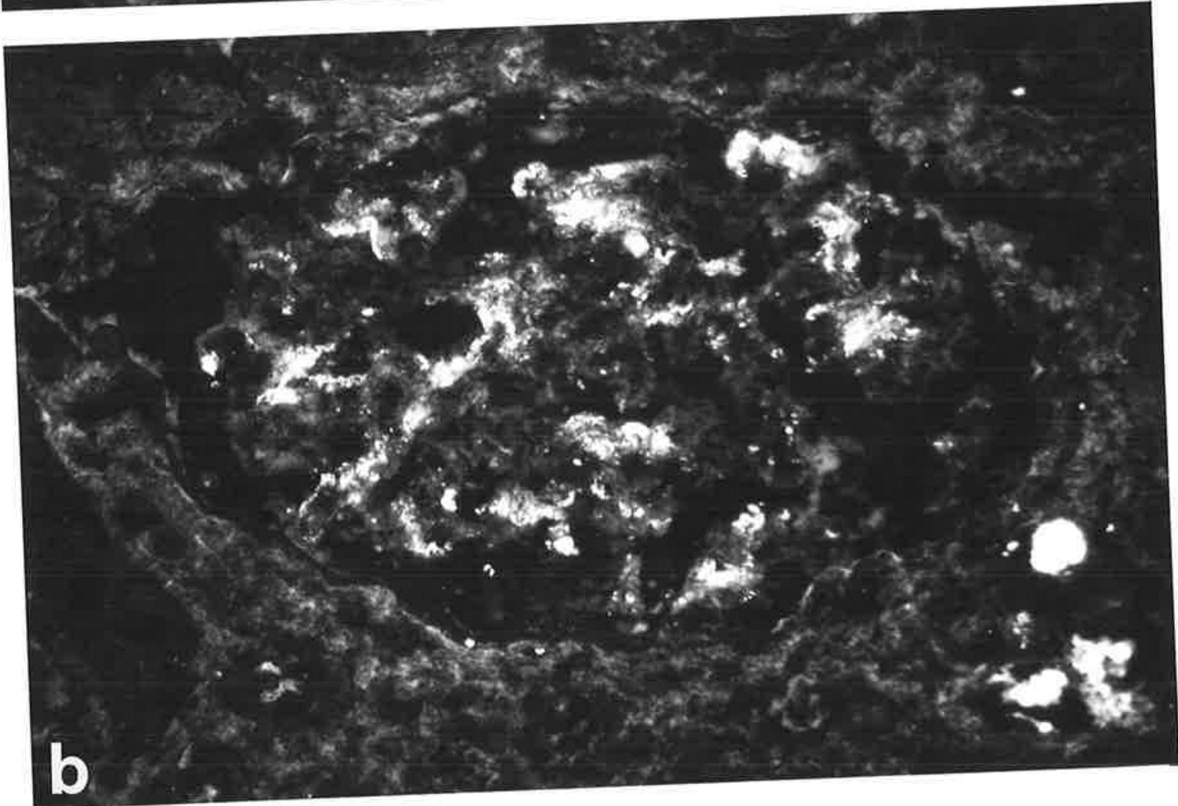


FIGURE 3.10
Glomerulus of a patient with ALD (50203) demonstrated the capacity to bind free SC. Both sections are stained with absorbed rabbit anti-human SC and FITC goat anti-rabbit IgG (IIF, timed print exposures, X1250).

a After incubation with PBS there is no staining for SC.

b After incubation with free SC there is a 4+ intensity of SC staining.

DISCUSSION

In this study, renal tissue from 39 patients with mesangial IgA deposits was examined by IF to elucidate the immunochemical nature of the IgA. With monoclonal antibodies it was shown that A_1 was present in all but four of the 39 patients, with no significant differences in the four disease categories examined. IgA_2 was less common, occurring in only 10-20% of patients with primary IgA nephropathy, mesangial IgA nephritis secondary to alcoholic cirrhosis, and HSP. IgA_2 occurred however, in three of the five patients with SLE.

A_1 and A_2 subclasses have also been sought by Conley et al (1980b) and André et al (1980) with conflicting results. Using monoclonal reagents, Conley et al (1980b) examined 10 patients with primary IgA nephropathy, 11 with HSP and nine with SLE. None showed IgA_2 staining, and, whilst present, J-chain staining correlated only with the intensity of IgM staining and not with that of IgA. André et al (1980) found that rabbit antiserum (Nordic) to A_1 stained the mesangium of five of 10 patients with primary IgA nephropathy, eight of nine with alcoholic cirrhosis, and all of those with HSP and SLE. In contrast to Conley et al (1980), André et al (1980) using rabbit antiserum (Nordic) to A_2 demonstrated staining in all 10 patients with primary IgA nephropathy, all nine patients with alcoholic cirrhosis, both of those with HSP, and all but one with SLE. Subsequently Tomino et al (1981b) examined seven patients with primary IgA nephropathy using both Nordic antisera and monoclonal reagents and found A_1 throughout using both systems, but A_2 in

only two instances using monoclonal antibody. Tomino et al 1982b) further examined five patients with HSP finding A_2 in none.

The data presented here support the suggestion of Tomino et al (1981b) that the reported differences relate to the specificity of the reagents. The sheep antiserum to A_2 (Nordic), evaluated in this study, cross-reacted with IgA_1 myeloma cells. The relatively high incidence of mesangial A_2 staining with this antiserum was probably caused by this cross-reactivity.

Monoclonal antibodies are of greater specificity, and the differences between this study and that of Conley et al (1980b) may be related to the relative insensitivity of the conventional IIF technique when compared to the avidin-biotin method. Biotin is a vitamin which is widely distributed in mammalian tissues. It has a molecular weight of 244.3 and binds avidly, but non-covalently, to avidin which is a basic glycoprotein of 68,000 molecular weight. This study confirmed the findings of Warnke and Levy (1980) in showing an avidin-biotin bridge to be more sensitive than conventional immunohistochemical techniques. The major disadvantage of the system is cross-reactivity with endogenous - particularly nuclear - biotin, as noted here. In practice, this staining is reduced by the blocking action of non-specific carrier protein (Wood and Warnke 1981) present in commercial reagents, and is not at a level where it can be confused with specific fluorescence.

Current data from Delacroix et al (1982a) have shown that a simple separation of serum from secreted IgA on the basis of the

presence of A_2 subclass, is not possible. Although polymers comprise only 13% of serum IgA compared with as much as 95% of secretions. A_2 subclass was present in all samples and sizes of IgA tested (Delacroix et al 1982a). The presence of A_2 subclass in mesangial deposits would not necessarily indicate either polymerisation or a mucosal origin.

J chain has been sought in mesangial IgA deposits as a means of identifying polymeric IgA (Bené et al 1982). It has been suggested, however, that staining for J chain in patients with IgA nephropathy may represent activity against J chain in the associated IgM deposits (Conley et al 1980b). The findings of mesangial J-chain staining in 10 of the 13 IgM-negative cases and its absence in four of the 26 IgM-positive cases, does not support this suggestion. As in other studies (Dobrin et al 1975), SC could not be demonstrated in the mesangial deposits of IgA. The antiserum to human SC cross-reacted with IgA. Absorption with NHS, which contains very little free SC (Delacroix and Vaerman 1982), affected a considerable reduction in cross-reactivity with plasma cell IgA and this procedure resulted in negative staining of all except one kidney with mesangial IgA deposits. This patient had unusually extensive deposits of IgA which may have cross-reacted with the antiserum to human SC. Similar cross-reactivity with this commercial reagent has been noted by Bené et al (1982).

The study of SC binding to jejunum showed that the SC bound specifically to plasma cells, most of which in this location might be expected to be producing dimeric IgA. Using this

technique, SC binding was observed in 13 of 16 kidneys from patients with alcoholic cirrhosis, one of 10 patients with primary IgA nephropathy, none of six patients with HSP, and three of four patients with SLE. The ability of the IgA deposits in alcoholic cirrhosis to bind SC suggests that these are polymeric (Brandtzaeg 1974a). However, IgA often is associated with IgM and it is necessary to interpret SC binding with caution. Five of the 10 primary IgA nephropathy patients and two of those with alcoholic cirrhosis had significant amounts of IgM and failed to bind SC. In contrast, two patients with alcoholic cirrhosis but no IgM, bound SC. There was a marked difference in the SC binding capacity of mesangial IgA deposits in primary IgA nephropathy and alcoholic cirrhosis although there was no significant difference in mesangial IgM content. As might be expected however, in cases with abundant IgM there tended to be more SC binding although the correlation was poor. This may be explained by the fact that IgM polymers have a variable capacity to bind free SC. Low molecular weight polymers (19 S) are thought by some to be deficient in this regard, SC binding being a property of only the high molecular weight polymers (Rádl et al 1971).

It has been suggested that primary IgA nephropathy is mediated by the deposition of IgA polymers from the circulation (Lopez-Trascasa et al 1980; Egido et al 1980). To support this postulate, increased concentrations of circulating J chain containing high molecular weight polymers with the ability to bind SC have been found in such patients (Lopez-Trascasa et al 1980). Similar properties have been recognised in the IgA

eluted from mesangial deposits in patients with primary IgA nephropathy (Tomino et al 1982a). In addition, it has been shown that the *in situ* mesangial deposits in primary IgA nephropathy, HSP and alcoholic cirrhosis are capable of binding SC whilst those from patients with SLE are not (Egido et al 1980; Sancho et al 1981).

In this study, the ability of mesangial deposits in primary IgA nephropathy, alcoholic cirrhosis, HSP and SLE, to bind SC were compared, and it was shown that SC binding was almost exclusively restricted to kidneys from patients with alcoholic cirrhosis or SLE. This finding suggests that only the deposits in alcoholic cirrhosis or SLE are polymeric. These data significantly differ from the findings of Egido et al (1980) and Sancho et al (1981) in failing to demonstrate SC binding capacity in the deposits of patients with primary IgA nephropathy. These authors found such binding consistently and concluded that it was produced by the deposition of IgA polymers.

Some groups, however, do not support a polymeric basis for primary IgA nephropathy, and have failed to show increased levels of serum IgA polymers in such patients (Woodroffe et al 1980; Lesavre et al 1982; Delacroix et al 1983a).

By contrast the IIF finding of J chain independent of IgM in all of the disease categories examined, implies the presence of some IgA polymers throughout. The dissociation of these two characteristics of IgA polymers, by the relative absence of SC binding in the presence of J chain, is not easily explained, except by

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suggesting a deficiency in the J-chain antiserum specificity. The SC binding data demonstrates a functional difference between the IgA deposits in primary IgA nephropathy, and mesangial IgA deposits secondary to ALD, which is independent of antibody and antisera specificity.

This work suggests that the mesangial deposits secondary to alcoholic cirrhosis are polymeric, and although not predominantly of A_2 subclass, possibly of gastro-intestinal origin.

CHAPTER 4

The immune complex nature of the mesangial IgA deposits in alcoholic liver disease

INTRODUCTION

The role of immune complexes in mesangial IgA deposition in patients with alcoholic liver disease: an outline of possible antigenic mechanisms

A The antigenicity of food proteins

B The antigenicity of absorbed bacterial organisms

C The role of auto-immunity in hyperglobulinaemia and IC production

INTRODUCTION

There is considerable circumstantial evidence in support of an IC origin for the mesangial IgA deposits occurring secondary to alcoholic cirrhosis. While animal studies may be used to test hypotheses of the aetiopathogenesis of such IgA deposits, it is necessary to study human deposits to determine their origin and constituents.

The liver is important in the sequestration of antigen, IC and IgA polymers. There is a precedent for believing that patients with ALD have increased absorption of macromolecules (Worthington et al 1978) and floral protein (Woodroffe et al 1980). Impaired hepatic sequestration of gut-derived antigens and IgA-class IC may be responsible for the occurrence of mesangial IgA deposits in patients with alcoholic cirrhosis. This hypothesis would be supported if the IgA could be shown to be gut-derived by a preponderance of A_2 subclass, or polymeric IgA (Delacroix et al 1982a). Although the studies described in Chapter 3 did not indicate a predominance of A_2 subclass in the deposits of patients with ALD compared with primary IgA nephropathy, there was a significantly greater ability to bind SC, suggesting that the deposits were polymers.

In patients with ALD there may be also auto-immunity directed towards liver proteins. The nature of the hyperglobulinaemia in patients with ALD might be elucidated by demonstrating antibody activity in the serum and an IC origin for the deposits would be suggested by the finding of CIC in the serum of such patients. The most conclusive data supporting an IC origin for

such deposits would be the identification of antigen and specific antibody in the deposits.

The identification of such antigen-antibody systems can be attempted either by examination of tissue sections or elution of antibody. The materials collected in the autopsy study of patients with ALD allowed such studies. Serum taken from these patients during life was sometimes available from the Division of Clinical Chemistry, IMVS, but had been kept at 4°C or -20°C and was therefore unsuitable for IC assay. Optimally stored serum from hospital patients with overt ALD was available and CIC were measured in this group of patients by the $SpC1_q$ assay. The renal deposits were examined both in situ and after elution by acid treatment of isolated glomeruli. The efficacy of dissociation of IgA by this means was demonstrated by DIF after slide elution of frozen sections. Antibody activity was sought in the eluates against food proteins, gut flora, Mallory bodies and mesangial antigens. The reasons for investigating these antigens are discussed briefly below.

A The antigenicity of food proteins in ALD

Gastro-intestinal absorption of macromolecules and particularly food proteins is prevented, to a large extent, by an adequate sIgA response. The proteins most investigated, perhaps because of recognised clinical intolerance, are those of milk. Non-specific milk protein precipitins are found, by gel diffusion, in the serum of 75% of IgA-deficient patients (Buckley and Dees 1969). In such patients, specific antibodies have been found by ELISA to be directed against casein, bovine gamma globulin

and BSA (Cunningham-Rundles et al 1979).

Anti-BSA activity in patients with ALD

André et al (1978b) found that alcohol abusers especially those with CIC frequently had antibodies to food substances. They found CIC by polyethylene glycol precipitation in seven of the 15 patients studied and noted that the levels rose postprandially in six. Furthermore, elevated anti-BSA activity in the serum has been detected by RIA in patients with alcoholic cirrhosis (Woodroffe et al 1980).

In this study, anti-BSA activity was assayed (after Woodroffe et al 1980) in eluates and, where possible, in serum from patients with mesangial IgA nephritis and ALD. A greater titre of antibody reactivity per unit immunoglobulin (Ig) in eluates was taken as an indication of specific antibody concentration in the deposits rather than serum entrapment. In addition, frozen sections of kidney were treated with fluorosceinated BSA to test for *in-situ* anti-BSA antibody. This experiment was controlled by examination of autopsy or biopsy material from kidneys with no abnormality by light or fluorescent microscopy, and from kidneys of patients with either primary IgA nephropathy or GN with other Ig deposits. Deposits of BSA were sought in frozen sections with FITC-conjugated goat antiserum to BSA.

B The antigenicity of intestinal bacteria in ALD

The serum of patients with ALD frequently demonstrates antibody activity against common gut bacteria (Prytz et al 1973; Woodroffe et al 1980). Such elevated antibody titres are sig-

nificantly more common in alcoholic than non-alcoholic chronic liver disease (Turunen et al 1981). This may be presumed to reflect both increased absorption of organisms and impaired clearance. Activity against gut-derived bacteria was sought in this study in two ways. First, a slide technique (Thomason 1974) was used in which a range of E coli serotypes were coated on glass slides and incubated with eluates before staining with antisera to human Ig. Second, eluates were tested against a battery of fractured bacterial coat proteins in an ELISA system. The bacteria used in this assay were a selection of those common in human intestinal flora.

C Auto-immune mechanism in ALD

The progression of liver disease to cirrhosis may be accelerated by the development of cellular or humoral immunological mechanisms (Canalese et al 1981). The morphological pattern of alcoholic hepatitis with degenerate hepatocytes, often containing Mallory bodies, surrounded by neutrophils and associated with a variable lymphocyte and macrophage infiltrate, could be compatible with this hypothesis. Moreover, immunological mechanisms can be implicated to explain the hyperglobulinaemia, low titre anti-smooth muscle and anti-nuclear antibodies (Bailey et al 1976) as well as the reversible impairment of delayed hypersensitivity (Berenyi et al 1974).

The mechanism(s) for these immune abnormalities are uncertain. Although a decrease in peripheral T cells in ALD, has been observed by some workers (Bernstein et al 1974), an impairment of T-cell regulation of B-cell activity has not been demon-

strated. Wands et al (1981) have investigated Con A-induced suppression of PWM-induced IgG production and showed no difference between patients with ALD and controls. It would appear, therefore, that the increase in humoral activity in such patients is not due to faulty suppressor T-cell function.

In addition to the production of auto-antibodies towards liver antigens, patients with ALD may have antibodies directed towards serum components such as albumin (Hauptman and Tomasi 1974b). Mesangial antigenicity has been implicated in patients with primary IgA nephropathy (Lowance et al 1973) and this mechanism may be important in patients with ALD.

i) Mallory bodies

Mallory bodies (MB) (Mallory's hyaline, alcholic hyaline) are often found in the hepatocytes of alcoholics. They are found occasionally in patients with diabetes, Wilson's disease, primary biliary cirrhosis, Indian childhood cirrhosis and hepatocellular carcinoma (reviewed by French 1981); and in a murine animal model, after prolonged griseofulvin administration (Denk et al 1976). Originally described in 1911 (Mallory 1911), MB can be shown to consist ultrastructurally, of intracellular fibrillar protein (Yokoo et al 1972), and, by various techniques to be immunoreactive. In patients with alcoholic hepatitis, MB can be shown to induce a leucocyte migration inhibition factor (MIF) which was not found in other patients with hepatocyte necrosis or in inactive alcoholic cirrhosis (Zetterman et al 1976; Triggs et al 1981). In addition, MB can be shown to induce blast transformation (Zetterman and

Leevy 1975) and lymphocyte cytotoxicity (Kakumu and Leevy 1977).

The demonstration of anti-MB activity in serum from patients with ALD has been disputed. Kanagasundaram et al (1977), examining sera from patients with alcoholic hepatitis, described circulating hyaline and anti-hyaline activity using complement fixation and immune adherence agglutination. Kehl et al (1981) using a solid phase RIA (Sp RIA) failed to confirm these results, finding neither MB antigen nor anti-MB antibody activity in the sera of 32 patients with alcoholic hepatitis.

Kanagasundaram and Leevy (1976), however, have also found anti-MB antibody activity of IgA and IgG class in the presumed IC eluted from the liver of patients with alcoholic hepatitis, thus adding further support to speculation that such antibody may be responsible for liver damage.

ii) Liver membrane antibodies

Both IgG and IgA class antibodies to liver membrane determinants, termed liver membrane auto-antibodies (LMA) have been described by Hopf et al (1976). One study of patients with ALD showed IgA and IgG class LMA in about a quarter of those with hepatitis, a third of those with active cirrhosis, and two-thirds of those with inactive cirrhosis (Burt et al 1982). MacSween et al (1981) have suggested that alcohol renders hepatocytes immunoreactive. In an IIF system with patients' serum and isolated rabbit hepatocytes, these authors demonstrated an increased incidence of LMA using isolated hepatocytes from animals pre-treated with intravenous alcohol (74%)

compared with those from untreated rabbits (28%) (Anthony et al 1983). Using a normal isolated rabbit hepatocyte system, they found LMA were rare in normal subjects and in other forms of acute or chronic liver disease and that their incidence never rose using alcohol pre-treated hepatocytes.

Another auto-antibody system under investigation is that directed against a liver membrane lipoprotein termed liver specific protein (LSP). Since rabbits immunised with liver fractions containing LSP develop hepatic necrosis and infiltration, it has been suggested that these antibodies to LSP in man are hepatotoxic and not epiphenomena (Meyer zum Büschenfelde et al 1972). Perperas et al (1981), using a liquid phase RIA with staphylococcal clumping factor, found abnormal levels of antibody to LSP in none of six patients with inactive alcoholic cirrhosis and only one of nine with alcoholic hepatitis. Anti-LSP was detected consistently only in active cirrhosis (60%) with or without hepatitis. In that study, anti-LSP levels seemed to correlate with the degree of lymphocyte infiltration and piecemeal necrosis in the liver sections. Anti-LSP levels seem to be related to periportal lymphocytic infiltration and piecemeal necrosis and are probably not alcohol-specific (Perperas et al 1981).

iii) Antibodies to altered albumin

Hauptman and Tomasi (1974a) described a monoclonal IgM protein with affinity for native and denatured human serum albumin (HSA). They postulated that this antibody functioned in some way in normal albumin metabolism and suggested that it was found rarely

in normal sera because of the antigen excess. For that reason, they examined a group of patients with alcoholic cirrhosis who had hypo-albuminaemia and found that, using a haemagglutinating system with heat-aggregated HSA (heat-Agg HSA), 40% had anti-albumin activity (Hauptman and Tomasi 1974b). Using a heat-Agg HSA immunoadsorbent column, they showed the active serum component to be an IgA-class antibody.

Subsequent work performed by Lenkei and Ghetie (1977) utilised glutaraldehyde-aggregated HSA (glut-Agg HSA). This Agg-HSA was used in radial immunodiffusion (RID) and haemagglutination systems, where the presence of antibodies was said to correlate with the severity of liver cell damage in viral hepatitis. In a group of cirrhotics, in whom the aetiology was not stated, precipitating antibodies were always present but haemagglutinating antibodies were rare (Lenkei et al 1981). These antibodies to glut-Agg HSA differ from those to heat-Agg HSA in being of IgM and IgG class (Lenkei et al 1981). In this respect they are similar to the IgG and IgM class - albumin complexes described in patients with HBsAg positive, chronic active hepatitis (Zhen et al 1983).

Lenkei and Ghetie (1977) have demonstrated receptors for Agg-HSA on liver cells and a possible function for these may relate to the postulated metabolism of Agg-HSA proposed by Onica et al (1978). The latter authors suggested that HSA polymers, produced normally *in vivo* as a result of aging, are cleared by the liver, but that if clearance is inadequate they may induce autoantibodies.

iv) Antibodies to renal mesangium in ALD

Kaufman et al (1982) showed that 20% of patients with alcoholic cirrhosis have up to two, and 34% have three or more, organ and non-organ specific auto-antibodies. Antibody with anti-mesangial activity has not been described in such cases, however the eluate from one patient with primary IgA nephropathy has been shown to bind weakly but specifically to a frozen section of a normal kidney (Lowance et al 1973).

In this study auto-antibody activity was sought in the glomerular eluates against MB, renal mesangium and both heat- and glut-Agg HSA. Anti-MB activity was examined by incubation of frozen liver sections containing hyaline with eluates, followed by staining with FITC-conjugated antisera to human Ig. The system was controlled by the use of human serum containing a high titre of anti-smooth muscle antibody, which cross-reacts with MB (Virtanen et al 1979). Antibody activity towards HSA was examined in two ways. First, in the eluates by immunoprecipitation with heat- or glut-Agg HSA and second, in the deposits by the binding capacity of fluoresceinated HSA to mesangium in frozen sections. HSA in the deposits was sought by DIF with FITC-conjugated antiserum to HSA. Anti-mesangial activity was sought in the eluates by incubation with frozen sections of normal kidney followed by staining with FITC-conjugated antisera to human Ig.

MATERIALS AND METHODS

I MATERIALS

II METHODS

A SpCl$_q$ assay.

B Preparation of glomeruli from whole kidneys.

C Citrate elution of immune deposits from isolated glomeruli.

D Citrate elution of immune deposits from tissue sections.

E Quantitation of Ig in glomerular eluates.

F The demonstration of anti-BSA activity in the serum and glomerular eluates from patients with ALD.

- i) RIA for anti-BSA activity in serum and eluates.
- ii) A search for BSA and BSA binding in mesangial deposits.

G The demonstration of anti-bacterial activity in eluates.

- i) IF technique for the demonstration of anti-E coli antibodies.
- ii) ELISA technique for demonstration of anti-bacterial coat antibodies.

H The demonstration of auto-antibody activity in eluates.

- i) IF technique for the demonstration of antibody activity to MB in liver sections.
- ii) Immunoprecipitation technique for the demonstration of antibody activity to Agg-HSA.
- iii) A search for HSA and HSA binding in mesangial deposits.
- iv) The binding of eluates to normal mesangia.

I MATERIALS

Sera

Sera from 28 patients with severe ALD were obtained from the stores of the Renal Unit, RAH, frozen at -70°C. These sera, coded 1-28, had been obtained from patients with overt ALD, and details of renal or liver biopsy diagnosis were obtained.

Kidneys

Kidneys were selected from those taken at autopsy and labelled and stored in plastic bags at -20°C. Nine kidneys were available from patients with ALD and mesangial IgA deposition, (Chapter 2) one from a normal control patient and two from patients with GN. Autopsy patients' code numbers were shortened by removing the final two digits which designated the year in which they died.

II METHODS

A SpC1_q assay

The modification of the SpC1_q assay (Tung et al 1978) was used. Vinyl 96 well flexible plates (COSTAR, Cambridge, MA) were coated with C1_q prepared from NHS (Yonemasu and Stroud 1971). Serum samples were tested in duplicate and their C1_q binding expressed in terms of micrograms of heat-aggregated human IgG (μg AHG).

The purification of human C1_q, the coating of plates, the preparation of aggregate and the performance of the assay is described in Appendix VI.

B Glomerular preparation

1 Each kidney was thawed overnight in a 4°C refrigerator.

2 The kidney was sliced and the capsule was stripped and discarded together with all non-cortical tissue. This was performed in a glass petri dish resting on ice.

3 Cortical tissue, including the columns of Bertin, was retained and weighed.

4 The cortex was roughly chopped in a beaker with scissors and finely diced using a micro-homogeniser (Sorvell, DuPont Newton, CT).

5 The tissue was then pushed through a 180 μm sieve (Endecotts Ltd, Johnson and Firth Bown, London, UK) with a small glass conical flask washing with up to 300 ml of PBS at 4°C.

6 The wash was centrifuged at 250 *g* for two minutes. The first supernatant from this procedure was retained and regarded as approximating serum in its content.

7 The pellet was re-suspended and centrifuged at 250 *g* for two minutes, five times and the supernatants discarded.

8 The final precipitate was re-suspended in 750 ml 49% sucrose in PBS with a specific gravity of 1.23.

9 The suspension was centrifuged at 2000 *g* for five minutes, and the supernatant discarded.

10 The pellet was washed in 300 ml PBS, centrifuged twice at 2000 *g* for five minutes, and re-suspended to give a final volume of 20 ml in PBS.

11 One drop was placed on a glass slide with a cover slip and examined by LM to confirm the presence of glomeruli and the purity of the isolate.

12 The suspension was centrifuged for 10 minutes at 2000 *g* and the supernatant was discarded. This washing manoeuvre was repeated six times.

13 The pellet, which consisted predominantly of glomeruli, was weighed.

C <u>Elution of immune deposits from isolated glomeruli</u> (Woodroffe and Wilson 1977).

1 To each gram of the glomerular pellet, 20 ml 0.02 M citrate buffer at pH 3.2 (Appendix IX) was added, and the suspension stirred at 37°C for 90 minutes.

2 The suspension was centrifuged at 1500 *g* for 10 minutes at room temperature, and the supernatant retained as the eluate.

3 The acid eluate was neutralised dropwise with 0.1 M sodium hydroxide.

4 The eluate was allowed to stand at 4°C for 18 hours, and was then centrifuged at 8000 *g* for 10 minutes.

5 The supernatant was concentrated to less than 2 ml, and frozen at -70°C in aliquots of 100 μl.

D <u>Elution of immune deposits from tissue sections</u>

1 Sections of frozen unfixed renal tissue were cut at 2 μm and washed for five minutes in PBS.

2 Adjacent sections were incubated for four hours and 30 minutes with 0.02 M citrate buffer at pH 3.2 or PBS at room temperature.

3 The sections were washed for 20 minutes in PBS and then stained with either FITC-labelled antiserum to human IgA

or C_3 (Wellcome) for 30 minutes at a dilution of 1:10 in PBS/BSA.

4 The sections were washed for 15 minutes in PBS and mounted in glycine buffered glycerol pH 8.6.

5 The sections were examined with a Leitz Orthomat Epiploem Microscope, and adjacent sections photographed with the same time exposure.

E Quantitation of Ig in glomerular eluates

A radioimmune assay using antiserum to human Fab was used to measure total Ig (after Roffe et al 1981).

Sp RIA for the measurement of total Ig

1 The wells of flexible vinyl microtitre plates (Linbro, Flow Laboratories, Hamden, CO) were filled with 200 µl of 1:250 antibody diluted in PBS and left overnight at 4°C.

2 The plates were aspirated and washed three times in PBS.

3 Human IgG (Human Immunoglobulin CSL, Australia) was purified by ammonium sulphate precipitation and ion-exchange chromatography. A standard curve was prepared from the human IgG at 43.26 mg/ml by 10-fold dilutions in 1% BSA in PBS with BSA/PBS zero blank.

4 100 µl of standard curve solutions and specimen samples were plated in duplicate and left for one hour at 37°C.

5 The plate was transferred to 4°C for nine hours.

6 The plates were aspirated and washed four times in PBS.

7 Affinity purified (by Dr P Ey, Department of Microbiology, The University of Adelaide) goat anti-human Fab antibody (Atlantic Antibodies, Scarborough, ME) was radiolabelled

as described in Appendix V.

8 Radiolabelled antibody was diluted in 0.05% Tween 20 in PBS (BDH Laboratory reagents) so that 100 µl contained 100,000 counts per minute (cpm). This volume was added to each well and the plate then left for one hour at 37°C.

9 The plate was transferred to 4°C and left overnight.

10 The wells were aspirated and washed six times in PBS.

11 The dried wells were cut and counted in a multidetector gamma counter (NE 1600, Nuclear Enterprises, Edinburgh, UK).

12 The total Ig was calculated from the mean counts obtained from each sample with reference to the standard curve and expressed in µg IgG equivalent (µg IgG equiv)/ml.

F The demonstration of anti-BSA activity in patients with ALD

Anti-BSA activity was examined using a liquid-phase radioimmune assay to test kidney eluates and available serum from patients with ALD. Each assay was controlled by the inclusion of a standard curve of dilutions of goat anti-BSA whole serum and the results expressed as a % of trichloroacetic acid (TCA) precipitable radioactivity. In addition, the mesangial deposits were examined *in situ* using FITC-conjugated BSA to demonstrate antibody activity to BSA, and FITC-conjugated antiserum to BSA to demonstrate bound antigen in the deposits.

i) Anti-BSA RIA

1 The samples were diluted as required, in duplicate in 0.1 M borate buffer at pH 8.4 (Appendix IX).

2 ^{125}I-BSA (Appendix V) was diluted in 1% HSA in 0.1 M borate buffer at pH 8.4 such that 1 ml contained 6 µg

^{125}I-BSA.

3 The percentage TCA precipitable ^{125}I-BSA was calculated for the duplicated test samples with reference to the precipitate obtained from 1 ml of ^{125}I-BSA solution mixed with 1 ml 20% TCA. This duplicated mixture was spun at 1500 *g* for 10 minutes, the supernatants discarded and the pellets counted in a multidetector gamma counter (NE 1600) for 60 seconds.

4 1 ml ^{125}I-BSA mixture was added to 1 ml of each appropriately diluted sample, mixed, placed at 37°C for 30 minutes and then at 4°C for 60 minutes.

5 Equal volumes of saturated ammonium sulphate (ie 2 ml) were added to each test sample and the mixture centrifuged (500 *g*) for 20 minutes at 4°C.

6 The supernatants were discarded and the pellets counted for 60 seconds in a gamma counter (NE 1600).

7 Each assay included a goat anti-BSA whole serum standard curve (as a gift from Dr P McKenzie, Renal Unit, RAH) which was diluted in duplicates from 1:10 to 1:2560 with a zero blank containing PBS. Each duplicate pair from the standard curve was treated in the same way as the samples.

<u>Assay application</u>

. The above protocol was used to assay 20 normal human sera obtained from staff of the RAH (arbitrarily coded from 31-50) at a dilution of 1:10.

. Serum samples from 17 patients with alcoholic cirrhosis (arbitrarily coded 101-117) were tested as above, at a dilution of 1:10 to demonstrate the frequency of anti-BSA anti-

body activity in ALD.

.Nine eluates from patients with ALD, onc from a normal kidney, and two from patients with other forms of GN (membranous nephropathy) were tested at a dilution of 1:10. Those that were positive were further diluted to determine an end-titre.

.The washes were tested at a dilution of 1:2 and 1:10.

ii) To demonstrate BSA binding capacity and in situ BSA in the mesangial deposits

Fluoroscein-conjugated BSA and goat anti-BSA antiserum

1 BSA (Sigma, St Louis, MO) powder was dissolved at 20 mg/ml in distilled water with a magnetic stirrer and dialysed against 10 times its own volume of 0.1 mg/ml FITC in 0.025 M carbonate buffer at pH 9.4 (Appendix IX) overnight. Unbound FITC was then removed by dialysis against repeated changes of PBS.

2 The fluoroscein:protein (F:P) ratio of fluorosceinated albumin was calculated from the protein concentration calculated using the bromocresol purple method (Appendix IV) and corrected fluorescein concentration calculated from the emission at 495 nm (after Nairn 1976).

3 10 mg/ml of ammonium sulphate-cut and ion-exchange chromatographed goat anti-BSA IgG was FITC-conjugated as above except that the final solution, after equilibration with 0.25 M PB, was purified by stepwise elution from a DE-52 column (Pharmacia, Uppsala, Sweden) and the 0.05 M fraction retained.

To examine the BSA binding capacity of IgA deposits in renal tissue

1 Sections of frozen unfixed renal tissue were cut at 2 µm and washed for five minutes in PBS.
2 The sections were incubated for 45 minutes at room temperature in a humid chamber with centrifuged FITC-BSA at a dilution of 1:8 in PBS.
3 The sections were washed for 15 minutes in PBS, mounted in glycine buffered glycerol pH 8.6, and examined with a Leitz Ploem epi-illumination Orthomat Microscope.

To confirm the specificity of BSA binding to mesangia by blocking

1 Sections of frozen unfixed renal tissue were cut at 2 µm and washed for five minutes in PBS.
2 Sections known to bind FITC-BSA were incubated with BSA at 20 mg/ml in PBS in a humid chamber for 30 minutes. Blocking was controlled by incubation of an adjacent section with PBS.
3 Sections were washed for 15 minutes in PBS, and incubated for 45 minutes in a humid chamber with centrifuged FITC-BSA at a dilution of 1:8 PBS.
4 Sections were washed for 15 minutes in PBS, mounted in glycine buffered glycerol pH 8.6, and examined using a Leitz Ploem epi-illumination Orthomat Microscope.

To demonstrate BSA deposits in renal tissue using FITC-conjugated goat anti-BSA IgG

1 The sections were cut as above, washed and stained for 45 minutes with FITC-conjugated goat anti-BSA absorbed with 1:10 NHS.

2 The sections were washed for 15 minutes in PBS, mounted in glycine buffered glycerol pH 8.6, and examined using a Leitz Ploem epi-illumination Orthomat Microscope.

G <u>The demonstration of anti-bacterial antibody activity in kidney eluates</u>

The eluates were tested for antibody activity in two ways. First, a simple IF system was used to demonstrate binding to a slide preparation of <u>E coli</u> (Thomason 1974). Second, eluates and washes were tested by an ELISA.

i) <u>IF technique (after Thomason 1974) for the demonstration of antibodies to E coli in kidney eluates</u>

<u>Preparation of E coli coated Teflon slides</u>

1 <u>E coli</u> broths of serotypes 01, 02, 04, 06, 018, 025, 075, 077 individually, and as a mixture, were smeared onto Teflon-coated multiwelled slides (Cel-line Associates Inc, Newfield, NJ).

2 The slides were air dried, and fixed for three minutes in Kirkpatrick's fixative (six parts absolute alcohol, three parts chloroform, one part 37% formaldehyde).

3 The slides were rinsed in 95% ethanol, and air dried at room temperature before being stored in sealed boxes at $-20^{o}C$.

<u>Fluorescein antibody identification of anti-E coli antibodies</u>

1 The slides were brought to room temperature and rinsed in absolute alcohol to remove moisture.

2 Serotype-specific rabbit antisera or eluates were incubated

with bacteria in slide wells for 30 minutes at room temperature in a moist chamber.

3 The slides were washed for 10 minutes in PBS three times, and air dried.

4 The test wells were incubated with FITC-conjugated antiserum to human or rabbit Ig for 45 minutes.

5 As 3.

6 The slides were air dried and mounted in a small amount of glycine buffered glycerol to prevent the bacteria from floating into adjacent wells.

7 The slides were examined with a Leitz Ploem epi-illumination Orthomat Microscope.

Application of the fluorescein anti-E coli antibody test

- The efficacy of the technique and the integrity of each serotype was shown using rabbit antisera to specific serotypes at 1:4 and 1:8 (stage 2 above) with single serotypes and the mixed wells. Binding was demonstrated using FITC-conjugated bovine anti-rabbit IgG at 1:10 (stage 4 above) diluted in BSA/PBS. Staining was controlled by the use of normal rabbit serum at stage 2 above.
- The mixed system was then tested using 1:4 human serum from a patient who died after an *E coli* septicaemia as a positive control and NHS as a negative control. Antibody activity was demonstrated by FITC anti-human IgG at 1:8 (Wellcome) as the second layer (stage 4).
- The mixed system was then used as a target for the nine eluates from patients with ALD and mesangial IgA deposition. A positive control was serum from a patient with *E coli* septicaemia

whilst NHS was used as a negative control. FITC-conjugated anti-human IgA, IgG and IgM (Dakopatts) at 1:8 were used as the second layer (stage 4).

ii) Anti-bacterial coat - ELISA

The ELISA system used to identify anti-bacterial antibody involved the sensitisation of plates with fractured bacterial envelopes followed by incubation with eluates or washes whose binding was identified by affinity-purified goat anti-human Fab conjugated with alkaline phosphatase, and subsequently quantitated by spectrophotometry. This assay was performed by Richard Harries under the supervision of Professor D Rowley, Microbiology Department, The University of Adelaide. Nine glomerular eluates from patients with ALD and mesangial IgA deposits, one from a normal control kidney and one from a patient with membranous nephropathy (MGN) were tested. Where available first glomerular washes were tested. In view of the paucity of available eluate and the assumption that washes contained more Ig, a single limited assay was performed using eluates at dilutions from 1:8 to 1:64, washes from 1:400 to 1:3200 and the positive control serum from 1:2 to 1:16. The detailed methodology is stated in Appendix VIII.

H Auto-antibodies in eluates

Auto-antibodies to MB, aggregated HSA and normal mesangial antigens were sought in the eluates.

i) The demonstration of antibody activity to MB in kidney eluates from patients with mesangial IgA deposits and ALD by IIF

1 Frozen sections of the liver of a patient (50432.79) with cirrhotic ALD and extensive MB without mesangial IgA deposition was sectioned at 4 μm.

2 The sections were incubated for 30 minutes with undiluted eluates or two known high titre anti-smooth muscle antibody (SMA) sera at a 1:10 dilution, as positive controls. Sections were also incubated with either PBS, serum from a patient with acute alcholic hepatitis at a dilution of 1:10, serum from a patient with an IgA myeloma at 1:20 or with 1:10 NHS.

3 The sections were washed for 15 minutes in PBS, air dried, and incubated in a moist chamber with FITC-conjugated rabbit anti-human IgA, IgG or IgM (Dakopatts) at a dilution of 1:8 for 45 minutes.

4 The sections were then washed for 15 minutes in PBS, air dried, and mounted in glycine buffered glycerol pH 8.6.

5 The sections were examined using a Leitz Orthomat Microscope with Ploem epi-illumination.

ii) To demonstrate antibody activity to aggregated serum albumin by immunodiffusion

HSA was aggregated by glutaraldehyde treatment or heating. Agg-HSA was then tested in a double immunodiffusion system against kidney eluates or serum. HSA was obtained, buffered concentrated, having been prepared by Cohn's ethanol process

from the Commonwealth Serum Laboratories (CSL Melbourne).

a) Preparation of glut-Agg HSA (after Onica et al 1978)

1 100 mg of HSA was made up to 9 ml in 0.1 M PB pH 6.8.

2 1 ml 2.5% aqueous glutaraldehyde was added and stirred at room temperature for two hours.

3 The solution was dialysed against PBS with four changes over two days.

4 The solution was loaded on a G-300 (Sephacryl Pharmacia) column 1.4 cm in diameter, equilibrated with 0.2 M Tris(hydroxymethyl)-aminomethane (Tris)-HCl buffer at a flow rate of 0.3 ml/minute.

5 The peaks were collected and compared with calibration elutions of albumin, IgG and blue Dextran to select albumin.

6 This fraction was then concentrated to approximately 3 ml, aliquoted and stored at -20°C.

b) Preparation of heat-Agg HSA (Hauptman and Tomasi 1974b)

HSA at 10 mg/ml was aggregated by heating to 65°C for seven minutes in 0.05 M Tris buffer at pH 8.1, cooled to room temperature and aliquoted and stored at -20°C.

The demonstration of antibody activity against Agg-HSA by double immunodiffusion

. The system was tested using diluted goat anti-HSA against doubling dilutions of heat- or glut-Agg HSA from 0.5 to 5.0 mg/ml.

.Serum samples from four patients with alcoholic cirrhosis were similarly tested.

.Nine kidney eluates from patients with ALD and mesangial IgA deposits, one from a patient with MGN and one from a normal kidney were tested in agarose against Agg-HSA prepared by both methods at concentrations from 0.5 to 5.0 mg/ml.

iii) The reactivity of mesangial deposits towards HSA

FITC-HSA, and FITC-conjugated goat anti-HSA were prepared in the same way as the BSA equivalents and tested against unfixed frozen sections of kidneys from patients with mesangial IgA deposits and ALD.

iv) The binding of eluates to normal mesangia

To identify antibody activity towards renal mesangial constituents in Ig eluted from kidneys with IgA deposits and ALD, the eluates were incubated with frozen sections from a kidney with no abnormality by light or immunofluorescence. Binding was sought by FITC-conjugated antisera to human IgA, IgG and IgM, and controlled by the staining of sections previously incubated with PBS.

1. Sections of frozen unfixed normal kidney were cut at 2 μm and washed for five minutes in PBS.
2. Three sections were incubated for 30 minutes with each of nine undiluted eluates from patients with mesangial IgA deposits, or PBS.

3 The sections were washed for 20 minutes in PBS and stained with FITC-conjugated antisera to human IgA, IgG and IgM for 30 minutes.

4 The sections were washed for 20 minutes, and mounted in glycine buffered glycerol pH 8.6 and examined with a Leitz Orthomat Microscope with Ploem epi-illumination.

RESULTS

A $SpC1_q$ assay for CIC in patients with ALD.

B Elution of immune deposits from tissue sections of kidney.

C Elution of immune deposits from renal glomerular preparations.

D Anti-BSA activity in patients with ALD.

i) RIA for anti-BSA activity in serum, eluates and washings.

ii) BSA binding capacity of IgA deposits in renal tissue.

E Anti-bacterial antibody activity in the glomerular eluates.

F Auto-antibody activity.

i) Anti-MB activity in eluates.

ii) Anti-HSA activity in eluates.

iii) Binding capacity of eluates to normal mesangium.

RESULTS

A CIC in patients with ALD

The SpC1_q assay was performed using purified C1_q to coat plates. The yield of C1_q from 80 ml serum was 2.57 mg. For the assay, aggregated human globulin (AHG) was used to produce a standard curve and the results were expressed in μg of AHG/ml.

Of the 28 patients examined with severe ALD, all but three had CIC (89%). Twelve had biopsy-proven cirrhosis and eight, biopsy-proven mesangial IgA deposition (Table 4.1). The mean plus two standard deviations (+2SD) CIC level in the group was 37 $\pm$ 31 μg AHG/ml. Seven of the eight with biopsy-proven mesangial IgA deposition, and all four patients with both cirrhosis and mesangial deposits, had CIC.

B Elution of immune deposits from tissue sections of kidney

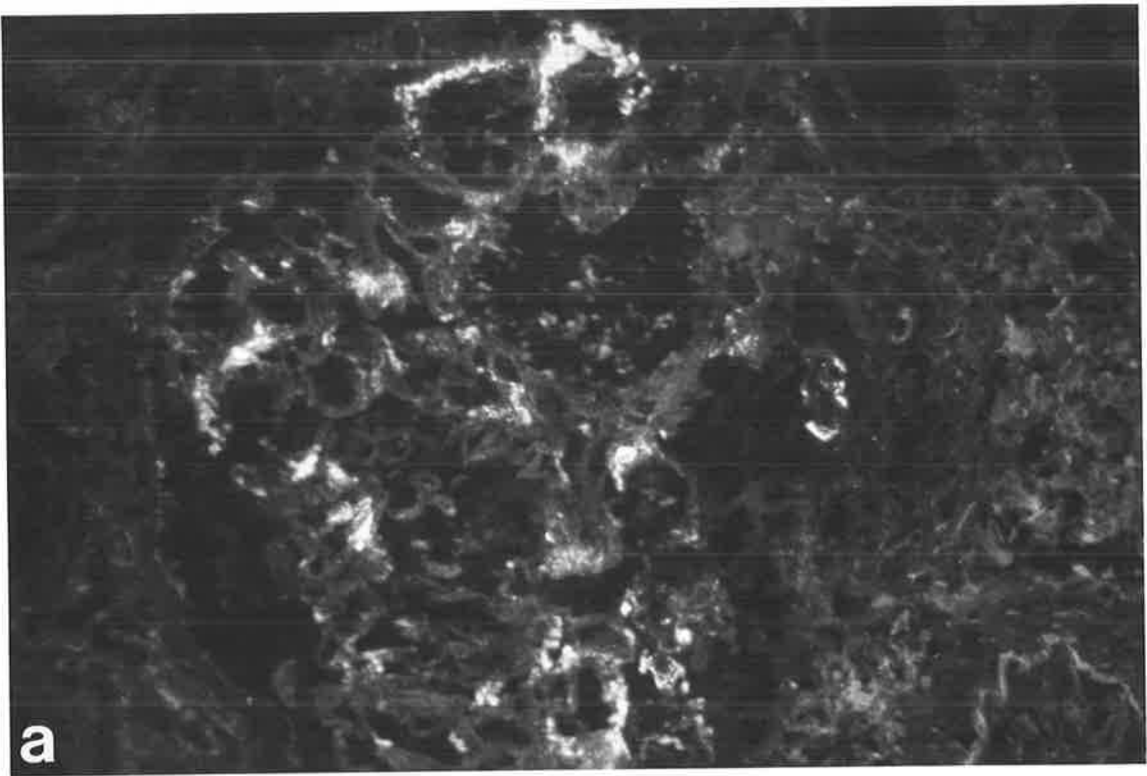
Citrate elution of frozen sections on a glass slide resulted in marked diminution of IgA from a grade 2 to 0 staining, and C_3 from grade 3 to 1 (Figure 4.1-4.2). The technique was capable therefore of removing mesangial deposits.

C Elution of immune deposits from renal glomerular preparations

The glomerular preparation yielded between 10-37 g of glomeruli from each kidney (Table 4.2), representing 9-32% of the cortical weight. The Ig concentration was calculated with reference to the standard curve in the SpRIA (after Roffe et al 1981) (Figure 4.3). The Ig yield was greatest in the kidneys with the lowest cortical weight and low glomerular

Table 4.1 The levels of CIC in the serum from 28 patients with ALD from the stored renal unit collection, measured in the solid phase Cl_q assay

Patient No.	Biopsy proven cirrhosis	Mesangial IgA deposition	CIC in μgAHG/ml
1	+	-	14
2	-	+	5
3	+	-	11
4	+	-	20
5	-	-	0
6	-	-	46
7	-	-	110
8	+	-	115
9	+	-	36
10	-	-	24
11	+	+	28
12	-	+	80
13	+	-	4
14	+	+	10
15	+	-	4
16	-	-	35
17	-	-	7
18	-	-	0
19	-	-	23
20	+	+	20
21	+	+	13
22	-	-	25
23	-	-	110
24	-	-	60
25	+	-	64
26	-	+	0
27	-	+	46
28	-	-	4



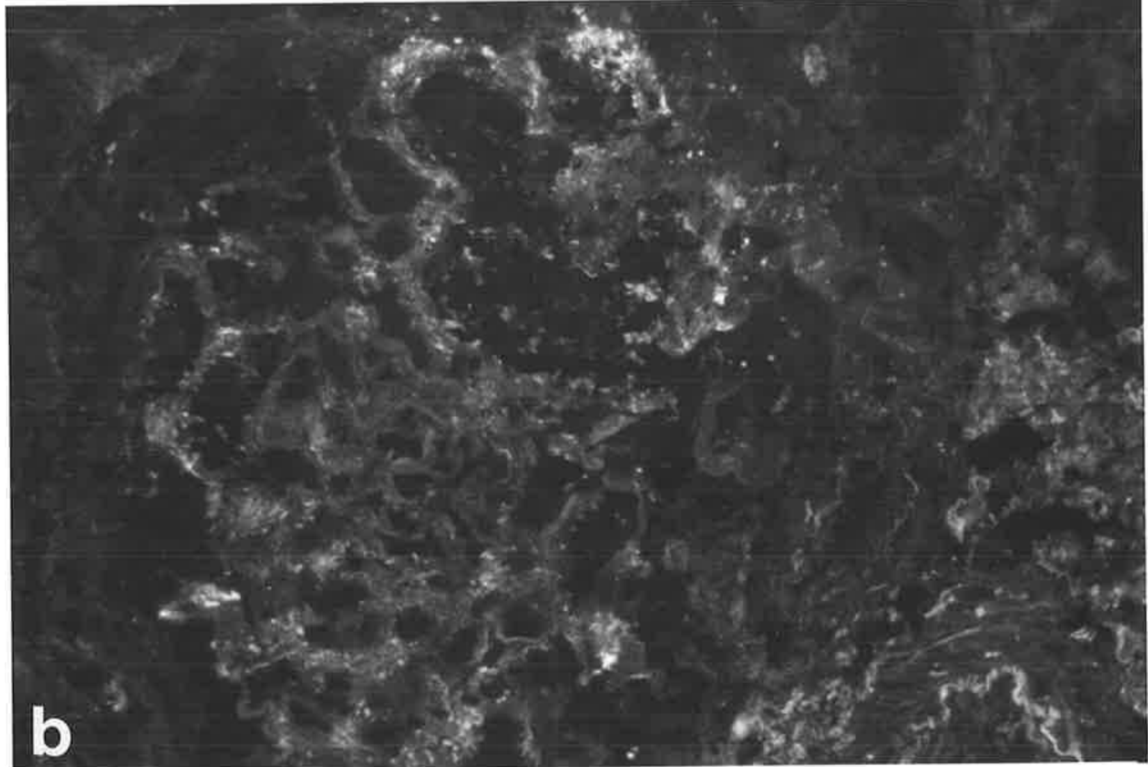
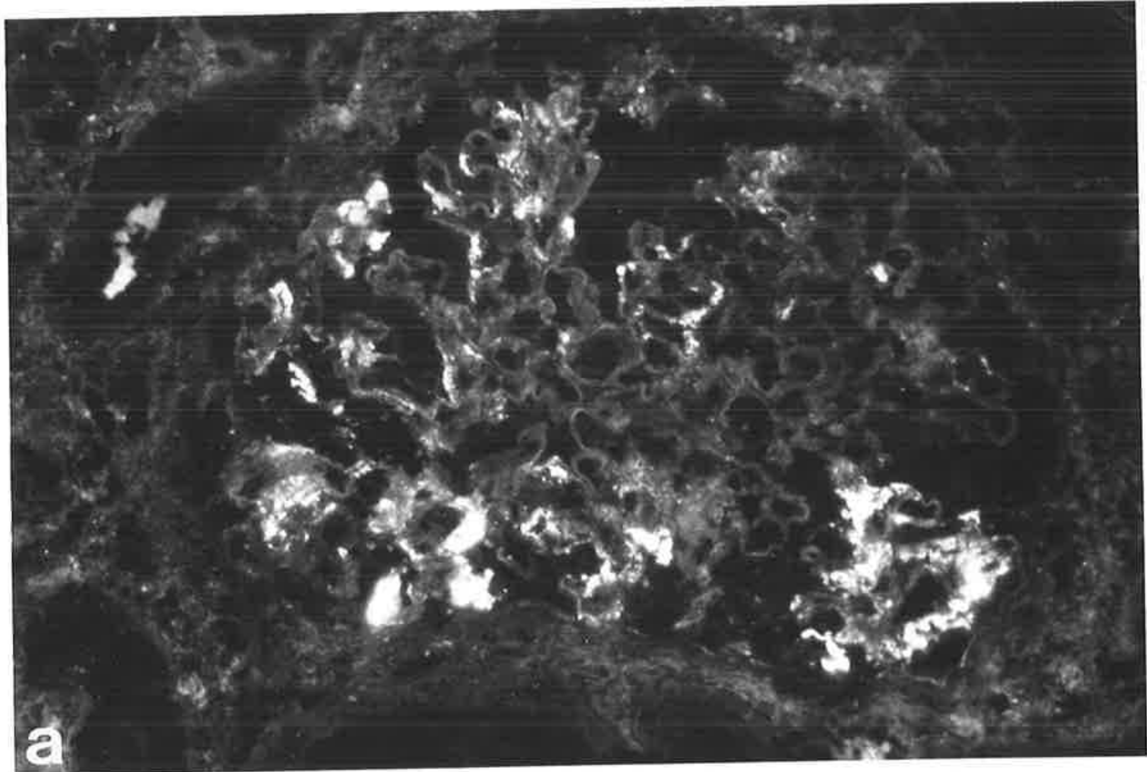


FIGURE 4.1
Renal glomeruli demonstrating slide elution by citrate buffer. Stained with FITC-conjugated rabbit anti-human IgA. There is a diminution in intensity of staining from 2+ in the section incubated with PBS (a) to barely a trace after incubation with citrate buffer (b). (DIF, X1400).



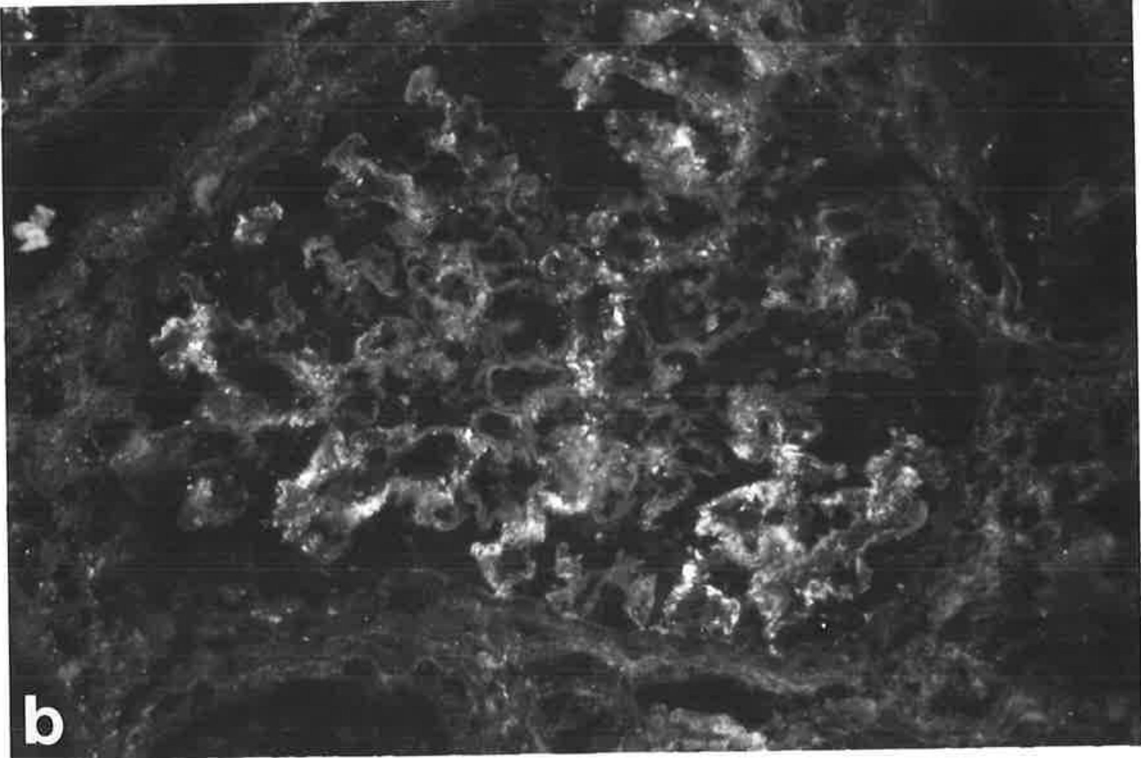


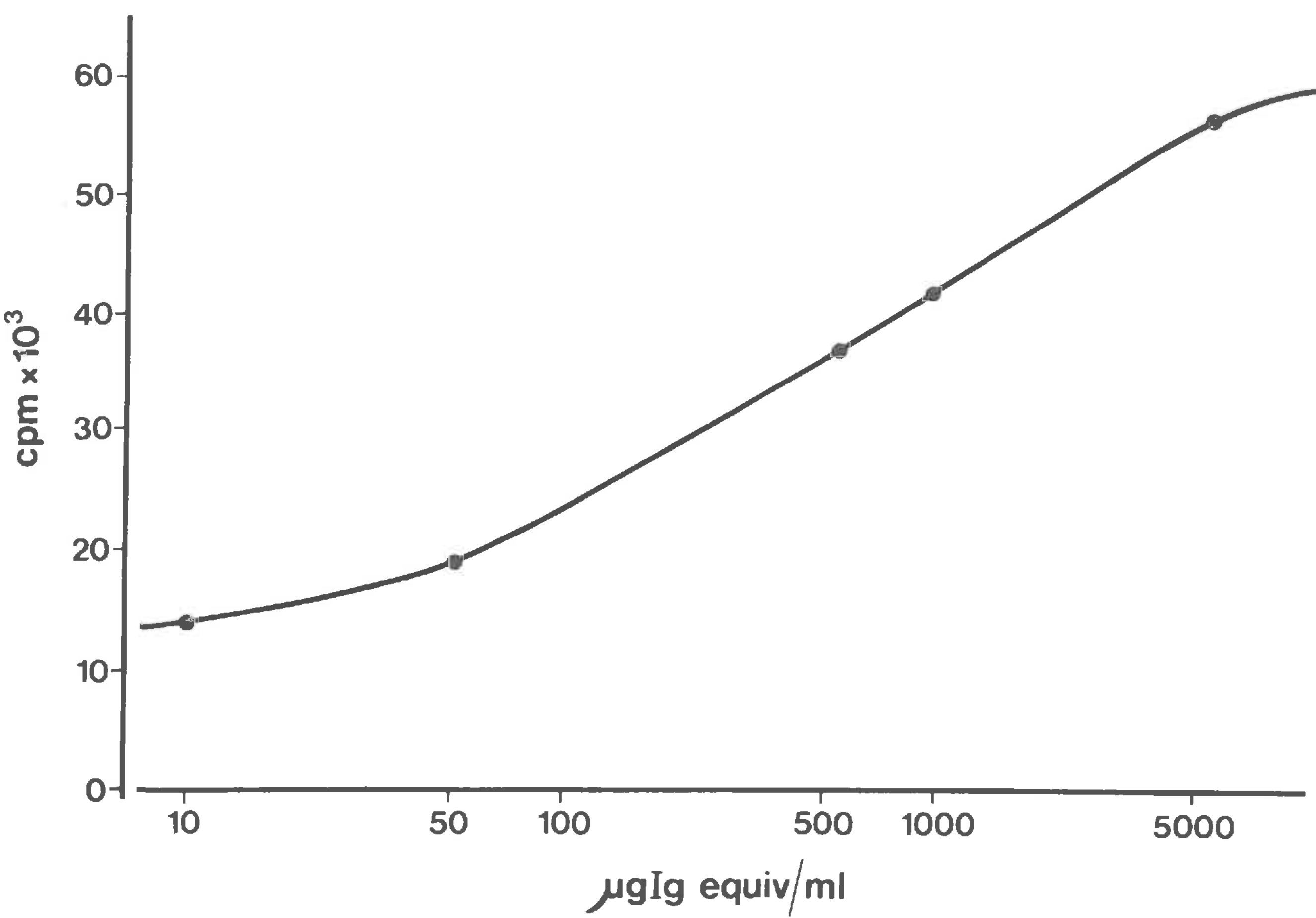
FIGURE 4.2
Slide elution by citrate buffer. Stained with FITC-conjugated rabbit anti-human C_3. There is diminution of staining from 3+ in the section incubated with PBS (a), to 1+ after incubation with citrate buffer (DIF, X1400).

Table 4.2 The glomerular preparation and acid elution of immunoglobulin from one control fluorescence-negative kidney (4783) and nine kidneys with mesangial IgA deposits from patients with ALD. The total immunoglobulin concentration was measured by RIA

Results (units)	Autopsy number									
	50203	50329	50331	50358	50439	50442	50493	50512	50730	4783 (Control)*
Cortex weight (g)	133.38	119.60	57.71	111.40	132.91	103.00	34.43	150.00	114.00	102.50
Glomerular weight (g)	30.85	31.43	11.39	10.37	21.78	23.00	8.80	35.25	36.13	24.38
Immunoglobulin (μg IgG equiv/ml)	31	522	743	36	260	254	840	186	328	6
Total Ig (μg IgG equiv/g of glomeruli)	4.02	4.09	101.04	8.68	47.75	27.60	267.27	13.19	27.23	0.54

* = IF negative kidney

Figure 4.3 Dose-response curve of IgG in a plate pre-coated with anti-Fab antibody



yield (50493; 50331). The results of Ig estimations performed on available washings are shown in Table 4.3.

D Anti-BSA activity in patients with ALD

i) RIA for anti-BSA activity of glomerular eluates, washings and where available serum

Results of the fluid phase anti-BSA assay were expressed as a percentage of TCA precipitable counts. With each assay, a standard curve of goat anti-BSA serum was used as a control for the system (Figure 4.4). The mean +2SD of anti-BSA activity in 20 healthy controls was 9.7%. The serum specimen (# 36) which had the nearest anti-BSA activity (9.8%) to the mean +2SD value was used in the subsequent assays to indicate the upper limit of normal for sera. PBS was used as the upper limit of normal for the assessment of eluates in the assay.

The assay was used to test 17 sera stored in the Renal Unit, RAH from patients known to have alcoholic cirrhosis. The results are shown in Table 4.4 and show that, with reference to the baseline serum, all were positive.

The results of the assays performed on kidney eluates, serum and washings are shown in Table 4.5. Two of the nine kidney eluates from patients with ALD showed levels of anti-BSA activity regarded as positive, being greater than that of PBS. Eluate 50329 had an anti-BSA activity of 4.7% compared with 2.5% for the PBS baseline marker, and in a different assay eluate 50512 had an anti-BSA activity of 4.5% compared with 1.7% for the PBS baseline marker. The upper limits for normal

Table 4.3 The total immunoglobulin concentrations of washes of glomeruli from patients with ALD measured by RIA

Autopsy Number	Immunoglobulin concentration (mg of IgG equiv/ml)
50203	15.80
50329	6.90
50331	0.60
50358	2.50
50439	4.05
50442	1.46
50493	6.70
50730	2.28
4783 (Control)	9.40

Figure 4.4 Curve obtained with increasing dilutions of goat anti-BSA serum in the fluid phase anti-BSA assay expressed as a percentage of available precipitable counts

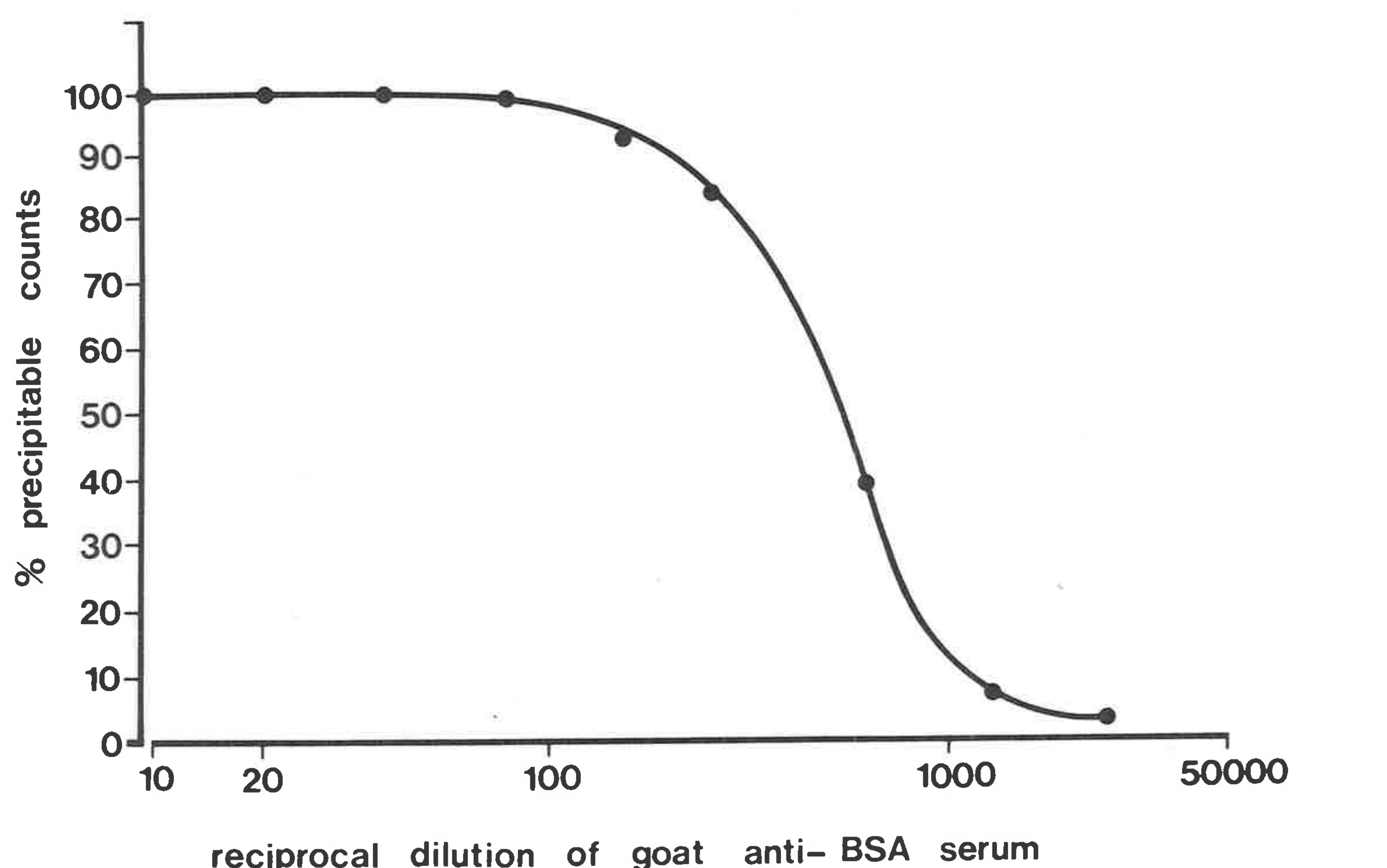


Table 4.4 The results of the anti-BSA fluid phase RIA performed upon sera from 17 patients with alcoholic cirrhosis and the control serum 36 (mean +2 SD)

Patient Number	Anti-BSA activity as % TCA precipitable counts
101	10.9
102	11.0
103	8.7
104	8.9
105	11.6
106	13.4
107	12.9
108	8.4
109	12.2
110	9.6
111	11.9
112	9.2
113	10.2
114	10.7
115	10.0
116	11.1
117	7.3
36 (mean +2 SD)	6.9

Table 4.5 The results of the anti-BSA fluid phase RIA performed upon glomerular eluates, washes and sera from patients and controls, measured at the dilutions stated

Patient category	Number	Anti-BSA activity in % TCA precipitable counts			
		Glomerular wash		Glomerular eluate	Serum
		1:10	1:2	1:10	1:10
Alcoholic liver disease	50331	NA	NA	1.7	NA
	50358	NA	NA	2.2	NA
	50439	1.0	2.2	2.0	10.3
	50442	0.9	3.2	2.1	9.1
	50203	1.6	3.4	1.8	NA
	50329	1.2	2.9	4.7	NA
	50730	1.0	2.4	1.3	NA
	50493	1.0	2.3	1.5	NA
	50512*	NA	NA	4.5	5.6
Controls					
Normal kidney	4783	1.3	2.7	1.5	4.5
Membranous GN	1	NA	NA	1.4	NA
	2	NA	NA	1.7	NA
Standards	36 (mean +2SD)	4.5	4.5	7.3	7.3
	PBS	0.4	0.4	2.5	2.5

NA = sample not available
* = specimens from this patient were obtained after the performance of the other assays. The standard samples assayed with these specimens gave results as follows: 36(mean +2SD)=6.4 PBS=1.7 expressed as anti-BSA activity in % TCA precipitable counts

sera in these assays were 7.3% and 6.4% respectively.

The two eluates from patients with MGN and the eluate from the normal control kidney (4783) showed anti-BSA activity below that of the PBS baseline marker. Two of the three available sera from patients with ALD whose kidneys were eluted showed levels of anti-BSA activity greater than the mean +2SD serum baseline (50439; 50442). All seven first glomerular washes, including that from the control kidney (4783), showed results greater than the PBS blank (0.4%) but less than the mean +2SD for serum (4.5%).

The two positive eluates 50329 and 50512 were titrated out in a subsequent anti-BSA assay. 50512 had serum available and 50329 only a first wash. These fluids were similarly titrated and the results are shown in Figure 4.5 and 4.6, with reference to the same PBS baseline marker. For kidney 50329 the eluate: washing end-titre ratio of anti-BSA antibody concentration/unit of Ig was 1.7. For kidney 50512 the eluate:serum end-titre ratio of anti-BSA antibody concentration/unit of Ig was 106 (Table 4.6). The significance of the titrations is discussed later.

ii) <u>BSA binding capacity of mesangial IgA deposits</u>

In eight of 11 autopsy patients with ALD and mesangial IgA deposits, fluoresceinated BSA with an F:P ratio of 2.68, bound to the mesangium as discrete globules. This appearance was reproducible (Figure 4.7). The same staining was seen in five of eight patients biopsied with ALD and mesangial IgA deposits

Figure 4.5 The titration of the eluate and washing from kidney 50329 in the liquid phase anti-BSA assay

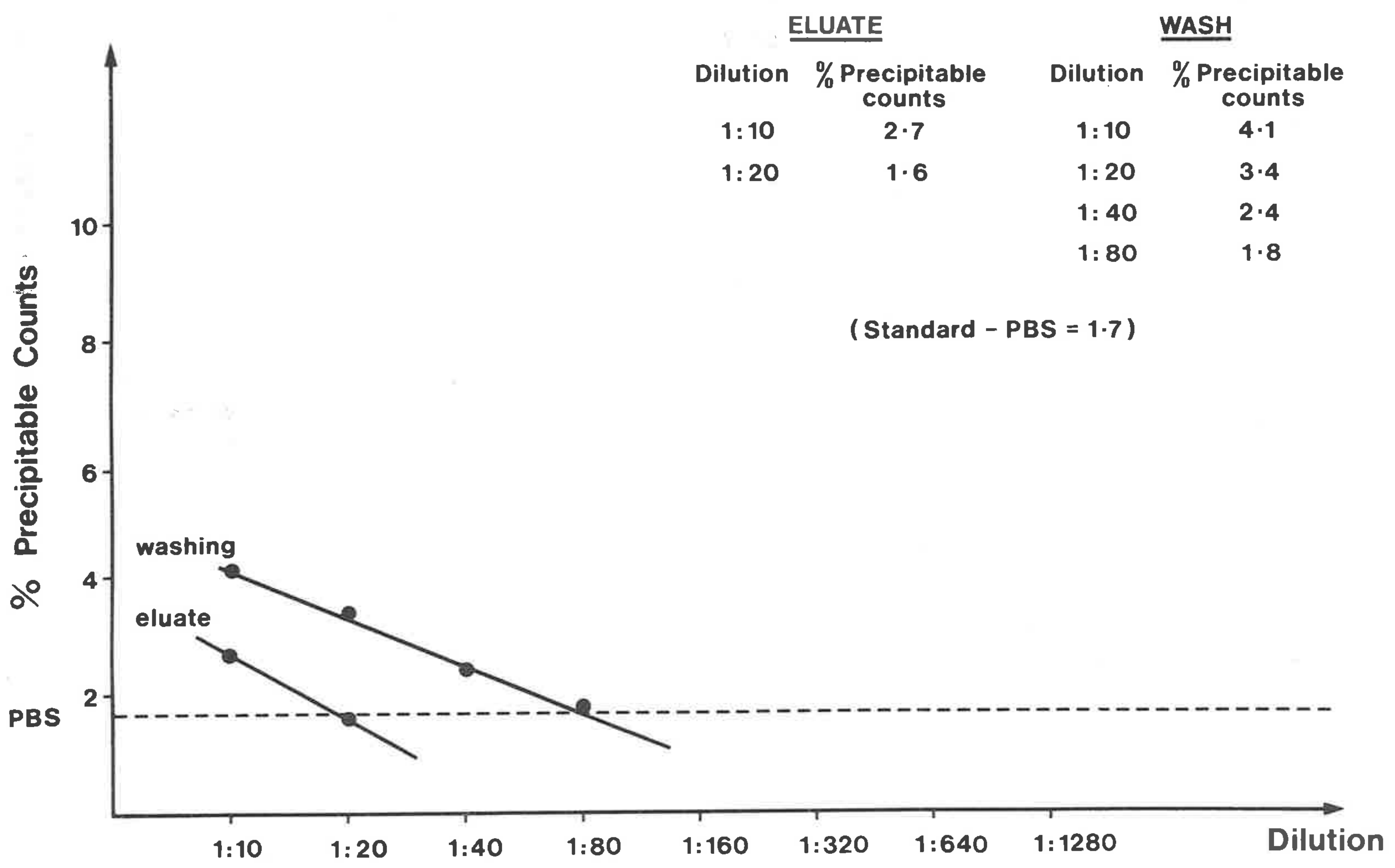
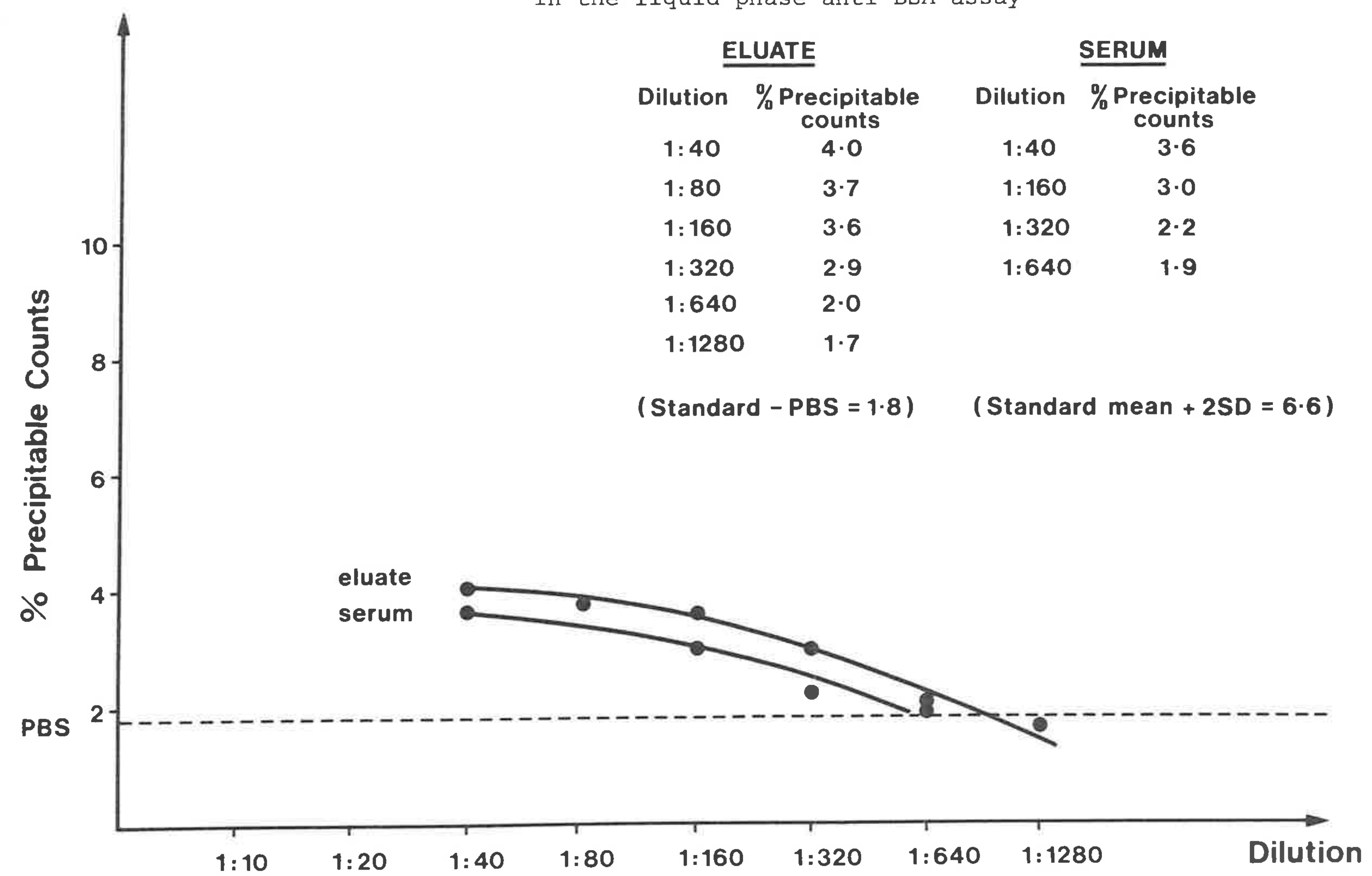


Figure 4.6 The titration of the eluate and serum from patient 50512 in the liquid phase anti-BSA assay



ELUATE

Dilution	% Precipitable counts
1:40	4·0
1:80	3·7
1:160	3·6
1:320	2·9
1:640	2·0
1:1280	1·7

(Standard - PBS = 1·8)

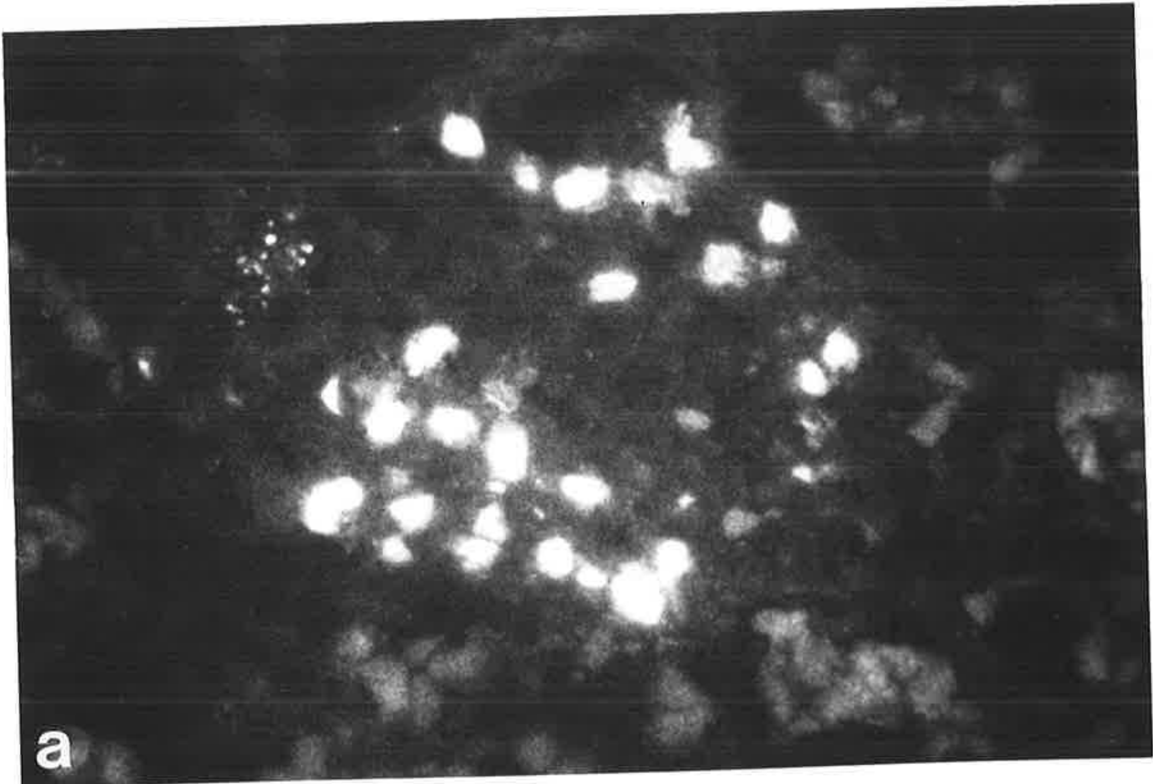
SERUM

Dilution	% Precipitable counts
1:40	3·6
1:160	3·0
1:320	2·2
1:640	1·9

(Standard mean + 2SD = 6·6)

Table 4.6 The immunoglobulin concentrations and end-titre concentration ratios of the two glomerular eluates demonstrating anti-BSA activity

Sample	End-titre in anti-BSA assay	Immunoglobulin concentration at end-titre in μg IgG equiv/ml	Specific antibody concentration ratio Eluate: wash or serum
50329			
Eluate	1:10	52.2	1.7
Washing	1:80	86.2	
50512			
Eluate	1:640	0.3	106
Serum	1:640	31.7	



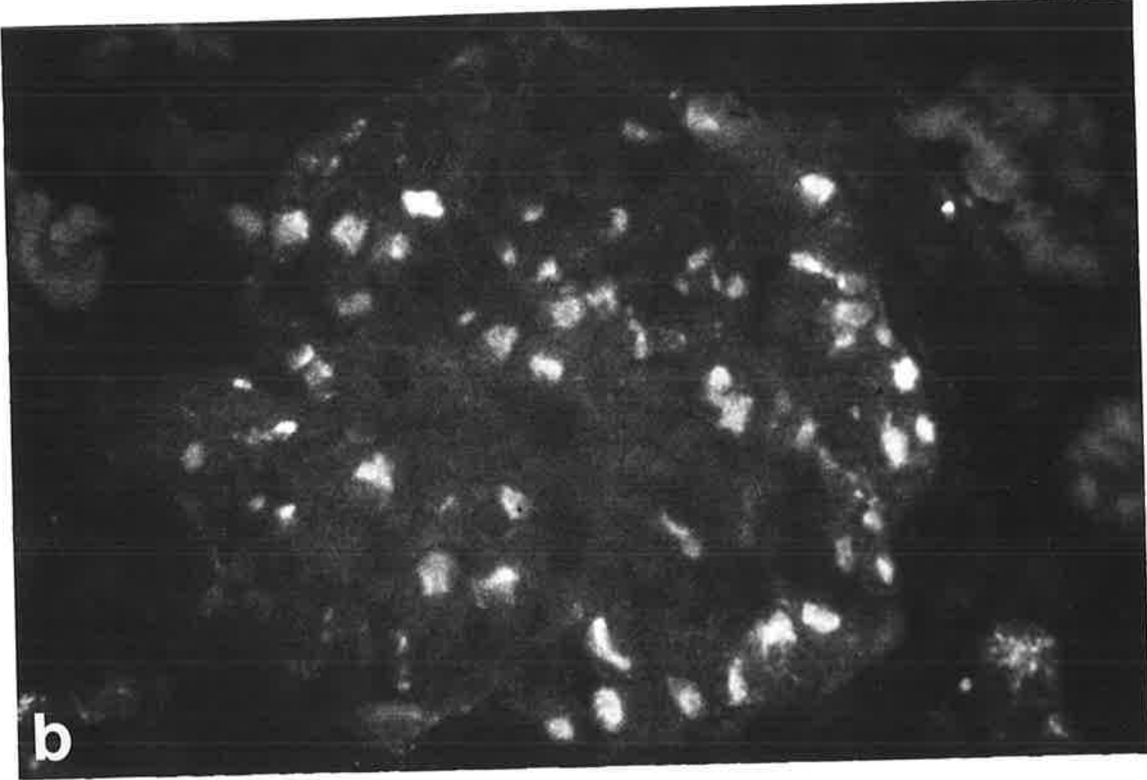


FIGURE 4.7
Glomeruli from a patient with mesangial IgA deposition and ALD, incubated with FITC-conjugated BSA (a), or FITC-conjugated HSA (b). Note the globular mesangial binding in both. (DIF, X900).

but in only two of eight kidney biopsies with primary IgA nephropathy. Twenty-one Ig-negative kidneys were studied. While seven of the 14 obtained at autopsy had globular BSA staining, no staining was observed in the seven obtained by biopsy.

FITC-BSA bound to kidney biopsies from patients with SLE (five of six) and HSP (two of four) as well as four of eight other forms of GN (mesangiocapillary glomerulonephritis (MCGN), dense deposit disease (DDD) and postinfectious glomerulonephritis (PIGN)) (Table 4.7). Blocking experiments repeated at increasing concentrations of BSA up to 30 mg/ml failed to prevent subsequent FITC-BSA binding.

iii) The demonstration of BSA in kidneys with mesangial IgA deposits

FITC-conjugated goat anti-BSA produced no mesangial staining in any of the kidneys tested. However, there was always linear glomerular basement membrane accentuation, the significance of which will be discussed.

E Anti-bacterial antibody activity in the glomerular eluates

i) IF technique for the identification of antibodies to E coli in eluates

The positive control IIF system with rabbit specific anti-serotypes showed between 2-3+ fluorescence when used at 1:4 and 1:8 dilutions on plates made with single serotypes in each well. Incubation of sero-specific antisera with mixed wells produced similar results. Incubation of plates with

Table 4.7 Mesangial binding of FITC-BSA in various disease groups

Source of tissue	Positive staining (Number of subjects)					
	Controls	Mesangial IgA and ALD	Primary IgA nephropathy	HSP	SLE	OTHER GN
Biopsy	0 (7)	5 (8)	2 (8)	5 (6)	2 (4)	4 (8)
Autopsy	7 (14)	8 (11)	NT	NT	NT	1 (1)

normal rabbit sera produced no staining with FITC-conjugated antiserum to rabbit IgG by IIF.

The positive control serum from a patient with E coli septicaemia showed 1+ fluorescence affecting half of the rods with FITC antisera to IgG, IgA and IgM. The nine eluates from kidneys of patients with ALD and one normal control were tested in parallel with NHS as a negative control, and the serum from the patient with septicaemia as a positive control. The latter test gave a 2+ reaction for IgG (Figure 4.8-4.9). The slides incubated with NHS and each of the eluates were all negative when stained for IgA, IgG and IgM.

ii) ELISA technique for the demonstration of anti-bacterial coat antibodies

The anti-bacterial coat ELISA showed reactivity of five eluates against E coli strain 221.58. Three of these also showed reactivity against E coli B, and one of these latter eluates (50493), against S typhimurium and the mixed Bacteroides species (Table 4.8). The eluate from a patient with MGN showed no significant conjugate binding. At the lowest dilution of the washes tested, only two washes showed reactivity (50442 and 50493). The end-titres are shown in Table 4.9. The assay was highly sensitive, recording reactivity in fluids with Ig concentrations of less than 20 μg/ml. In the single assay performed, end-titres were not recorded for all of the washes. However, at the lowest dilutions tested they could be shown in some cases to be non-reactive at a concentration greater than the end-titre of the corresponding eluate. This limited study shows preferential concentration of anti-bacterial reactivity in the

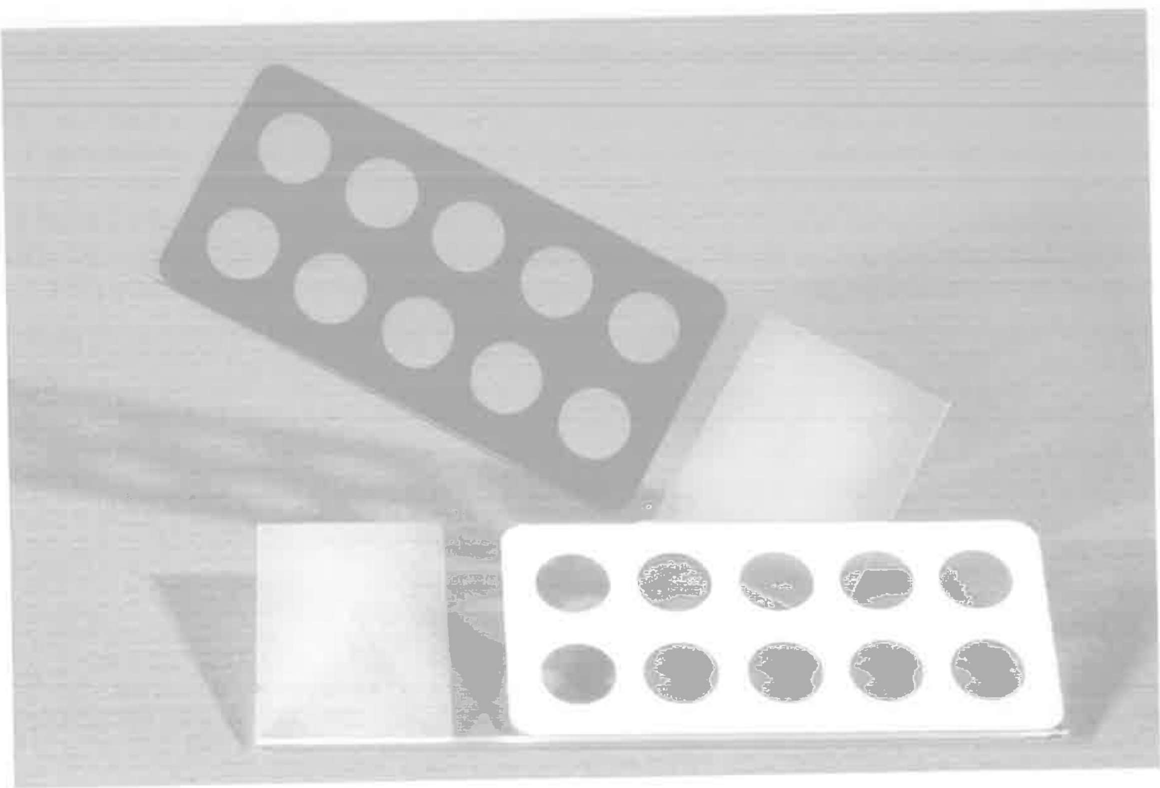


FIGURE 4.8
Teflon coated multi-welled slides which were coated with E coli broths and used to identify fluids with anti-bacterial activity.

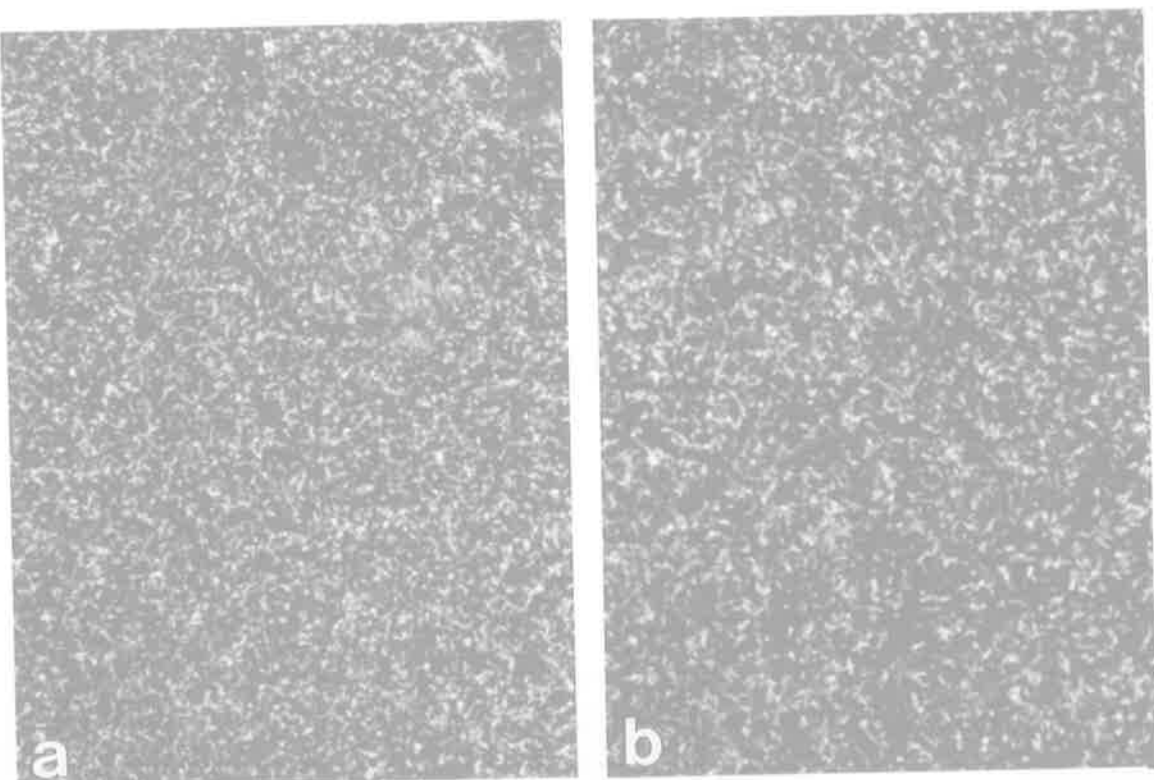


FIGURE 4.9
E coli coated slides. (a) Incubated with the serum from a rabbit immunised with E coli 016 (stained with FITC-conjugated anti-rabbit IgG). (b) Incubated with serum from a patient who died from an E coli septicaemia (stained with FITC-conjugated anti-human IgG). The rods show a 2-3+ staining reaction in both instances (IIF, X90).

Table 4.8 Results of anti-bacterial coat enzyme linked immunosorbent assay performed upon nine kidney eluates from patients with ALD associated with mesangial IgA deposits; one normal control kidney (4783) and one patient with membranous nephropathy (MGN)

Bacterial coat activity	Reactivity of kidney eluates										
	50203	50329	50331	50358	50439	50442	50493	50512	50730	4783	MGN
E coli 221.59	-	-	-	-	-	-	-	-	-	-	-
221.58	-	-	-	-	+	+	+	+	+	-	-
B	-	-	-	-	-	+	+	-	+	-	-
BV	-	-	-	-	-	-	-	-	-	-	-
LC	-	-	-	-	-	-	-	-	-	-	-
S typhimurium	-	-	-	-	-	-	-	-	-	-	-
enteroides	-	-	-	-	-	-	-	-	-	-	-
minnesota	-	-	-	-	-	-	+	-	-	-	-
adelaide	-	-	-	-	-	-	-	-	-	-	-
P vulgaris	-	-	-	-	-	-	-	-	-	-	-
Psd pyogenes	-	-	-	-	-	-	-	-	-	-	-
Mixed Clostridia	-	-	-	-	-	-	-	-	-	-	-
Mixed Bacteroides	-	-	-	-	-	-	+	-	-	-	-

Table 4.9 The end-titre Ig concentration in μg IgG equiv/ml of anti-bacterial coat activity in acid eluates and washes from five patients with ALD measured by ELISA

Sensitising bacterial coat	Eluates: washes end-titre Ig concentration				
	50439	50442	50493	50512	50730
	E:W	E:W	E:W	E:W	E:W
E coli 221.58	4:>10(p)	4:2	5:8 (p)	3:NA	20:>6
E coli B	-	8:2	5:>17(p)	-	10:>6
S minnesota	-	-	5:>17(p)	-	-
Mixed Bacteroides	-	-	5:>17(p)	-	-

(p)=preferential concentration in eluates

Ig of eluates 50439 and 50493. Similar preferential concentrations may exist in the eluates from patients 50512 and 50730.

F Auto-antibody activity

i) Anti-MB activity in glomerular eluates

The high-titre SMA sera used as controls were graded as having titres of 2+ to 3+. Two of the higher titre anti-SMA sera tested, showed IgA hepatocyte staining arbitrarily graded as 2+ on a scale of 0-3. This staining was in a perinuclear rope-like pattern in some hepatocytes conforming in number and distribution to the LM distribution of MB-containing hepatocytes. Two of the sera were used as positive controls in the subsequent experiments to investigate the ability of eluted Ig to bind to MB.

The eluates showed similar binding in seven of nine cases (Table 4.10). Anti-MB staining was also seen using serum from a patient with acute alcoholic hepatitis. The positive fluorescence in all cases was restricted to IgA. IIF was negative following incubation with a normal control eluate, PBS, an IgA myeloma serum and NHS. Positive hepatocyte staining was distinguished from the staining of plasma cell cytoplasm on morphological grounds (Figure 4.10). IgA, IgG and IgM plasma cells were observed predominantly in the fibrous connective tissue.

ii) Antibody activity in eluates to Agg-HSA

Immunoprecipitation to both heat- and glut-Agg HSA by goat anti-HSA was seen to a serum dilution of 1:128 and HSA dilution of

Table 4.10 The binding of glomerular eluates and sera to MB in liver sections, demonstrated by IIF and graded 0-3+

Specimen type		Number	MB binding of IIF		
			IgA	IgG	IgM
		50439	1	0	0
		50358	2	0	0
		50493	2	0	0
Eluates:		50331	1	0	0
From patients with ALD		50730	0	0	0
		50329	3	0	0
		50203	1	0	0
		50442	1	0	0
		50512	0	0	0
From normal control patient		4783	0	0	0
Serum:	SMA positive	44532	3	0	0
	SMA positive	43875	2	0	0
	ALD		2	0	0
	IgA myeloma		0	0	0
	NHS		0	0	0

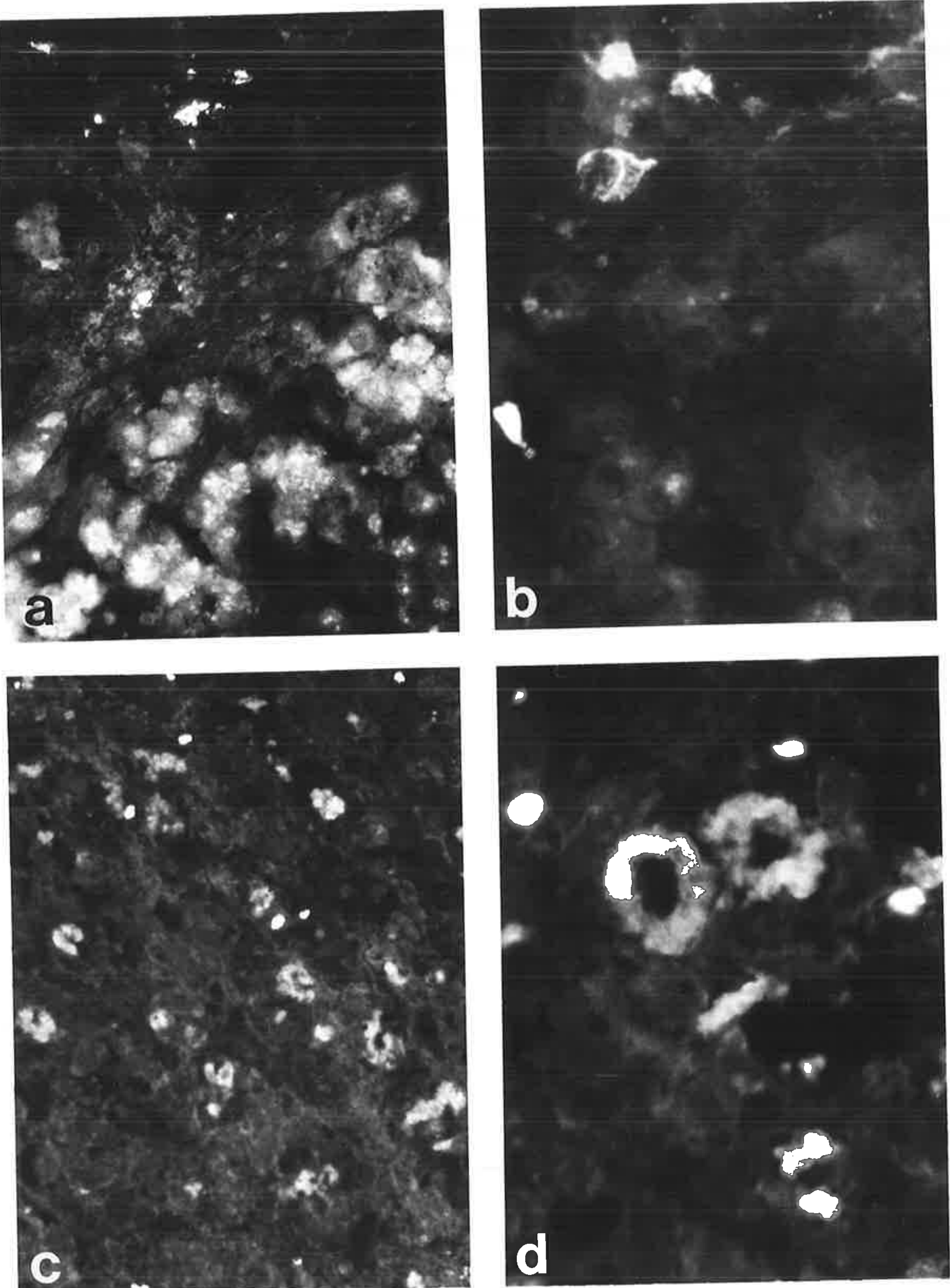


FIGURE 4.10
IIF of liver sections with abundant MB stained with FITC-conjugated antiserum to human IgA. (a) After incubation with high titre SMA there is extensive intracytoplasmic staining suggesting the contours of MB. A plasma cell is seen in the connective tissue (X750).
(b) Incubation with NHS, shows only plasma cell staining (X2000).
(c) Incubation with the eluate of a patient with ALD (50331) shows intracytoplasmic staining (X750) which at higher power, (d) has the characteristic conformation of MB (X2000).

0.5 mg/ml. Sera from four patients with alcoholic cirrhosis and eluates from nine kidneys from patients with ALD were all non-reactive.

iii) HSA and HSA binding in mesangial deposits

Anti-HSA activity demonstrated by FITC-HSA binding to mesangial areas mirrored exactly the appearances seen with FITC-BSA but with less intensity. Similarly, FITC-conjugated anti-HSA produced only faint glomerular basement membrane accentuation.

Blocking experiments using increasing concentrations of HSA up to 90 mg/ml produced a marginal diminution of subsequent FITC-HSA binding where the F:P ratio was 4.68.

iv) The binding capacity of eluates to normal mesangium demonstrated by IIF

None of the eluates showed any anti-mesangial reactivity. All eluates produced accentuation of the glomerular basement membrane with antisera to IgG and one produced tubular basement membrane accentuation (50730). Only one (50329) produced any staining with antisera to IgA, and this was only an accentuation of the glomerular basement membrane.

DISCUSSION

This series of experiments was designed to elucidate the IC nature of the mesangial deposits in IgA nephropathy secondary to ALD, and to demonstrate the specificity of the constituent Ig. In support of a CIC origin for the mesangial deposits, a high incidence of CIC was found in the sera of patients with ALD, especially in those with associated mesangial IgA deposition when CIC were almost always present. Mesangial Ig was removed by an elution technique shown by Woodroffe and Wilson (1977) to retain antibody activity. The slide elution experiment performed here showed that mesangial IgA and C_3 were dissociated by incubation with citrate, and subsequent reactivity in several systems showed that the Ig had retained some of its activity. Two acid eluates had anti-BSA activity in the fluid phase RIA, at least two had anti-bacterial coat reactivity, and seven had anti-MB activity. BSA and anti-BSA activity could not be shown in the <u>in situ</u> mesangial deposits, and no auto-antibody activity towards HSA or normal mesangium could be shown in the eluates.

Acid elution of deposits from post-mortem kidneys required first, the preparation of glomeruli and second, their incubation with acid citrate buffer. The variation in glomerular yields found in the kidneys studied could reflect the varying size of glomeruli or their degree of retention in the filter mesh. Very large glomeruli may have been retained in the mesh but the smallest glomerular yields were from those kidneys without glomerulomegaly (50331; 50493) suggesting a correlation between

yield and individual glomerular size. The Ig yield per gram of glomeruli showed marked variation and may have been related to the Ig content or the degree of mesangial sclerosis or hypercellularity. However, the highest Ig yield per gram of glomeruli was from kidneys with only a 2-3+ mesangial IgA intensity by IF whilst the kidney (50442) with the most extensive and greatest intensity of staining had a relatively low yield. The two greatest yields in terms of Ig per gram of glomeruli were from kidneys without diffuse mesangial cell proliferation, and it seems that intrinsic glomerular structure may affect elution.

The anti-BSA assay was performed as described using the serum whose activity was nearest to the mean +2SD for normal serum, as the upper limit of normal. Although this method is valid under normal circumstances when studying sera, its validity when examining low concentration eluates is less certain. For this reason, a PBS baseline was introduced as another standard. Eluates from two patients showed anti-BSA activity in the RIA by these criteria. The anti-BSA activity in both instances was below that of the serum standard. The validity of the PBS baseline for evaluation of low protein fluids was supported by the observations in eluates from one normal kidney and two kidneys from patients with MGN. In these three instances, anti-BSA activity would not be expected and the levels were equal to or below the PBS baseline.

Only one of the eluates with anti-BSA activity (50512) had corresponding pre-mortem serum. In the other (50329), the

first PBS wash was the only available fluid and this was presumed to approximate serum. Following this assumption, the first PBS washes were examined in the anti-BSA assay in relation to the serum standard and all were negative. However, few of the washings were fairly judged against this standard because of their relatively low Ig concentrations. Indeed, two of the pre-mortem serum samples available had elevated anti-BSA titres despite the low levels found in the first PBS washes from the same patients' kidneys. It would seem therefore, inappropriate to use PBS glomerular washes as a substitute for serum in such assays. Since these washes seem to behave as greatly diluted sera, for comparison with a glomerular eluate, the end-titre concentrations of Ig in these fluids were calculated with regard to the PBS baseline.

For case 50329, the kidney eluate had an anti-BSA antibody concentration 1.7 times that of the PBS washing. The eluate from kidney 50512 had an anti-BSA antibody concentration approximately one hundred times higher than the serum. These data imply that the anti-BSA antibody was concentrated in the eluates, and was not a reflection of non-specific entrapment of serum proteins. It is of interest that neither of the two cases with pre-mortem sera showing anti-BSA activity showed similar activity in the acid glomerular eluates. This suggests either that not all such antibodies undergo mesangial entrapment, or that serum anti-BSA correlates poorly with mesangial IgA deposition.

As an extension of this work, and in order to demonstrate _in situ_ anti-BSA activity within the mesangial deposits, BSA was fluores-

ceinated and incubated with kidney sections from patients with mesangial IgA deposition secondary to ALD. There was distinctive globular mesangial binding of FITC-BSA without any extraglomerular binding in patients with ALD. This group was compared with patients with primary IgA nephropathy, other diseases with IgA deposits such as HSP and SLE, and diseases such as PIGN, kappa light chain disease and MGN, normally not associated with IgA deposits. There were inherent problems in comparing these disease categories, in that the material from patients with ALD was obtained post-mortem, while material from all but one patient with MGN was obtained during life by percutaneous needle biopsy. Eight percutaneous needle biopsies from patients with alcoholic cirrhosis were retrieved from the files of the Division of Tissue Pathology, IMVS and examined together with seven biopsies having no fluorescent or light microscopical abnormalities, six autopsy blocks from normal Coronial autopsies, and eight control hospital autopsies. FITC-BSA binding under these circumstances seemed to be related to the presence of any Ig, irrespective of class or disease type and furthermore, to be more common in autopsy material. The failure of blocking experiments demonstrated the non-specific nature of this binding, although the nature of the Ig binding is unclear.

The simple means used to demonstrate anti-E coli activity in the eluates by IIF failed. An ELISA method using fractured bacterial coat particles provided a more sensitive test system and showed antibody activity in five eluates. Each of these five eluates reacted with one or more E coli strains and one showed reactivity against S minnesota and mixed Bacteroides species.

As with the anti-BSA activity described above, it was necessary in the ELISA system to perform titration studies with the eluates and washings to show that antibody activity was concentrated in the eluates in terms of specific antibody per unit of Ig. In one instance (50512) neither washing nor serum was available but the end-titre for the eluate in the anti-E coli 221.58-ELISA was only 3 µg Ig equiv/ml, suggesting a specific reaction. Three end-titre Ig concentrations for washes were calculated, in two, for 50442, these were lower than that of the eluate, and in one for 50493 (E coli 221.58) it was greater than in the corresponding eluate. In all other instances, the lowest dilution of wash tested showed no reactivity in the assay so that the endpoint was presumed to be at a high concentration.

Assessed in this fashion, there was specific reactivity in the eluates 50439 and 50493. The reactivity in eluate 50442 was not specific and those of 50512 and 50730 could not be assessed by the same criteria, either because no wash was available, or the highest concentration of wash tested was non-reactive and more dilute than the eluate end-titre. In assessing specificity of antibody activity by comparing eluates with either washes or serum, one may be inadvertently disregarding significant activity in an eluate if that patient died with terminal infection. This was common in patients with ALD (Chapter 2) but was only specifically noted in case 50493 where, however, there was still a greater specific antibody concentration in the glomerular eluate.

The anti-bacterial coat antibody concentration per unit Ig was more than 2.5 times higher in the eluate than the washing of case 50439 and more than 3.4 times higher for some antigens in case 50493. These data suggest that in at least two cases, anti-bacterial antibody activity was concentrated within the mesangial eluates of patients with ALD. The finding of multiple bacterial specificities within the eluates indicates further the heterogeneity of the IgA antibodies involved.

Patients with ALD have high titres of antibodies against floral antigens (Prytz et al 1973; Woodroffe et al 1980). Antibody to E coli has been demonstrated in the CIC of patients with ALD, and in cryoprecipitates, but preferential concentration was not found, so that they were not implicated in their pathogenesis (Kaufman et al 1982). This ELISA study is the first documentation of the concentration of anti-bacterial activity in the deposits of patients with ALD.

Auto-antibody activity is common in patients with ALD. In this study, several systems were investigated. Human SMA cross-reacts with MB (Virtanen et al 1979) and was used as a positive control in the experiments to demonstrate anti-MB activity in the eluates. Incubation of liver sections with high titre-SMA sera showed intracellular binding demonstrated by subsequent staining with fluoresceinated antiserum to human IgA. The reason for the variability of this property amongst high titre-SMA sera is unclear. Seven of the nine eluates showed similar binding to hepatocyte cytoplasm demonstrated by FITC antiserum to IgA in a structured form similar to the LM contours of MB.

There was no hepatocyte surface membrane staining above the level of the PBS control incubations. The antibody activity demonstrated was of IgA class in both the eluates and positive control systems tested. There was no IIF staining for IgG and IgM.

The immunoreactivity of MB has been demonstrated in ALD by studies of leucocyte migration inhibition, blast transformation and lymphocyte toxicity. Irrespective of the role of such reactivity in hepatic destruction, it has been suggested that antibody activity to MB may play a part in the frequent CIC formed in such patients (Penner et al 1978). Only Kanagasundaram et al (1977) have identified MB and anti-MB in the serum of patients with ALD and these findings have been disputed (Kehl et al 1981).

Zinneman (1975) has shown by IIF, that IgA purified from the serum of alcoholic cirrhotics will bind to MB. He demonstrated that there was however, already by DIF, some background IgA bound to MB. This type of background MB staining by antiserum to human IgA presumably, representing native anti-MB activity, was not detected after PBS incubation in the liver sections used in this study, nor noted in the IF study of liver (Chapter 2). In this study, serum from a patient with an IgA myeloma was not reactive in the IIF system for anti-MB activity, suggesting that MB does not preferentially bind IgA from patients without ALD.

The significance of MB in the mesangial deposits, and therefore

by inference in the CIC of patients with ALD, has been re-affirmed by the finding of IgA anti-MB activity in the eluates of seven of nine glomerular eluates. A complementary study by Burns et al (1983) showed MB antigen in such deposits using a monoclonal antibody named JMB2. They found three of nine patients with alcoholic cirrhosis to have mesangial IgA deposition. JMB2 antigen was associated with these deposits immuno-histochemically in two patients. There seems compelling evidence that auto-immunity to MB is instrumental in the development of mesangial IgA deposition in patients with ALD.

The minor degree of accentuation of normal glomerular basement membrane incubated with eluted IgA was taken as a non-specific reaction. The inability of eluted IgA to bind to normal mesangium confirms the work of Tomino et al (1981). From their work it would appear that the IgA deposits of an individual with IgA nephropathy have a specific activity towards autologous mesangial antigens but in addition show some cross-reactivity with similar antigens in other patients. These antigens may be an integral part of the IC deposits.

SUMMARY

These experiments have demonstrated the heterogeneous nature of the mesangial IgA deposits in ALD, with specificity towards bacterial antigens, BSA and MB. The hypotheses put forward in the introduction to this Chapter, suggesting possible roles for auto-immunity and hyperimmunisation to absorbed food proteins or bacterial organisms have been substantiated. Auto-antibody

activity towards HSA and the mesangium, however, could not be demonstrated. In the limited experiments performed, no antigens could be detected in mesangial deposits. In the absence of demonstrable antigens, one cannot conclusively state that the deposits are the result of the deposition of circulating antigen-antibody complexes akin to those demonstrated in experiments undertaken by Dixon et al (1961). Until recently, the only circumstances where antigen had been demonstrated within the deposits were those where massive experimental or infective loads of antigen had been imposed, acute staphylococcal bacterial endocarditis in man being one such instance (Yum et al 1978). The description by Burns et al (1983) of MB-antigen in the mesangial deposits of patients with ALD is particularly exciting because it demonstrates the validity of our concepts about CIC deposition in these patients and confirms the finding of anti-MB activity in the eluates presented in this thesis. Germane to the aetiopathogenesis of mesangial IgA deposition in ALD is the recognition of the heterogeneity of antibody specificities in such deposits. The morphological result of deposition of antibodies of diverse specificity is the same, and it would appear that such patients have a multiplicity of antibodies or IC with an underlying abnormality in the control of antibody production or IC clearance. On a more practical level, the finding of anti-BSA activity in both eluates and serum has necessitated modification of all laboratory procedures ranging from fluorescence, to radiolabelling and $SpC1_q$ assays. BSA has been used routinely as a non-specific stabilising protein in many laboratory procedures but should be avoided on theoretical grounds since it may affect the accuracy of many subsequent tests.

CHAPTER 5

The establishment of a reproducible model of IgA nephropathy secondary to liver disease in the rat

INTRODUCTION

Development of an animal model for IgA nephropathy

Mesangial IgA deposition can be induced by both active and passive models of CIC deposition, by the induction of chronic infections and after portacaval shunting or the administration of hepatotoxins.

A Active or passive models of IC-mediated mesangial IgA deposition

Rifai et al (1979) developed a model of IC disease in which mice were injected with pre-formed dinitrophenyl and MOPC 315 mouse myeloma (DNP-MOPC 315) IgA complexes which then localised in the mesangium. An *in vivo* model of IC disease, induced by intraperitoneal injection of neutral dextran is of further interest because this carbohydrate, generally thought to be of low antigenicity, induced mesangial IgA deposition, morphologically of IC type (Isaacs et al 1981). Emancipator et al (1983) have further supported the proposed role of mucosal immunity in IgA associated IC GN by producing a murine model of mesangial IgA deposition by oral immunisation with foreign proteins.

B Chronic infection as a cause of IC-mediated mesangial IgA deposition

Experimental schistosomiasis with, presumably, a persistent antigenic load in conjunction with some degree of portal obstruction results in mesangial IgA deposition, and CIC (reviewed by Digeon et al 1979). Aleutian disease in the mink is a viral infection characterised by IC-mediated proliferative

GN and vasculitis (Porter and Larsen 1974). It is of considerable economic importance and in a series of experimentally infected sapphire minks, all 28 animals were shown to have glomerular IgA and C_3 deposits (Portis and Coe 1979).

These models share the effect of a continuing systemic antigenic load and are curious in producing predominantly IgA-class deposits. Only in schistosomiasis, with its proclivity for portal vein localisation can portacaval shunting be postulated, as a cause for the deposition of a mucosal type antibody.

C   Models involving portacaval shunting or hepatotoxicity

Partial ligation of the portal vein (Cheever and Warren 1963) has been used to enhance the consequences of hepatosplenic schistosomiasis. However, used alone on a group of uninfected control animals in a study of schistosomal GN by van Marck et al (1977), this procedure produced mesangial deposits of IgA, IgM, IgG and C_3 in 71% of animals.

Although IF was not performed, Sakaguchi et al (1964) showed that cirrhosis in rats caused by CCl_4 and ethionine caused mesangial sclerosis and electron dense deposits. Gormly et al (1981) modified this model in Adelaide by administering the CCl_4 by inhalation and produced diffuse mesangial deposits of IgA, IgM, IgG and C_3. In the study reported here, sc injections of CCl_4 were used to avoid operator hazards and to measure more accurately the dose of hepatotoxin administered. Injections were given biweekly at 0.05 ml/100 g body weight (after Sakaguchi et al 1964).

Gormly et al (1981) used Lewis rats which are no longer available locally. Two alternative inbred strains of rats, JC and DA were used in the pilot study. The model was designed to have mesangial IgA deposition as the endpoint with serial serology to monitor Ig levels. Slide elution of mesangial deposits was performed with citrate buffer to confirm the IC nature of the deposits.

MATERIALS AND METHODS

A Animal maintenance and the administration of hepatotoxin

Eighteen inbred JC and DA male rats aged 2-3 months and weighing 180-240 g were divided into two groups, experimental and controls. The animals were weighed at weekly intervals. Experimental rats received sc 0.05 ml CCl_4/100 g body weight twice weekly. The CCl_4 was diluted 1:4 in an inert diluent, olive oil, to facilitate the injections by increasing the volumes. The diluent was high grade Spanish olive oil (ex Star Grocery, Hindley Street, Adelaide) and consisted of one batch which was used in this and all subsequent experiments. Control rats received twice weekly the same volume of olive oil in terms of volume per 100 g body weight as the experimental rats.

The rats were marked individually by toe amputation coding and weighed weekly. All rats were bled via the orbital sinus under halothane (Fluothane ICI, Australia) and nitrous oxide anaesthesia at the beginning of the experiment and then at lunar monthly intervals. The rats were anaesthetised in a non-air-tight box attached to an anaesthetic machine (Midget 3, Commonwealth Industrial Gas, Australia) with a halothane delivery unit (Fluotec Mark 2 Cyprone and Keighly, UK). The anaesthetic was delivered at 800 ml/minute nitrous oxide and 500 ml/minute oxygen so that there was a halothane concentration in the box of approximately 6%. Blood was allowed to clot at room temperature for one hour and then spun, at 1600 *g* at room temperature. The serum was aliquoted and stored at -70°C.

The rats were maintained in groups of 4-5 animals and allowed to eat and drink ad libidum. They were fed standard pellets. Three animals were killed from each group at monthly intervals. At sacrifice each rat was exsanguinated under halothane inhalation anaesthesia and the kidneys were removed before death was induced by 0.5 ml of intracardiac sodium pentobarbitone (Valobarb, VR Laboratories, Syntax, Australia) at 300 mg/ml. Throughout this and subsequent animal studies months refer to lunar months of four weeks.

B Pathology

i) IIF upon rat kidney

Tissue and reagents

Kidney blocks for IF were prepared and stored as previously described (Chapter 2). The indirect technique used goat antisera directed against IgG, IgM, IgA and C_3 with FITC-conjugated rabbit anti-IgG (Nordic). The specificity of the primary reagents was tested by IEP (Appendix VII), against whole rat serum (Figure 5.1).

The FITC-conjugated antiserum was prepared in the following way. Rabbit anti-goat IgG (Nordic) was purified to obtain an IgG fraction by repeated precipitation with 50% ammonium sulphate followed by ion exchange chromatography on a DE-52 (Pharmacia) column, equilibrated with 0.01 M phosphate buffer (PB) (Appendix IX) at pH 7.5. The IgG fraction was then conjugated with fluorescein by dialysis against FITC dissolved in carbonate buffer at pH 9.4 (Appendix IX). Free FITC was then removed by repeated dialysis and stepwise elutions from a

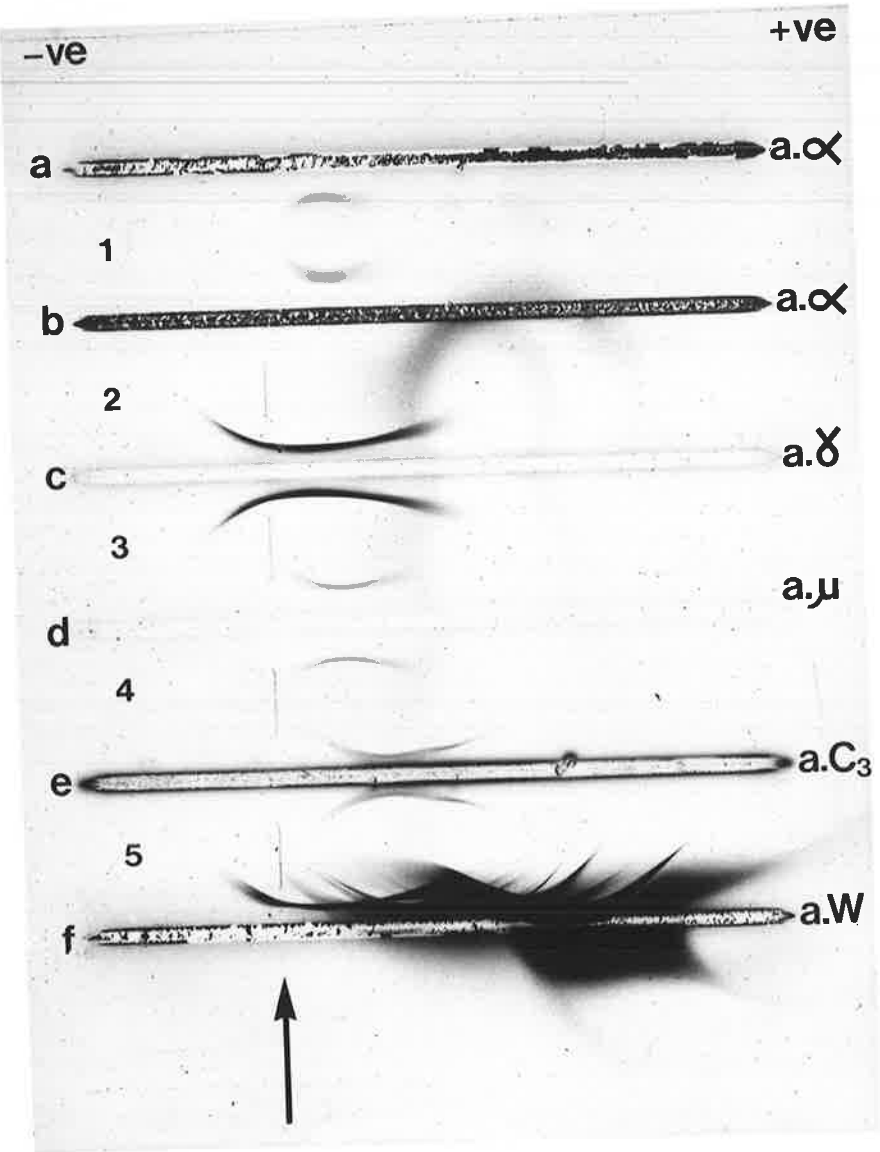


FIGURE 5.1
Immunoelectrophoresis of whole rat serum showed single immunoprecipitation lines with antisera to α-chains (a and b), γ-chains (c), μ-chains (d), C_3 (e). Whole (W) rat serum (f). The serum in well 1 was from a cirrhotic rat since there was insufficient IgA in normal rat serum to produce a precipitin line. Stained with Coomassie blue (X1).

DE-52 column. Fractions were retained and after testing in the IIF system, the 0.05 M fraction was used. Although the original antiserum showed no immunoprecipitation in gel against the constituents of whole rat serum, the conjugated antibody was absorbed with 1:10 normal rat serum in 2% BSA in PBS (BSA/PBS), and spun prior to use.

IIF upon rat kidneys

Unfixed frozen sections were cut at 2 μm, and placed on gelatin and formalin coated slides (Appendix II). The sections were washed in PBS for five minutes and stained for 30 minutes with 1:5 goat anti-rat antisera, or PBS as a control. The sections were washed in two changes of PBS for 10 minutes and stained for 45 minutes with 1:8 FITC-conjugated rabbit anti-goat IgG. The sections were washed in two changes of PBS for 10 minutes and mounted in glycine buffered glycerol at pH 8.6. The sections were examined with a Leitz Orthomat Microscope fitted with Ploem epi-illumination, and the reactions were graded subjectively from 0 to 4.

Slide elution of frozen kidney sections

Two frozen sections of rat kidney showing intense IgA and C_3 deposition were incubated for four and a half hours at room temperature with citrate buffer, as described in Chapter 4, with adjacent sections incubated with PBS. The sections were then stained for IgA or C_3 and resulting intensity of staining compared and photographed with the same time exposure.

ii) <u>Preparation of tissue for LM</u>

Blocks of kidney cortex and liver were fixed in 10% neutral buffered formalin and processed to wax. Sections of liver cut at 4 µm were stained by H&E, an adaptation of Fouchet's stain with Sirius red and Gordon and Sweet's reticulin stain. Sections of kidney cut at 2 µm were stained with H&E and PAS. These staining procedures are described in Appendix I.

iii) <u>Preparation of tissue for EM</u>

The tissue was diced into 1 mm cubes, and fixed in 4% formaldehyde with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (McDowell and Trump 1976). The cubes were then post-fixed in 2% osmium tetroxide in cacodylate buffer. The tissues were stained <u>en bloc</u> with 2% aqueous uranyl acetate and then dehydrated in ethanol before being embedded in RD-2 epoxy resin (Tarb Laboratories Ltd, Reading, UK). Thin sections were cut with an ultramicrotome (Sorvall MT2-B, Dupont Instruments, Newtown, CO) using a diamond knife (Diatome, Wild/Leitz, Sydney). The sections were then stained with lead citrate and uranyl acetate and examined in an AEI 801 transmission electron microscope. Photographs were taken on Kodak 4489 film.

C <u>Serology</u>

i) <u>Urea and creatinine</u>

Urea and creatinine were measured by an automatic spectrophotometric serum analyser (Astra, Beckman, CA) by Mr G Hall (Division of Clinical Chemistry, IMVS) and the results expressed in mmol/L, and µmol/L respectively.

ii) Immunoglobulin and C_3 levels

Serum Ig and C_3 levels were determined by single radial immunodiffusion described in detail in Appendix VII (after Mancini 1951). Goat antisera to rat C_3, IgM and IgG were used (Nordic). Goat anti-rat IgA was a gift from Dr Graham Jackson (Department of Microbiology, The University of New South Wales). It had been absorbed with normal rat serum and showed no cross-reactivity with other serum components in gel (IEP and double immunodiffusion, Appendix VII). The antisera used were diluted between 1:25 and 1:1000 times in 1% agarose in PBS at 56°C in a water bath and applied to 0.05% agar coated glass slides. 5-10 µl of serum were loaded into each 2 mm punched well. The plates were kept at room temperature overnight and washed in 3% saline for three hours followed by water for 24 hours. After drying overnight in a 37°C oven, the plates were stained with Coomassie blue and washed in a destaining solution to facilitate measuring the diameter of the precipitin rings using a calibrated eyepiece (Peak Scale lupe 7x). IgM and C_3 levels were expressed as a percentage of the concentration in a standard pool of normal serum. IgG and IgA were expressed in mg/ml in relation to the same purified standards throughout the whole study.

Pure rat IgA was a gift from Mr Paul Drew in the Department of Medicine, The University of Adelaide. Rat IgG was prepared by ammonium sulphate precipitation and ion exchange chromatography. Both Ig standards had been tested by IEP and double immunodiffusion (Appendix VII). Protein estimations of these standards was determined after Lowry et al (1951) (Appendix IV).

D Statistics

The data were assessed using a Hewlett Packard Desk-top Computer (HP 9825, Fort Collins, CO), and significance calculated with a Non-parametric Statistics Pack 3 (Software code 15020, Hewlett Packard) using a Mann-Whitney U ranked sums test. "Z" values were calculated and then two tailed probabilities read from tables of mean standard deviants (Siegel 1956).

RESULTS

A Weight gain and general condition

The results of the weekly weight recordings in the form of means and one standard deviation (1SD) for each group of rats are shown in Table 5.1 At the end of three months, CCl_4 treated JC and DA rats had gained 45.9% and 27.0% respectively in body weight. This was less than the 70.4% and 32% respectively gained by control rats treated with sc olive oil only. These differences were significant only in JC rats ($p<0.05$). The general condition of all rats in control and experimental groups was good.

B Indirect immunofluorescence

The IIF performed on the rat kidneys of both strains after three months of CCl_4 treatment demonstrated mesangial IgA staining that ranged in intensity from 2 to 3 (Table 5.2). In addition, IgG and IgM with C_3 were seen in a similar distribution but with lesser intensity (Figure 5.2-5.3). The fluorescence was in all cases diffuse, affecting greater than 80% of glomeruli, and global, affecting all mesangial areas.

Control rats at three months showed no mesangial IgA, but frequent segmental deposits of IgM were present (Figure 5.4). The first appearance of IIF changes in the treated rats was at two months, when all of the treated rats showed IgA staining graded 1 to 2. The two strains of rats differed in that the DA rats showed more intense staining reactions (Figure 5.5-5.6). Acid elution of frozen sections showed diminution of

Table 5.1 The mean (+1SD) weekly weights in g of the two strains of rats given bi-weekly CCl_4 and the mean total weight gain expressed as a percentage of the weight at the start of the experiment. Controls are in parentheses

Time in weeks	Strain of rats			
	JC		DA	
Baseline	220 ± 10	(223 ± 9)	215 ± 17	(219 ± 13)
1	235 ± 15	(240 ± 10)	223 ± 16	(231 ± 12)
2	250 ± 15	(260 ± 10)	231 ± 16	(238 ± 12)
3	265 ± 12	(277 ± 13)	233 ± 16	(252 ± 9)
4	270 ± 10	(290 ± 13)	244 ± 14	(260 ± 10)
5	292 ± 7	(302 ± 8)	242 ± 14	(256 ± 7)
6	286 ± 8	(302 ± 5)	250 ± 16	(269 ± 8)
7	301 ± 9	(323 ± 7)	245 ± 16	(268 ± 7)
8	312 ± 8	(336 ± 9)	258 ± 15	(280 ± 8)
9	311 ± 17	(348 ± 8)	276 ± 10	(280 ± 11)
10	308 ± 22	(358 ± 11)	278 ± 7	(285 ± 15)
11	318 ± 15	(373 ± 11)	275 ± 11	(283 ± 19)
12	321 ± 21	(380 ± 12)	273 ± 22	(289 ± 22)
Mean weight gain	45.9%	(70.4%)	27.0%	(32.0%)
	$p<0.05$		NS	

Table 5.2 The results of renal IIF in rats of two strains treated with biweekly CCl_4 compared with controls. Three rats (treated and control) of each strain were killed at three intervals of one month. The intensity of fluorescence was graded from 0-4 with a mere trace coded as tr

Rat strain	Treatment Group	Renal IIF											
		1 month				2 months				3 months			
		IgA	IgG	IgM	C_3	IgA	IgG	IgM	C_3	IgA	IgG	IgM	C_3
JC	Experiment	0	0	0	0	1	1	2	1	2	2	1	2
		0	0	0	0	1	1	1	tr	2	1	3	1
		0	0	0	0	1	1	1	tr	2	0	0	3
	Control	0	0	0	0	0	0	tr	0	0	0	1	0
		0	0	0	0	0	0	0	0	0	0	tr	0
		0	0	0	0	0	0	tr	0	0	0	0	0
DA	Experiment	0	0	0	0	2	1	1	1	3	2	2	1
		0	0	0	0	2	1	1	1	3	2	2	1
		0	0	0	0	1	1	1	0	3	2	2	1
	Control	0	0	0	0	0	0	tr	0	0	0	1	0
		0	0	0	0	0	0	0	0	0	0	2	0
		0	0	0	0	0	0	tr	0	0	0	2	0

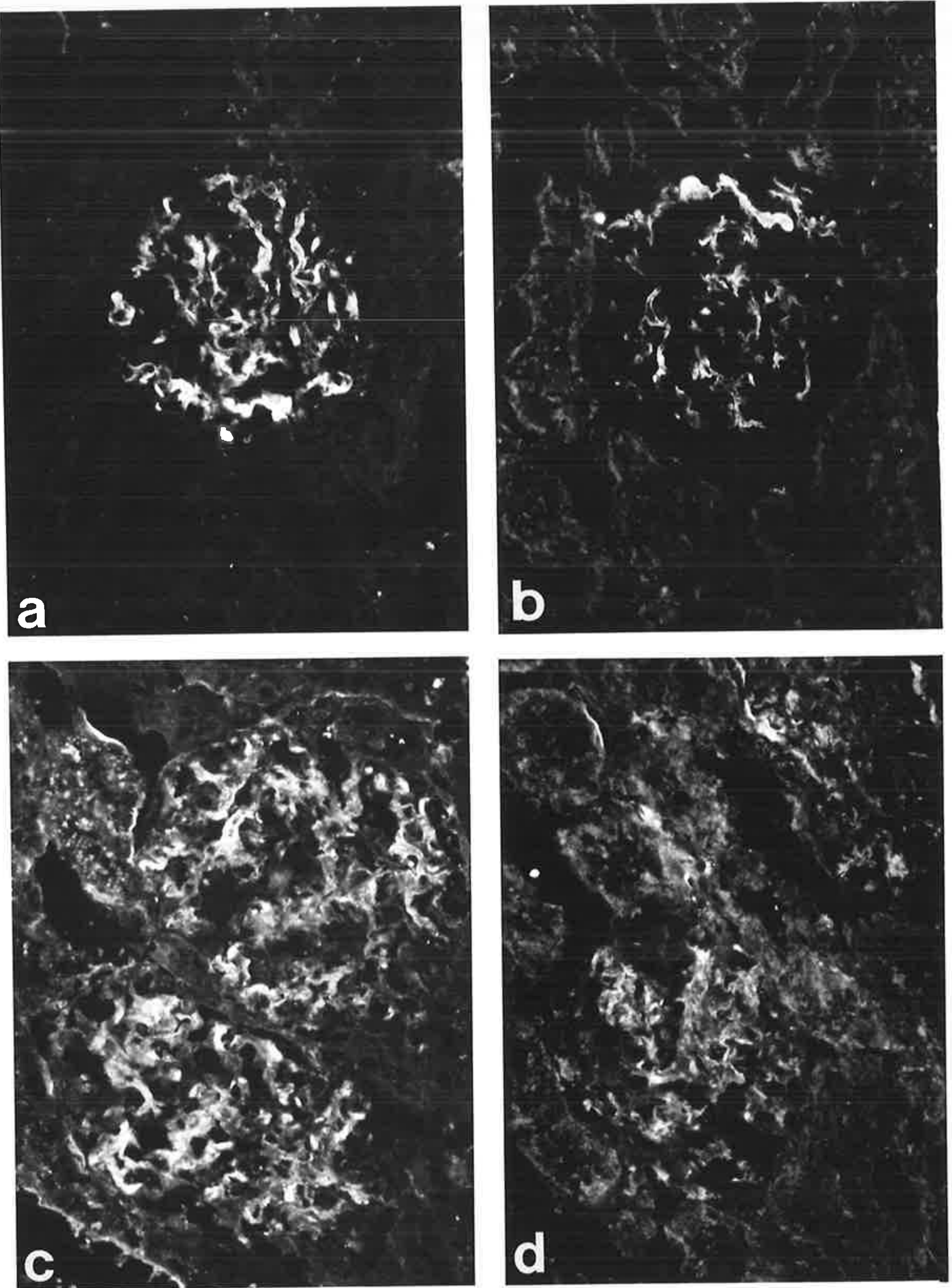


FIGURE 5.2

Kidney from a DA rat given biweekly sc CCl_4 for three months. Stained with goat monospecific antisera and FITC-conjugated rabbit anti-goat IgG. Mesangial fluorescence was graded 3+ for IgA (a), 2+ for IgG (b), 2+ for IgM (c), and 1+ for C_3 (d) (IIF, X1250).

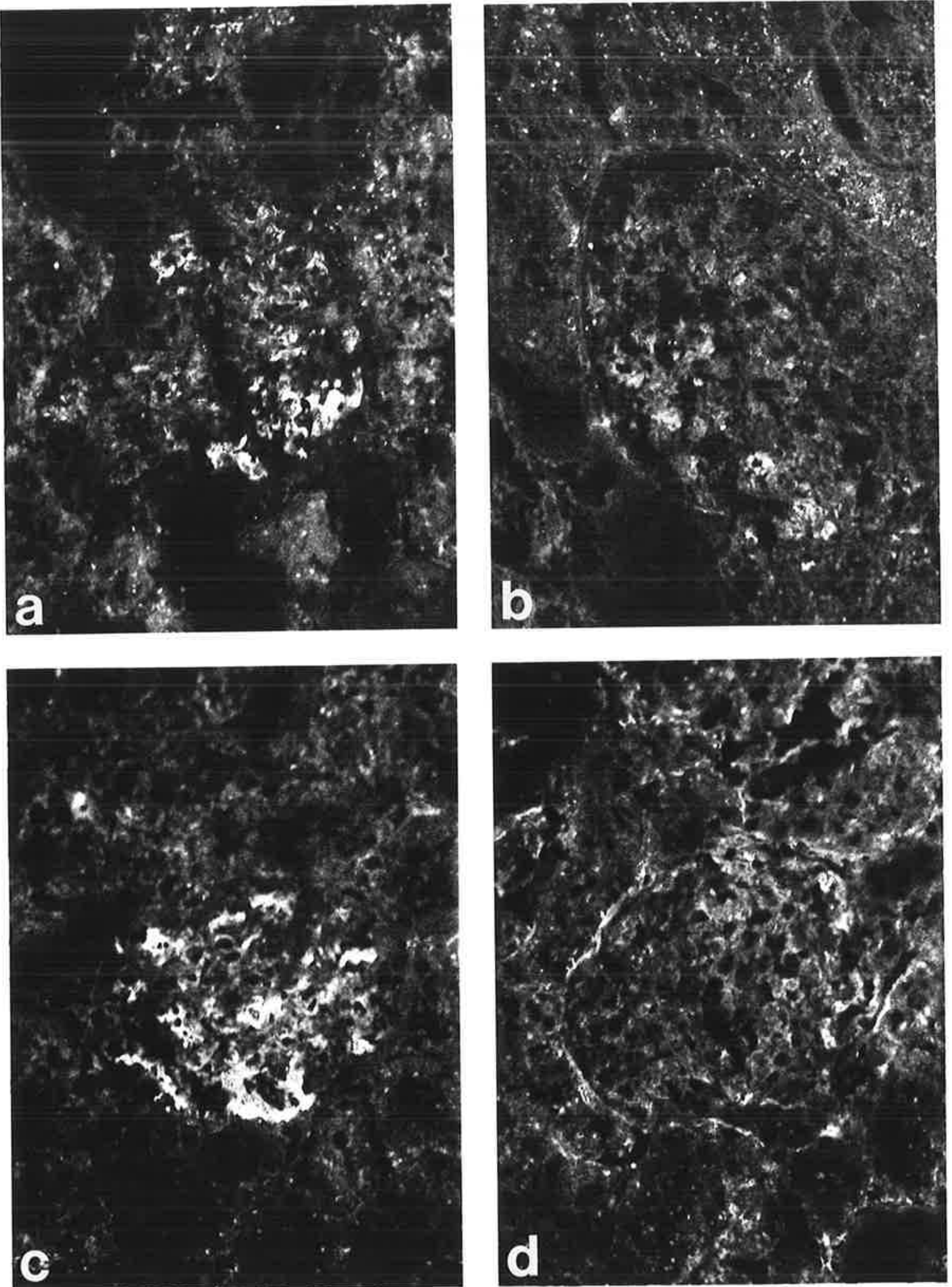
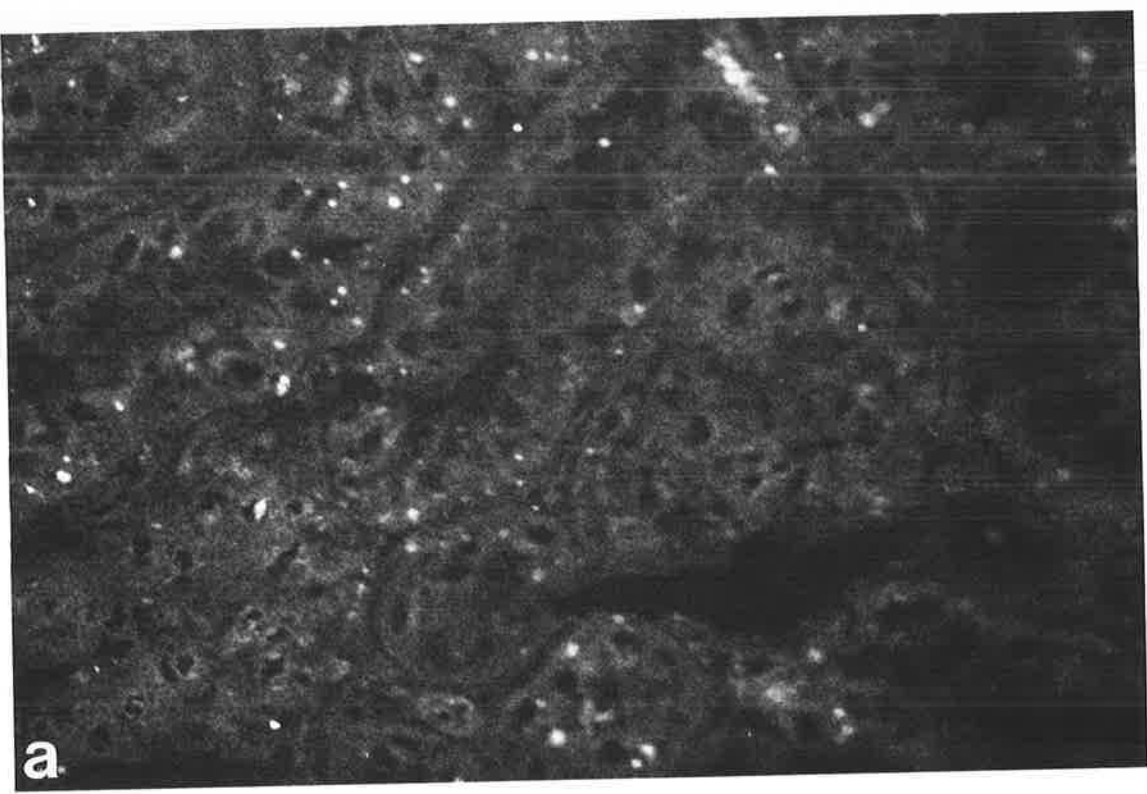


FIGURE 5.3
Glomeruli from a JC rat given biweekly sc CCl_4 for three months. Stained with goat anti-rat antisera and FITC-conjugated rabbit anti-goat IgG. Mesangial staining was observed, (a) IgA graded 2+, (b) IgG graded 1+, (c) IgM graded 3+, (d) C_3 graded 1+ (IIF, X1250).



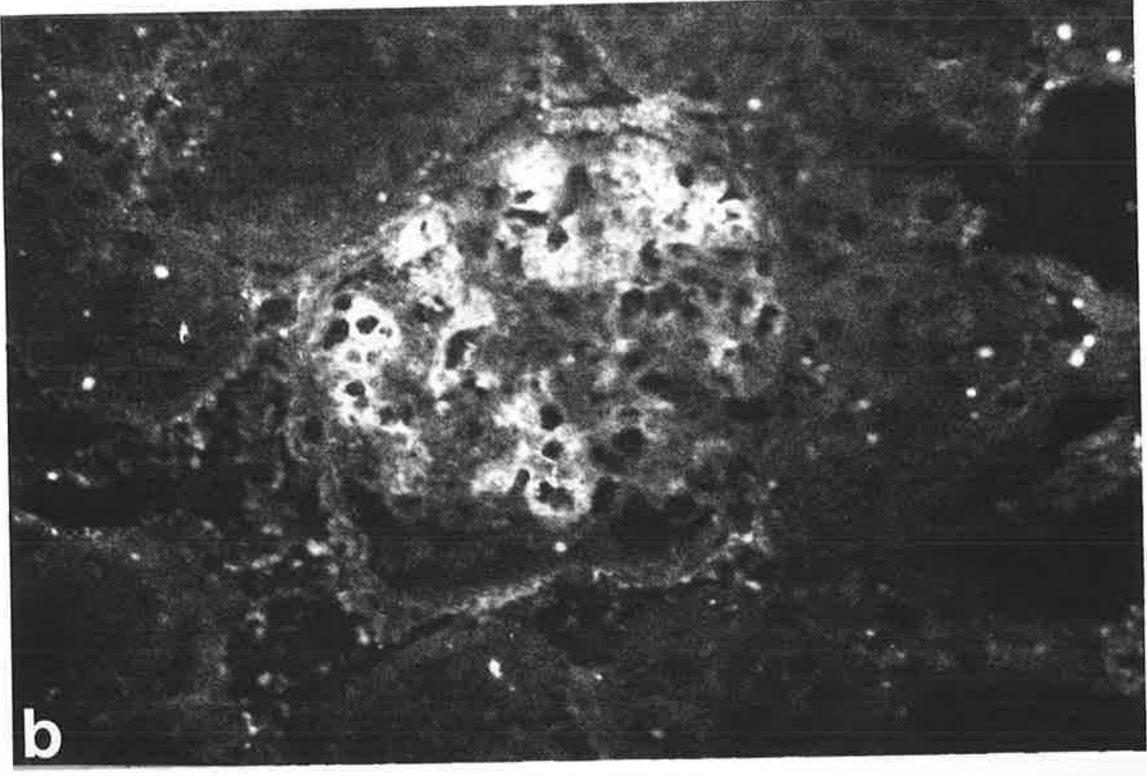
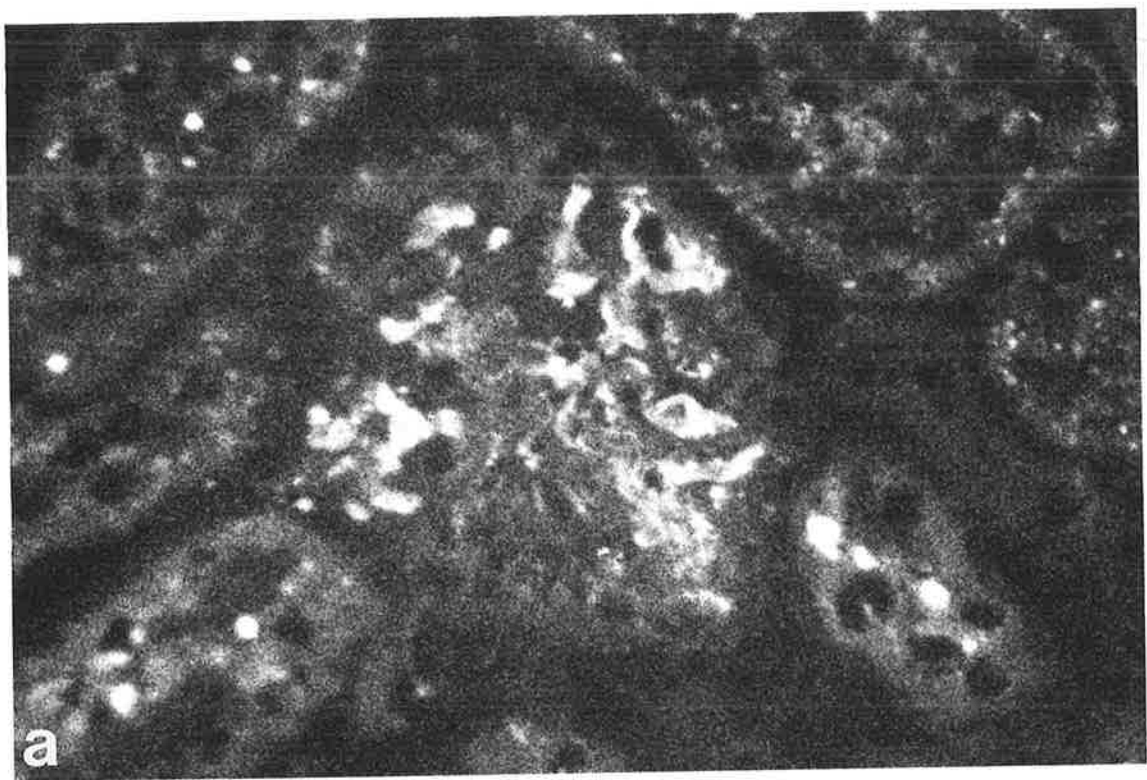


FIGURE 5.4
Glomeruli from a control DA rat after three months. There is no mesangial IgA staining (a), but a 2+ mesangial reaction for IgM (b). Stained with monospecific goat antisera and FITC-conjugated rabbit anti-goat IgG (IIF, X1250).



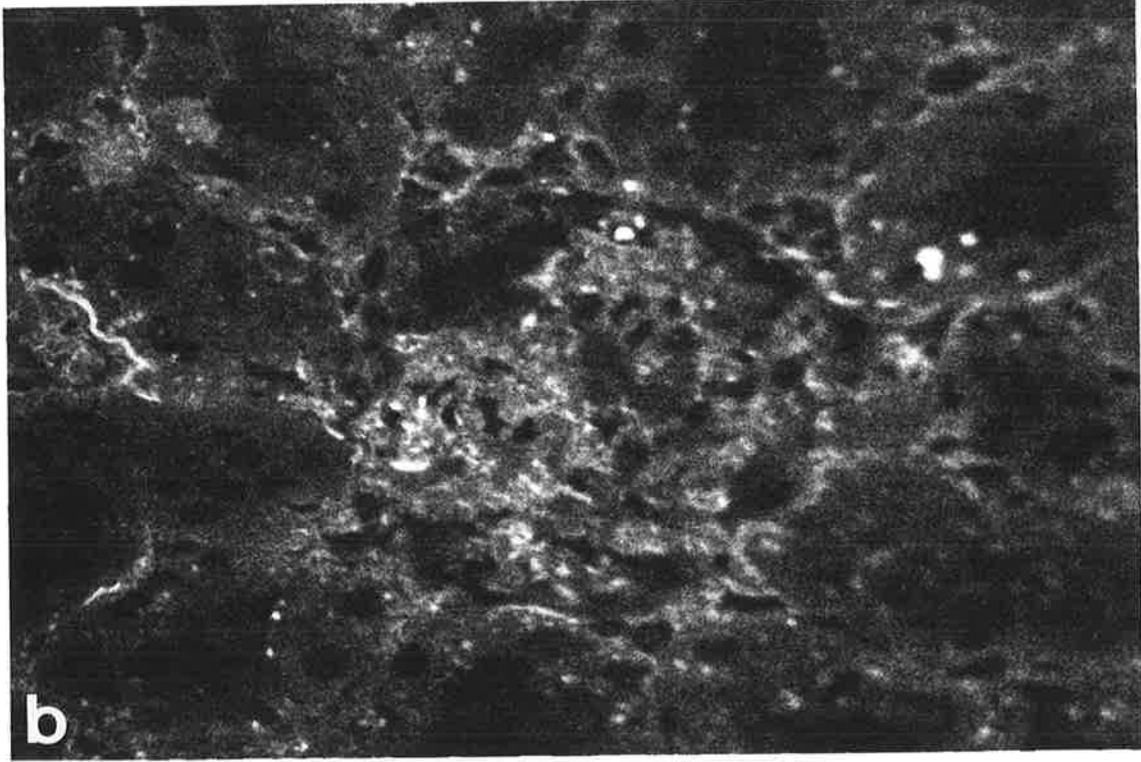


FIGURE 5.5
Glomeruli of a DA rat after two months sc CCl_4 stained with goat monospecific antisera and FITC-conjugated rabbit anti-goat IgG. Mesangial staining was observed (a) IgA graded 2+, (b) C_3 graded 1+ (IIF, X1100).

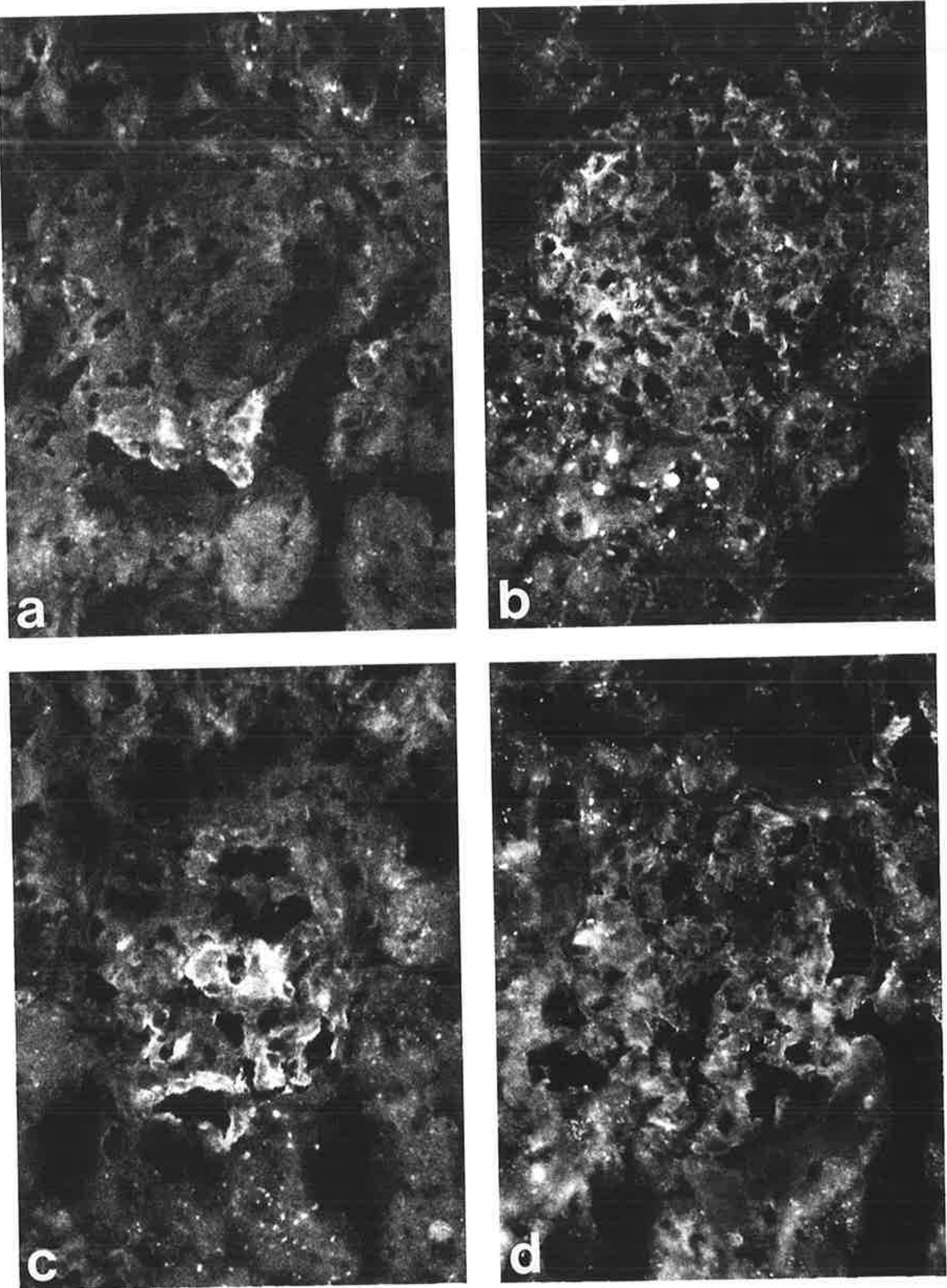


FIGURE 5.6
Glomeruli of a JC rat after two months sc CCl_4. Stained with goat monospecific antisera and FITC-conjugated rabbit anti-goat IgG. The mesangial reactions overall were (a) IgA graded 1+, (b) IgG graded 1+, (c) IgM graded 2+, (d) C_3 graded 1+ (IIF, X500).

the intensity of staining of both IgA and C_3 (Figure 5.7).

C Light microscopy

The results of the LM examination of the livers of the rats of both strains are shown in Table 5.3. At the end of the experiment all of the CCl_4 treated rats had cirrhosis, and marked steatosis. All of the control rats had normal liver architecture without steatosis. The two strains developed liver damage at a different rate. The JC rats developed some degree of steatosis with minor degrees of fibrosis in two of three rats killed after one month. At two months all three killed showed fibrosis, associated in two with bridging (Figure 5.8a). Although the DA rats showed no steatosis but some degree of fibrosis after one month, by two months they all showed fibrosis and one had cirrhosis (Figure 5.8b).

The changes in the kidneys were minor. Two of the rats, one in each strain, treated with CCl_4 had a segmental hypercellularity, but this was not prominent (Figure 5.9). There was no sclerosis and the most consistent change noted was the finding of hyaline mesangial nodules in all of the DA rats and all but one of the JC rats at three months. The kidneys of control rats appeared normal throughout the experiment.

D Electron microscopy

Limited EM examination of the kidneys was performed. One DA rat treated with CCl_4 after three months showed an increase in mesangial matrix and cells, with electron dense deposits in mesangial and paramesangial areas as well as in subepithelial

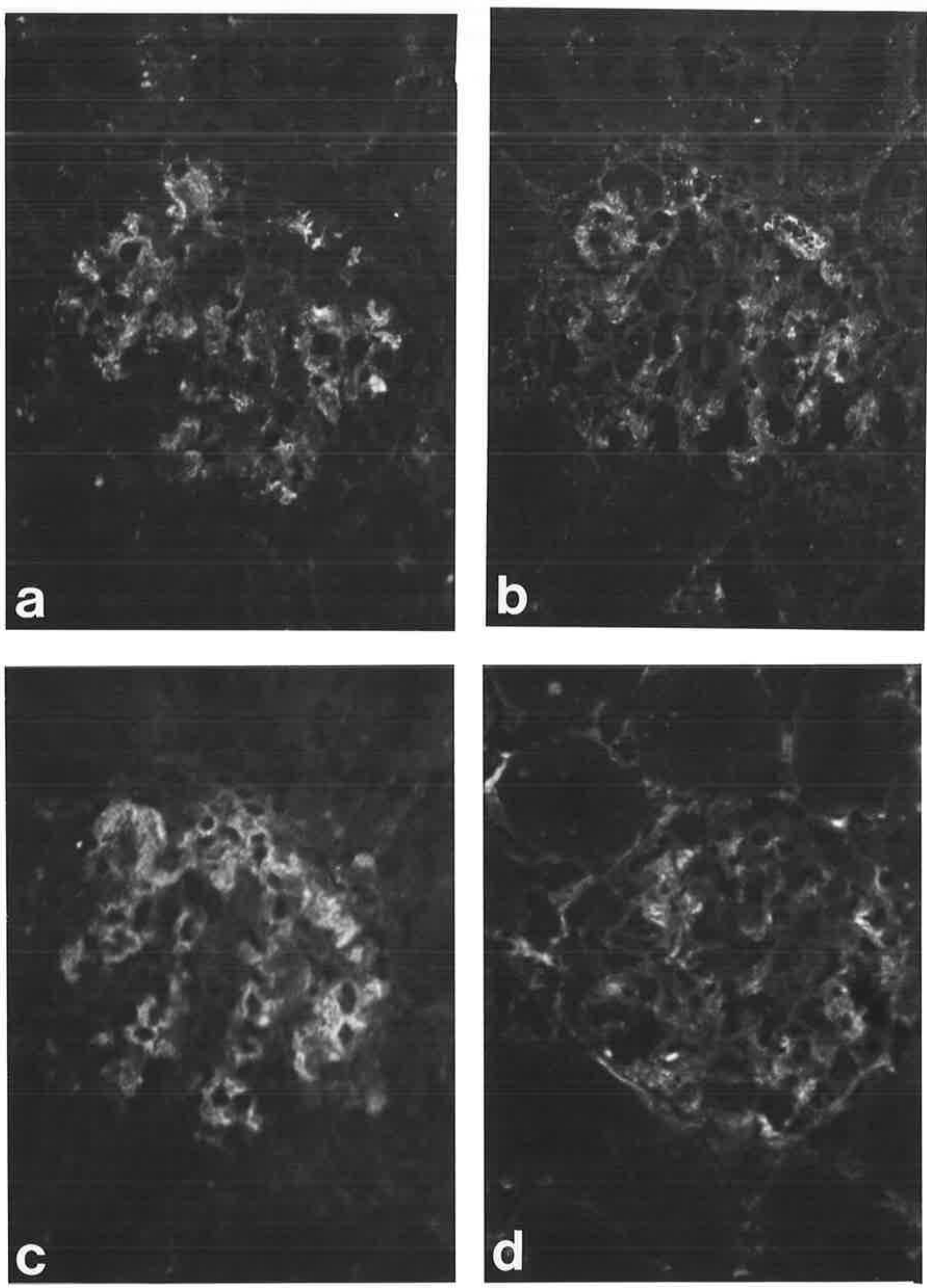


FIGURE 5.7

Adjacent sections of rat kidney treated with PBS (left) or citrate (right) for four and a half hours and then stained with goat anti-rat IgA (a and b) or goat anti-rat C_3 (c and d). Timed exposures illustrate a slight diminution in staining for IgA and C_3 after citrate elution (IIF, X550).

Table 5.3 The results of light microscopic examination of livers of rats of two strains treated with biweekly CCl_4 compared with controls. Three rats (treated and control) of each strain were killed at three intervals of one month. Cirrhosis and bridging fibrosis (B) were recorded as present or absent but the other features were arbitrarily graded as 0-4

Rat strain	Treatment group	Light microscopical features of rat livers								
		One month			Two months			Three months		
		Steat	Fib	Cirr	Steat	Fib	Cirr	Steat	Fib	Cirr
JC	Experiment	2	1	-	2	2(B)	-	2	4	+
		2	1	-	2	2(B)	-	2	4	+
		0	0	-	2	2	-	2	4	+
	Control	0	0	-	0	0	-	0	0	-
		0	0	-	0	0	-	0	0	-
		0	0	-	0	0	-	0	0	-
DA	Experiment	0	1	-	0	2(B)	-	2	4	+
		0	1	-	2	3	+	2	4	+
		0	0	-	0	2	-	2	4	+
	Control	0	0	-	0	0	-	0	0	-
		0	0	-	0	0	-	0	0	-
		0	0	-	0	0	-	0	0	-

Steat=steatosis; Fib=fibrosis; Cirr=cirrhosis

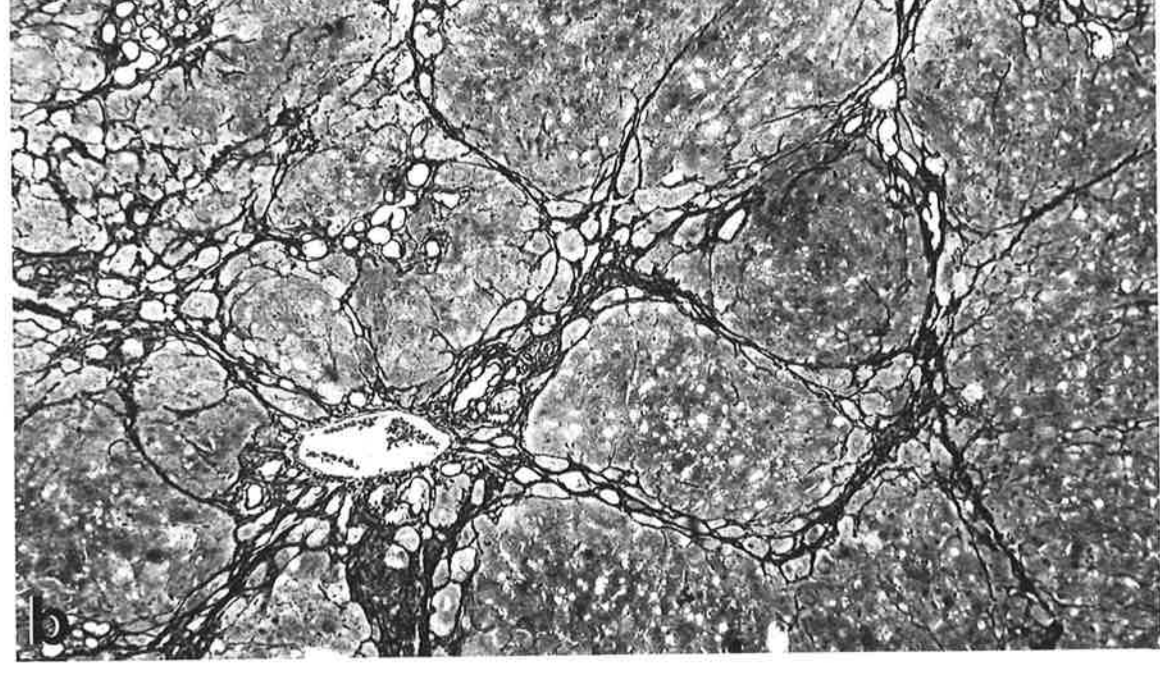
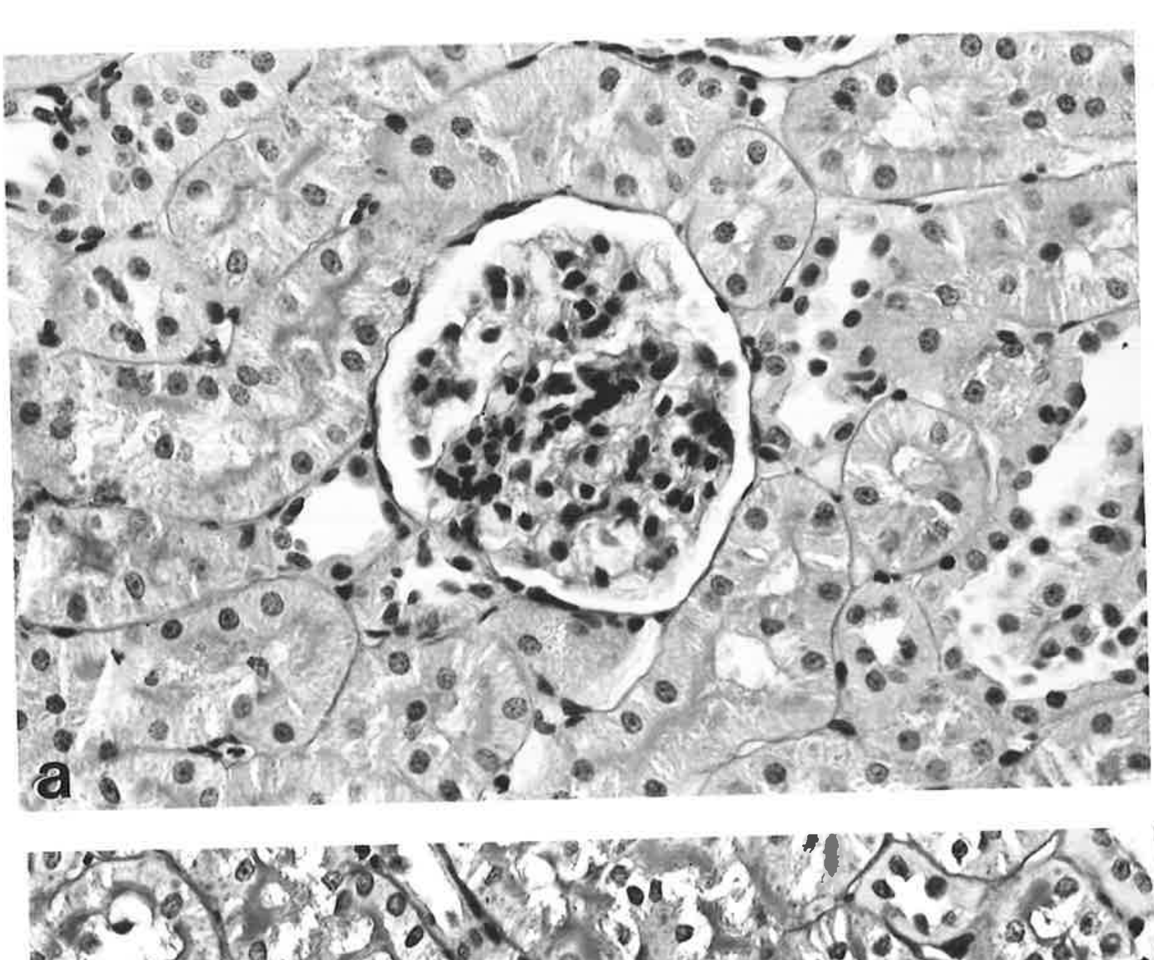


FIGURE 5.8
Rat livers after two months of biweekly sc CCl_4. (a) JC rat liver shows early bridging fibrosis, (b) DA rat liver shows established cirrhosis (Reticulin stain, X40).



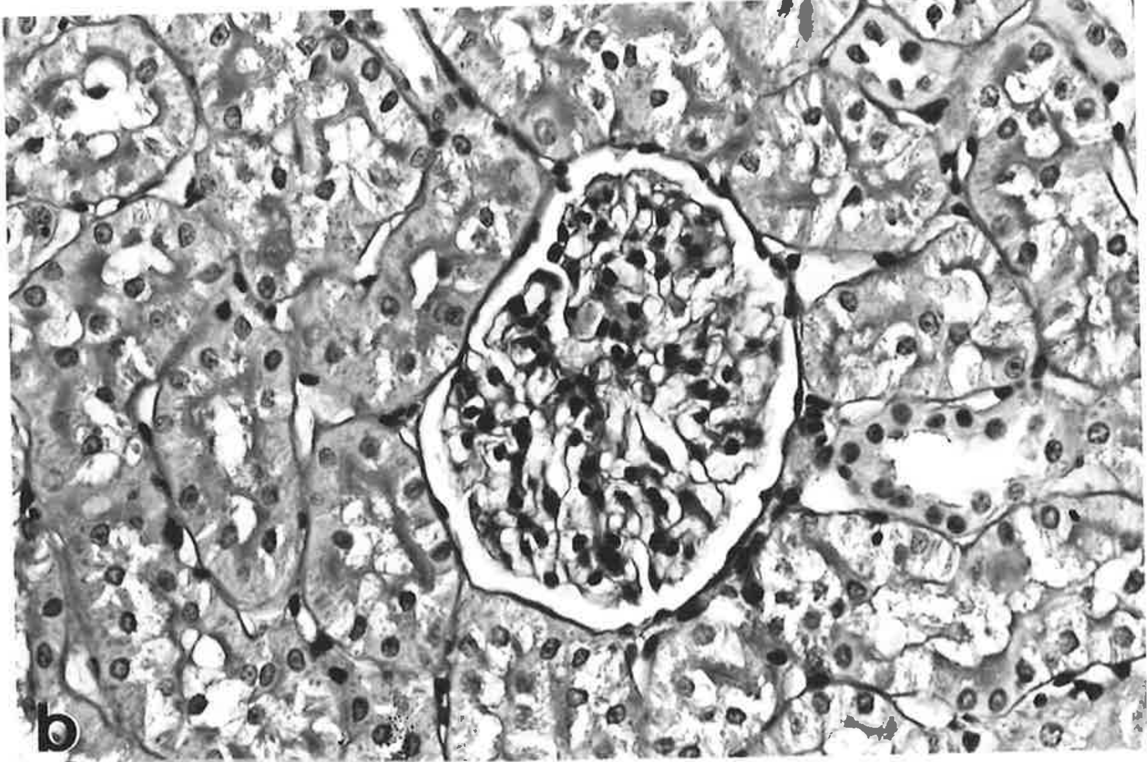


FIGURE 5.9
The glomerular appearances of (a) a DA rat given biweekly sc CCl_4 and (b) a control DA rat given inert diluent. The cirrhotic rat shows minor mesangial expansion and hypercellularity compared to the control (PAS, X400).

sites (Figure 5.10). There was no foot process fusion or evidence of "etching" (Spargo et al 1980). Two control rats studied by comparison showed no deposits.

E Serum urea and creatinine levels

The results of serum chemistry showed (Table 5.4) that there was no evidence of renal failure in any of the rats and that, although the mean levels of creatinine rose in CCl_4 treated rats, they also rose in the control rats of both strains.

F Serum immunoglobulin and complement levels

The results of the Ig and C_3 quantitation by radial immunodiffusion are shown in Table 5.5-5.7. Table 5.5 shows that there was an initial significant difference in IgA and IgM levels between the two strains ($p<0.005$), both Ig being approximately twice as high in the DA strain.

The CCl_4 treated JC rats (Table 5.6) developed a seven-fold increase in IgA levels compared with controls at three months, a slight decrease in IgG levels and a two-fold increase in IgM levels. The C_3 levels changed by 0.05%. The CCl_4 treated DA rats (Table 5.7) after three months developed a 12.8-fold increase in IgA levels compared with controls, a 20% increase in IgG levels, a 2.2-fold increase in IgM levels. The mean C_3 levels changed by 2% only. The alterations in mean levels at the end of three months were based on only three rats.

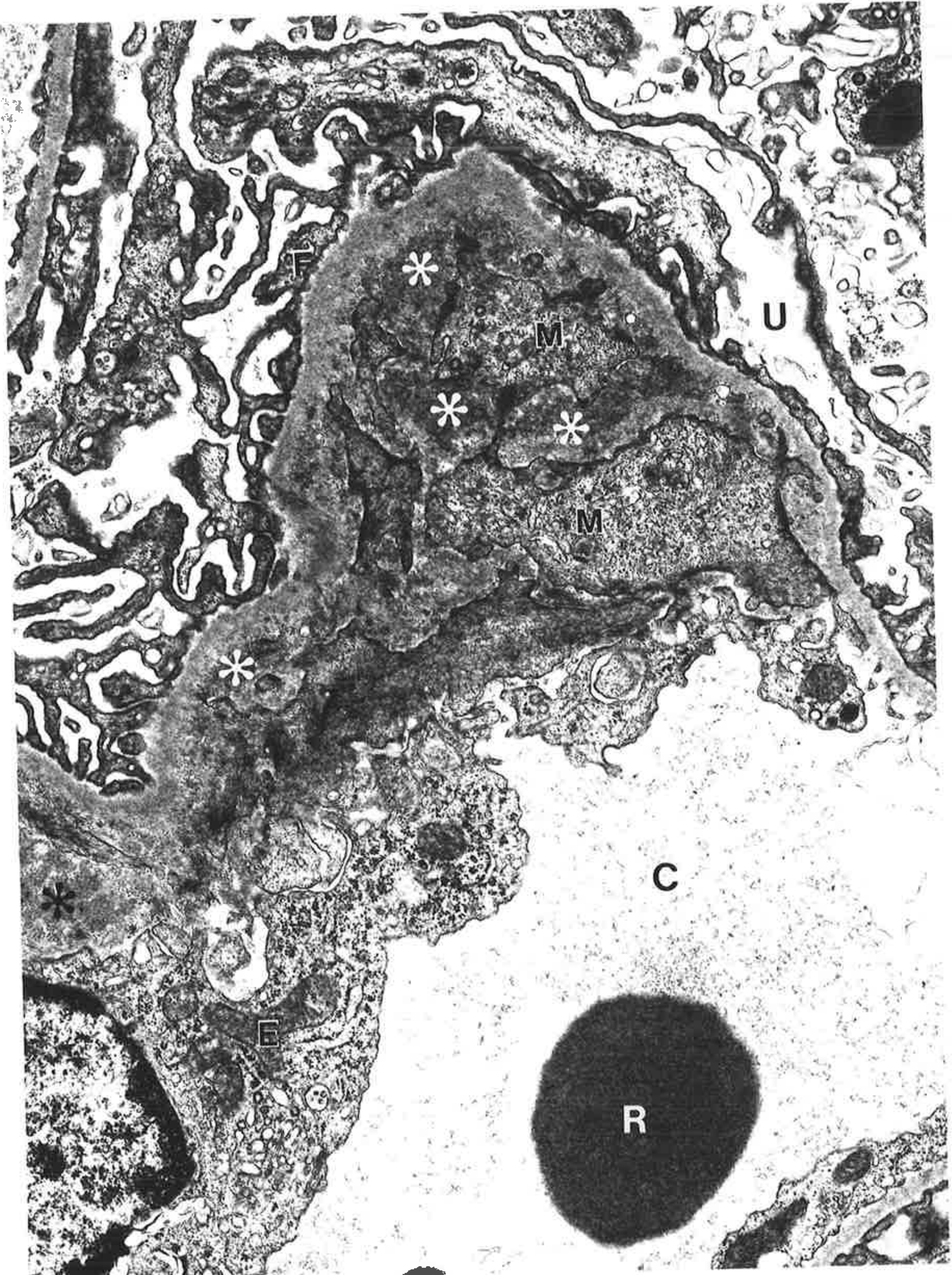


FIGURE 5.10

Electron micrograph of glomerulus from a DA rat given three months of biweekly sc CCl_4. There are electron dense mesangial and paramesangial deposits(*). The urinary space (U), capillary lumen (C), mesangial cells (M), foot processes (F), endothelial cells (E) and a red blood cell are marked (X19,000).

Table 5.4 Mean (+1SD) of serum urea in mmol/L, and creatinine in μmol/L, in rats of both strains at monthly intervals, given biweekly CCl_4. Controls in parenthesis

	JC				DA			
	Urea		Creatinine		Urea		Creatinine	
Baseline	5.6 ± 1.1		38.93 ± 12.9		5.9 ± 0.6		41 ± 0.4	
1 month	6.9±0.9	(7.3±0.7)	44.3±12.7	(44.7±10.3)	6.8±0.6	(6.3±0.4)	43.0± 5.0	(43.0±5.0)
2 months	7.6±0.4	(7.7±0.4)	44.0± 4.8	(46.6± 4.7)	6.5±0.8	(5.7±0.2)	55.0±12.2	(48.3±4.0)
3 months	6.2±0.6	(7.8±0.8)	46.6± 4.7	(63.3±12.4)	5.4±0.8	(6.0±0.3)	70.0± 0	(83.3±5.7)

Table 5.5 A comparison between the mean (+1SD) serum immunoglobulin and C_3 levels in the two strains of rats at the start of the study

Serum concentrations	JC	DA	
IgA in mg/ml	0.035 $\pm$ 0.015	0.065 $\pm$ 0.016	*
IgG in mg/ml	16.94 $\pm$16.03	12.71 $\pm$ 5.36	NS
IgM % pool	44 $\pm$24	95 $\pm$20	*
C_3 % pool	88 $\pm$40	115 $\pm$24	NS

*p <0.005

Table 5.6 The serum immunoglobulin and C_3 levels of JC rats given biweekly CCl_4 injections compared with controls (in parenthesis) and expressed as a mean +1SD. The baseline results represent all JC rats in this study

Time	IgA in mg/ml	IgG in mg/ml	IgM % standard pool	C_3 % standard pool
Baseline	0.035±0.015	16.94±16.03	44±24	88±40
4 weeks	0.051±0.010 (0.059±0.029)	26.72± 4.59 (38.50± 8.45)	68±18* (59±11)	86±41 (87±19)
8 weeks	0.174±0.042 (0.132±0.060)	39.33± 8.07* (51.83± 9.50)	54± 7* (58± 8)	169±18 (158± 9)
12 weeks	0.700±0.416 (0.100±0)	39.15± 9.87 (47.83± 5.48)	101±50 (54±22)	157±39) (116±14)

*p<0.05

Table 5.7 The mean serum immunoglobulin and C_3 levels of DA rats (measured by RID) given biweekly CCl_4 injections compared with controls (in parenthesis) and expressed as as mean +1SD. The baseline results represent all DA rats in this study

Time	IgA in mg/ml	IgG in mg/ml	IgM % standard pool	C_3 % standard pool
Baseline	0.065±0.017	12.71± 5.37	95±20	115±24
4 weeks	0.204±0.074* (0.073±0.019)	36.36± 9.58 (40.31± 7.19)	82±28 (91±21)	127±18 (115±17)
8 weeks	0.532±0.540* (0.079±0.006)	37.33± 3.34* (49.00± 8.00)	93±13 (96± 3)	137±19 (136± 9)
12 weeks	1.290±0.413 (0.093±0.036)	53.3± 5.03 (44.0±22.27)	129±34* (59±15)	154±20 (157±29)

*significant $p<0.05$

DISCUSSION

Both strains of rats developed cirrhosis of the liver after three months of twice weekly CCl_4 injections. In conjunction with this there was mesangial deposition of IgA with - to a lesser extent - IgG, IgM and C_3. By two months all experimental rats had some degree of hepatic fibrosis and steatosis and the kidneys showed weak but identifiable IgA staining.

The LM features in the kidneys at the end of the experiment were limited to a minor increase in mesangial cells in those rats with cirrhosis and IgA deposition in the kidney. No sclerosis was noted but occasional hyaline nodules similar to those seen in humans with IgA nephropathy were recorded. The control rats injected with diluent only showed neither morphological changes in the liver nor IgA deposits in the mesangium. The limited EM study confirmed the finding of electron dense deposits in the mesangium of rats with CCl_4 induced cirrhosis described by Gormly et al (1981).

There was no difference between the concentrations of urea and creatinine levels in the experimental or control rats. The numbers of rats reaching three months were small in this pilot study, but serum Ig assays showed similar features to those seen in cirrhotic humans. Cirrhotic JC rats showed a seven-fold increase in IgA levels and a two-fold increase in IgM levels. DA rats showed a 13-fold increase in IgA and a two-fold increase in IgM levels. C_3 levels did not alter significantly in either strain. The IgG levels were not significantly

increased at the end of three months in either strain.

The DA rats demonstrated at three months, a greater change in serum Ig levels, and after two months, brighter IgA staining and more pronounced liver changes. It was therefore decided that this strain should be used as the experimental model to be manipulated in the following study.

CHAPTER 6

Therapeutic manipulation of the animal model of IgA nephropathy

INTRODUCTION

The model of IgA nephropathy developed using biweekly sc CCl_4 injections was reproducible and produced significant renal IF without overt renal failure or morbidity (Chapter 5).

The model was manipulated with five drugs in order to test the hypotheses linking cirrhosis with mesangial deposits. The drugs used were: phenytoin, which in humans lowers serum IgA levels; d-penicillamine, which lowers CIC levels and alters B-cell function; dapsone, which reduces the intensity of complement deposits associated with IgA deposits in dermatitis herpetiformis (DH); neomycin, which alters gut flora; and prostaglandins, which have been shown to protect animals against IC nephritis.

A Prostaglandin E_1

In experimental models of IC-mediated disease, prostaglandins have proved potent anti-inflammatory agents. This action was demonstrated first in the NZB/W hybrid mouse which spontaneously develops an SLE-like GN with eventual death.

Long-term treatment of NZB/W mice with large pharmacological doses (200 μg bd) of prostaglandin (PG) E_1 (Zurier et al 1978), prevents progression of the IC GN and prolongs survival. Kelley et al (1979), using the same model, showed that PGE_1 caused reduced glomerular changes and proteinuria but no alteration in serum Ig levels or specific antibody titres. PGE_1 is similarly effective in reduced IC deposition in murine GN

induced by daily intraperitoneal injections of apoferritin (McLeish et al 1980).

Kunkel et al (1979) have shown the potency of the PGE_1 analogue, and stable derivative, 15-(s)-15-methyl PGE_1, in inhibiting IC-induced vasculitis produced in the rat by intradermal injection of antibody followed by intravenous injection of the antigen BSA, that is, a reverse Arthus reaction. This group has shown that only a single dose of either 250 μg (sc) or 500 μg (orally) of the analogue is necessary for this inhibition. In rat adjuvant arthritis, the effective oral dose has been shown to be even lower, so that 50-100 μg will produce a dose-dependent reduction in inflammation (Kunkel et al 1981). The efficacy of such tiny physiological amounts of 15-(s)-15-methyl PGE_1 has been re-affirmed by Zurier et al (1980) who used 4 μg twice daily (bd) sc (ie 200 μg/kg/day) to retard progression of the murine NZB/W lupus model.

Thus, there is a precedent for the use of PGE_1 in IC nephritis. In this model of mesangial IgA deposition the initial sc dose of the analogue was 250 μg bd. In subsequent discussion, the analogue is called PGE_1.

B D-penicillamine

Penicillamine is a powerful chelating agent used in rheumatological practice on the basis of its ability, as a sulphydryl reducing agent, to dissociate IgM-rheumatoid factor *in vitro* (Jaffe 1962). Clinically, the drug does not affect an instant reduction in circulating rheumatoid factor levels. It may take

several months to lower the level of this marker of disease, and clinical improvement may be independent of such alterations (Huskisson et al 1974).

The range of toxic side effects of this drug are protean and prevented wide clinical use until a pure dextro form with reduced toxicity became available (Rothermich et al 1981). There is still, however, a risk of drug-induced auto-antibody formation, with or without the development of a variety of auto-immune diseases which include: SLE-like syndromes with anti-nuclear factor and renal lesions; a Goodpasture's-like syndrome; myasthenia gravis; and Graves' disease (reviewed by Dawkins et al 1981). There are often moderate reductions in circulating Ig levels (reviewed by Wollheim 1981) and occasionally drug-induced IgA deficiency (Hjalmarson et al 1977). D-penicillamine is also of therapeutic benefit in primary biliary cirrhosis where it was originally used as a copper chelating agent. Primary biliary cirrhosis may, however, be an IC disease producing bile duct damage by activation of the classical complement pathway with circulatory spillover causing systemic disease (Thomas et al 1977). D-penicillamine has been shown by Epstein et al (1979) in a controlled trial to lower CIC levels in primary biliary cirrhosis, and may exert its beneficial effect by this means. Experimentally, d-penicillamine has been shown to alleviate chronically induced adjuvant arthritis in rabbits (Hunneyball et al 1977). During the course of treatment, rabbits demonstrated suppression of delayed cutaneous hypersensitivity, inhibition of leucocyte migration, and an increase in the phagocytic capacity of macrophages as

judged by carbon clearance (Hunneyball et al 1978).

The cellular effects of d-penicillamine may be brought about by a direct effect upon T lymphocytes (Hunneyball et al 1978) and the demonstration of inhibition of helper T-cell function in PWM studies (Lipsky 1981), may account for some of its humoral actions.

Humans are given from 250-1000 mg/day (Mohammed et al 1976; Rothermich et al 1981; Epstein et al 1979) that is 3-14 mg/kg/day. Hunneyball et al (1977) treated rabbits successfully at 15 mg/kg/day, whilst in rats up to 50 mg/kg/day have been used in experiments lasting one month (Arrigoni-Martelli and Binderup 1981).

D-penicillamine, either by altering B-cell function or reducing CIC levels, might be expected to affect the cirrhotic model of mesangial IgA deposition. To avoid toxicity, 10 mg/kg/day was given to the rats in this study.

C Neomycin

Neomycin is a water soluble aminoglycoside with broad spectrum anti-microbial activity. Common gram-negative species are highly sensitive, whilst many gram-positive organisms have their growth inhibited. The drug is used in the pre-operative preparation of patients for bowel surgery and as an adjunct to the therapy of hepatic coma (Sande and Mandell 1980). Neomycin is poorly absorbed from the gastro-intestinal tract; 95% of the usual dose escapes absorption and for this reason it acts preferen-

tially upon the gut flora. Neomycin does not sterilise the gut but allows the growth of resistant organisms. Therefore, the most important complication of oral administration, is intestinal malabsorption and superinfection. Patients occasionally develop a sprue-like syndrome marked by diarrhoea and malabsorption (Dobbins et al 1968).

There is a precedent for believing that absorbed gut floral antigen may be significant in the aetiopathogenesis of mesangial IgA deposition secondary to cirrhosis in man or animals. Neomycin has been shown to be capable of reversing hyperglobulinaemia in rats with surgically induced portacaval shunts (Keraan et al 1974). The effect of neomycin upon the cirrhotic model was studied, to see whether mesangial IgA deposition could be prevented. Neomycin was given at a dose of 25 mg/rat/day (after Keraan et al 1974).

D Phenytoin

Phenytoin (diphenylhydantoin) is one of the hydantoins commonly used as an anticonvulsant in epilepsy. Low serum IgA levels (Grob and Herold 1972) and a depressed phytohaemagglutinin response (Sorrell and Forbes 1975) have been found in 25% of patients. Some groups suggest that primary epilepsy itself may be associated with low serum IgA levels, that are accentuated rather than produced *de novo* by phenytoin treatment (Seager et al 1975; Fontana et al 1976).

Several groups have described a fall in serum IgA levels in patients with primary IgA nephropathy after phenytoin therapy

(Lopez-Trascasa et al 1980; Clarkson et al 1980). Although some have suggested a clinical improvement (Egido et al 1981), no objective clinical or morphological change has been seen by others (Clarkson et al 1980). No clinical trial of phenytoin in mesangial IgA deposition secondary to liver disease, has been reported. The range of dosage used in man is 3-5 mg/kg/day (Clarkson et al 1980; Grob and Herold 1972; Sorrell and Forbes 1975). In the present study, phenytoin was given at a dose of 5 mg/kg/day.

E Dapsone

Dapsone (Bis (4-aminophenyl) sulphone) is an alcohol soluble anti-bacterial agent used in leprosy and to suppress DH. DH is a condition characterised by severe pruritis and a papular, vesicular rash on extensor surfaces. Histologically, there are subepidermal neutrophilic micro-abscesses with fibrin deposition and papillary oedema. Both affected and unaffected skin have granular IgA and C_3 along the dermo-epidermal junction (Fry and Seah 1974). Patients with DH often have gluten sensitive enteropathy (coeliac disease) which may be asymptomatic. Over 70% of patients with DH have either HLA B8 or DRW 3 histocompatibility antigens (Seah et al 1976; Reunela et al 1976). Thus, although there is a similarity between the nature of the immune deposits in IgA nephritis and DH, the two conditions do not share a common histocompatibility profile (Berthoux et al 1978; Fauchet et al 1980).

It is of interest that GN has been described in several patients with DH (Pape et al 1978; Decoteau et al 1973; Bartoli et al

1982) as well as in two patients with coeliac disease (Katz et al 1979; Helin et al 1983) and three patients with both diseases (Moorthy et al 1978; Helin et al 1983). Where performed, IF of the renal lesions has shown mesangial IgA and C_3 (Pape et al 1978) although Bartoli et al (1982) found linear IgA without C_3 in a patient with DH and crescentic GN.

The presence of systemic disease in DH with coeliac disease and deposits in skin (Fry et al 1974) and - occasionally - glomeruli is suggestive of an IC aetiology in DH. To support this, IgA class IC (IgA-IC) were found in approximately 30% of patients with DH using a Raji-cell assay and an IgA-class endpoint (Zone et al 1982). These IgA-IC increased in concentration after a wheat challenge (Zone et al 1982).

The immune deposits in the skin and kidney may be related to antibodies directed against wheat or non-wheat antigens absorbed because of increased permeability of the damaged bowel. In support of this hypothesis, glomerular function was shown to improve after gluten withdrawal in the patient described by Katz et al (1979). Furthermore, the cutaneous deposits of IgA have been shown by IIF to contain J chain in the absence of IgM and to bind free SC, both characteristic properties of IgA dimers (Unsworth et al 1982). Some workers have demonstrated similar findings in the glomerular deposits of primary IgA nephropathy (Egido et al 1980). In Chapter 3, such qualities were found specifically in mesangial IgA deposits of patients with alcoholic cirrhosis.

A gluten free diet, whilst causing a reduction in cutaneous C_3 deposits, has no effect upon IgA deposition (Fry et al 1980; Van der Meer et al 1979). The effect of dapsone is similar, in responsive cases, preventing only C_3 deposition and having little influence upon IgA deposits (Van der Meer et al 1979; Fry et al 1980). Treatment results in a rapid reduction in pruritis with cessation of fresh crops of vesicular eruptions which, however, re-appear within 48 hours of stopping treatment.

There is considerable patient variation in response to dapsone. Effective therapeutic doses have ranged from 100-300 mg/day (Fry and Seah 1974) and very occasionally up to 700 mg/day (Swain et al 1983). The usual dose is 4 mg/kg/day but 10 mg/kg/day may be used. There is precedent for using dapsone in a disease mediated by IgA deposition and in this trial dapsone was administered at 20 mg/kg/day.

MATERIALS AND METHODS

A Animals and their maintenance

DA male rats aged two to three months and weighing 180-220 g were numbered and identified by toe amputation. The rats were maintained in groups of up to 10 rats and allowed to eat and drink *ad libidum*. They were fed pellets. All rats were bled at the start of the experiment and at monthly intervals until death as described in Chapter 5. At weekly intervals, the rats were weighed. The weekly testing of urine, with Albustix (Ames, Miles Laboratories Pty Ltd, Australia) proved impracticable and insensitive.

Experimental rats were given 0.05 ml CCl_4/100 g body weight sc, twice weekly. The CCl_4 was diluted 1:4 in olive oil (Chapter 5). The CCl_4 was prepared and drawn into numbered syringes in a fume cupboard to avoid the risk of inhalation by the operators. Double rubber gloves were worn at all times to avoid accidental self-inoculation. Control rats received the same volume per gram body weight of olive oil, sc, twice weekly. The drugs or diluents were given daily by gavage or sc.

Gavage was performed using a French 5 gauge intravenous cannula with an outer diameter of 1.65 mm (Portex, Seton Products, Hythe, Kent, UK) attached to a 1 ml syringe. A separate gavage tube was used and labelled for each drug. Each gavage tube was marked at a point shown, by autopsy on a rat of the size used in the study, to correspond to the distance from incisors to the gastric fundus. This technique prevented lipoid pneumonia

by allowing the tube to be positioned in the stomach rather than the trachea, which is shorter. For gavage, the rats were anaesthetised as described (Chapter 5), removed from the box, laid flat and straight on their backs and the tube passed backwards and very slightly posteriorly just to one side of the incisors. The time taken to gavage a rat was approximately five seconds so that the depth of anaesthesia required was very light.

The animals were killed by exsanguination under halothane anaesthesia. For this purpose, the gas mixture was delivered by single mask to each rat. To preserve architecture, the kidneys were removed before death was induced by 0.5 ml of intracardiac sodium pentobarbitone (Valobarb) at 300 mg/ml. Kidney cortex blocks were taken for LM, EM and IF as previously described (Chapter 5). Liver tissue was taken for LM. Urine removed from the bladders after death, by intravesical puncture, was stored and assayed for protein after Lowry et al (1951).

B   Histopathology

IIF was performed on unfixed frozen renal tissue using goat antisera to rat IgG, IgM, IgA and C_3, stained with fluoresceinated rabbit anti-goat IgG as described in Chapter 5.

Formalin-fixed wax-embedded tissue was sectioned at 5 μm. Sections of kidney were stained with H&E and PAS, and sections of liver with H&E, Gordon and Sweets reticulin stain and an adaptation of Fouchet's bile stain with a Sirius red collagen counterstain. These staining techniques are described in

Appendix I.

Glutaraldehyde-fixed renal tissue from two control rats, three given PGE_1 only and three given PGE_1 and CCl_4 was processed and examined ultrastructurally (as described in Chapter 5).

C Therapeutic agents

PGE_1

PGE_1 was obtained as a gift from Dr John Pike of Upjohn Co (Kalamazoo, MI). Initially (Experiment I) 250 μg bd was given sc to the rats in this trial; however, toxicity after three days was such that the regime was reduced to 225 μg/day. Later, (Experiment IV) 150 μg/rat/day was administered. PGE_1 substance or the solution in 100% ethanol were stored at -20°C. Daily aliquots were mixed with PBS to give a 10% ethanol-PBS solution at pH 6.8. The final injection volume was 0.2 ml.

D-penicillamine

D-penicillamine substance was obtained as a gift from Mr J Glasby (Lilly Ltd, West Ryde, NSW ex Dista). The powder is water and alcohol soluble, but in order that the same control rats could be used as in the PGE_1 experiments, the d-penicillamine was dissolved in 10% ethanol in PBS. D-penicillamine is unstable in solution and was mixed daily for use, having been stored as a powder at 4°C. Daily aliquots were dissolved to make a solution of 100 mg/ml in 100% ethanol and then diluted in PBS, pH 6.8 to make a 10% ethanol-PBS solution at pH 6.8. D-penicillamine was administered at 10 mg/kg/day with a final injection volume of 0.2 ml.

Neomycin

Neomycin sulphate is water soluble and was dissolved in PBS at 100 mg/ml and stored at room temperature. Neomycin was given by gavage at a dose of 25 mg/rat/day in a final volume of 0.25 ml.

Phenytoin

Phenytoin is stable in solution at a high pH (11-12) but is poorly water soluble; for this reason it was mixed with propylene glycol. Ampoules at 100 mg/ml were diluted 1:10 in propylene glycol and stored at room temperature. Phenytoin was given by gavage at a dose of 5 mg/kg/day in propylene glycol in a final volume of 0.2 ml.

Dapsone

Dapsone substance was a gift from ICI (Cheshire, UK), with the assistance of Mr J Varigos (ICI, Melbourne). Dapsone substance was light sensitive and stored at room temperature in the dark. The substance was dissolved in propylene glycol at a concentration of 20 mg/ml and kept in foil protected aliquots at $4^{o}C$. Dapsone was given by gavage at a dose of 20 mg/kg/day in propylene glycol in a final volume of 0.2 ml.

D Serology

Urea and creatinine were measured at monthly intervals as previously described (Chapter 5).

Estimations of serum Ig and C_3 levels were measured by Mancini's technique (Chapter 5).

E Experimental design

The drugs were tested in a control trial which included controls for the vehicles in which the hepatotoxin and drugs were delivered. The experiments were carried out in four batches consisting of not more than 50 rats. Each batch contained tests of up to two drug regimes using the same diluent and route of administration.

In the first instance, the drugs were administered to 10 rats simultaneously with biweekly CCl_4 in olive oil. This was controlled by giving biweekly olive oil and daily, the diluent alone (control group-eight rats) or the drug in its diluent (drug only group-five rats). In addition, a positive control group developing cirrhosis and mesangial IgA deposits was included in each experiment (CCl_4-only group-10 rats), and this group also received the drug diluent daily (Table 6.1).

Rats in Experiment I took part in the trial of treatment with PGE_1 and d-penicillamine. The rats in Experiment II formed a trial of phenytoin and neomycin treatment. Experiments I and II started one week apart in 1981. Mortality was such that in subsequent experiments "CCl_4 and drug" group sizes were increased to 15 animals, and control groups to 10 rats (Experiments III and IV). Experiment III consisted of a controlled trial of dapsone treatment. The last experiment consisted of rats treated with PGE_1 at a lower dose (150 μg daily) both concurrently with CCl_4 and starting three months after CCl_4, which was continued (Experiment IV). Experiments III and IV started one week apart in 1982. The individual rat numbers were

Table 6.1 The experimental design of the controlled trial of therapeutic agents in the biweekly sc CCl_4 model of mesangial IgA deposition

Experiment	Rat groups (numbers)		Drug	Diluent	Daily dose/rat	Route
I	Control	(8)	-	Ethanol/PBS	-	sc
	PGE_1 only	(5)	PGE_1	Ethanol/PBS	225 mg	sc
	Penicillamine only	(5)	Penicillamine	Ethanol/PBS	2 mg	sc
	CCl_4 only	(10)	-	Ethanol/PBS	-	sc
	CCl_4 + PGE_1	(10)	PGE_1	Ethanol/PBS	225 mg	sc
	CCl_4 + Penicillamine	(10)	Penicillamine	Ethanol/PBS	2 mg	sc
II	Control	(8)	-	Propylene-glycol	-	g
	Neomycin only	(5)	Neomycin	Propylene-glycol	25 mg	g
	Phenytoin only	(5)	Phenytoin	Propylene-glycol	1 mg	g
	CCl_4 only	(10)	-	Propylene-glycol	-	g
	CCl_4 + Neomycin	(10)	Neomycin	Propylene-glycol	25 mg	g
	CCl_4 + Phenytoin	(10)	Phenytoin	Propylene-glycol	1 mg	g
III	Control	(10)	-	Propylene-glycol	-	g
	Dapsone only	(5)	Dapsone	Propylene-glycol	4 mg	g
	CCl_4 only	(10)	-	Propylene-glycol	-	g
	CCl_4 + Dapsone	(15)	Dapsone	Propylene-glycol	4 mg	g
IV	CCl_4 only	(10)	-	Ethanol/PBS	-	sc
	CCl_4 + PGE_1	(15)	PGE_1	Ethanol/PBS	150 mg	sc
	CCl_4 + late PGE_1*	(6)	PGE_1	Ethanol/PBS	150 mg	sc

* = started after three months
g = gavage

1-200 in 1981 and 1-200 in 1982.

F   Statistics

"p" values were calculated using a Mann-Whitney U test for non-parametric independent samples on a Hewlett Packard Desk-top Computer (HP9825, Fort Collins, CO) with a Non-parametric Statistics Pack 3 (Software code 15020). "Z" values were calculated and then two tailed probabilities read from tables (Siegel 1956) of mean standard deviants.

RESULTS

CCl_4 alone produced cirrhosis in each of the experimental batches. Control groups of drug diluent only, whether given sc or by gavage under halothane anaesthesia, showed, no hepatic damage.

CCl_4 alone always resulted in mesangial IgA deposits with - to a lesser extent - IgG, IgM and C_3. None of the drugs or diluents given alone resulted in mesangial IgA deposition although mesangial staining for IgM was common (Table 6.2). The results for all of the experimental batches with the production of cirrhosis and the presence of mesangial IgA deposits are shown in Table 6.3. There was no significant difference between the serum urea and creatinine or urinary protein concentrations in any of the treatment groups after 12 weeks (Table 6.4-6.5).

Forty-six rats received CCl_4 alone for three months and had serial serum Ig and complement levels assayed. Although 16 of these rats subsequently continued into the delayed trial of PGE_1, for three months this group of rats provided a useful overall "cirrhotic" profile of serum Ig and complement levels (Table 6.6). Cirrhotic rats (from Experiments I-IV) after 12 weeks of biweekly CCl_4 showed a mean 21-fold rise in serum IgA levels. Serum IgG levels rose only 1.2-fold in those cirrhotic rats treated by gavage. Serum IgM rose 2.3-fold and C_3 levels fell by up to 0.3 times in all cirrhotic rats. The fall in C_3 levels was most marked in those rats treated by gavage ($p=0.02$). In subsequent discussion the results of serum Ig assays will be

Table 6.2 Renal IIF of control groups of rats from all experiments

Rat groups (numbers)		No. rats with renal IIF staining			
		IgA	IgG	IgM	C_3
Controls	(24)	0	0	9	0
PGE_1 only	(5)	0	0	0	0
Penicillamine only	(5)	0	0	4	0
Neomycin only	(1)	0	0	0	0
Phenytoin only	(4)	0	0	2	0
Dapsone only	(5)	0	0	2	0
CCl_4 only	(23)	23	23	23	18

Table 6.3 The presence of mesangial IgA and cirrhosis in all of the experimental groups

Experiment	Rat groups (numbers)	Survival	No. with mesangial IgA	No. with cirrhosis
I	Controls	8 (8)	0	0
	PGE_1 only	5 (5)	0	0
	Penicillamine only	5 (5)	0	0
	CCl_4 only	10 (10)	10	10
	CCl_4 + PGE_1	4 (10)	0	1
	CCl_4 + Penicillamine	9 (10)	9	9
II	Controls	8 (8)	0	0
	Neomycin only	1 (5)	0	0
	Phenytoin only	4 (5)	0	0
	CCl_4 only	7 (10)	7	7
	CCl_4 + Neomycin	9 (10)	9	9
	CCl_4 + Phenytoin	6 (10)	6	6
III	Controls	8 (10)	0	0
	Dapsone only	5 (5)	0	0
	CCl_4 only	6 (10)	6	6
	CCl_4 + Dapsone	13 (15)	13	13
IV	* CCl_4 + PGE_1	11 (15)	3	3
	$ CCl_4 + late PGE_1	5 (6)	5	5
	$† CCl_4	0 (10)	3	3

* = these are not the same rats as those with mesangial IgA deposition; of those three, only one had cirrhosis

$ = extended studies longer than three months duration of the other experiments

† = all rats in this group died before the end of 20 weeks; histology refers to postmortem tissue obtained between 12 and 17 weeks

Table 6.4 Serum urea and creatinine estimations in all groups of rats after 12 weeks expressed as mean +1SD (the number of samples available). The results of the serum estimations of rats to be treated with delayed PGE_1 and CCl_4-only rat groups were combined at 12 weeks*

Experiment	Rat groups	(numbers)	Urea (mmol/L)	Creatinine (mmol/L)
I	Controls	(8)	6.6 ± 0.8	0.047 ± 0.007
	PGE_1 only	(4)	6.5 ± 0.9	0.050 ± 0.014
	Penicillamine only	(5)	6.7 ± 1.3	0.056 ± 0.009
	CCl_4 only	(9)	7.2 ± 3.3	0.070 ± 0.094
	CCl_4 + PGE_1	(4)	7.2 ± 1.2	0.048 ± 0.019
	CCl_4 + Penicillamine	(9)	7.6 ± 1.0	0.049 ± 0.009
II	Controls	(6)	6.5 ± 0.8	0.053 ± 0.005
	Neomycin only	NT	NT	NT
	Phenytoin only	NT	NT	NT
	CCl_4 only	(7)	5.8 ± 1.1	0.047 ± 0.005
	CCl_4 + Neomycin	(9)	5.7 ± 0.9	0.046 ± 0.005
	CCl_4 + Phenytoin	(6)	6.4 ± 1.8	0.042 ± 0.008
III	Controls	(8)	6.6 ± 0.6	0.056 ± 0.008
	Dapsone only	(4)	7.4 ± 0.6	0.042 ± 0.007
	CCl_4 only	(6)	6.1 ± 1.2	0.050 ± 0.007
	CCl_4 + Dapsone	(12)	7.1 ± 1.0	0.059 ± 0.012
IV	CCl_4 only	(16)*	6.9 ± 2.2	0.050 ± 0.014
	CCl_4 + PGE_1	(5)	6.8 ± 1.5	0.053 ± 0.017

Table 6.5 Urinary protein concentration of each treatment expressed as a mean +1 SD in mg/ml. Number of samples in parenthesis

Experiment	Treatment group		Protein in mg/ml
I	Controls	(4)	8.5 ± 3.3
	PGE_1 only	(2)	13.1 ± 4.1
	Penicillamine only	(2)	11.9 ± 5.8
	CCl_4 only	(9)	8.6 ± 4.5
	CCl_4 + PGE_1	(4)	7.5 ± 2.4
	CCl_4 + Penicillamine	(7)	12.6 ± 4.6
II	Controls	(8)	12.0 ± 4.4
	Neomycin only	(1)	NA
	Phenytoin only	(3)	21.6 + 9.8
	CCl_4 only	(5)	8.5 ± 6.2
	CCl_4 + Neomycin	(8)	11.3 ± 2.0
	CCl_4 + Phenytoin	(4)	7.0 ± 3.6
III	Controls	(5)	15.1 ± 3.8
	Dapsone only	(3)	18.0 ± 10.2
	CCl_4 only	(6)	22.6 ± 13.6
	CCl_4 + Dapsone	(10)	15.5 ± 8.3
IV	CCl_4 + PGE_1	(10)	6.1 ± 1.2

Control = daily diluent only

Table 6.6 The mean (±1SD) serum immunoglobulin and C_3 concentrations after 12 weeks of biweekly CCl_4 compared with control rats given biweekly olive oil (IgA and IgG in mg/ml, IgM and C_3 as percentage of level in a pooled sample)

Rat treatment	Serum concentrations			
	IgA	IgG	IgM	C3
All control rats	0.082 ± 0.055	43.2 ± 12.5	88 ± 21	126 ± 29
Cirrhotic rats (daily ethanol/PBS)	1.57 ± 1.20	48.3 ± 14.9	193 ± 76	108 ± 28
Cirrhotic rats (daily gavage)	2.01 ± 0.67	51.7 ± 24.8	225 ± 81	76 ± 17
All cirrhotic rats	1.70 ± 1.08	49.4 ± 18.9	203 ± 79	98 ± 29

compared with the control groups in each individual experiment.

Survival of rats treated with CCl_4 beyond three months (Experiment IV) was poor. None of the 10 CCl_4-only rats survived to week 20 and the last four died in the 17th week. Five of six rats treated with late PGE_1 survived to the 17th week and the last five were killed in week 18 since no positive controls remained. Insufficient urine for protein estimations was obtained from the rats in this extended experiment. Serum urea and creatinine levels in the CCl_4 and late PGE_1 treated rats were not significantly different.

The results of IIF and LM are given separately in regard to each therapeutic agent.

1 EXPERIMENT I

1.1 Controlled trial of PGE_1 (225 µg/day) administration in the animal model of IgA deposition

Survival and weight gain

The rats treated with PGE_1, whether alone or with CCl_4, exhibited marked morbidity and mortality. Within three days, three rats had died. All rats developed diarrhoea, appeared sick and had spiky fur. They were generally irritable, becoming excessively so within 30 minutes of drug administration. Subsequently, they were difficult to handle and pugnacious. They were of necessity kept in individual cages to prevent fighting. An hour after the PGE_1 injections, they became somnolent and remained in that state for some two hours. Those that died did so in this period and autopsy showed no obvious cause of death

but demonstrated excessively distended bladders which may have accounted for some of their discomfort. The rats treated with the diluent only showed no such changes in well-being, when given either ethanol/PBS injections alone or in combination with CCl_4. Control rats given daily injections of ethanol/PBS had a mean gain of 56.7% in body weight (Table 6.7). Those rats given PGE_1 only gained 17.9% in body weight ($p<0.005$). The cirrhosis only rats gained 32.1% in weight ($p<0.005$), whilst those given CCl_4 and PGE_1 only gained 23.2% body weight over the three months of the experiment. PGE_1 significantly reduced weight gain in control rats but did not affect significantly the rate of weight gain in rats given CCl_4. This may have been because of ascites in all CCl_4 treated rats.

Light microscopy

LM was performed on all of the available kidneys and livers from rats treated with PGE_1 and CCl_4, or PGE_1 alone. The livers from rats treated with CCl_4 and the sc ethanol/PBS, all showed steatosis, 3+ fibrosis and cirrhosis. They were not distinguishable from rats given CCl_4 alone. However, only one of the livers of the four surviving rats treated with CCl_4 and PGE_1 showed cirrhosis. Two of the others showed bridging fibrosis and the fourth only steatosis with a mild increase in fibrous tissue (Figure 6.1). The kidneys were not significantly different from those of control rats.

Renal immunofluorescence

IIF of rat cortex was performed on all rats killed and on some of the rats which died during working hours. The overall

Table 6.7 The change in weight during three months treatment with concurrent PGE_1 at 225 μg/day, with or without CCl_4 compared with controls (Experiment I)

Treatment groups	mean % weight gain	p value	
Controls	56.7	0.0035 (S) (Controls vs PGE_1 only)	0.0014 (S) (Controls vs CCL_4 only)
PGE_1 only	17.9		
CCL_4 only	32.1	0.289 (CCL_4 only vs CCL_4 + PGE_1)	
CCL_4 + PGE_1	23.2		

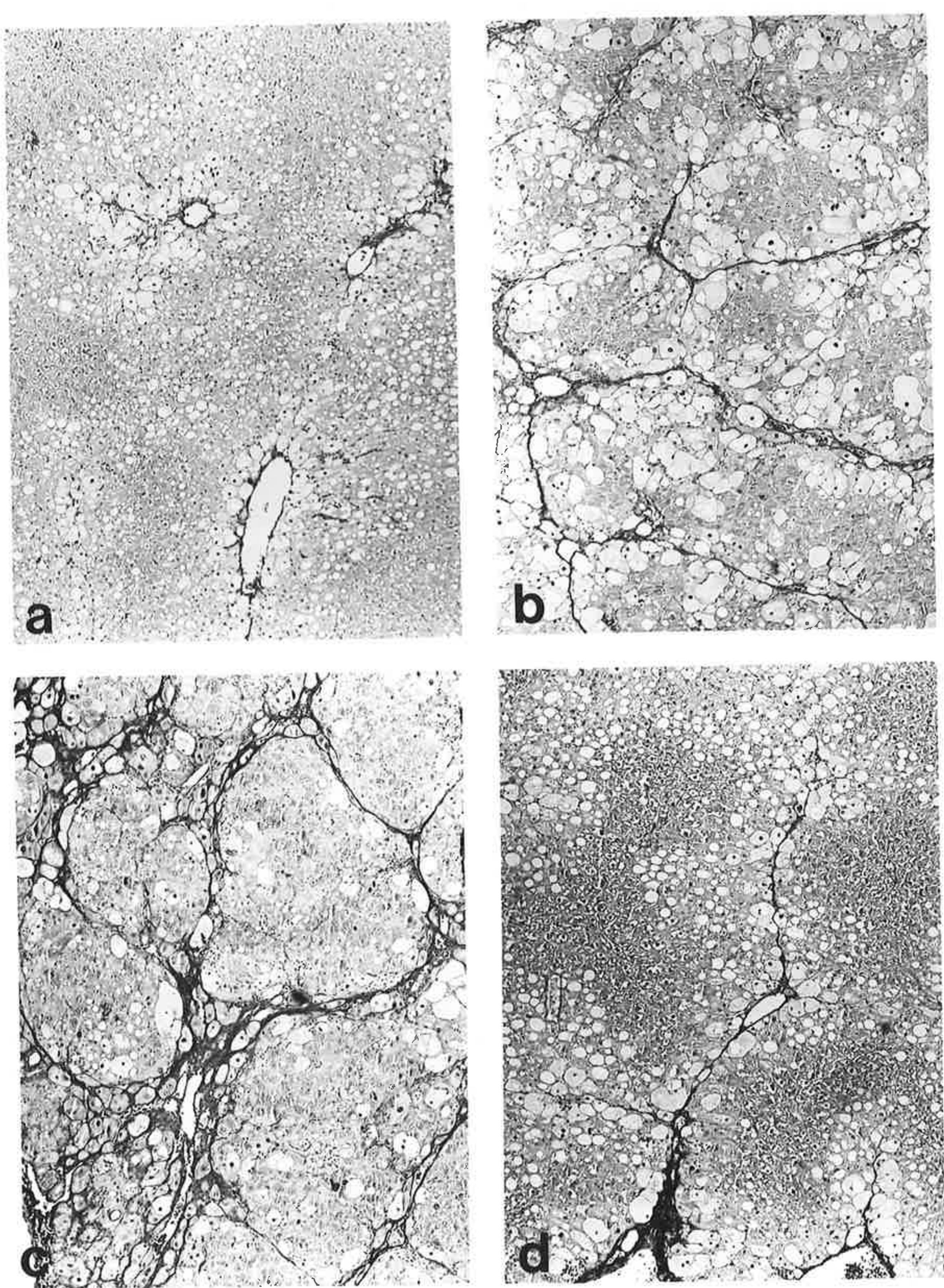


FIGURE 6.1
PGE_1 (225 μg/day) modified the liver damage produced by biweekly CCl_4 injections. The livers from the four surviving rats given PGE_1 and CCl_4 showed only one (c) to have cirrhosis (rat 68), two had variable degrees of fibrosis with bridging-(b) rat 66 and (d) rat 70. One rat, 54 (a) had only steatosis and a minor increase in fibrosis (Fouchet-Sirius red stain, X280).

results are shown in Table 6.3 and the results for individual rats in relation to liver pathology in Table 6.8. None of the four rats which survived three months of simultaneous sc CCl_4 and sc PGE_1 showed mesangial IgA deposition. Occasionally there was IgM staining, as in the controls (Figure 6.2).

Electron microscopy

EM studies were performed in a limited number of cases, the results of which are shown in Table 6.9. In the small numbers of rat kidneys examined, animals treated with PGE_1 alone, appeared to have no increase in mesangial cells, but an apparent expansion of mesangial matrix.

Serology

The results of serum Ig and C_3 estimations are shown in Figure 6.3-6.4 and Table 6.10. PGE_1 treated rats had similar mean IgG, IgM and C_3 levels to CCl_4-only rats. IgA levels were depressed in PGE_1 treated rats but in view of the small numbers of surviving rats, the effect was not significant.

1.2 The results of a controlled trial of d-penicillamine administration in the animal model of mesangial IgA deposition

Survival and weight gain

The rats treated with sc d-penicillamine in ethanol/PBS exhibited normal behaviour and showed no clinical signs of toxicity. Their weight gains are shown in Table 6.11 where it can be seen that rats treated with d-penicillamine alone

Table 6.8 The liver histology and renal IIF (graded 0-4+) in rats treated with biweekly CCl_4 and daily PGE_1 at 225 μg/rat/day (Experiment I)

Rat number	(Survival in weeks)	Liver histology				Renal IIF			
		S	F	B	C	IgA	IgG	IgM	C_3
10	(1)	NT	NT	NT	NT	NT	NT	NT	NT
54*	(12)	+	+	+	0	0	0	0	0
58	(1)	NT	NT	NT	NT	NT	NT	NT	NT
63	(8)	+	0	0	0	0	0	0	0
66*	(12)	+	+	+	0	0	0	0	0
67	(2)	NT	NT	NT	NT	NT	NT	NT	NT
68*	(12)	+	+	+	+	0	1	2	0
69	(11)	NT	NT	NT	NT	NT	NT	NT	NT
70*	(12)	+	+	0	0	0	0	1	0
76	(9)	+	0	0	0	0	0	0	0

S = steatosis
F = fibrosis
B = bridging
C = cirrhosis
NT = not tested
* = surviving rats

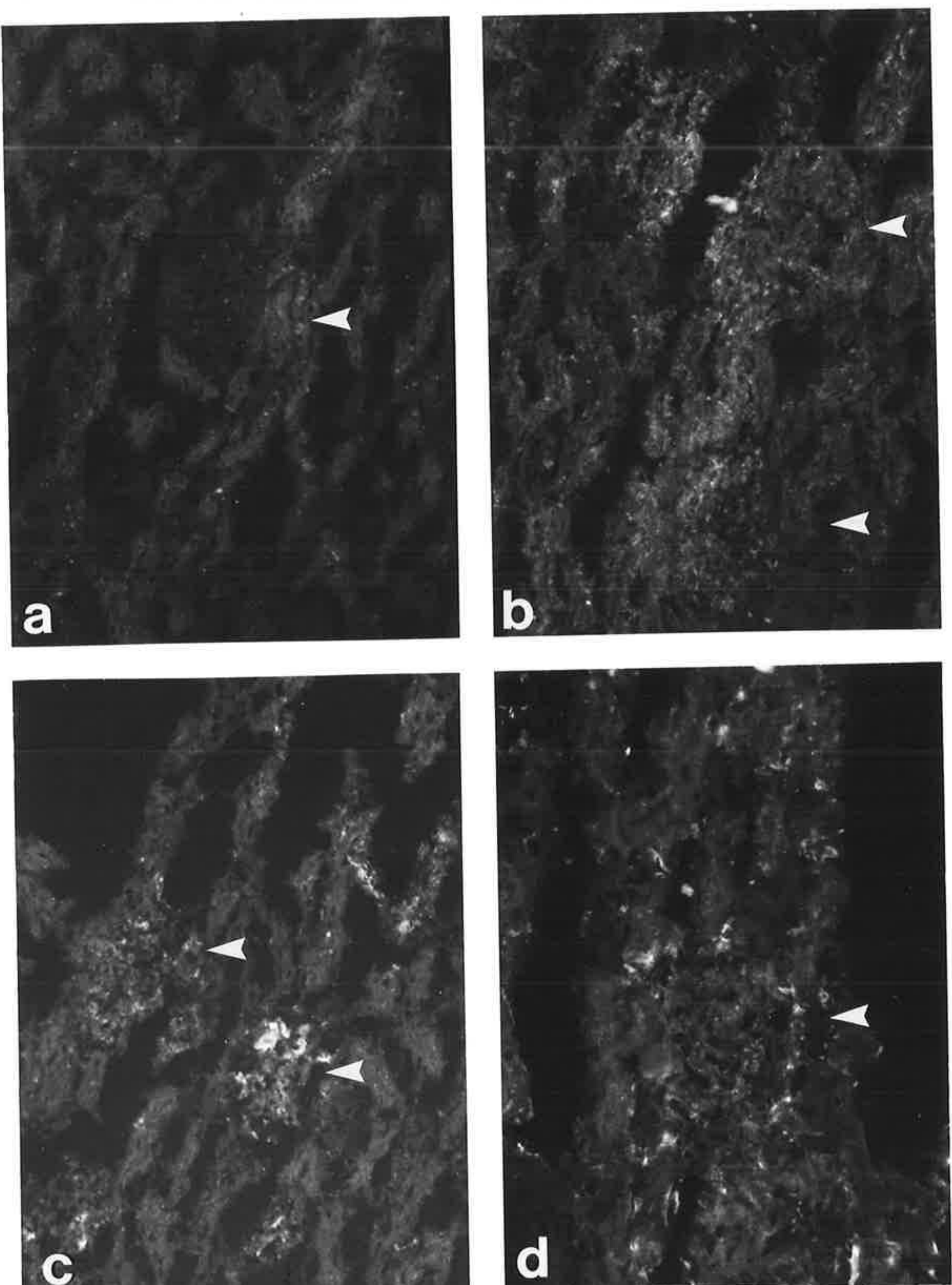


FIGURE 6.2

Glomeruli (arrowed) of rat 54 given biweekly sc CCl_4 and daily sc PGE_1. Stained with goat anti-rat IgA (a), IgG (b), IgM (c), C_3 (d) and FITC-conjugated rabbit anti-goat IgG. Reactions are negative apart from weak mesangial staining for IgM (IIF, X370).

Table 6.9 Ultrastructural features of kidneys of rats treated with PGE_1 compared with controls and those also given CCl_4

Treatment groups	Rat number	Enlargement of mesangial matrix	Electron dense deposits
PGE_1 only	85	+	0
	88	+	0
	89	+	0
PGE_1 + CCl_4	54	0	0
	70	+	+
	76	+	0
Controls	1	0	0
	2	0	0

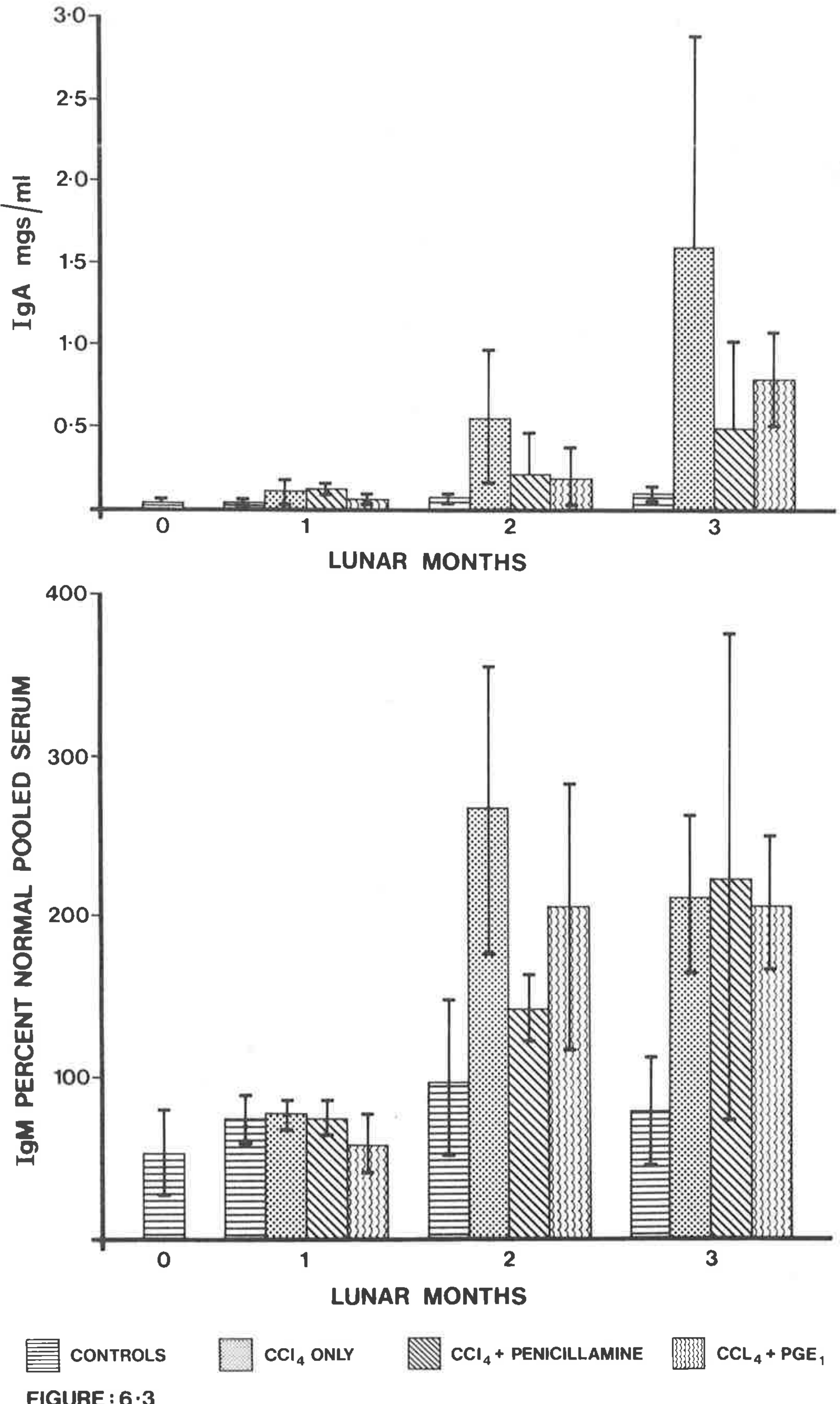
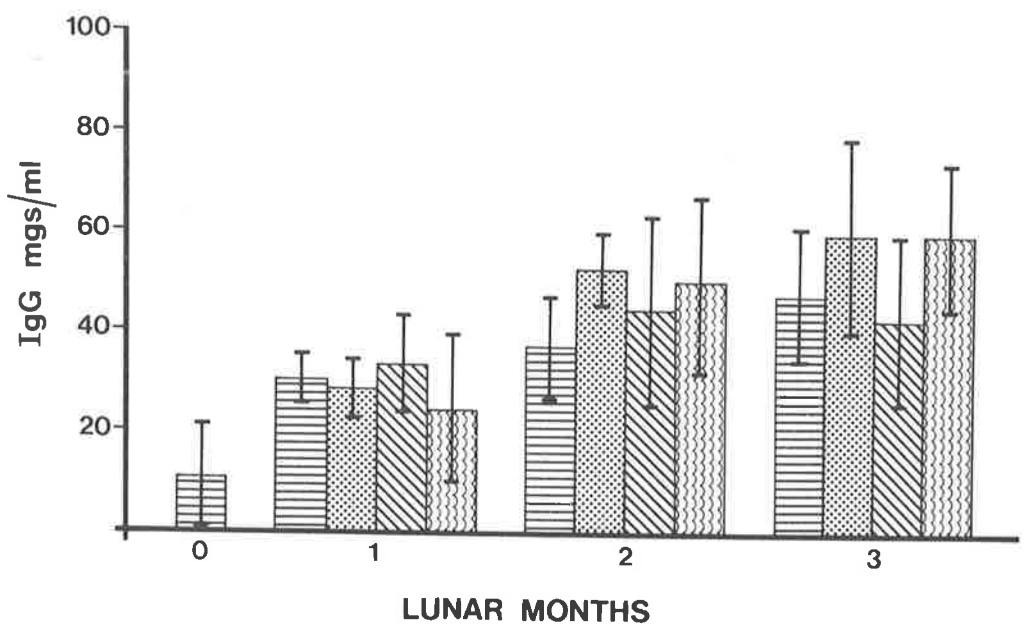
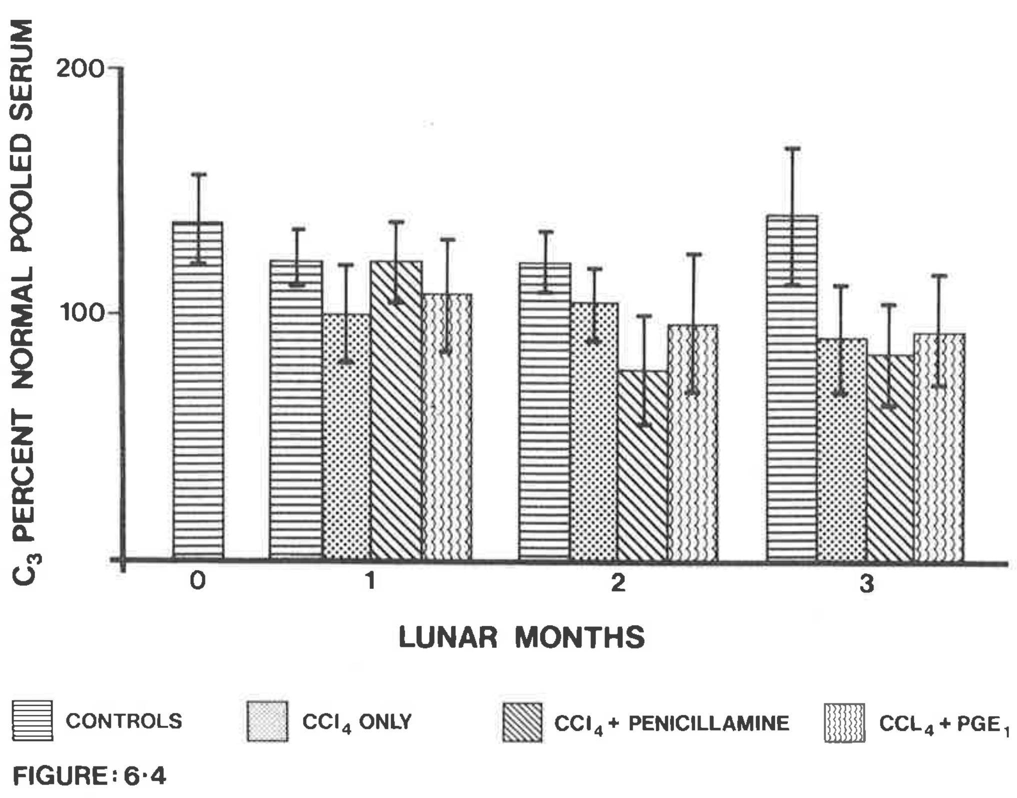


FIGURE: 6·3

Serum IgA and IgM levels in gavaged rats given biweekly CCl_4 and treated with d-penicillamine or PGE_1 (225 µg) compared with control and CCl_4 only rats. Each bar represents the mean of the group with one standard deviation.





CONTROLS　CCl_4 ONLY　CCl_4 + PENICILLAMINE　CCL_4 + PGE_1

FIGURE: 6·4

Serum IgG and C_3 levels in gavaged rats given biweekly CCl_4 and treated with d-penicillamine or PGE_1 (225 µg) compared with control and CCl_4 only rats. Each bar represents the mean of the group with one standard deviation.

Table 6.10 Mancini calculations performed on serum from Group I and II rats expressed as mean and 1SD of each group

Groups	1 MONTH				2 MONTHS†				3 MONTHS			
	IgA	IgG	IgM	C_3	IgA	IgG	IgM	C_3	IgA	IgG	IgM	C_3
I												
Controls	0.044±0.007	31.5± 4.0	74±13	138±17	0.073±0.023	37.7±10.6	98±44	123±14	0.097±0.047	48.4±13.5	78± 28	144±28
CCl_4 only	0.111±0.079	29.6± 6.4	76± 7	101±20	0.570±0.490	53.3± 7.6	266±89	106±14	1.610±1.620	60.5±19.6	219± 49	92±22
CCl_4+PGE_1	0.059±0.025	25.0±17.8	57±20	109±23	0.199±0.193	50.7±18.0	206±78	98±28	0.800±0.28	60.2±14.3	207± 43	94±24
CCl_4+Pen	0.120±0.090	34.5± 9.6	74±13	124±17	0.22 ±0.260	44.8±17.6	142±21	79±24	0.49 ±0.67	42.9±15.8	223±151	86±22
II												
Controls	0.069±0.052	30.1±15.3	84±13	114±16	0.071±0.050	42.5±11.8	102±21	102±14	0.072±0.056	46.3±13.7	95± 25	130±27
CCl_4 only	0.308±0.155	29.6±11.9	98±16	105±26	0.800±0.367	52.7± 5.7	153±51	128±22	2.243±0.786	67.5±30.8	290± 75	79±21
CCl_4+Neo	0.495±0.580	20.9±10.3	87±18	78±23	0.693±0.420	26.8± 7.5	158±60	101±14	1.625±1.082	31.0±24.9	248±109	66±22
CCl_4+Phen	0.172±0.09	24.4± 8.0	112±23	89±16	0.192±0.010	54.0± 0	110±0	111±50	1.560±1.04	82.3±11.7	226±126	83±28

CCl_4 = carbon tetrachloride
PGE_1 = prostaglandin E_1
Pen = Penicillamine

Neo = Neomycin
Phen = Phenytoin
† = these results are based on less than five rats per group; see text

Table 6.11 The mean percentage weight gain during three months treatment with d-penicillamine with and without CCl_4 compared with controls (Experiment I)

Treatment groups	mean % weight gain	p value	
Controls	56.7	0.364 (Controls vs Penicillamine only)	0.0014 (S) (Controls vs CCl_4 only)
Penicillamine only	48.7		
CCl_4 only	32.1	0.384 (CCl_4 only vs CCl_4 + Penicillamine)	
CCl_4 + Penicillamine	28.8		

gained as much weight as control rats. The weight gain in CCl_4-only treated rats was significantly different from that of control rats ($p<0.005$), and there was no significant difference between the CCl_4 treated rats with or without d-penicillamine.

Light microscopy

D-penicillamine treatment did not modify the development of cirrhosis in the rats treated concurrently with CCl_4. All showed cirrhosis. The rats given only daily d-penicillamine showed no abnormalities in the liver.

Renal immunofluorescence

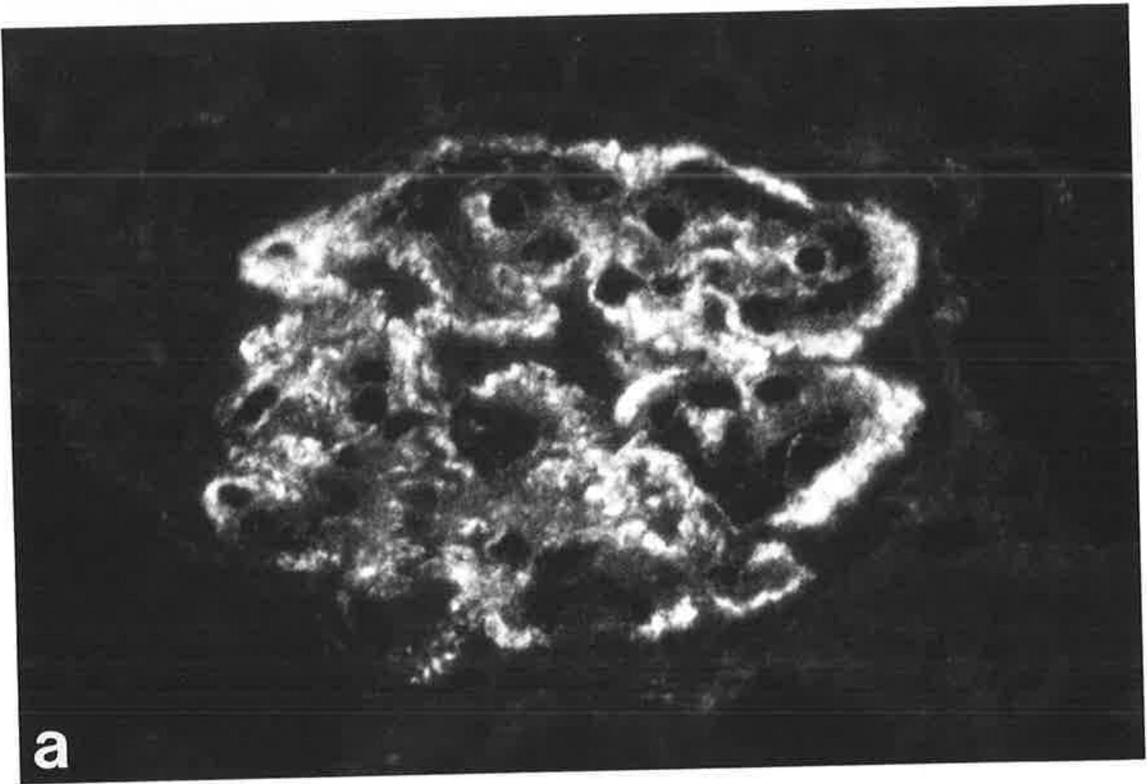
IIF results are shown in Table 6.12. Rats treated only with d-penicillamine showed no renal deposits, but all of those given CCl_4 and d-penicillamine had mesangial deposits of IgA, IgG, IgM and C_3. The group of rats given d-penicillamine and CCl_4 differed from those treated with CCl_4-only in having more prominent capillary wall staining for IgA, C_3 and IgG (Figure 6.5).

Serology

The results of serum Ig and C_3 estimations are shown in Table 6.10 and illustrated in Figure 6.3-6.4. The cirrhotic rats treated with d-penicillamine showed a five-fold rise in serum IgA after three months, compared with a 16-fold rise in untreated Experiment I, cirrhotic rats ($p=<0.05$).

Table 6.12 The renal IIF (graded 0-4+) of rats treated with biweekly CCl_4 and daily d-penicillamine (Experiment I)

Rat number	Intensity of renal IIF			
	IgA	IgG	IgM	C_3
51	2	2	3	2
52	2	2	2	1
87	3	2	3	2
90	2	2	2	1
91	1	1	2	1
92	2	2	2	1
95	2	2	2	1
96	2	2	2	2
97	2	2	1	1
99	3	2	2	1



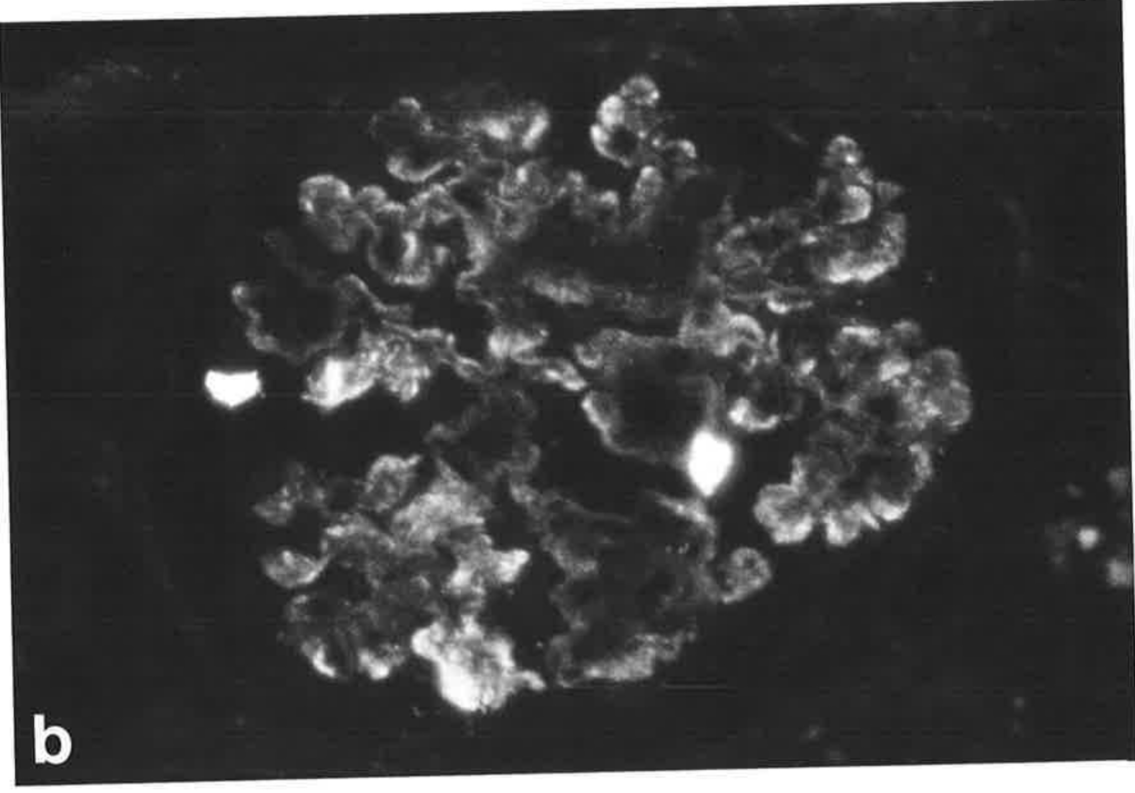


FIGURE 6.5
Glomeruli of a rat given biweekly sc CCl_4 and daily d-penicillamine. Stained with goat anti-rat IgA (a) and C_3 (b). The fluorescence is mesangial with granular extension along the capillary loops (IIF, X2000).

2 EXPERIMENT II

2.1 Controlled trial of phenytoin administration in the animal model of mesangial IgA deposition

Survival and weight gain

The survival of the rats treated with phenytoin is shown in Table 6.3. One of the phenytoin-only rats and four of 10 CCl_4-only rats died before the end of the experiment. The deaths all occurred shortly after anaesthesia and were assumed to be related to the gavage technique. In this experiment, three of the 10 rats treated with CCl_4-only but given also daily gavage died.

The general health of the rats in this experiment was good. The mean percentage weight gains in the group are shown in Table 6.13. The administration of phenytoin alone did not affect significantly the mean weight gain (20%) compared with that in controls (23.4%). Although there was a significant difference between the control and CCl_4 treated rats, this latter group's weight gain was not significantly different from that of the rats treated with CCl_4 and phenytoin.

Light microscopy

LM performed on livers from six rats treated with CCl_4 and neomycin showed cirrhosis to develop in the same manner as those given CCl_4 alone (Table 6.3). The four rats treated with phenytoin-only had no evidence of liver disease. The kidneys from four rats treated with phenytoin alone were the same as those from control rats. The kidneys from six rats treated with biweekly CCl_4 and daily phenytoin were similar.

Table 6.13 The mean percentage weight gain in rats treated with phenytoin and neomycin compared with controls (Experiment II)

Treatment groups	mean % weight gain	p value		
Controls	23.4			
Phenytoin only	20.0		0.144 (Controls vs Phenytoin only)	
Neomycin only	10.0	0.027 (S) (Controls vs Neomycin only)		
CCl_4 only	-1.6			0.002 (S) (Controls vs CCl_4 only)
CCl_4 + Phenytoin	9.0		0.238 (CCl_4 only vs CCl_4 + Phenytoin)	
CCl_4 + Neomycin	-0.4	0.548 (CCl_4 only vs CCl_4 + Neomycin)		

Renal immunofluorescence

IIF performed on the six surviving rats treated with phenytoin and CCl_4 showed IgA, IgG and IgM in all cases and weak C_3 in three (Table 6.14).

Serology

The results of Ig and complement assays are shown in Figure 6.6-6.7 and in Table 6.10. Rats given biweekly CCl_4 with daily phenytoin showed a mean 21-fold rise in serum IgA after three months of treatment, compared with a 31-fold rise in rats in Experiment II given CCl_4 alone. The difference, however, was not significant. Serum IgG, IgM and C_3 levels were similar to those of rats treated with CCl_4 alone.

2.2 The results of a controlled trial of neomycin administration in the animal model of mesangial IgA deposition

Survival and weight gain

The survival of the rats treated with neomycin is shown in Table 6.3. Only one of the five rats treated by daily gavage with neomycin alone survived. Fortunately only one of the 10 rats treated with biweekly CCl_4 and daily neomycin died. Deaths were all in the immediate post-anaesthetic period. The rats treated with neomcyin tended to have diarrhoea and to be moderately sick with spiky fur. The mean percentage weight gains in the group are depicted in Table 6.13 which shows a significant reduction in weight gain in rats treated with neomycin alone compared with the control group ($p=0.027$).

Table 6.14 The intensity of renal IIF (graded 0-4+) of rats treated with biweekly CCl_4 and daily phenytoin (Experiment II)

Rat number	Intensity of renal IIF			
	IgA	IgG	IgM	C_3
6 †	-	-	-	-
24 †	-	-	-	-
30	2	2	2	0
32	2	2	2	1
33	2	3	2	2
46 †	-	-	-	-
47	1	1	1	1
55 †	-	-	-	-
71	2	1	2	1
72	3	3	3	2

† = died; no IIF performed

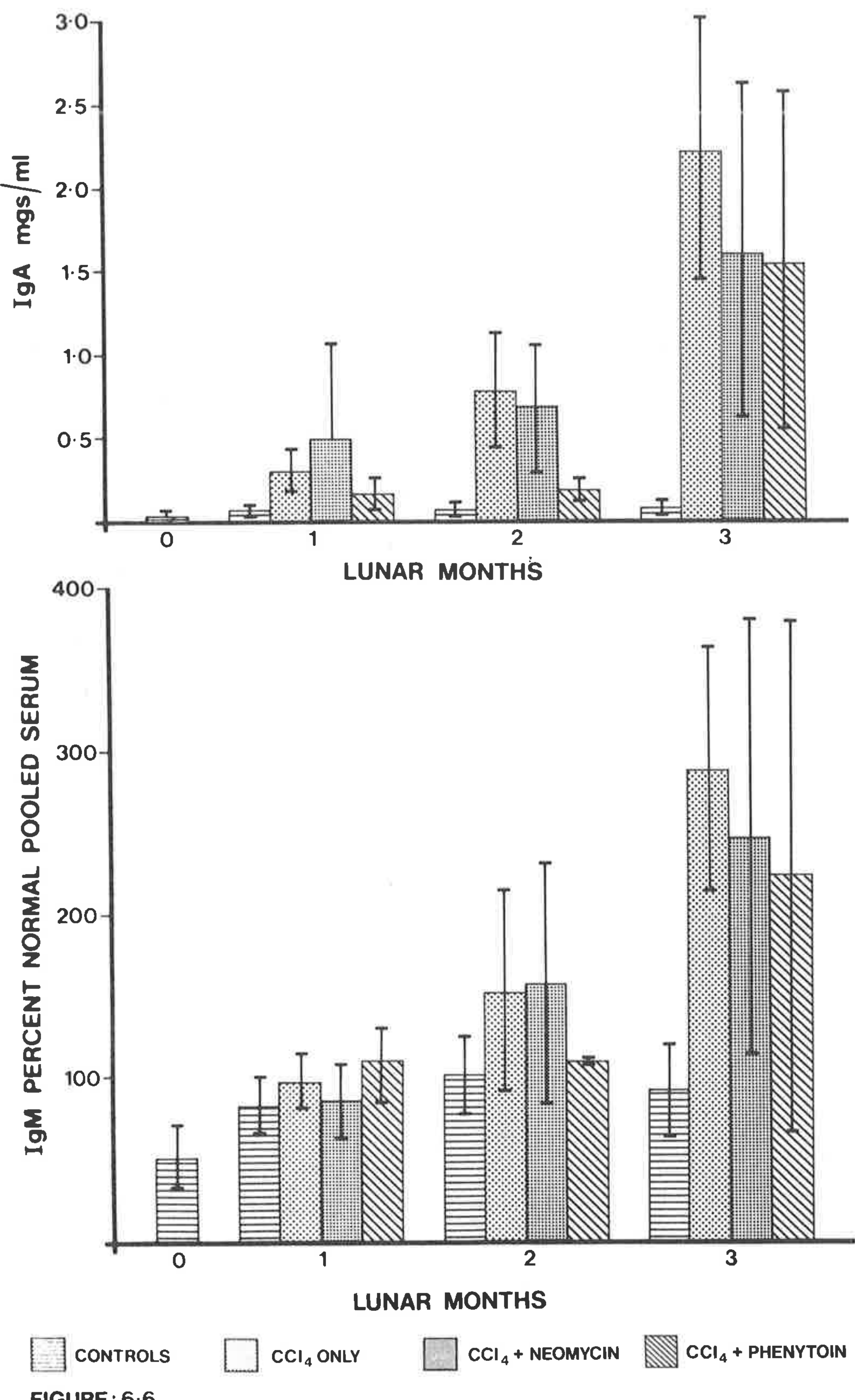
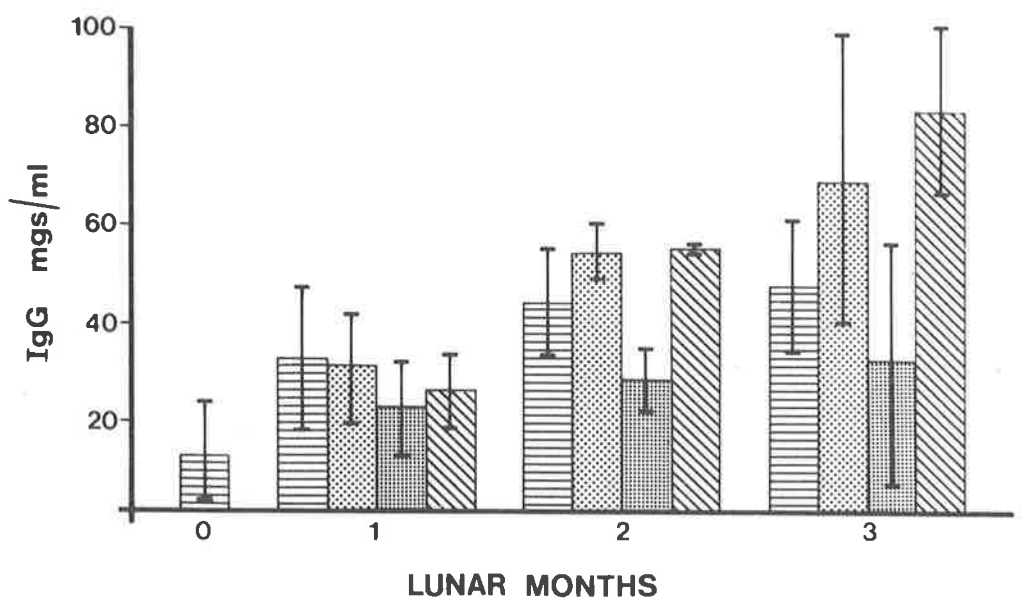
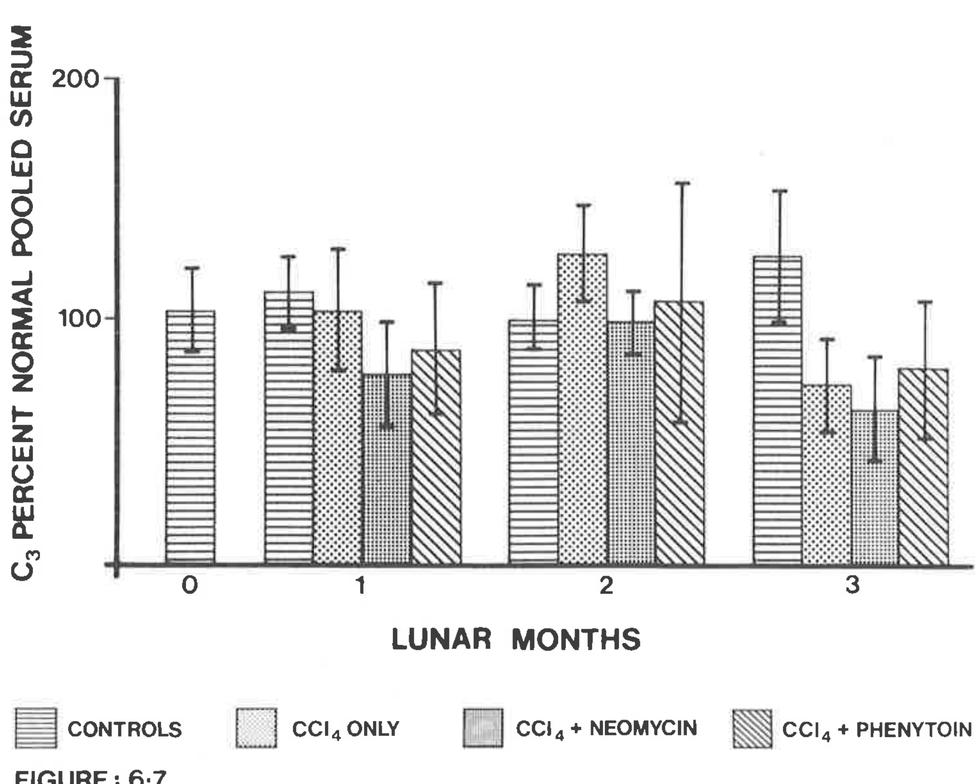


FIGURE: 6·6

Serum IgA and IgM levels in rats given biweekly CCl_4 and treated with Neomycin or Phenytoin compared with gavaged control and CCl_4 only rats. Each bar represents the mean of the group with one standard deviation.





CONTROLS CCl_4 ONLY CCl_4 + NEOMYCIN CCl_4 + PHENYTOIN

FIGURE: 6·7

Serum IgG and C_3 levels in rats given biweekly CCl_4 and treated with Neomycin or Phenytoin compared with gavaged control and CCl_4 only rats. Each bar represents the mean of the group with one standard deviation.

Light microscopy

LM performed on the livers of the six surviving rats treated with CCl_4 and neomycin confirmed that cirrhosis had developed. The one surviving rat treated with neomycin alone showed a normal liver. LM of the kidneys showed that all rats treated with biweekly CCl_4 and daily neomycin demonstrated the same minor changes seen in rats given CCl_4 only.

Renal immunofluorescence

IIF performed on the kidneys of the nine surviving CCl_4 and neomycin treated rats showed that all had mesangial IgA, IgG and IgM and six had C_3 (Table 6.15).

Serology

The results of Ig and complement assays are shown in Figure 6.6-6.7 and Table 6.10. There was a significant reduction in IgG increment after three months in rats treated with biweekly CCl_4 and neomycin compared with CCl_4 alone ($p=0.02$). This did not appear to have any effect on the intensity of mesangial IgG deposition. The 21-fold rise in serum IgA after three months was not significantly different from the 31-fold rise in those given biweekly CCl_4 only.

3 EXPERIMENT III

3.1 The results of a controlled trial of dapsone administration in the animal model of mesangial IgA deposition

Survival and weight gain

The survival of rats treated with dapsone is shown in Table 6.3. The general condition of the rats was good. Two of the

Table 6.15 The intensity of renal IIF (graded 0-4+) performed in rats treated with biweekly CCl_4 and daily neomycin
(Experiment II)

Rat number	Intensity of renal IIF			
	IgA	IgG	IgM	C_3
20	3	2	3	0
35	3	2	3	2
36	3	2	3	1
37	3	1	2	0
39	2	1	2	0
40	2	2	2	2
41	2	1	2	1
42	2	1	1	0
49	2	1	2	1
56 †	-	-	-	-

† = rat died before the end of the experiment

10 controls, four of 10 CCl_4-only rats, and two of the 15 CCl_4 and dapsone treated rats died. All deaths occurred shortly after anaesthesia and in the last four weeks of the drug trial. The mean percentage weight gains in dapsone treated rats and controls are shown in Table 6.16. There was no difference in weight gain between dapsone treated and untreated rats although there was a significant difference in weight gain between those rats given CCl_4-only and the control animals.

Light microscopy

LM performed on 15 livers from rats treated with CCl_4 and dapsone showed all to be cirrhotic. None of those treated with dapsone alone had liver abnormalities. The kidneys of rats treated with dapsone only were within normal limits and those of rats given CCl_4 alone showed the minor changes described earlier.

Renal immunofluorescence

IIF performed on kidneys from 13 surviving dapsone treated rats (Table 6.17) showed IgA, IgG and IgM in all mesangia and C_3 in all but one instance.

Serology

The results of Ig and C_3 serum estimations are shown in Figure 6.8-6.9 and Table 6.18. Dapsone tended to lower serum IgA levels compared with those of rats given biweekly CCl_4 alone but the reduction was not significant. There was, however, a significant amelioration of the depression in serum C_3 levels seen in such rats (p=0.012).

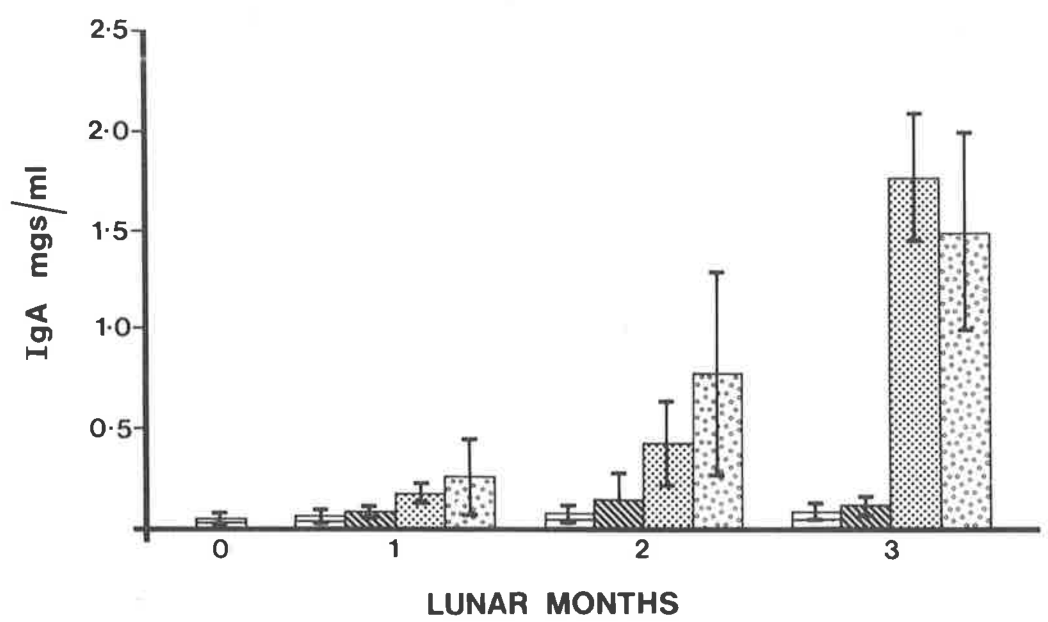
Table 6.16 The mean percentage weight gain in rats treated with dapsone compared with controls (Experiment III)

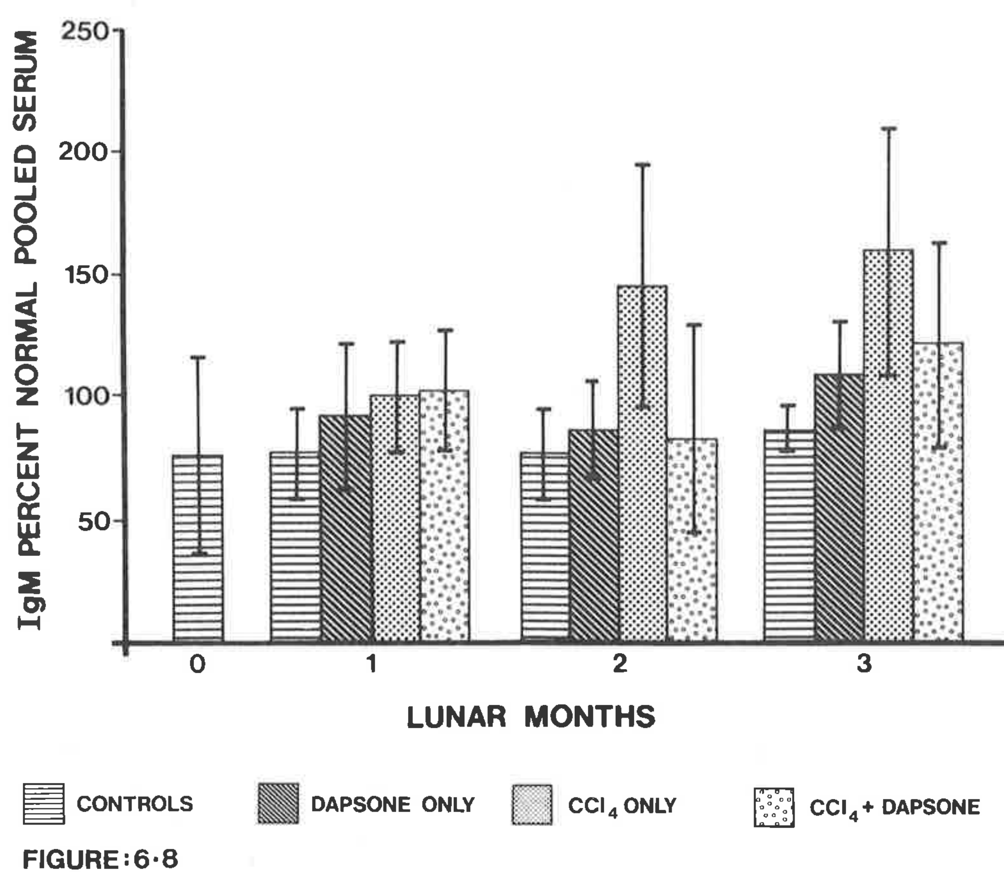
Treatment groups	mean % weight gain	p value	
Controls	21.8		
Dapsone only	17.3	0.711 (Controls vs Dapsone only)	
CCl_4 only	6.8		0.002 (S) (Controls vs CCl_4 only)
CCl_4 + Dapsone	10.9	0.3576 (CCl_4 only vs CCl_4 + Dapsone)	

Table 6.17 The intensity of renal IIF (graded 0-4+) in rats treated with biweekly CCl_4 and daily dapsone (Experiment III)

Rat number	Intensity of renal IIF			
	IgA	IgG	IgM	C_3
58 †	-	-	-	-
59	3	1	2	1
61	2	1	3	2
62	3	0	1	1
64	3	2	3	1
66	1	0	1	0
68	3	1	1	1
69	2	1	1	1
70	3	3	3	3
71	3	2	3	2
72	2	2	2	1
75	3	2	2	2
89 †	-	-	-	-
94	3	2	3	1
95	3	2	3	3

† = rat died before the end of the experiment

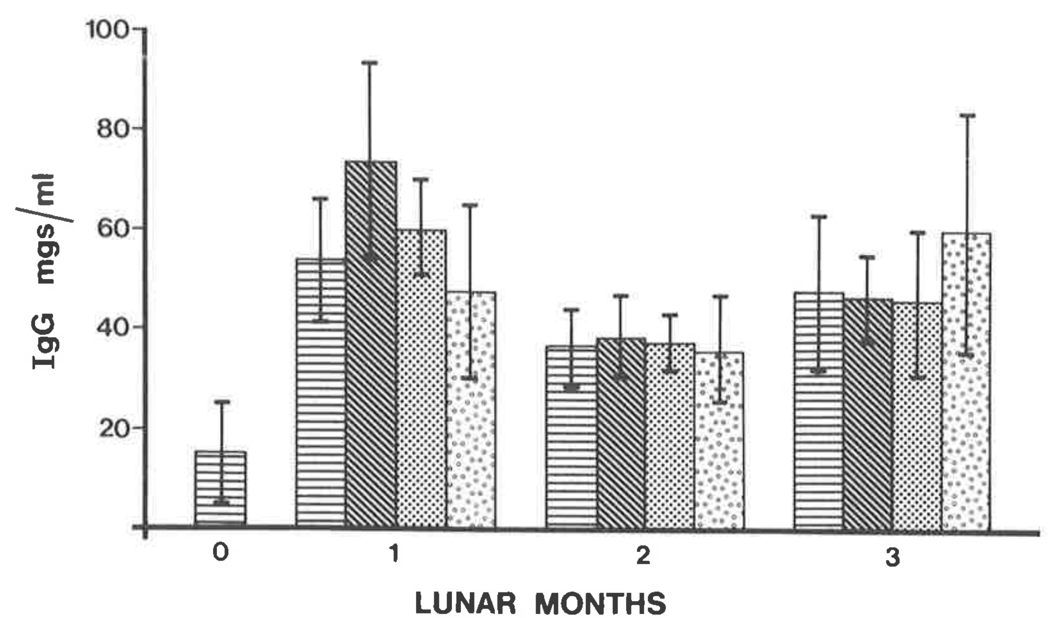


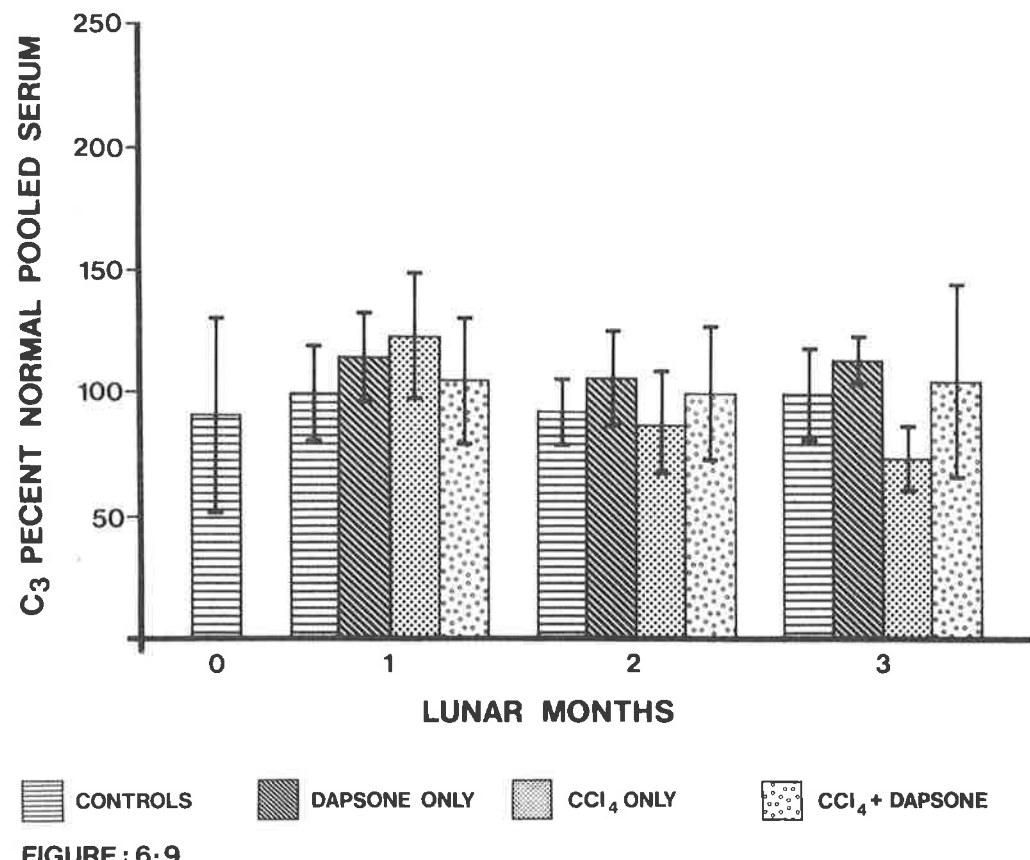


CONTROLS DAPSONE ONLY CCl_4 ONLY CCl_4 + DAPSONE

FIGURE: 6·8

Serum IgA and IgM levels in gavaged rats given biweekly CCl_4 and treated with Dapsone compared with gavaged control and CCl_4 only rats. Each bar represents the mean of the group with one standard deviation.





CONTROLS DAPSONE ONLY CCl_4 ONLY CCl_4 + DAPSONE

FIGURE: 6·9

Serum IgG and C_3 levels in gavaged rats given biweekly CCl_4 and treated with Dapsone compared with gavaged control and CCl_4 only rats. Each bar represents the mean of the group with one standard deviation.

Table 6.18 Immunoglobulin and C_3 results of Mancini estimations performed on serum from Group II and IV rats up to three months

Groups	1 MONTH				2 MONTHS				3 MONTHS			
	IgA	IgG	IgM	C_3	IgA	IgG	IgM	C_3	IgA	IgG	IgM	C_3
III												
Controls	0.055±0.034	54.2±12.9	78±18	99±19	0.075±0.035	36.7± 7.9	78±18	92±15	0.078±0.60	48.0±16.6	87± 9	100±19
Dap only	0.083±0.018	73.4±20.5	94±30	115±19	0.140±0.143	39.1± 8.6	87±21	106±19	0.115±0.058	47.0±8.8	111±22	114±11
CCl_4 only	0.176±0.047	59.9± 9.6	102±23	123±27	0.444±0.216	37.7± 6.0	146±49	86±20	1.796±0.574	46.2±14.2	161±49	73±15
CCl_4+Dap	0.262±0.195	47.5±17.0	103±25	105±27	0.789±0.486	36.0±11.0	84±48	99±28	1.494±0.976	60.5±24.6	123±43	105±39
IV												
CCl_4 only*	0.241±0.297	37.1±12.9	102±24	111±25	0.830±0.392	46.0±15.8	115±37	112±25	1.553±1.073	43.9± 8.7	182±85	116±29
CCl_4+PGE_1†	0.083±0.018	29.6± 8.5	88±12	108±23	0.248±0.256	42.3±14.4	117±56	124±25	0.443±0.616	51.3± 6.4	132±41	125±37

Dap = Dapsone

* = includes those rats later to be given PGE_1

† = concurrent PGE_1 (150 μg/day)

4 EXPERIMENT IV

4.1 The results of a controlled trial of concurrent PGE_1 administration at 150 μg/day in the animal model of IgA deposition

Survival and weight gain

The rats treated with PGE_1 at a dose of 150 μg/day concurrently with the biweekly CCl_4 injections exhibited less toxicity than those in an earlier experiment given 225 μg/day. There was still diarrhoea and drowsiness but extreme irritability was not seen. The rats did not fight and were kept in groups of up to 10 animals. The PGE_1 treated rats gained only 0.5% in mean weight compared with 19.9% in the CCl_4-only rats ($p=0.0006$).

Renal and hepatic morphology

The results of IIF of kidneys and the examination of the livers of rats treated with PGE_1 concurrently with CCl_4 are shown in Table 6.19. Rats given CCl_4-only in this experiment all showed cirrhosis (Table 6.2) whilst of the 11 surviving rats treated with CCl_4 and PGE_1, only three developed cirrhosis (Figure 6.10). As shown in Table 6.19 only three of the surviving PGE_1 treated rats had mesangial IgA deposits. Only one of the three PGE_1 treated rats with cirrhosis and two of eight without cirrhosis had mesangial IgA deposits (Figure 6.11-6.12).

Serology

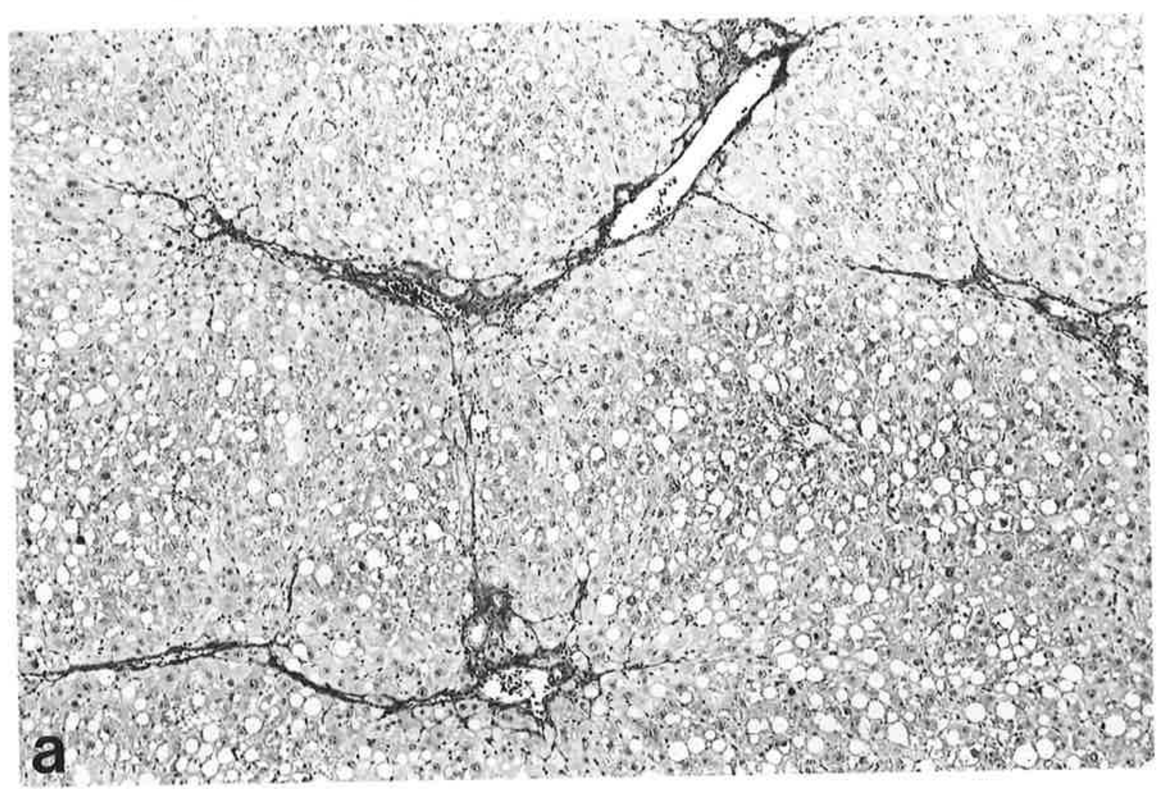
The results of Ig and C_3 estimations are shown in Table 6.18 and Figure 6.13-6.14. There was a significant reduction in serum IgM ($p=0.036$) and IgA ($p=0.00006$) levels in PGE_1 treated, compared with untreated rats given biweekly CCl_4.

Table 6.19 The liver histology and renal IIF (graded 0-4+) of rats treated with biweekly CCl_4 and concurrent PGE_1 at 150 µg/rat/day (Experiment IV)

Rat Number	Liver Histology		Intensity of renal IIF			
	Cirrhosis	Bridging	IgA	IgG	IgM	C_3
1	+	NA	0	0	0	0
5	0	+	0	0	1	0
11	+	NA	0	1	1	1
13	0	+	2	1	2	1
15	0	+	1	0	1	0
27	0	0	0	0	0	0
28	+	NA	1	0	1	0
29 †	-	-	-	-	-	-
34	0	0	0	0	1	0
51	0	+	0	0	1	0
52 †	-	-	0	0	0	0
53 †	-	-	-	-	-	-
55	0	+	0	0	0	0
56 †	-	-	-	-	-	-
57	0	0	0	0	1	0

† = rat died before the end of the experiment

IIF graded as absent 0-3+
Histological features graded as absent 0 or present +
Bridging fibrosis was not assessed in the presence of cirrhosis



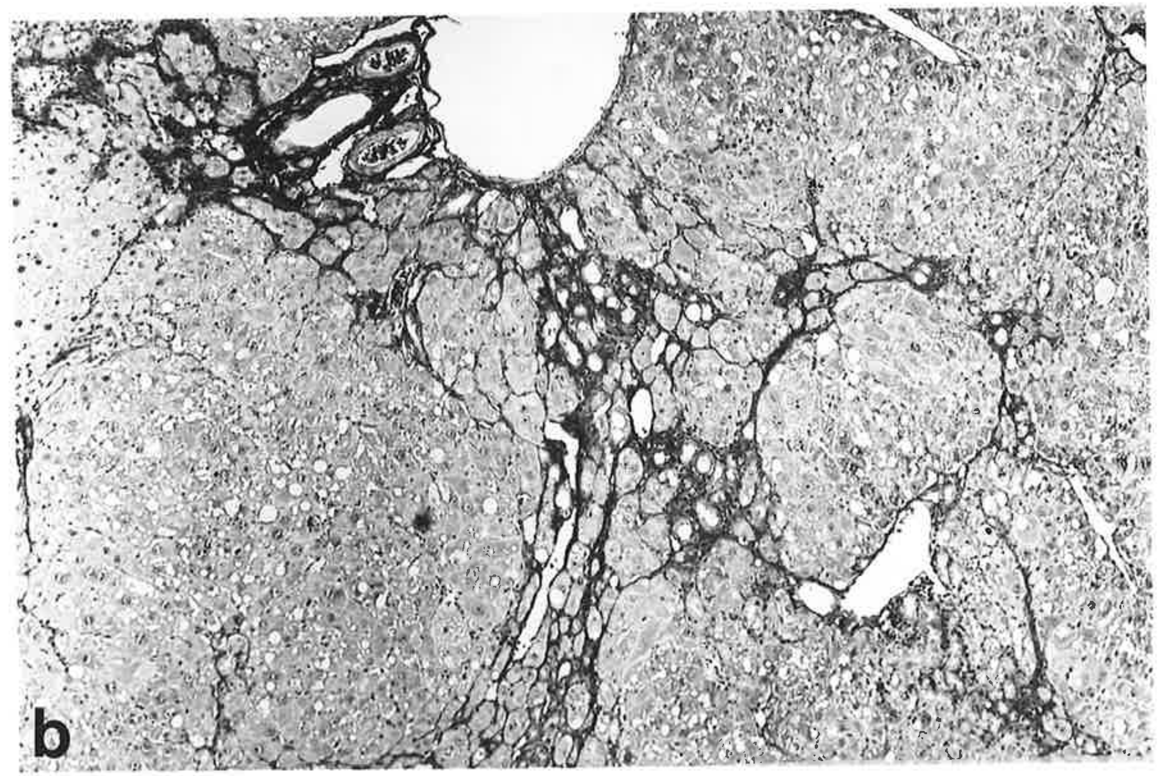


FIGURE 6.10
PGE_1 at 150 μg/day modified the development of cirrhosis in rats given biweekly CCl_4. There was (a) steatosis with some degree of fibrosis and bridging (rat 13), whilst some showed (b) the development of cirrhosis with nodule formation and loss of architectural relations (rat 1) (Fouchet-Sirius red stain, X540).

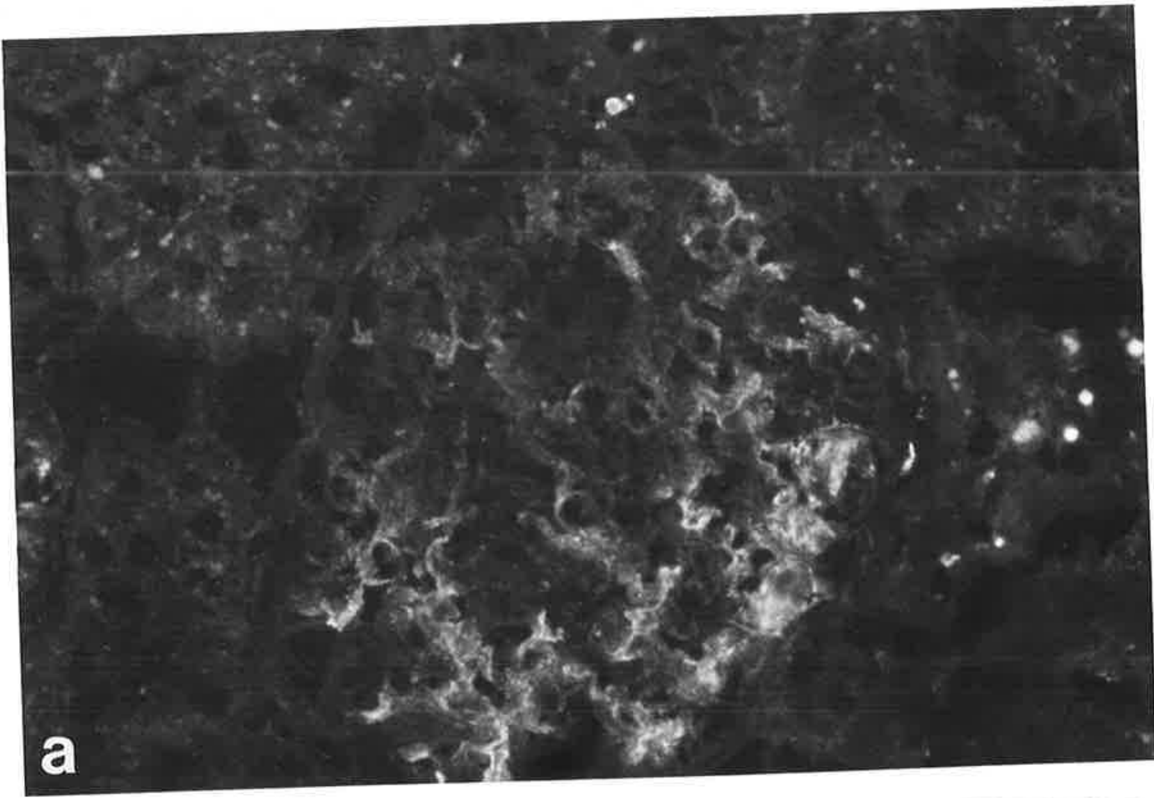
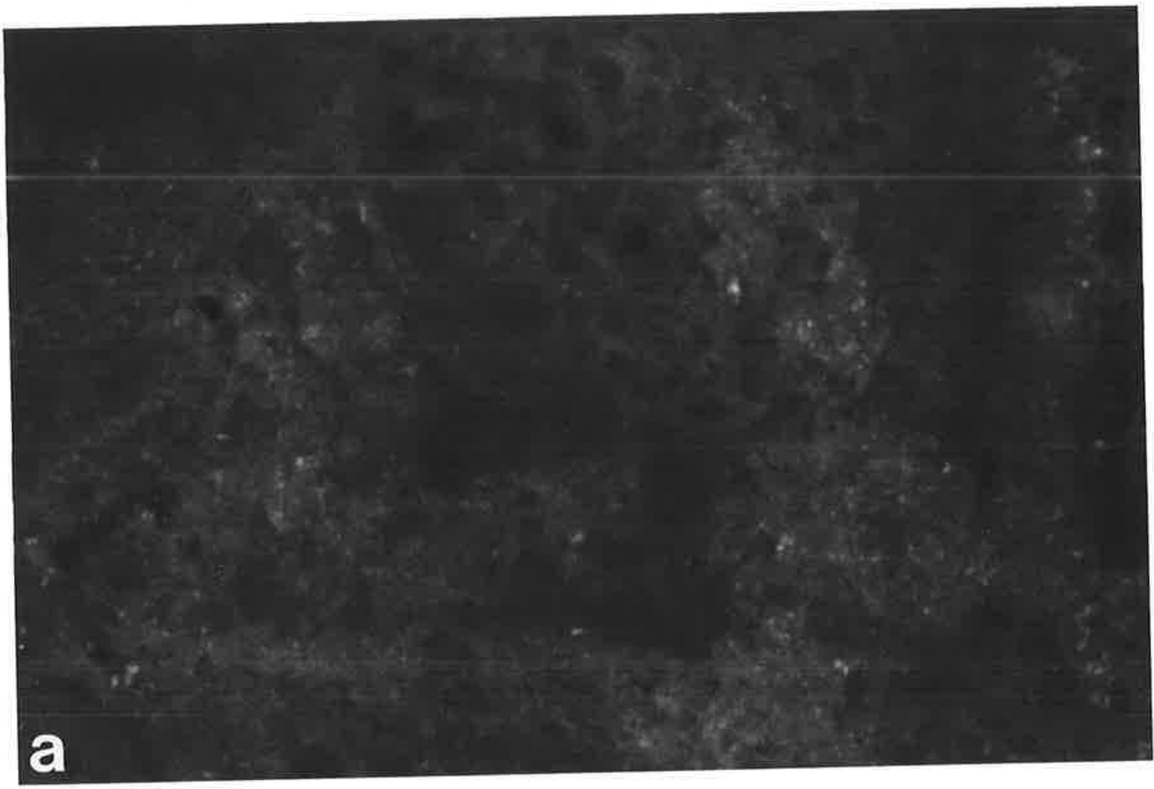


FIGURE 6.11
Glomeruli of a rat treated with 150 μg PGE_1 and CCl_4 for three months. This rat (15) was not cirrhotic but showed weak mesangial IgA staining (a) without C_3 (b). Stained with goat anti-rat antisera and FITC-conjugated rabbit anti-goat IgG (IIF, X1300).



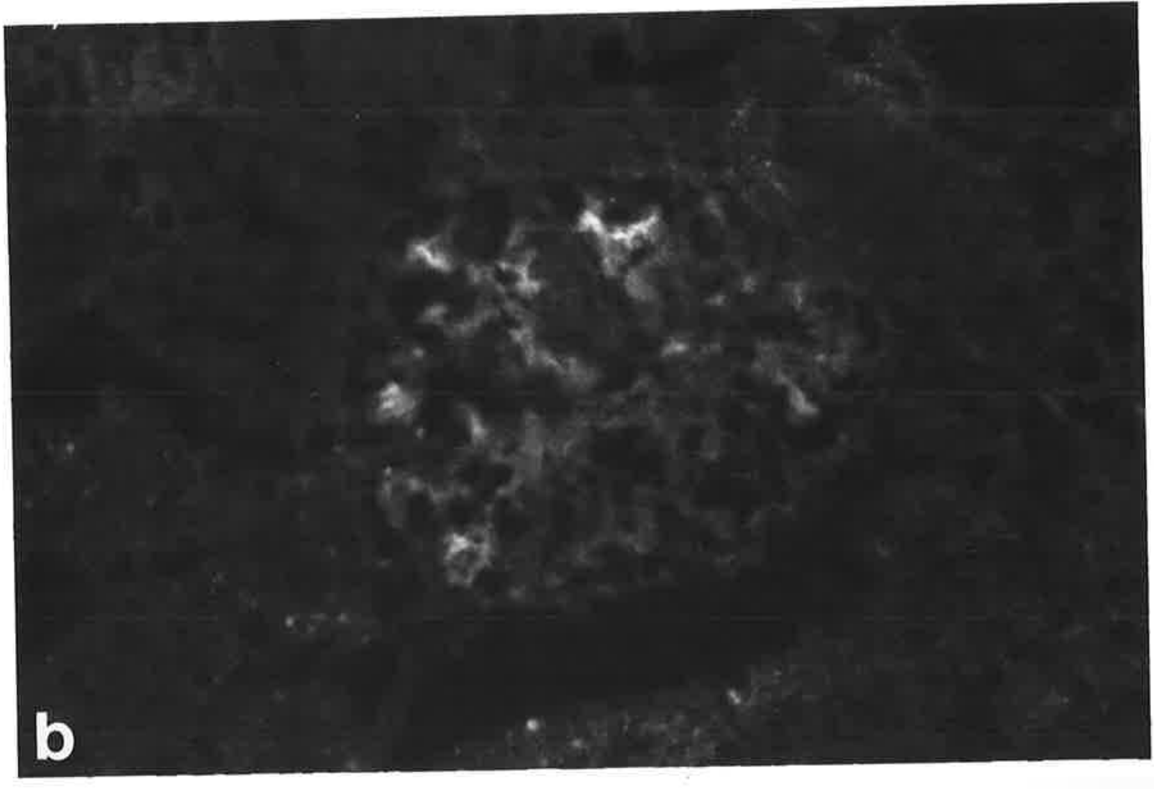


FIGURE 6.12
Glomeruli from a rat treated with 150 μg PGE_1 and CCl_4 for three months. This rat (11) was cirrhotic but showed no IgA (a) and a trace of IgM (b). Stained with goat anti-rat antisera and FITC-conjugated rabbit anti-goat IgG (IIF, X1346).

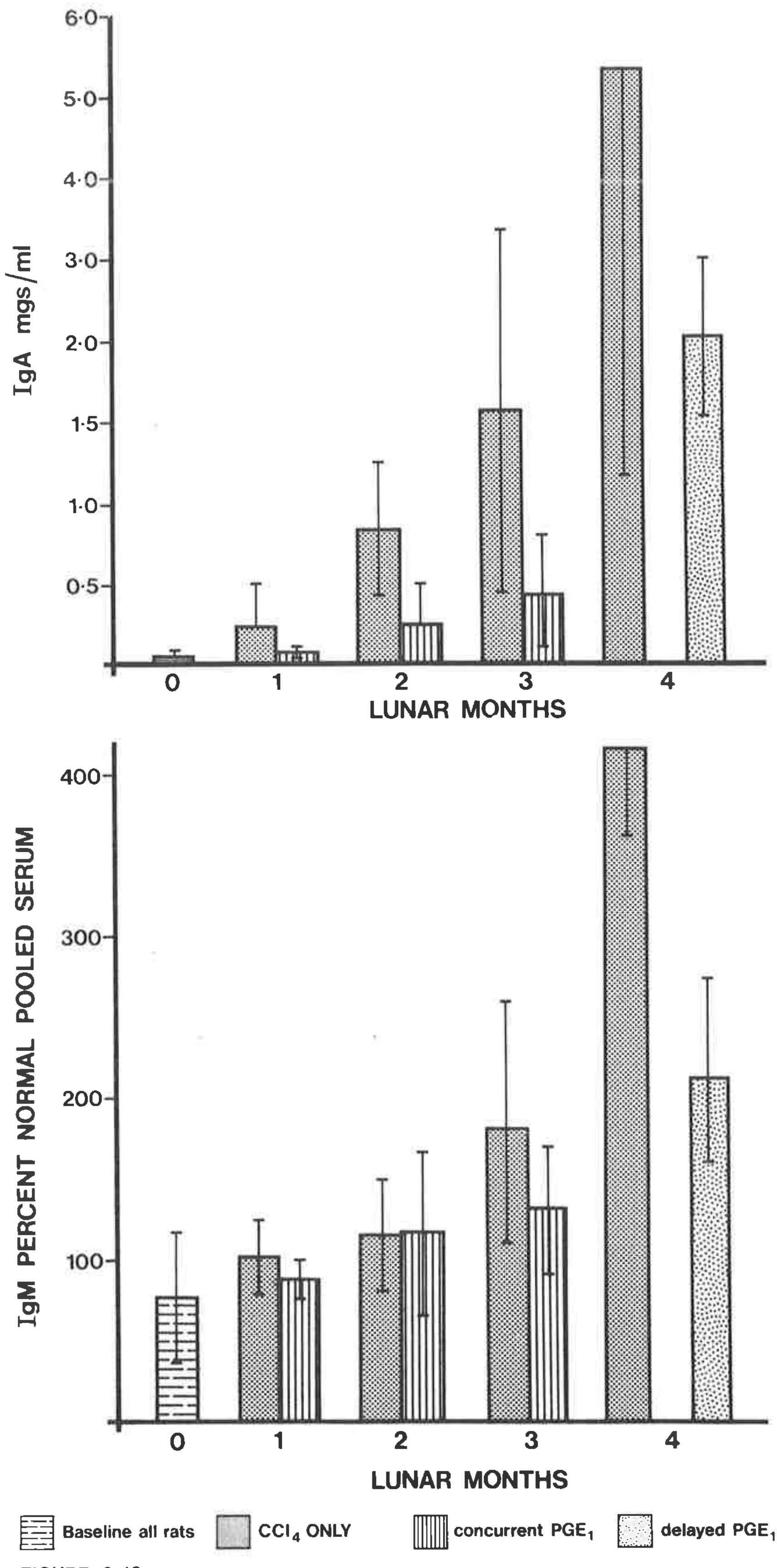
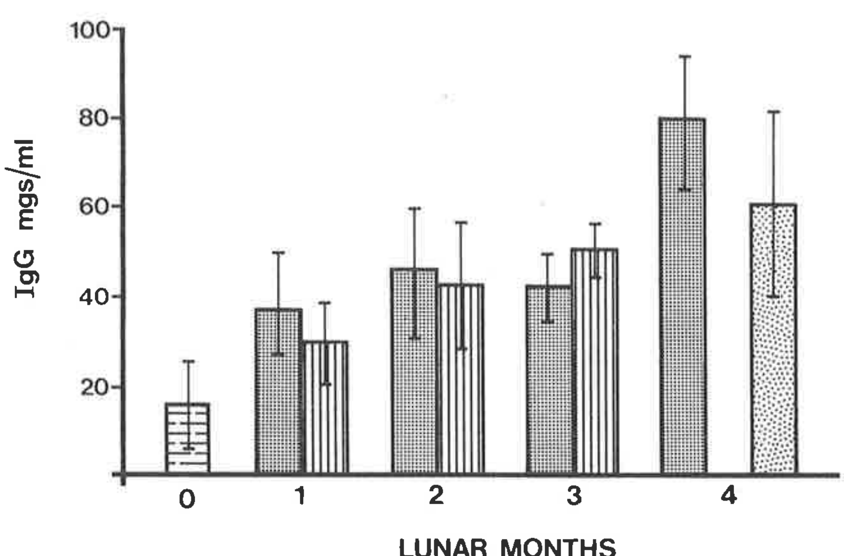
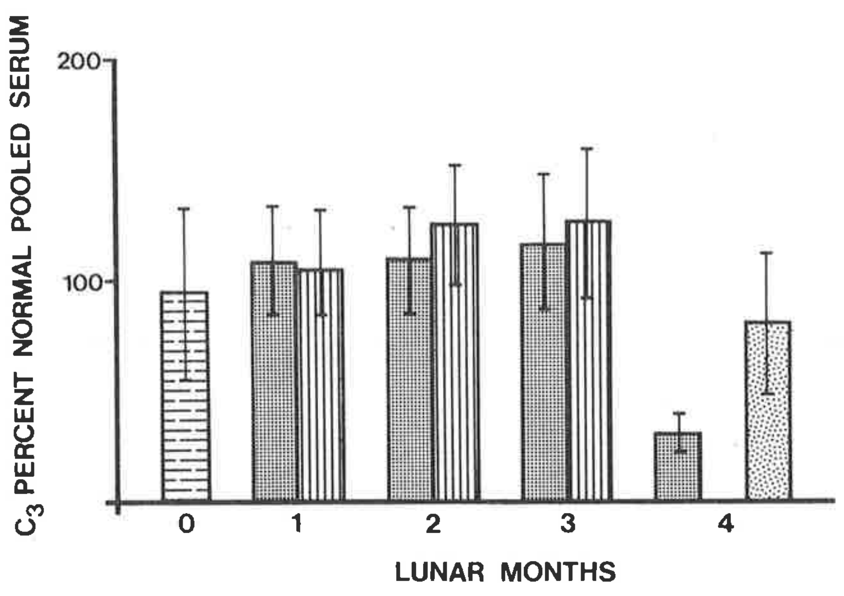


FIGURE: 6·13

Serum IgA and IgM levels in rats given biweekly CCl_4 alone compared with those treated with concurrent PGE_1 or with PGE_1 starting after three months. Each bar represents the mean of the group with one standard deviation.





Baseline all rats CCl_4 ONLY concurrent PGE_1 delayed PGE_1

FIGURE: 6·14

Serum IgG and C_3 levels in rats given biweekly CCl_4 alone compared with those treated with concurrent PGE_1 or with PGE_1 starting after three months. Each bar represents the mean of the group with one standard deviations.

4.2 The results of a delayed trial of PGE_1 administration at 150 µg/day in the animal model of IgA deposition

Rats given biweekly sc CCl_4 were started on daily PGE_1 injections after three months of hepatotoxin which was then continued. The six rats given CCl_4-only became increasingly lethargic and developed spiky fur. One of these died in week 14, one in week 16 and the last four in the 17th week. At autopsy all of these CCl_4-only rats had ascites and jaundice. All of the six rats given delayed PGE_1 were alive at this point. One rat treated with delayed PGE_1 died in the 18th week and in view of the loss of the entire positive control group, the remaining five rats treated with PGE_1 were killed. Thus, five of the six PGE_1 treated rats survived to the 18th week, while all of the untreated CCl_4-only rats died. All of the rats given delayed PGE_1 with a continuation of CCl_4 had cirrhosis.

Renal immunofluorescence

IIF performed upon kidneys from all rats in this extended study showed mesangial IgA deposition together with IgG, IgM and C_3 (Table 6.20). Thus, PGE_1 increased animal survival in cirrhotic rats, but after six weeks of administration, did not alter mesangial Ig deposition.

Serology

The serum Ig and C_3 levels of these treated rats at four months were similar to the CCl_4-only rats at three months (Figure 6.13-6.14) but in view of the low numbers of surviving CCl_4-only rats, the results were not significant.

Table 6.20 The intensity of renal IIF (graded 0-4) in rats treated with biweekly sc CCl_4 and delayed PGE_1 at 150 μg/rat/day

(Experiment IV)

Rat number	Intensity of renal IIF			
	IgA	IgG	IgM	C3
33 †	3	2	3	2
37	2	2	2	2
42	2	2	2	1
44	3	1	3	1
47	3	2	2	1
48	3	2	3	2

† = rat died before the end of the experiment

DISCUSSION

The animal model was manipulated by administration of drugs known to affect the parameters thought to be involved in mesangial IgA deposition in patients with ALD. Only PGE_1 had any effect upon mesangial IgA deposition in the model. Neomycin and phenytoin tended to reduce serum IgA levels but a reduction was only significant in rats treated with PGE_1 and d-penicillamine. Dapsone was found to ameliorate the serum reduction in C_3 levels found in cirrhotic rats.

During the course of the four experiments, all rats given biweekly CCl_4 were rendered cirrhotic except those treated concurrently with PGE_1. Two of the 26 control rats and seven of the 30 rats given CCl_4-only died. The greatest mortality in the three month studies was amongst those rats given daily gavage. Of the 80 rats gavaged daily, 21 died before the end of these experiments, from presumed anaesthetic related causes. Apart from those rats receiving PGE_1, all rats treated with biweekly CCl_4 showed mesangial IgA deposition with, to a lesser extent, IgG, IgM and C_3. These findings re-affirm the consistency of the model in producing cirrhosis and mesangial deposits. As previously demonstrated, mesangial IgM was a common finding in control rats. In the model, mesangial IgA, IgG and C_3 deposits were not associated with renal impairment. Extended studies demonstrated that the original study period had been optimal in this cirrhotic model, since after 12 weeks, CCL_4 treated rats rapidly began to decompensate and die. Cirrhotic rats after three months of sc CCl_4 exhibited a mean 21-fold

rise in serum IgA and a 2.3-fold rise in IgM levels when compared with control rats given diluents only. IgG was not significantly changed and C_3 levels tended to fall by up to 30%, depending on whether the rats were gavaged or given daily injections of ethanol diluent.

Dapsone was the only drug which affected serum C_3 levels, preventing the usual fall recognised in the larger groups of rats used in Experiment IV. Complement levels in primary IgA nephropathy are normal but in severe chronic liver disease they may be depressed, probably due to impaired liver synthesis (Finlayson et al 1972). Increased catabolism of C_3, however, has been implicated in primary biliary cirrhosis (Potter et al 1976). In addition, Van der Meer et al (1979) have shown that dapsone removes C_3 from the skin of patients with biopsy-proven DH. Serum levels were not measured in that study and the mechanism for this effect is unclear. In the cirrhotic rat model of mesangial IgA deposition, complement deposits were not affected by dapsone therapy although there was apparently a significant effect upon complement metabolism.

Dapsone has been used with good results in DH and rheumatoid arthritis (McConkey et al 1976). Notwithstanding its failure to prevent mesangial deposits in this model, its known effect upon complement deposits in DH, together with the effect on circulating complement levels demonstrated here, suggest that it may be a useful adjunct to therapy in other forms of GN.

There was a tendency to lower the serum levels of IgA in rats

treated with neomycin and phenytoin, but these levels were reduced significantly, only in rats treated with PGE_1 or d-penicillamine. The failure of phenytoin and neomycin to affect a significant reduction was partly because of the very wide range of serum IgA levels in cirrhotic rats but also because of the dramatic rise in serum IgA seen in this model. There was, however, a significant fall in IgG levels in rats given neomycin. Keraan et al (1974) stated that neomycin prevented the rise in serum Ig in portacaval shunted rats, but they assayed only total gamma globulin levels. Even in cirrhotic rats, the major class of Ig in the serum is IgG, with an IgG:IgA ratio of up to 50:1. Although serum IgA would theoretically be expected to have risen in the untreated portacaval shunted rats, the major class depressed in the study of Keraan et al (1974) must have been IgG. Phenytoin significantly reduces the serum level of IgA in both epileptic and normal patients without reducing IgG, IgM or complement levels (Bardana et al 1983). In the animal model, there was a similar tendency to lower serum IgA levels in cirrhotic rats but this reduction was not significant ($p=0.14$), probably because of the magnitude of the rise. Other Ig were not affected.

The effect of d-penicillamine was interesting because there was no reduction in mesangial IgA deposition, despite producing a significant and profound fall in serum IgA levels. In fact, the deposits of IgA, IgG and C_3 were more intense and widely distributed in d-penicillamine treated rats. The deposits extended around capillary loops in a granular pattern resembling the appearance of MCGN (type I) in humans. In humans, d-penicill-

amine is known to cause MGN (Spargo et al 1980) and even on occasions a Goodpasture's-like syndrome (Sternlieb et al 1975). The pattern of capillary wall fluorescence seen in the d-penicillamine treated cirrhotic rats, however, was not seen in the control rats given d-penicillamine only.

Of the drugs tested, only PGE_1 prevented mesangial IgA and the associated IgG and C_3 deposition. However, at the first dose investigated, there was an associated morbidity and high mortality rate (60%). Kunkel et al (1981) described diarrhoea and somnolence in rats given 200 μg daily of the stable analogue but did not report a high mortality. None of the surviving rats treated with PGE_1 at the higher dose had mesangial IgA deposits and only one had cirrhosis. This protection of an organ, such as the liver, from a toxic agent is termed cytoprotection (Stachura et al 1981). To overcome apparent cytoprotection and reduce morbidity, PGE_1 was administered at a lower dosage, both concurrently with CCl_4 and starting after three months of hepatotoxin, when cirrhosis and mesangial deposition should have developed. The lower dose (150 μg/day) was less toxic, having a 20% mortality rate, but was still associated with hepatic cytoprotection and a reduced incidence of mesangial IgA deposition. However, mesangial IgA deposition was seen in the absence of cirrhosis and *vice versa* so that, to some extent, mesangial deposition had been dissociated from hepatic cytoprotection. Delayed administration of PGE_1 did not affect the mesangial IgA deposits, although it did prolong survival. None of the other drugs had any effect upon the development of mesangial IgA deposits. The results of the trial of d-penicillamine

treatment show that depression of serum IgA levels alone was not associated with prevention of mesangial IgA deposition. This suggested that PGE_1, which also reduced serum IgA levels, may have prevented mesangial IgA deposition in another way.

The other working postulates in this study have been that increased gastro-intestinal absorption, portacaval shunting and depressed reticulo-endothelial (RE) function are related to mesangial IgA deposition. There is no evidence that systemic hepatotoxins or sc ethanol could affect gastro-intestinal absorption, although PGE_1 might be effective in such circumstances by protecting the gastric mucosa from ulceration (Gibinski et al 1977). However, it would appear more likely that PGE_1 prevented or ameliorated mesangial deposits by other mechanisms such as: its cytoprotective effect upon the liver, thus preventing cirrhosis and portacaval shunting; by promoting RE clearance; or by affecting the haemodynamics of the kidney.

The effect of PGE_1 on RE function is controversial but under some circumstances PGE_2 may enhance macrophage functions *in vitro* (Bonta and Parnham 1982). RE function may be tested *in vivo* by assessing the clearance dynamics of radiolabelled Ig aggregates. Keane and Raij (1981) using IgG aggregates could not demonstrate any effect of large doses of PGE_1 upon splenic or liver clearance in the rat. Similar results were gained in our laboratory in the mouse using physiological doses of PGE_1 (A Cornelius, personal communication) and IgA aggregates (after Egido et al 1982c).

It has been suggested that some protection of the kidney by PGE_1 may be mediated by a reduction in blood flow (Kelley et al 1979). However, Keane and Raij (1981) studying their aggregated-IgG model of IC GN in the rat, could show no effect upon kidney uptake, suggesting that there was no alteration in renal blood flow.

Although PGE_1 did not produce an alteration in specific antibody titres in the NZB/W model (Kelley et al 1979), it has been suggested that in the apoferritin model it may bring about a reduction in renal IC deposition by a reduction in antibody production (McLeish et al 1983). PGE_1 seems in fact to have different modes of action in different circumstances. In the nephrotoxic nephritis model, Kunkel et al (1982) showed that - without reducing antibody binding - PGE_1 was capable of preventing proteinuria and hypercellularity. In that instance, PGE_1 may act by reducing vascular permeability or neutrophil chemotaxis.

The remaining possible mechanism for the effect of PGE_1 upon mesangial IgA deposition in the cirrhotic animal model, is hepatic cytoprotection. Only one of four surviving rats treated with CCl_4 and PGE_1 at 225 μg/day showed cirrhosis. None had mesangial IgA deposition. Three of 11 surviving rats treated with 150 μg/day had cirrhosis. Only one of these cirrhotic rats and two of the non-cirrhotic rats had mesangial deposits. Although PG is known to protect the gastric mucosa of rats and humans (Gibinski et al 1977), it was not until after the start of the presently reported work that its protective effect upon

the liver was demonstrated.

Stachura et al (1981) and Ruwart et al (1982) have shown biochemical and histological evidence that PGE_2 prevents CCl_4 induced acute hepatic necrosis. This is, however, the first extended study of the effect of PG upon a CCl_4 induced model of cirrhosis. It was hoped that, as in the study of Ruwart et al (1982), the dose of PGE_1 could be reduced to a level where there was no hepatic protection. In that model using massive doses of CCl_4, there was acute renal failure which could be alleviated by doses of PGE_2 too small to affect hepatic function.

In this study only four cirrhotic rats could be produced after two trials of PGE_1, administered concurrently with biweekly CCl_4, and only one of these animals showed mesangial IgA deposition. All rats given CCl_4 alone had cirrhosis and all of these had mesangial IgA deposits. Furthermore, in Chapter 5 rats were shown after two months of sc CCl_4 to have mesangial IgA, IgG and C_3 deposition despite showing only bridging fibrosis. Conversely, two of 11 PGE_1 treated rats showed mesangial IgA deposits despite the absence of cirrhosis. It may not be possible to completely dissociate the renal effects of PGE_1 from the hepatic changes in this cirrhotic model.

It seems clear that in this model of mesangial IgA deposition, as in the human (Clarkson et al 1980), reduction of circulating IgA levels is not a useful therapeutic manoeuvre. Understanding of the mechanism of action of PGE_1 in this animal

model, however, has been complicated by dramatic hepatic cyto-protection. Despite this reservation, only 25% of cirrhotic rats treated with concurrent PGE_1 showed mesangial deposits, compared with 100% of cirrhotic rats which were untreated or treated with other therapeutic agents.

Further studies of PGE_1 treatment are warranted, in the cirrhotic model at lower doses, in an attempt to reduce the cyto-protective effects of the drug, and, in other models of IgA nephropathy not dependent upon hepatotoxicity. Studies of the effect of PGE_1 upon RE function and renal blood flow might elucidate the role played by these factors in PGE_1 therapy of IC GN.

PGE_1 has exciting therapeutic implications in all forms of human GN. Preliminary trials in humans offer promising results. Infusion of 200 μg PGE_1 daily, that is around 3 μg/kg/day, in patients with chronic renal failure, resulted in a statistical improvement in creatinine clearance, which persisted for up to seven months after cessation of therapy (Niwa et al 1982); these patients had chronic GN of heterogeneous types. The same effect has been seen in a patient with acute crescentic PIGN whose improvement continued after cessation of therapy (Niwa et al 1983). There may also be a place for PGE_1 in hepatology, perhaps initially in the treatment of acute toxicity.

CHAPTER 7

The aetiopathogenesis of mesangial IgA deposition in alcoholic liver disease: a discussion

DISCUSSION

The data presented in the preceding chapters have defined the incidence of mesangial IgA deposits in an autopsy series of patients with ALD; characterised the IgA deposits; and, in elution studies, identified some of their specificities. In addition, an animal model of mesangial IgA deposition secondary to CCl_4-induced cirrhosis was developed and characterised before being manipulated in a drug trial.

The results of each part of this study have briefly been discussed at the end of each chapter. Here, the results of the study as a whole are discussed with particular reference to the aetiology of mesangial IgA deposition secondary to alcohol ingestion, and the therapeutic implications of the drug trial.

The association between IgA deposition and ALD

The autopsy series presented here showed that 50% of patients with alcoholic cirrhosis had mesangial IgA deposits. This was significantly higher than the 4% incidence found in the control population studied here and subsequently confirmed by Sinniah et al (1983). Although the association between chronic liver disease and mesangial IgA deposition is well documented (Callard et al 1975; Berger et al 1978), few studies have been performed outside France (Nakamoto et al 1981) and only one study has been restricted to those patients with ALD (Nochy et al 1976).

Mesangial IgA deposition was found by Berger et al (1978) in

61% of 100 patients with cirrhosis, of whom 90 were alcoholic, and by Nakamoto et al (1981) in 53% of 43 patients with cirrhosis of various aetiologies. Although the patients in the studies of Berger et al (1978) and Nakamoto et al (1981) were heterogeneous, the liver disease was predominantly of alcoholic origin and both groups showed a similar incidence of mesangial IgA deposition to that found in this study.

Only Nochy et al (1976) restricted their study to patients with ALD and further selected those with overt GN. All had proteinuria, and just over half had elevated serum creatinine levels. They found mesangial IgA deposition in 86% of 22 patients with cirrhosis and 58% of 12 with non-cirrhotic ALD. These figures are higher than the 50% and 33% recorded respectively in this thesis, where patients were not selected on the basis of renal function.

Callard et al (1975) studied the kidneys of patients with cirrhosis undergoing portacaval shunting procedures, and found mesangial IgA deposition in nine of 10 cases. Amongst these patients, eight had alcoholic cirrhosis but none had proteinuria, elevated serum creatinine or hypertension, and only one had haematuria. The study of Callard et al (1975) in patients fit enough to undergo portacaval shunting illustrates the occult nature of mesangial IgA deposits in some patients with ALD. This is similar to the apparently subclinical forms of primary IgA nephropathy.

In Chapter 2, renal failure and abnormal urinalysis were found

frequently in decompensating patients dying in hospital with ALD. These features, however, did not correlate significantly with the presence of mesangial IgA deposits. In this autopsy study, overt GN was recognised during life in three of 20 patients with ALD and mesangial IgA deposition. The renal condition was rarely of clinical significance amongst the plethora of other medical problems and reduced life expectancy of such patients. The animal model was similar in this respect to the patients with alcoholic cirrhosis, in that mesangial IgA deposition did not produce biochemically-evident renal insufficiency.

Admixed in the series of Nakamoto, Berger and Callard there were a small number of patients with cirrhosis of non-alcoholic aetiology. In this study, a group of six patients with non-alcohol-induced serious liver diseases were examined separately and found to show a much lower incidence of mesangial IgA deposition (17%) than patients with alcoholic cirrhosis. Although this series is small, the data suggest that mesangial IgA deposition is related specifically to alcohol abuse rather than liver damage.

One feature of ALD previously recorded (Nochy et al 1973), but not widely appreciated, was the increased incidence of mesangial IgA deposition in the absence of cirrhosis (33%). This finding has important implications for an understanding of the pathogenesis of mesangial IgA deposition secondary to alcoholic cirrhosis. Significant portacaval shunting does not occur in the absence of cirrhosis (Israel et al 1982) so that porta-

systemic loading of antigens, antibodies or complexes cannot be a major factor in such patients. By implication, porta-systemic loading may be the final insult rather than the major pathogenetic mechanism for the mesangial deposits in alcoholic cirrhosis.

The other possible mechanisms advanced earlier to explain mesangial IgA deposits in ALD were: a failure in RE and particularly Kupffer cell clearance; an increase in gastro-intestinal absorption; and the deposition of preformed IC. These mechanisms are interdependent but will be discussed separately.

The failure of phagocytic function in alcohol abuse

Kupffer cell clearance may be rendered ineffective by shunting, but may also be affected by alcohol itself. Alcohol, in fact, affects the function of both neutrophils and macrophages, accounting in alcoholics for their long recognised predisposition to infections (Ratnoff and Patek 1942). This susceptibility, demonstrable by elevated enteric-bacterial coat antigens, is greater in patients with alcoholic cirrhosis than in patients with other serious liver diseases (Turunen et al 1981). To further illustrate this point, in Chapter 2, it was shown that nine of 41 patients with ALD died from septicaemia. Holdstock et al (1982b) have shown defects in circulating monocyte function in alcoholic cirrhosis, with impaired spreading, chemotaxis, bacterial phagocytosis and killing. These defects in patients' cells could be corrected by incubation with normal peripheral serum and, conversely, could be induced in normal

cells by using portal serum obtained at laparotomy from patients with no evidence of liver disease. A single dose of alcohol in humans can be shown to reduce neutrophil migration (Brayton et al 1970) and promote serum inhibitory factors causing both reduced bactericidal activity of normal serum and reduced phagocytosis by normal neutrophils (Johnson et al 1969). Kupffer cell clearance in laboratory animals can be monitored by the use of endotoxin or whole bacteria. The effect of chronic alcohol ingestion on endotoxin clearance has been shown in several ways. Nolan et al (1980) fed rats 25% ethanol in drinking water and found that after three weeks the serum levels of endotoxin following an intraperitoneal challenge were more than twice as high in ethanol treated rats than in controls. Similarly, Di Luzio and Williams (1980) gave rats a fluid diet of 35% ethanol and found after 30 days that an intravenous S aureus challenge killed 90% in three days compared with 10% of controls.

Radiolabelled, heat-Agg HSA can be used to measure phagocyte function and, because of the relatively large blood flow to the liver, the results are largely a reflection of Kupffer cell activity (Benacerraf et al 1957). Ali and Nolan (1967) used this technique in a rat model of acute ethanol ingestion and found that oral administration of 0.5 ml of 100% ethanol resulted in a 40% prolongation of the time taken to clear half of an intravenously injected dose. Liu (1979) tested half-time clearance of radiolabelled Agg-HSA in alcoholic patients admitted acutely without overt ALD. By injecting both a significant load and a tracer dose of labelled protein, they demon-

strated significantly prolonged half-time clearance for the large dose but a normal half-time clearance for the tracer dose, implying a normal tissue perfusion rate. The same tests were repeated seven days later and showed a reversion to normal, so that depression of Kupffer cell function in this group of patients was temporary and unrelated to blood flow. It is, therefore, possible to invoke RE failure in the alcohol abuser as an explanation for mesangial IgA deposition in the absence of portacaval shunting.

There is now evidence that elevated levels of serum IgA in humans may be responsible in some degree for susceptibility to infection and even the development of CIC. Van Epps et al (1976) demonstrated that IgA myeloma serum, particularly of polymeric-type, inhibits polymorphonuclear leucocyte chemotaxis. This ability was significantly reduced by reduction-alkylation which converts polymers to monomers and by pepsin digestion, suggesting that it required an intact Fc portion.

Further studies by the same group suggested a concentration-dependent suppression of neutrophil bactericidal capacity by IgA polymers (van Epps et al 1978). Thus, it seems possible that such phagocytic failure, already illustrated in patients with primary IgA nephropathy (Egido et al 1982b; Sato et al 1983), may play a part in the persistence of CIC in patients with high serum levels of polymeric IgA.

There seem, therefore, to be several mechanisms by which alcohol abusers may acquire a defect in both the polymorphonuclear and

monocytic phagocytic systems without the necessity for porta-caval shunting.

Altered gastro-intestinal permeability in alcohol abuse

One functional abnormality which may be specific to the liver disease of alcohol abusers is an increase in mucosal permeability (Worthington et al 1978). This would partly explain the observed increase in anti-BSA and E coli titres seen in patients with ALD (Woodroffe et al 1980) and would be in keeping with the finding of CIC (Zone et al 1982) and mesangial deposits in conditions such as DH and coeliac disease (Pape et al 1978; Katz et al 1979).

The studies of eluted antibody in Chapter 4 implicated gastro-intestinal absorption of antigens in the pathogenesis of mesangial IgA deposits in ALD, by the demonstration in some eluates of anti-BSA activity in an RIA and activity against gut flora in an ELISA system. One of the eluates with these reactivities was from a patient without cirrhosis. The mechanism of such macromolecular uptake has recently been illustrated ultrastructurally in the rat where duodenal alcohol infusion weakened intercellular enterocyte adherence and allowed horse-radish peroxidase to enter the intercellular spaces (Draper et al 1983).

Deposition of CIC in ALD

The other important proposed aetiology for secondary mesangial IgA deposition in ALD is the deposition of CIC. Dixon et al (1961) suggested that antibody-antigen complexes form in the

circulation of rabbits with serum sickness and later become deposited in the tissues where they induce inflammation. The deposition of CIC in human GN, although controversial, is suggested by: their presence in the serum; the deposition of Ig and complement at extrarenal vascular sites; and morphological appearances in humans similar to those recognised in animal models (reviewed Cameron 1982a). The formation of CIC would be favoured by the increase in gastro-intestinal absorption and failure in RE clearance discussed above.

CIC in serum

The presence of CIC in the circulation of patients with ALD is well documented (Penner et al 1978; Gluud and Jans 1982; Kaufman et al 1982). Here, as in other studies, CIC were shown to be common in patients with ALD, especially those with mesangial IgA deposition.

Extrarenal deposits of Ig

Extrarenal deposits are said to be characteristic of GN of an IC origin. The extrarenal clinical manifestations, such as arthritis, neuropathy and cutaneous vasculitis associated with diffuse IC deposition, are not seen in ALD (Kaufman et al 1982). This, however, does not exclude their deposition since they may be non-phlogogenic (Cameron 1982a). The autopsy study provided the opportunity to examine skin and choroid plexus specimens by IF. Deposition in such sites was not significantly more common in patients with ALD than in controls, and did not correlate well with mesangial IgA staining. Thus, there is no supportive evidence in this study for a correlation between

renal and extrahepatic capillary bed IgA deposits. Hepatic IgA appeared to be more common in patients with ALD than controls but did not show a positive correlation with mesangial IgA deposits. Other workers have examined simultaneous skin and liver biopsies and shown a positive correlation between superficial dermal capillary IgA staining and ALD with continuous hepatic IgA staining (Swerdlow et al 1982b). Dermal IgA deposition in the study reported here, as shown previously, does not identify those patients with mesangial deposits (Thompson et al 1980; Moatamed et al 1981) nor does it identify those patients with ALD. It would appear that CIC and hepatic IgA deposits are common in ALD but that only a proportion of patients develop mesangial deposition.

Immunochemical characterisation and origin of mesangial deposits

The studies designed to characterise the mesangial IgA deposits were more conclusive. They indicated a possible mucosal origin for those found in ALD, in contrast to those in other forms of GN with mesangial IgA deposition.

Because of the relatively low concentration of IgA in serum compared with secretions, workers have often postulated a mucosal origin for the mesangial deposits in IgA nephropathy. However, studies of gastro-intestinal IgA-producing plasma cells have not shown a quantitative difference between patients with IgA nephropathy and controls (Westberg et al 1983). One marker of mucosal origin may be the presence of SC. Even if SC were found, however, its significance would be uncertain since a recent study suggests that circulating SC-Ig complexes

may form by intravascular binding of polymers with the small quantities of free SC released from mucosal sites (Delacroix and Vaerman 1982). Notwithstanding this observation, SC was not found in mesangial IgA deposits by Dobrin et al (1975) or in Chapter 3. Although IgA of mucosal origin comprises 95% J-chain containing polymers and 32% A_2 subclass, serum IgA is 13% polymeric and 21% IgA_2 subclass, so that these are not exclusive markers (Delacroix et al 1982d). The specificity of J chain is further reduced by the fact that it is an integral part of IgM which is often present in association with mesangial IgA deposits (Spargo et al 1980).

The controversy surrounding the contradictory mesangial subclass studies, whereby some workers fail to find any IgA_2 subclass (Conley et al 1980b) whilst others find it consistently (André et al 1980), has partially been resolved by the use of more consistent reagents. The intermediate results reported in Chapter 3, with the finding of A_2 subclass in the mesangial deposits of some patients in all the disease categories examined, is to be expected in the light of the results of the study by Delacroix et al (1982d). They showed the ubiquity of A_2 subclass which formed a minor but consistent percentage of all sera ($\simeq$ 19%) and secretions (< 41%) sampled. Statistically, serum SC, pIgA and IgA_2 are unlinked parameters and it would appear that A_2 subclass is not a highly specific marker of mucosal origin (Delacroix et al 1983a). More discrimination is afforded by the identification of polymers which comprise 95% of salivary, intestinal, lacrimal and milk IgA, and 65% of biliary IgA compared with only 13% of serum IgA (Delacroix et

al 1982d). Elution studies have demonstrated presumed IgA polymers in the deposits of primary IgA nephropathy by ultra-centrifugation (Sancho et al 1981; Tomino et al 1982). This system cannot differentiate between aggregated monomers and polymers but the capacity of the deposits to bind SC will identify polymers (Brandtzaeg 1976). SC binding has been demonstrated in the deposits of primary IgA nephropathy (Egido et al 1980; Bené et al 1982) suggesting that they are polymers, and perhaps of mucosal origin.

These SC binding studies would be consistent with the finding of raised serum polymers in such patients (Egido et al 1980). There is, however, controversy about the presence of raised levels of serum polymers in patients with primary IgA nephropathy and some workers have failed to demonstrate this elevation by ultracentrifugation (Lesavre et al 1982; Woodroffe et al 1980; Delacroix et al 1983a). As in eluates and frozen sections, the most sensitive method for assaying polymers in serum is by SC binding capacity. Newkirk et al (1983) have developed a radiolabelled SC-IgA binding assay and showed patients with primary IgA nephropathy to have an absolute rise in pIgA when there was an overall rise in IgA but a selective increase, if present at all, only in monomers. The workers in Madrid found high circulating levels of pIgA in patients with primary IgA nephropathy, HSP and ALD (Lopez Trascasa et al 1980; Egido et al 1980; Sancho et al 1981) and postulated a common immuno-aetiopathogenesis for these diseases. However, the normal control values of pIgA recorded by Lopez Trascasa et al (1980) were almost double those found by Newkirk et al

(1983) using the SC binding assay. The molecular size of IgA affects its assay by various techniques (Delacroix et al 1982c). It seems likely that assays utilising sucrose density gradient centrifugation and RIA may overestimate polymers and that an SC binding assay would be more accurate (Newkirk et al 1983).

If mesangial deposits merely reflected high levels of circulating IgA polymers, one might expect more patients with IgA myelomas to present with secondary IgA nephropathy. Dosa et al (1980) reported such a case but did not record ultracentrifugation so that the deposits may not have been predominantly polymeric.

The data presented in Chapter 3 show a difference in the functional characteristics of the deposits in ALD, compared with other diseases characterised by mesangial IgA deposition. Only the deposits in patients with ALD (13 of 16) and SLE (three of four) showed the capacity to bind free SC compared with one of 10 with primary IgA nephropathy and none of six patients with HSP. The similarity between SLE and ALD in this respect is not easily explained. Circulating polymers are not consistently raised in SLE (Delacroix et al 1983a) and, furthermore, although not a linked parameter, the percentage of A_2 subclass is, if anything, decreased (Delacroix et al 1983a; Conley and Koopman 1983). Previous studies have found SC binding in the majority of cases of primary IgA nephropathy (Egido et al 1980; Bené et al 1982) as well as patients with ALD (Sancho et al 1981). These different results could be explained by: a lack of sensitivity in the

binding test used here; differences in the selection procedure of patients; or a geographical difference between populations. The latter postulate seems inadequate to explain such marked differences. The criteria used for selection of the patients with primary IgA nephropathy in this study were rigid, excluding any patient with evidence of excessive alcohol intake, not just those with overt ALD. SC binding studies are less dependent upon antibody specificity than subclass studies and, unlike ultracentrifugation, do not rely upon size with the inevitable overlap between IC, aggregates and polymers. It is difficult to assess the comparative sensitivity of SC binding tests used in other work so that the dramatic differences are not easily explained.

The data in this study would be consistent with the presence of IgA polymers in the mesangial deposits of patients with ALD and SLE only. Further, they are consistent with the presence of monomers in HSP and primary IgA nephropathy. Both monomers and polymers may be present as aggregates or antigen-antibody complexes. Again, it might well be argued that these differences, like those of the subclass studies, may be quantitative rather than qualitative. However, the sensitive assays of Newkirk et al (1983) support a serological difference between patients with primary IgA nephropathy and ALD. They showed in a radiolabelled SC-IgA binding assay that there was a selective elevation in pIgA in patients with ALD but not in those with primary IgA nephropathy.

Recent studies by Delacroix et al (1983a) show that the elevated

serum IgA levels of patients with IgA nephropathy predominantly comprise monomers with an increased proportion of A_1 subclass whilst, in common with the serum of patients with Crohn's disease, that of patients with alcoholic cirrhosis had a higher proportion of polymers of A_2 subclass. The increase in A_2 related to the total IgA was significantly greater in alcoholic cirrhosis than in other forms of chronic liver disease. These data from Delacroix et al (1983a) are important since, together with those of Newkirk et al (1983), they show qualitative differences between the sera of patients with primary and secondary IgA nephropathy and add support to the findings present here.

The polymeric nature of mesangial IgA deposits in ALD, suggested by the SC binding studies reported here, would be in keeping with some of the "enteric" specificities observed in Chapter 4. Activity towards gut-derived protein would be expected to be associated with mucosal-type polymeric IgA. Some of the eluates showed multiple activities towards food and bacterial antigens. This is not unexpected since, in ALD, a basic immunological defect might occur, producing a polyclonal abnormality rather than the specific antigenic stimulus found in such conditions as the GN of infective endocarditis.

Auto-antibodies and the heterogeneous antigenic specificity of mesangial deposits in ALD

The presence of a basic immunological defect in patients with ALD is supported by the presence of auto-antibodies as well as antibodies to gut flora and food proteins in the glomerular eluates. In Chapter 4, neither anti-HSA nor anti-mesangial

antibody activity could be demonstrated in the eluates but anti-MB activity was frequently seen. Previously, anti-HSA (Hauptman and Tomasi 1974b) and anti-MB (Kanagasundaram et al 1977) antibodies have been recognised in the sera of patients with ALD. Kanagasundaram et al (1977) also described circulating MB. Kaufman et al (1982) found that patients with ALD tended to have multiple auto-antibodies but that these were not concentrated within cryoprecipitates, implying that they were not instrumental in CIC formation. This may be also true of anti-HSA activity which could not be demonstrated in eluates using the test systems employed.

The only previous report of anti-mesangial antibody activity was by Lowance et al (1973) who eluted antibody from four renal samples from patients with IgA nephropathy and showed one to have weak but specific mesangial binding of IgA class. Other studies of eluted IgA have focused upon unspecified but common antigenic specificity in mesangial deposits of IgA, demonstrated by the ability of eluates to re-combine with _in situ_ fixed antigen. Tomino et al (1981b, 1983a) eluted IgA and demonstrated that it re-combined most effectively with autologous acid-treated kidney sections from patients with HSP (100%) or primary IgA nephropathy (83%). In that study, re-combination of eluates from HSP patients was not seen using sections from other forms of GN (four) or normal control kidneys (four). The only cross-reaction was between HSP eluates with primary IgA nephropathy sections (67%) and _vice versa_ (50%).

It would be interesting to try re-combination of IgA acid

eluates from patients with ALD and heterologous sections from patients with ALD with and without mesangial deposits. Alcohol may affect mesangial antigenicity in the same way that MacSween et al (1981) have suggested that it affects that of hepatocytes.

Some of the most interesting data in this study were the finding of anti-MB activity in the eluates of patients with ALD. Zinneman (1975) studied MB auto-immunity in patients with alcoholic cirrhosis. He demonstrated the ability of IgA purified from patients with alcoholic cirrhosis, to bind to MB in frozen sections, and also showed that prior to such incubation that was native IgA bound to MB. He could not show such binding by myeloma or normal IgA suggesting specific antibody to MB within the serum of patients with ALD. Subsequently Kanagasundaram and Leevy (1976) eluted IgG and IgA from livers of patients with alcoholic hepatitis. They demonstrated anti-MB activity in such eluates, and Kanagasundaram et al (1977) subsequently showed such patients to have circulating MB and anti-MB antibody. This latter work, although not confirmed (Kehl et al 1981), suggests that antibody activity to MB may play a part in liver damage and the formation of CIC.

The concept of MB-anti MB CIC in ALD is supported by the finding of similar constituents in mesangial deposits. Anti-MB activity was demonstrated in seven of nine mesangial eluates from patients with ALD in Chapter 4. Subsequently, Burns et al (1983) have shown MB antigen in such deposits using monoclonal antibody. These two findings suggest that MB partici-

pate in the formation of mesangial IgA deposits which may represent the deposition of CIC. The antibody demonstrated in Chapter 4 was of IgA class. It would be speculative to suggest that such MB antibodies would be of a particular size or subclass, but they may play a part in the progression of chronic liver disease.

The normal human liver contains few plasma cells. Kronborg et al (1982) have studied *in vitro* Ig production in homogenised liver biopsies by ^{3}H-leucine incorporation. They demonstrated low Ig production by hepatic plasma cells in normal livers. An IgA-class rise predominated in alcoholic hepatitis in contrast to the IgG elevation seen in chronic active hepatitis and viral hepatitis. The mechanism of such a class specific production of plasma cells in the liver is not clear. Nor is it apparent whether this hepatic-IgA production is important in the elevation in serum IgA levels found in such patients.

Several studies have examined liver staining by immunochemical techniques. These have shown sinusoidal IgA staining (Kater et al 1979) in a continuous and apparently specific pattern in patients with ALD (Swerdlow et al 1982a). These deposits, moreover, show a preponderance of A_2 subclass (Swerdlow and Chowdhury 1983). In the study reported in Chapter 2 using autopsy tissue, the finding of IgA staining was less specific for ALD. Chandy et al (1983) also examined autopsy livers and noted a "fainter and more diffuse pattern" of IgA staining than that described previously, and explained it as an effect of post-mortem autolysis. Chandy et al (1983) also described IgA

in the walls of bile canaliculi, probably analogous to the pattern described in Chapter 2 as spider-like. The spider-like, bile canalicular staining in this study, however, showed a predominance of IgG class. This could be explained on the basis of the non-SC dependent nature of Ig hepatocyte uptake in humans. In humans, SC has rarely been seen on hepatocytes (Hsu and Hsu 1980) and the consensus of opinion is that most SC-dependent pIgA transport into bile is through the bile ducts. This, perhaps, accounts for the less avid biliary clearance of injected pIgA and the smaller percentage of serum derived IgA within bile in humans compared with rats (Delacroix et al 1982b).

There are several facets of IgA metabolism in normal humans and alcoholics which await elucidation. It is, however, recognised that much IgA is produced at mucosal sites, from where it is secreted via an SC-dependent mechanism. If polymers, aggregates or IC gain entry to the circulation, they are cleared by the RE system, especially Kupffer cells, and that which is not directly catabolised is re-excreted via the biliary system.

The alcoholic patient, with or without cirrhosis, has a higher incidence of mesangial IgA deposition than normal subjects. Such patients frequently show CIC, which may localise in extrarenal sites, and frequently have hepatic staining for IgA. The mesangial deposits can be shown to possess a variety of specificities and represent both auto-immune antibodies

and immunity to absorbed antigens. Although subclass studies have been inconclusive, the SC binding studies reported here indicate that the deposits in ALD differ from those in primary IgA nephropathy in being largely polymeric.

The therapeutic implications of the drug trial

There are inherent dangers in extrapolating from animal studies to explain human data. There are important inter-species differences in biliary transport of IgA particularly between humans and rats (Delacroix et al 1983b). Firstly, circulating IgA in humans is mostly monomeric, whilst that of the rat is almost entirely dimeric. Secondly, it seems that most human biliary IgA is derived from local synthesis. This has been deduced from the strikingly slower biliary clearance of injected radiolabelled-pIgA in humans (< 1%) compared with rats (> 20%) (Dooley et al 1982) and the finding that only 50% of biliary pIgA in humans was serum derived (Delacroix et al 1982c).

With due recognition of these important differences, an animal model of mesangial IgA deposition produced by CCl_4-induced cirrhosis was used. The model shared with the human disease a reduction in liver bulk and portacaval shunting. It lacked the alcohol specific depression of RE function and a defective mucosal barrier.

The induction of cirrhosis

CCl_4 has been said to produce unpredictable liver damage

requiring phenobarbitone induction to elevate the success rate to some 75% (Proctor and Chatamra 1982). The method described by Sakaguchi et al (1964) used here, however, produced cirrhosis in all animals after three months. In addition, mesangial IgA deposition with, to a lesser extent IgG, IgM and C_3, were consistently found.

Repeated halothane exposure is known to produce hepatitis in humans and may be considered an additional complicating factor in this study. However, the damage in halothane hepatitis in humans is idiosyncratic, of a hypersensitivity-type and not thought to induce chronic liver damage (Maddrey et al 1980). Halothane anaesthesia in this study was not associated with histological evidence of liver damage or mesangial IgA deposition in the rats not treated with CCl_4.

The effects of ethanol

The rats given sc drugs were subjected to ethanol which might have effected an induction of hepatotoxicity or a decrease in macrophage function as described earlier.

However, the dramatically increased sensitivity of rats to S aureus challenge after a 35% ethanol diet for 30 days (Di Luzio et al 1980) and the failed clearance of endotoxin after three weeks of 25% ethanol in drinking water (Nolan et al 1980) represent the effects of massive pharmacological loads. The vehicle for d-penicillamine and PGE_1 administration in this study was 10% ethanol in PBS. The rats given diluents alone

or with CCl_4 therefore received 0.2 ml of 10% ethanol daily for three months, representing 20 μl of absolute alcohol/day, and a cumulative dose of 1680 μl. It is unclear whether such a low dose could induce impaired macrophage function, as data in this dose range do not exist. In this study, however, control rats given diluents only, or any of the drugs in their diluents, showed neither hepatotoxicity nor mesangial deposits.

The effect of neomycin

Kupffer cell dysfunction may allow gut-derived endotoxin to produce both hepatic damage and extrahepatic phenomena (Nolan 1975). Endotoxin is absorbed from the bowel of normal animals but is usually cleared by the liver (Ravin et al 1960). Notwithstanding the role of an initial failure in endotoxin clearance, it would seem that endotoxins themselves are hepatotoxic. Evidence for this has been the finding that neomycin, and to a lesser extent absorbable antibiotics, will delay the onset of choline-deficiency induced cirrhosis (Rutenburg et al 1957). The development of fibrosis is complex but may in part be related to circulating endotoxin levels which can be shown to be reduced by neomycin. Anti-bacterial antibodies have been described in patients with chronic liver disease (Bjørneboe et al 1972; Triger et al 1972) and attempts at gut sterilisation in the portacaval shunted rat have produced a reversal of the hyperglobulinaemia (Keraan et al 1974). A similar study performed by Gans (1980) demonstrated circulating endotoxin in the portacaval shunted rat which was reduced to unmeasurable levels by neomycin, without, however, producing a total return to normal in the Ig levels. Macrophages, under the influence

of endotoxin lipopolysaccharides, have been shown to produce fibroblast stimulating factors. It is not always possible to predict Kupffer cell function from macrophage studies but they may be responsive to similar factors, and it is tempting to speculate that neomycin protection in the choline-deficient model of cirrhosis is mediated by a reduction in fibroblast stimulating factors. In view of the postulated reduction by neomycin in endotoxins, which, as described earlier are believed to induce release of fibroblast stimulating factors by macrophages, it is interesting that liver fibrosis was not reduced by neomycin in this study.

The effect of PGE_1

Only PGE_1 reduced mesangial IgA deposition, whilst serum IgA levels could be altered by other pharmacological agents. Alone, a reduction in serum IgA levels did not alter the renal immunopathological features taken as the endpoint for study. This was similar to the findings in humans, where Clarkson et al (1977) found phenytoin to affect a reduction in serum IgA levels but produce no immunopathological effect upon the mesangial deposits.

PGE_1 at the first dose used was highly toxic. Of the four surviving rats, none had mesangial deposits but only one had cirrhosis. The other three showed hepatotoxicity and varying degrees of fibrosis with bridging. This effect of PGE_1 upon the liver is termed cytoprotection, which is defined as the prevention of cellular damage upon exposure to injurious agents (Stachura et al 1981). Cytoprotection by PG was first noted

in hollow organs. It is, perhaps, not surprising that PGE_1 can prevent gastric bleeding induced by PG inhibitors such as aspirin (Cohen 1978) or indomethacin (Johansson et al 1980). It is now well recognised that PG will inhibit gastric acid secretion and prevent experimental ulcer formation (Robert et al 1979). Clinical endoscopic trials of the effect of oral PG upon peptic ulceration have shown an increased rate of healing (Fung et al 1974; Gibinski et al 1977; Vantrappen et al 1982). In this situation, the mechanism of increased healing is not clearly understood but altered acid or mucus secretion may play a part.

Cytoprotection by PGE_2 in the liver has been shown histologically and by serum chemistry in models of acute galactosamine (Tarnawski et al 1981) and CCl_4 hepatotoxicity (Ruwart et al 1982). It appears that PG does not affect CCl_4 absorption (Stachura et al 1981) and that the mechanism for reduced hepatocellular necrosis is at a cellular level. The endoplasmic reticulum is thought to be the site of CCl_3 free radical formation (Reynolds 1977). PGE_1 may also act upon Kupffer cells which have been shown in culture to respond to PG by the release of collagenase (Bhatnager et al 1982). This effect was opposed by indomethacin or bacterial lipopolysaccharides and was mediated via cyclic AMP. When PGE_1 was used at a lower dose, morbidity was reduced but hepatic cytoprotection was not abolished. Only three of 11 surviving treated rats developed cirrhosis, and only one of these had mesangial IgA deposition. Two of the eight without cirrhosis had mesangial IgA deposition. One approach to this data would be to repeat the experiment with

decreasing doses of PGE_1 until hepatic cytoprotection was abolished, and then re-assess the mesangial IF. Such an approach might allow the renal fluorescence to be assessed in the absence of cirrhosis but would not allow study of the kidney without any manipulation of hepatic function by PGE_1. Reduction of mesangial staining may be induced under such circumstances by altered RE or hepatocyte function, or by local mesangial or vascular factors within the kidney.

A different approach was attempted by the delayed administration of PGE_1 after the induction of cirrhosis by three months of CCl_4. There is a precedent for believing that IgA deposits in the mesangium are capable of dissolution. Cases of inadvertent transplantation of donor kidneys with mesangial IgA deposition have been recorded (reviewed by Cameron 1982b) and in some instances the disappearance of such deposits has been documented (Silva et al 1982).

IC may be solubilised by complement, especially those components in the alternate pathway. C_3 is a common mesangial IF finding in GN and may play a role in IC metabolism. Interest was focused on its presence when it was found to be taken up by the smaller complexes produced by the solubilisation of larger aggregates by fresh serum (Miller and Nussenzweig 1975). This solubilisation function of complement may actually be impaired in patients with mesangial IgA deposition. Tomino et al (1983) have shown that the solubilisation of mesangial IgA deposits in sections by fresh serum from patients with IgA nephropathy is significantly less than that produced by normal

serum. The short trial of delayed PGE_1 administration in the sc CCl_4 model of mesangial IgA deposition, however, did not produce a reduction in mesangial deposition once cirrhosis and deposition had occurred. The therapeutic implications of the drug trial were limited to the effects of PGE_1 and these may largely have been mediated through hepatic cytoprotection. The role of PGE_1 in renal blood flow may be important but was not specifically addressed.

The effect of d-penicillamine

D-penicillamine like PGE_1 has been found to affect collagen metabolism and has been used in progressive systemic sclerosis (scleroderma) where it produced a reduced rate of increase in dermal collagen assessed by skin thickness (Steen et al 1982). Whether this is a direct effect of the drug upon collagen metabolism or an indirect result of immunological changes is uncertain. In this drug trial, however, d-penicillamine, unlike PGE_1 had no effect upon liver fibrosis. D-penicillamine did lower serum IgA levels but without an effect upon mesangial deposition, implying that high IgA levels *per se* were not the only causes of such mesangial deposition.

This itself gives some insight into human IgA nephropathy. High serum IgA levels (Lesavre et al 1982) may just be a marker of an underlying immunological defect in such patients rather than the cause of the mesangial deposits and may not therefore be a suitable parameter for therapeutic manipulation. Such a conclusion was drawn from therapeutic trials of phenytoin (Clarkson et al 198) where serum IgA levels could be reduced

but no clinical or immunopathological change was detected.

The underlying immunological defect in primary IgA nephropathy could lie in B-cell function, with an increase in IgA-producing circulating cells (Nomoto et al 1979) or a decrease in suppressor T-cell function (Sakai et al 1979b). In any case, it seems that immunosuppressive therapy aimed at reducing serum IgA levels alone would not be an effective form of therapy.

The other facet of the animal study which gave similar results to those found in the study of human material was the recognition of mesangial IgA deposition in non-cirrhotic liver disease. Two rats without cirrhosis given 150 µg PGE_1 and CCl_4 had mesangial IgA deposition, a feature which was seen in patients with ALD and suggests that a continuum of liver destruction could produce renal disease.

How small an intake of alcohol might also result in mesangial IgA deposition in a susceptible individual is unclear.

APPENDICES

APPENDIX I

Histological staining methods

CONTENTS

i) Haematoxylin and eosin staining
(after Lillie 1942)

ii) Periodic acid-Schiff reaction for carbohydrates
(after McManus 1946)

iii) Periodic acid-Methenamine silver for basement membranes
(after Jones 1957)

iv) Silver impregnation of reticulin fibres
(after Gordon and Sweets 1936)

v) Bile pigment stain
(after Fouchet 1917 with a collagen counterstain
after Sweat et al 1964)

vi) Prussian blue reaction for ferric iron
(after Perls 1867)

All tissues were fixed in 10% neutral buffered formalin, processed to paraffin wax and sections of kidney cut at 2 μm and liver at 4 μm.

i) HAEMATOXYLIN AND EOSIN STAINING

(after Lillie 1942)

Introduction

The classical methodology has been adapted for use in a linear staining machine (Hunneywell CLS360-1). The linear staining machine carries the slides on a conveyor belt through each well for approximately 30 seconds. Stages lasting longer are comprised of multiples of 30 seconds by repeating the contents in adjacent wells.

Reagents

Haematoxylin

5 g haematoxylin was dissolved in a few ml of ethanol. 50 g aluminium ammonium sulphate (ammonium alum) was dissolved in 700 ml distilled water by gentle heating. These two solutions were then mixed, and the remaining reagents (300 ml glycerol; 1 g sodium iodate; 20 ml glacial acetic acid) added.

Dilute acid alcohol

700 ml ethanol; 300 ml distilled water; 3 ml concentrated hydrochloric acid. The reagent keeps indefinitely.

Scott's tap water substitute

20 g magnesium sulphate; 2 g potassium hydrogen carbonate; 1000 ml distilled water.

Acetified eosin-phloxine solution

50 ml 1% weight to volume (w/v) aqueous eosin; 390 ml 95% aqueous ethanol; 5 ml 1% (w/v) aqueous phloxine TS; 2 ml glacial acetic acid.

Method

Pre-stage.................................drying oven

Stage 1 xylene..............................90 seconds

Stage 2 ethanol.............................90 seconds

Stage 3 running tap water...................30 seconds

Stage 4 haematoxylin........................ 3 minutes

Stage 5 running tap water...................60 seconds

Stage 6 dilute acid alcohol.................30 seconds

Stage 7 running tap water...................30 seconds

Stage 8 Scott's tap water substitute........60 seconds

Stage 9 running tap water...................30 seconds

Stage 10 acetified eosin-phloxine...........90 seconds

Stage 11 ethanol............................ 2 minutes

Stage 12 xylene.............................30 seconds

Holding chamber xylene

The slides are removed from the machine and mounted by hand in resinous medium.

Results

Nuclei are stained blue and different tissue components are identified as varying shades of pink.

ii) PERIODIC ACID-SCHIFF REACTION FOR CARBOHYDRATES (after McManus 1946)

Introduction

The periodic acid-Schiff reaction (PAS) is based upon the Mala-pride reaction, in which 1, 2-glycols undergo oxidative cleavage

to form di,aldehydes, which are subsequently demonstrated by condensation with leuco-fuchsin (after Schiff 1866), producing a magenta reaction product. The nuclear stain utilised was Celestin blue (after Lendrum 1935).

Reagents

Oxidising agent

1% (w/v) aqueous periodic acid.

Schiff's leuco-fuchsin (after de Tomasi 1936)

Using moderate heat, 1 g of basic fuchsin dye was dissolved in 200 ml water, and the solution cooled to 50°C. 50 ml of 1 N hydrochloric acid was added, and the solution further cooled to 25°C. The solution was poured into a clean, dry reagent bottle already containing 1 g potassium metabisulphite, quickly stoppered, and inverted several times to mix. The bottle was then left to stand in the dark at least overnight, during which time the solution became a pale straw yellow colour. The reagent was stored at 4°C.

Nuclear stain (after Lendrum 1935)

1 g of Celestin blue B dye and 0.5 ml concentrated sulphuric acid were ground together to form a paste, and then left standing for five minutes. 100 ml 2.5% (w/v) aqueous ferric ammonium sulphate solution was then added slowly, mixed, and the solution decanted into a stock bottle. When all the paste had been dissolved, any remaining alum solution was added, followed by 14 ml of glycerol. The stain was filtered before use.

Method

1 Dewax sections with xylene.

2 Treat slides with ethanol.

3 Wash well in distilled water.

4 Oxidise in periodic acid solution (5 minutes).

5 Wash in distilled water.

6 Transfer to Schiff's reagent in a closed vessel (30 minutes).

7 Wash well in fast running tap water (5 minutes).

8 Rinse with distilled water.

9 Counterstain nuclei with filtered Celestin blue B (15 seconds).

10 Wash well in running tap water.

11 Rinse with distilled water.

12 Dehydrate with ethanol.

13 Clear in xylene.

14 Mount in resinous medium.

Results

Sites positive by PAS are magenta in colour: positive structures include - basement membranes, collagen (weakly), glycogen, lipofuscins (sometimes), amyloid (weakly), reticulin, elastin. Nuclei are stained blue.

iii) PERIODIC ACID-METHENAMINE SILVER FOR BASEMENT MEMBRANE (after Jones 1957)

Introduction

Basement membranes are composed of a highly hydrated gel con-

taining proteoglycan. After formal precipitation, these components readily produce non-labile aldehydes by oxidation with periodic acid. Using the methenamine (hexamine) silver nitrate solution of Gomori (1946) basement membranes are sharply delineated black structures. Essentially the method stains the same structures as the periodic acid-Schiff technique but with greater contrast. This is exaggerated by the gold toning procedure. As stressed by Jones (1957)

> "1-3 μm sections are a prerequisite for the visualisation of the fine details in the glomerulus".

Reagents

Oxidising agent

1% (w/v) aqueous periodic acid.

Silver impregnation solution

2 ml of the 10% (w/v) aqueous silver nitrate solution was added to 25 ml of 3% (w/v) aqueous methenamine solution, and shaken until the precipitate dissolved. 47 ml of distilled water was added and then 3 ml of 5% (w/v) aqueous sodium tetraborate solution were added, and shaken until the precipitate dissolved. The solution is unstable, and prepared fresh prior to use. The solution was placed in a water bath at 60°C 15 minutes prior to use.

Toning solution

0.2% (w/v) aqueous gold chloride (sodium chloroaurate).

Fixing solution

5% (w/v) aqueous sodium thiosulphate.

Method

1 Dewax sections with xylene.

2 Treat slides with ethanol.

3 Wash well with distilled water.

4 Oxidise in periodic acid for 45 minutes.

5 Wash well with distilled water.

6 Impregnate in methenamine silver solution at 60°C for 60 minutes.

7 Wash well with distilled water.

8 Tone with sodium chloroaurate for 5 minutes.

9 Wash well with distilled water.

10 Fix with sodium thiosulphate for 3 minutes.

11 Wash well with distilled water.

12 Dehydrate with ethanol.

13 Clear in xylene.

14 Mount in resinous medium.

Results

Basement membranes are sharply delineated black structures.

iv) SILVER IMPREGNATION OF RETICULUM FIBRES
(after Gordon and Sweets 1936)

Introduction

Selective demonstration of precollagenous reticulins, and stromal reticulins may be achieved using an argyrophilic reaction. By the action of Mallory's bleach sequence (potassium permanganate followed by oxalic acid), basement membranes may be excluded from the reaction, whilst the addition of catalytic

sulphuric acid to the permanganate permits oxidation of nuclear chromatin beyond reaction with the silver solution.

Reagents

Oxidising agent (mixed immediately prior to use)

95 ml 0.5% (w/v) aqueous potassium permanganate; 5 ml 3% (w/v) aqueous sulphuric acid.

Decolourising agent

1% (w/v) aqueous oxalic acid.

Sensitising agent

2.5% (w/v) aqueous ferric ammonium sulphate.

Ammoniacal silver impregnation solution

Ammonium hydroxide (strong ammonia) was added dropwise to 5 ml of 10.2% aqueous silver nitrate until the brown precipitate, at first formed, was just dissolved. 5 ml of 3.1% aqueous sodium hydroxide was added followed by further strong ammonia, dropwise until the brown precipitate again formed, was almost dissolved. The solution should be not quite clear and should not smell of ammonia. The solution was made to 50 ml with distilled water and filtered.

Reducing solution

10 ml 36% to 40% (w/v) aqueous formaldehyde; 90 ml distilled water.

Toning solution

0.2% (w/v) aqueous sodium chloroaurate.

Fixing solution

5% (w/v) aqueous sodium thiosulphate.

Nuclear stain

1% (w/v) aqueous neutral red acetified with one drop of glacial acetic acid per 100 ml.

Method

1 Dewax sections with xylene.
2 Treat slides with ethanol.
3 Wash well in distilled water.
4 Oxidise with acidified permanganate solution (1 minute).
5 Wash well in distilled water.
6 Decolourise with oxalic acid solution.
7 Wash well in distilled water.
8 Sensitise with ferric ammonium sulphate (10 minutes).
9 Wash well with distilled water.
10 Impregnate with ammoniacal silver solution (1 minute).
11 Rinse briefly with distilled water.
12 Reduce with formaldehyde solution (1 minute).
13 Wash well with distilled water.
14 Tone with sodium chloroaurate (5 minutes).
15 Wash well with distilled water.
16 Fix with sodium thiosulphate (3 minutes).
17 Wash well with distilled water.
18 Stain with filtered neutral red solution (2 minutes).
19 Drain slide and blot gently.
20 Differentiate and dehydrate with ethanol.
21 Clear in xylene.
22 Mount in resinous medium.

Results

Reticulum fibres are sharply delineated black, nuclei are stained red, and erythrocytes a clear pale yellow.

v) STAIN FOR BILE PIGMENTS

(after Fouchet et al 1917)

Introduction

The action of ferric chloride in the presence of trichloroacetic acid demonstrates bile, possibly by oxidation to a mixture of green biliverdin and blue-green cholescyanin. The greenish reaction product is stabilised against natural fading by enhancement with picric acid counterstaining. The picric acid-Sirius red combination of Sweat et al (1964) is employed, thus also visualising collagen and reticulin.

Reagents

Fouchet's reagent

25 ml 25% (w/v) aqueous trichloroacetic acid; 25 ml 10% (w/v) freshly prepared aqueous ferric chloride.

Connective tissue stain

10 ml 1% (w/v) aqueous Sirius red F3BA; 90 ml saturated aqueous picric acid. Mix the two solutions, then add a further excess of picric acid crystals to ensure saturation.

Method

A known positive control was always included with every batch of stained slides.

1 Dewax sections with xylene.

2 Treat slides with ethanol.

3 Wash well with distilled water.

4 Oxidise in Fouchet's reagent for 5 minutes.

5 Wash well with distilled water.

6 Counterstain with Sweat's stain for 30 minutes.

7 Blot gently.

8 Rapidly dehydrate with ethanol.

9 Clear in xylene.

10 Mount in resinous medium.

Results

Bile pigments are demonstrated as apple green deposits with cytoplasm showing a clear yellow. Collagen and larger reticulum fibres are sharply delineated red, and are especially suitable for black and white photography.

vi) PRUSSIAN BLUE REACTION FOR FERRIC IRON
(after Perls 1867)

Introduction

Ferric iron is hydrolysed from binding protein using dilute hydrochloric acid, and then captured with potassium ferrocyanide to form insoluble ferric ferrocyanide (Prussian blue).

Reagents

Hydrolysing reagent

1% (v/v) aqueous hydrochloric acid.

Capture reagent

1% (w/v) aqueous potassium ferrocyanide.

Nuclear stain

1% (w/v) aqueous neutral red, acetified with one drop of glacial acetic acid per 100 ml solution.

Method

A known positive control was included in every batch of slides to be stained.

1 Dewax sections with xylene.
2 Treat with ethanol.
3 Wash well with distilled water.
4 Place slides on a rack, and flood with the dilute hydrochloric acid.
5 Using a naked flame, heat the slides to just steaming, approximately 60°C, then allow to cool for 5 minutes.
6 Drain away the acid.
7 Replace with the ferrocyanide solution.
8 Re-heat the slides as above, allow to cool for 5 minutes.
9 Wash well in running tap water (5 minutes).
10 Wash well with distilled water.
11 Stain with filtered neutral red solution (1 minute).
12 Drain slide and blot gently.
13 Differentiate and dehydrate with ethanol.
14 Clear in xylene.
15 Mount in resinous medium.

Results

Haemosiderin deposits are localised as the intense colour of Prussian blue, nuclei are stained red and erythrocytes a clear pale yellow.

APPENDIX II

Immunofluorescence techniques

CONTENTS

i) Gelatin and formalin coated slides

ii) Glycine buffered glycerol mounting medium (pH 8.6)

i) Gelatin and formalin coated slides

Introduction

The coating of slides is designed to avoid loss of sections during washing especially when electric stirrers are used. Uncoated slides were never used however and the precaution may not actually have been necessary.

Method

1 The slides were placed in slide holders and washed in tap water.

2 The slide holders were drained and dipped in cold 2% gelatin in distilled water and dried for about an hour.

3 The slide holders were then dipped in 10% buffered formalin, left to dry overnight in a fume cupboard, and stored in their original boxes.

ii) 0.1 M glycine buffered glycerol pH 8.6 mounting medium

Introduction

The mounting medium is designed to be transparent, liquid and inert. It is kept at 4°C and used until it develops any change in colour.

Materials

.Glycine (Univar, Ajax Chemicals, Sydney).

.Glycerol (Univar, Ajax Chemicals, Sydney).

Method

200 ml of glycerol was mixed with 100 ml of glycine buffer at pH 8.6 (1.4 g glycine:0.07 g sodium hydroxide:1.7 g sodium chloride:0.1 g sodium azide:100 ml distilled water) and stored at 4°C.

APPENDIX III

Hepatitis serology

HBV status was tested using a commercially available RIA system (Abbott Laboratories, N Chicago, IL). The tests were performed by Mr Peter Lindschau in the Division of Virology, IMVS.

APPENDIX TABLE III

HBV status of 16 patients and three controls in the autopsy series

(graded as negative or positive, NT=not tested)

Patient category	Autopsy number	HBV status		
		HBsAg	Anti-HBs	Anti-HBc
Cirrhotic ALD	50975.78	-	-	-
	50010.79	-	+	+
	50078.79	-	NT	-
	50132.79	-	-	-
	50432.79	-	-	-
	50439.79	-	-	-
	50442.79	-	-	-
	50443.79	-	-	-
	50502.79	-	+	+
	50510.79	-	-	-
	50561.79	-	-	-
Non-cirrhotic ALD	50012.79	-	-	-
	50486.79	-	-	-
	50698.79	-	-	-
Other serious liver diseases	50892.79	-	-	-
	50137.80	-	-	+
Controls	50500.79	-	+	-
	50515.79	-	+	-
	50116.80	-	-	-

APPENDIX IV

Protein estimations

CONTENTS

i) Folin-phenol method (after Lowry et al 1951)

ii) Bromocresol purple method (after Pinnell and Northam 1978)

i) Protein estimation (after Lowry et al 1951)

Materials

. Solution 1

2% sodium carbonate in 0.1 N sodium hydroxide.

. Solution 2

Freshly mixed 1% sodium tartrate and 0.5% copper sulphate.

Method

1 1 ml of solution 2 was added to 50 ml of solution 1 and mixed (solution 3).

2 The test tubes and standard curve tubes were labelled, in the range of 0 to 500 µg/ml.

3 The test samples were diluted to within this range.

4 2.5 ml of solution 3 was added to 100 µl of the test or standard solutions, mixed and left for 10 minutes at room temperature.

5 Folins and Ciocalteu's phenol reagent (BDH) solution was prepared at 1 N (that is 5.0 ml reagent plus 4.7 ml water) and 250 µl added to each tube, which was mixed and left for 30 minutes at room temperature.

6 The spectrophotometer was adjusted to zero using the blank standard at 700 nm visible light and the absorbance of each sample measured.

7 The concentrations of test specimens were calculated, with reference to the standard curve.

ii) Albumin determination using bromocresol purple method (after Pinnell and Northam 1978)

Reagents

Stock bromocresol purple (BCP) solution was made by dissolving 0.54 g BCP (Merck, Darmstadt, W Germany) in 25 ml absolute ethanol, and stored at 4°C.

Stock acetic acid solution was made by diluting 15 ml analar grade glacial acetic acid to 100 ml with distilled water.

Briz-35 solution (Technicon Sydney).

Working BCP reagent consists of 1 g sodium acetate trihydrate dissolved in 80 ml distilled water; 1 ml stock acetic acid solution; 0.1 ml Briz-35 solution; 0.1 ml of stock BCP solution, made up to 100 ml with distilled water at pH 5.2 ± 0.03.

Method

1 20 µl of sample or standards at 10-55 mg/ml was added to 4 ml of working reagent.

2 The spectrophotometer was adjusted to zero with the blank standard at 600 nm and each test and standard sample's absorbance recorded.

3 The concentrations of the test samples were calculated with reference to the standard curve and dilution.

APPENDIX V

Radiolabelling

Introduction

The method described was an adaptation of the chloramine-T method described by McConahey and Dixon (1966). The entire procedure was performed in a Radioactive Safe area, inside a fume cupboard with the operator wearing gloves, apron, and monitoring badge (Monitored by an IMVS Safety Committee). All equipment was kept in the fume cupboard.

Method

1 The protein to be labelled was diluted to 10 mg/ml in 0.25 M PB.

2 1 mCi ^{125}I in NaOH at pH 7.11 (Amersham International, Amersham, Bucks, UK) (10 µl) was placed in a test tube and buffered with 25 µl of 0.25 M PB.

3 100 µl of protein was added followed by 25 µl chloramine-T (3.5 mg/ml in 0.25 M PB) and the incubation allowed to proceed for 60 seconds.

4 The reaction was stopped with 100 µl sodium metabisulphite (2.4 mg/ml in 0.25 M PB).

5 Unbound ^{125}I was separated by filtration over Sephadex G-25 (Uppsala, Sweden) (Column diameter 1 cm, length 12 cm) equilibrated with PBS or 1% BSA in PBS.

6 Twenty 1 ml fractions were collected and the radioactivity counted in a single well gamma counter (Logic Model 101 B Abbott Laboratories). Three or four of the most radioactive fractions were pooled. Aliquots were labelled appropriately and stored at $-70^{o}C$ for up to six weeks.

APPENDIX VI

Solid phase Cl_q assay

CONTENTS

i) Purification of human Cl_q from fresh serum

ii) Coating of Cl_q plates

iii) Preparation of aggregated human IgG

iv) RIA method

i) Purification of human Cl_q using fresh blood
(after Yonemasu and Stroud 1971)

All prospective Cl_q donors had preliminary hepatitis screening by the Division of Virology, IMVS.

Materials

The preparation used a series of salt and disodium ethylenediaminetetra-acetic acid (EDTA) solutions some of which were further diluted to have a relative salt concentration (RSC), Radiometer (Copenhagen) equal to that of specified concentrations of sodium chloride (NaCl). These were 0.04 M NaCl and 0.078 M NaCl. The salt solutions required were:

0.1 M disodium EDTA pH 5.0

0.1 M disodium EDTA pH 7.5

0.005 M disodium EDTA pH 7.5

0.3 M NaCl

0.75 M NaCl in 0.01 M EDTA pH 5.0

0.1 M disodium EDTA pH 7.5 diluted with distilled water to have a RSC equal to the 0.04 M NaCl

0.1 M disodium EDTA pH 5.0 diluted with distilled water to have a RSC equal to the 0.078 M NaCl

Serum

180 ml blood was obtained after an overnight fast and allowed to clot at room temperature for 1 hour and then placed at 4°C for 1 hour to retract. The clot was then removed by centrifugation at 0°C at 600 *g* and the supernatant then re-centrifuged at 30,000 *g* for 40 minutes at 0°C.

Method

1 The serum was placed in an ice bath with one quarter of its volume of 0.1 M EDTA pH 7.5.

2 The serum was incubated at 37°C for 10 minutes and then cooled to 0°C on ice.

3 The pH was adjusted to 7.5 using 1 N hydrochloric acid.

4 0.005 M EDTA pH 7.5 was added slowly to the serum while stirring, until the RSC reached the conductivity of 0.04 M NaCl.

5 The mixture was then placed on ice for 1 hour and stirred for 1 to 2 minutes every 20 minutes before centrifuging at 13,000 *g* for 30 minutes at 0°C.

6 The supernatant was discarded and the pellet re-suspended in a volume of diluted 0.1 M EDTA pH 7.5 equal to twice the original volume of serum, and centrifuged at 13,000 *g* for 30 minutes at 0°C.

7 This step was repeated.

8 The pellet was then dissolved in 12 ml of 0.75 M NaCl in 0.01 M EDTA at pH 5 and left at 4°C overnight.

9 The solution was centrifuged at 20,000 *g* for 30 minutes at 0°C to remove aggregates.

10 The supernatant was then dialysed against 1 L of diluted 0.1 M EDTA at pH 5.0 for 4 hours with one change of buffer.

11 The supernatant was then centrifuged with half the original serum volume of diluted 0.1 M EDTA at pH 5.0 at 13,000 *g* for 30 minutes at 0°C.

12 The pellet was then dissolved in half the original serum volume of diluted 0.1 M EDTA pH 5.0 and centrifuged at 13,000 *g* for 30 minutes at 0°C.

13 The pellet was dissolved in approximately 5 ml of 0.3 M NaCl and left overnight at $4^{o}C$.

14 The solution was centrifuged at 20,000 *g* for 30 minutes at $0^{o}C$ and the supernatant retained as pure Cl_q.

15 Purity of the Cl_q sample was tested by double immunodiffusion (Appendix VII) using antiserum to Cl_q, IgA, IgG, and IgM and by immunoaggregation where one drop of Cl_q preparation was added to one drop of aggregated human globulin at 2 mg/ml. Using heat inactivated Cl_q ($56^{o}C$ for 20 minutes) as a negative control.

16 The Cl_q preparation was used immediately.

ii) <u>Cl_q coating of microtitre plates</u>

1 The concentration of Cl_q (measured by optical density (OD) 280 nm) using E^{280} x 1.466 in mg/ml) was adjusted to 11 μg/ml in PBS, and kept at $0^{o}C$.

2 Each microtitre plate (Costar Vinyl 96 - well Assay plates, Cambridge, MA) requiring 10 ml of Cl_q preparation was prepared by rinsing with PBS.

3 After drying each well was filled with 100 μl of cold Cl_q solution and the plates placed in cardboard boxes with dividers and left overnight at $4^{o}C$.

4 Each well was aspirated and washed twice with cold PBS and patted dry.

5 The wells were filled with 1% BSA (Sigma, St Louis, MO) in PBS by flooding and left at room temperature for 2 hours.

6 The wells were aspirated and washed twice with cold PBS, tapped dry and stored at $-70^{o}C$ for up to six weeks.

iii) Preparation of aggregated human globulin

1 A 2% solution of normal human immunoglobulin (from CSL 160 mg/ml with 0.01% thiomersal) in PBS was heated at 63°C for 15 minutes, and then allowed to cool to room temperature.

2 Slowly 40 ml of 2.18 M sodium sulphate was added and stirred for 30 minutes at room temperature.

3 The solution was then placed at 4°C for 30 minutes followed by centrifugation at 3000 *g* for 30 minutes at 4°C.

4 The pellet was re-suspended in 20 ml PBS and dialysed against PBS at 4°C for 24 hours with two changes of buffer.

5 The solution was then centrifuged at 100,000 *g* for 90 minutes at 4°C.

6 The pellet was then re-suspended in 20 ml of PBS, and centrifuged at 5,000 *g* for 30 minutes at 4°C.

7 The supernatant was retained as aggregated human globulin (AHG) and its concentration calculated by the Folin phenol method (after Lowry et al 1951, Appendix IV).

iv) To perform the solid phase Cl_q assay (after Tung et al 1978)

1 120 µl of 0.2 M EDTA pH 7.5 and 60 µl of test serum were mixed and incubated for 30 minutes at 37°C.

2 200 µl of the normal control serum, shown in a previous assay to have the nearest Cl_q binding to the mean +2SD value for normal serum, and 400 µl of 0.2 M EDTA pH 7.5 were incubated for 30 minutes at 37°C.

3 A standard curve was prepared from AHG diluted in PBS to give between 1 and 5000 µg/ml when 1 ml of standard was

added to 100 μl of the mean +2SD serum in EDTA.

4 100 μl of the standard curve solutions were added in duplicate to the plate.

5 3 ml of PBS was added to each test serum, and 100 μl added in duplicate to the plate.

6 The plates were incubated at 37°C for 1 hour and then placed overnight at 4°C.

7 The sera were aspirated and the plates were washed three times with PBS 0.05% Tween 20 (polyoxyethylene (2) sorbitan mono-oleate, BDH Chemicals Ltd) then hit against a paper towel.

8 100 μl of radiolabelled goat anti-human IgG in 1% BSA in PBS-0.5% Tween 20 was added to each well (equivalent to approximately 50,000 cpm) and incubated at 37°C for 1 hour.

9 The plate was placed at 4°C for 30 minutes.

10 Test tubes marked in duplicate 500, 500 TCA and 100 were given appropriate volumes of the ^{125}I mixture.

11 500 μl of 20% TCA was placed into the tubes marked 500 TCA.

12 All test tubes were then centrifuged for 10 minutes at 1500 *g*.

13 The supernatant from the TCA precipitation was aspirated and the radioactivity in each duplicate pair correlated in a gamma counter (Nuclear Enterprises NE 1700, Edinburgh, UK).

14 The plates were washed three times in PBS-0.05% Tween 20 in a radioactive waste sink by aspirating without splashing.

15 The plates were cut and the individual wells placed in a multi well gamma counter (NE 1600).

16 The percentage of TCA precipitable counts in the ^{125}I mixture was calculated from the amount precipitated by 500 µl of TCA from 500 µl of the mixture. The available counts in 100 µl was then calculated from the duplicate 100 µl ^{125}I mixture.

17 For each duplicated sample and standard curve specimen, a mean percentage of available precipitable counts was calculated.

18 A standard curve was plotted on semilogarithmic paper with percentage counts bound on the linear scale.

19 The values for test samples were read from the curve and expressed as µg AHG/ml of serum.

APPENDIX VII

Immunoelectrophoresis and precipitation techniques

CONTENTS

i) Immunoelectrophoresis

ii) Single radial immunodiffusion

iii) Double immunodiffusion

i) Immunoelectrophoresis

1 1% agarose in 1 N veronal buffer at pH 8.6 was prepared by boiling and cooling. Aliquots were stored in screw top jars at $4^{o}C$. When required the agarose was melted at $63^{o}C$ in a water bath and poured onto a plastic eight troughed format using an agarose gel support medium (Gelbond FMC Marine Colloids Bioproducts, Rockland, MA).

2 After setting, the supported agarose was separated from the mould and placed on moistened glass in a moist chamber above a 1 N veronal buffer bath.

3 Wicks were placed into the buffer and on to the edges of the supported agarose.

4 1 µl of samples were placed in the wells and 100 volts applied for 65 minutes.

5 The wicks were removed and one drop of antiserum placed in each trough and the gel left overnight in the humid chamber.

6 The gel was washed in 3% saline for 3 hours followed by two changes of distilled water overnight using an electric stirrer.

7 The supported agarose was then dried overnight and stained with Coomassie blue (0.2% Coomassie Brilliant blue R250 in 45:45:10, alcohol:water:acetic acid) for five minutes. The plates were then decolorised in alcohol and acetic acid for approximately five minutes.

ii) Single radial immunodiffusion for the quantitation of proteins (after Mancini et al 1951)

1 1% agarose in 0.1 M PBS was prepared by boiling and cool-

ing to 55°C. Aliquots were stored in screw top jars at 4°C and were used after heating in a water bath to 55°C.

2 An appropriate volume of antiserum was added and stirred in warm agarose by swirling in the bath to prevent setting.

3 The agarose was then poured from the bottle smoothly onto a glass slide on which had been scratched an identification code. Poured plates were stored at 4°C in a moist chamber for several days.

4 Plates were slid into a frame and equidistant 2 mm holes punched in rows.

5 The wells were loaded in triplicate and sometimes at a range of dilutions using a micrometer pipette (Absoluter Micropipetting method, Tri-Continent Scientific Inc, Grass Valley, CA) and left at room temperature in a moist chamber overnight.

6 The plates were then washed in a multishelved slide rack in 3% saline for 6 hours and overnight in distilled water in a bath with an electric stirrer.

7 The plates were dried in an oven at 37°C and stained with Coomassie blue as described above.

8 The diameters of precipitin rings were measured using a calibrated eyepiece (Peak scale Lupe 7x) and the mean for each sample recorded.

9 The square of the diameter of the rings was plotted against the concentrations of the standards to construct a standard curve.

10 The concentration of the protein in the test sera was calculated from the standard curve.

iii) Double immunodiffusion

1 Aliquots of 1% agarose in 0.1 M PBS were heated and poured as described for single radial immunodiffusion but without the addition of antiserum.

2 Holes were punched in the gel in a circle around a central well.

3 Antiserum placed in the centre well could be tested against several antigen samples or vice versa.

4 Diffusion was allowed to progress for several days to visualise all of the antigen-antibody systems present.

5 A range of dilutions of antibody and antiserum was tested to unmask latent cross-reactivity or contamination.

6 The plates were washed, dried and stained as described above.

APPENDIX VIII

Enzyme linked immunosorbent assay to demonstrate anti-bacterial coat activity

Enzyme linked immunosorbent assay (ELISA) to demonstrate anti-bacterial activity in kidney eluates from patients with ALD and mesangial IgA deposits (performed by Richard Harries, supervised by Professor D Rowley in the Department of Microbiology, The University of Adelaide).

Materials

All strains of bacteria were from freeze dried stock of the Departments of Microbiology of The University of Adelaide, and the IMVS.

Gram-negative bacteria

E coli 221.59; 221.58; B; BV; LC

Salmonella typhimurium; *S enterotides*; *S minnesota*; *S adelaide*

Proteus vulgaris

Pseudomonas pyogenes

Gram-positive bacteria

Mixed *Clostridia* or mixed *Bacteroides*

Methods

A Small scale preparation of fractured bacterial envelope

i) Gram-negative strains

All enteric strains were obtained from freeze dried stock and grown on nutrient broths at 37°C and then re-cultured on agar to obtain single colonies. 10 ml of cells were taken and centrifuged at 7000 *g* for 10 minutes at 4°C. The pellet was re-suspended in 10 ml of Tris buffer (30 mM pH 8.1) and re-

centrifuged. The pellet was then frozen for 10 minutes at $-20^{o}C$, re-suspended in 0.2 ml cold 20% (w/v) sucrose in Tris-HCl (pH 8.1) and maintained at $0^{o}C$. The suspension was then supplemented with 20 μl of lysozyme at 1 mg/ml in 0.1 M EDTA pH 7.3, and followed after 30 minutes by 3 ml 3 mM EDTA pH 7.3 which led to rapid lysis of the organisms.

ii) Gram-positive strains

Clostridia and Bacteroides cell walls were extracted by fracturing in a French pressure cell (Paton Industries, SA) followed by further disruption in a sonicator (MSE 11.62) using repeated 30 second pulses. Uncracked bacteria were removed by centrifugation at 5000 *g* for 10 minutes at $4^{o}C$. The supernatant was then centrifuged at 25,000 *g* for 1 hour at $4^{o}C$. The cell wall pellet thus obtained was re-suspended in 5 ml of distilled water. The protein concentration of the cell wall preparation was estimated (after Lowry et al 1951).

B Performance of the assay

1 Individual assay plates (Costar Vinyl 96 - well Assay plates) were coated with the cell wall fractions from each bacterial strain at a protein concentration of 10 μg/ml using 80 μl per well overnight at room temperature.

2 The plates were washed twice with BSA/Tween (0.01% BSA-0.05% Tween 20) (Sigma) to block non-specific binding.

3 Antigen specific plates were incubated with 100 μl of glomerular eluates, positive control sera or BSA/Tween for 4-6 hours at room temperature. The eluates were tested at doubling dilutions from 1:8 to 1:64, the washes

from 1:400 to 1:3200 and sera from 1:2 to 1:16. The technique was controlled by incubation of cell wall and envelopes with the substrate to show that there was no inherent membrane bound alkaline phosphatase activity; and also with the conjugate followed by substrate to exclude cross-reactivity with the conjugated antibody.

4 The plates were washed twice with BSA/Tween.

5 The wells were incubated with 100 μl of alkaline phosphatase (Sigma) conjugated-affinity purified goat anti-human Fab (Atlantic Antibodies, Scarborough, ME) at 20 ng/ml. The purification and conjugation was performed by Dr P Ey, Department of Microbiology, The University of Adelaide.

6 The plates were washed twice with BSA/Tween.

7 160 μl of 0.01% o-nitrophenyl phosphate was added to each well and the plate was incubated at 37°C for 2-6 hours.

8 The absorbance was measured at 450 nm (Titertek Multiscan MC, Flow Laboratories, McLean, VA).

Results

Clostridia organisms could not be disrupted and were used whole.

APPENDIX IX

Buffers

i) Phosphate buffered saline

ii) Phosphate buffers

iii) Borate buffer

iv) Citrate buffer

v) Veronal buffer

vi) Carbonate buffer

All water used was double distilled.

i) Phosphate buffered saline pH 7.5 (PBS)

0.15 M sodium chloride

0.01 M phosphate buffer

ii) Phosphate buffers pH 7.5

Phosphate buffers were made from stock solutions of 0.5 M dipotassium hydrogen orthophosphate and potassium dihydrogen orthophosphate diluted to produce appropriate final molarities at pH 7.5.

iii) Borate buffer pH 8.4 (0.2 M)

0.2 M disodium tetraborate

0.2 M boric acid

iv) Citrate buffer pH 3.2 (0.02 M)

0.02 M sodium citrate

0.02 M citric acid

v) Veronal buffer pH 8.6

0.05 M sodium barbitone

0.01 M diethyl barbituric acid

vi) Carbonate buffer pH 9.4 (0.025 M)

0.025 M sodium carbonate

0.025 M sodium hydrogen carbonate

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