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MECHANISMS OF MEDIATION OF ALLERGIC GLOMERULAR INJURY

by

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CONTENTS

SUMMARY

The glomerular deposition of circulating immune complexes or antibody to glomerular basement membrane (GBM) is thought to be responsible for the majority of cases of human glomerulonephritis. As a consequence of these immune reactions in the glomerulus, mediator systems are activated and these induce glomerular injury. In this thesis I have investigated the mechanisms of mediation of glomerular injury in experimental crescentic glomerulonephritis.

Crescentic glomerulonephritis due to anti-GBM antibody (nephrotoxic nephritis: NTN) was induced in rabbits by the intravenous injection of nephrotoxic serum. Within twelve days a severe crescentic glomerulonephritis would develop in which glomerular fibrin deposition was prominent. Chronic immune-complex glomerulonephritis in rabbits was induced by the daily intravenous administration of bovine serum albumen (BSA). It was found that by preimmunising the animals with BSA in Freund's complete adjuvant and by adjusting the daily dose of BSA according to the antibody response, crescentic glomerulonephritis could be induced in 80% of animals within five to seven weeks.

The role of fibrin in experimental crescentic glomerulonephritis was investigated by examining the effect of anticoagulation with heparin of defibrination with ancrod. In NTN anticoagulation with heparin resulted in a reduction in glomerular fibrin deposition, extracapillary cell proliferation (crescent formation) and renal failure only when

heparin was administered in very large doses (2000 u/Kg/day). Defibrination with ancrod in NTN and chronic immune-complex glomerulonephritis provided protection superior to that provided by very large doses of heparin. In both models of crescentic glomerulonephritis proteinuria was unaffected by anticoagulation or defibrination. These findings suggest that glomerular fibrin deposition plays no part in the primary allergic events causing capillary damage and proteinuria but that it is a consequence of capillary damage.

Defibrination with ancrod in NTN, after glomerular fibrin deposition had occurred and crescents were developing also provided some degree of protection from crescent formation and renal failure. Sequential studies showed that once further fibrin deposition was prevented by defibrination, glomerular fibrin deposits were rapidly removed.

The function of polymorphonuclear leucocytes (PMN) in experimental crescentic glomerulonephritis was examined by depleting circulating PMN with a specific antipolymorph serum. Depletion of PMN in NTN not only prevented glomerular fibrin deposition, crescent formation and renal failure but also largely prevented proteinuria. It would seem that the PMN is the principal injurious agent in this disease and that glomerular fibrin deposition is a consequence of the PMN-induced glomerular damage.

Decomplementing animals with cobra venom factor did not reduce the glomerular fibrin deposition and crescent formation of NTN. This

indicates that complement is not important in the induction of glomerular PMN accumulation and subsequent damage.

These studies have defined a system of injury in experimental crescentic glomerulonephritis due to anti-GBM antibody, which is mediated by PMN and fibrin but is independent of complement. Although only the role of fibrin was examined in the crescentic glomerulonephritis of chronic immune-complex disease it seems likely that the mechanism of injury is also mediated by PMN.

The relevance of these findings to human crescentic glomerulonephritis and the possible therapeutic implications have been discussed.

CANDIDATE'S STATEMENT

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university and that 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.

NAPIER MAURICE THOMSON

The work described in this thesis has been reported in the following publications :

(1) Thomson, N.M., Simpson, I.J. and Peters, D.K. (1975)
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(2) Thomson, N.M., Simpson, I.J., Evans, D.J. and Peters, D.K. (1975)
Defibrination with ancrod in experimental chronic immune-complex nephritis.
Clin. exp. Immunol., 20, 527.

(3) Naish, P.F., Thomson, N.M., Simpson, I.J. and Peters, D.K. (1975)
The role of polymorphonuclear leucocytes in the autologous phase of nephrotoxic nephritis.
Clin. exp. Immunol., 22, 102.

(4) Simpson, I.J., Thomson, N.M. and Peters, D.K. (1975) Mediator systems in experimental allergic glomerular injury. In "Proceedings of the VIth International Congress of Nephrology": Karger - Basel (in press)

(5) Thomson, N.M., Simpson, I.J. and Peters, D.K. (1975) Anticoagulation in experimental glomerulonephritis. Abst. VIth International Congress of Nephrology

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HOW THIS THESIS ADVANCES MEDICAL KNOWLEDGE

This thesis advances medical knowledge in the following ways :

it defines a system of mediation of glomerular injury in experimental crescentic glomerulonephritis, dependent on polymorphonuclear leucocytes and glomerular fibrin deposition but independent of complement.

it leads to a better understanding of the mechanisms of glomerular fibrin-fibrinogen deposition and removal in crescentic glomerulonephritis.

it demonstrates the superiority of defibrination with ancrod over anticoagulation with heparin in preventing glomerular fibrin deposition in crescentic glomerulonephritis.

It is hoped that a better understanding of the pathogenesis and mechanisms of mediation of injury in glomerulonephritis will lead to specific methods of prevention and therapy.

Chapter 1

INTRODUCTION

It is now generally accepted that allergic processes participate in the pathogenesis of most cases of human glomerulonephritis; the deposition of circulating immune complexes in the glomerulus or the reaction of antibodies with antigens in the glomerular basement membrane is thought to be the pathogenetic mechanism in about 70% of all cases of human glomerulonephritis. Although evidence of these two pathogenetic mechanisms is lacking in the other 30% of cases other findings suggest the involvement of the immune response (Wilson and Dixon, 1974).

Studies of experimental glomerulonephritis in animals have established that glomerular disease similar to that found in humans may be induced by allergic mechanisms. These studies have led to considerable understanding of the factors that predispose to the deposition of immune complexes (or anti-basement membrane antibody) in the kidney and of the mechanisms involved in the mediation of glomerular injury resulting from the allergic reaction in the glomerulus.

This thesis is principally a study of the mechanisms of the mediation of glomerular injury which follow the primary immunological insult. In Part I, I have reviewed current knowledge of the immunopathogenesis of glomerulonephritis and described the models of experimental glomerulonephritis in animals.

The materials and methods I have utilised in studying mediation of glomerular injury have been described in Part II. The induction

and characterisation of two models of experimental glomerulonephritis, namely nephrotoxic nephritis and bovine serum albumen-induced chronic immune-complex nephritis in rabbits, are described in Part III.

In Part IV I have described and discussed the findings of experiments designed to delineate the role of glomerular fibrin deposition, polymorphonuclear leucocytes and complement in the mediation of glomerular injury in experimental crescentic glomerulonephritis. The interrelationship of these mediators and their relevance to the mediation of human glomerulonephritis is also discussed.

PART I

A REVIEW

Chapter 2

PATHOGENESIS AND MEDIATION OF INJURY IN GLOMERULONEPHRITIS

Introduction

Initial evidence for an immunological pathogenesis of glomerulonephritis came from observation made near the turn of the century. Nephrotoxicity of heterologous anti-kidney serum in animals was noted by Lindemann in 1900. Von Pirquet (1911) related the glomerulonephritis occurring in serum sickness to a "toxic compound" resulting from antibody production. Descriptions of other animal models of immunological renal disease followed. Chronic glomerulonephritis induced by repeated injection of foreign serum protein was noted by Longcope (1913). Rich and Gregory (1943) described acute serum sickness nephritis in rabbits. Cavelti and Cavelti (1945) induced chronic glomerulonephritis in rats by repeated immunisation with heterologous kidney. Further evidence for an allergic mechanism came with finding that serum complement was low in some forms of glomerulonephritis (Gunn, 1915 ; Kellett and Thomson, 1939). More direct evidence came with the demonstration by immunofluorescence that immunoglobulins were deposited in the glomeruli in systemic lupus erythematosus and chronic membranous glomerulonephritis (Mellors and Ortega, 1956).

In the past two decades detailed studies of experimental glomerulonephritis in animals and the application of techniques developed in these animal studies to human disease have established the existence of two different and distinctive mechanisms of allergic glomerular injury in

animals and man (Germuth, 1953; Germuth and Heptinstall, 1957; Dixon, Feldman and Vazquez, 1961; Unanue and Dixon, 1967; Dixon, 1968; Germuth and Rodriguez, 1973). In the first of these mechanisms, antibodies react with antigens in the glomerular basement membrane (GBM). In the second, antibodies capable of reacting with non-glomerular antigens combine with the antigens, leading to the formation of circulating antigen-antibody complexes ; these complexes then become trapped in the walls of the glomerular capillaries. As a consequence of these allergic reactions in the glomerulus, host factors (or mediators) are activated and induce glomerular injury.

In this chapter I shall describe the principal models of experimental glomerulonephritis and spontaneous glomerulonephritis in animals, summarise what is known of the pathogenesis and aetiology of human glomerulonephritis and finally consider the mechanisms of allergic glomerular injury.

MODELS OF EXPERIMENTAL GLOMERULONEPHRITIS

1 : Anti-GBM Glomerulonephritis

(a) Nephrotoxic Serum Nephritis (NTN)

The intravenous injection of heterologous antiserum (NTS) raised against either a crude renal extract or purified GBM, into an animal of the same species from which the antigen was taken has been shown to induce a proliferative glomerulonephritis in most species of animal so far examined (Lindemann, 1900; Pearce, 1903-4; Masugi, 1929, 1933, 1934; Masugi, Sato and Todo, 1935; Eisen and Pressman, 1950).

It has been shown that the glomerular damage occurs in two phases.

The immediate or heterologous phase follows within a few hours of the administration of the NTS and fixation of heterologous antibody on the host GBM. In most species this phase is characterised by the accumulation of polymorphonuclear leucocytes along the GBM and proteinuria. Regression of these changes usually occurs within 24-48 hours. Four to six days later, with the onset of host antibody production against the heterologous antibody, the second or autologous phase of injury commences. Host antibody fixes to the heterologous antibody along the GBM inducing glomerulonephritis, the severity of which varies with species. Rabbits, dogs and sheep develop a severe extracapillary proliferative (crescentic) glomerulonephritis in which glomerular fibrin deposition is prominent. Death from renal failure often occurs within 10-20 days. Rats tend to develop a mixed proliferative and membranous nephritis (Masugi, 1933) whilst mice usually show little proliferative response but develop intracapillary thrombi and mesangial and membranous alterations (Göbel-Schmitt, 1950; Reid, 1956).

The injury to the glomerulus in the heterologous phase, as measured by polymorphonuclear leucocyte infiltration of the glomeruli and proteinuria is directly related to the quantity of nephrotoxic antibody fixed in the kidney (Henson, 1971 a). Autologous-phase injury is dependent upon host-antibody formation to the heterologous antibody. Injury can be prevented by depression of antibody synthesis with X-irradiation (Kay, 1940, 1942) or by induction of tolerance to the heterologous immunoglobulin (Hammer and Dixon, 1963) and can be accelerated by measures which increase antibody response, such as pre-immunisation with the heterologous immunoglobulin (Unanue and Dixon, 1967).

On immunofluorescence microscopy during the heterologous phase, heterologous antibody is seen deposited in a smooth continuous linear fashion along the GBM and if the nephrotoxic antibody is complement fixing, deposition of complement (C) in a similar pattern is also seen. In the autologous phase, linear deposition of host antibody along the GBM is seen as well as the heterologous antibody. Linear deposition of immunoglobulin along the GBM has been found to be the hallmark of anti-GBM antibody-induced glomerulonephritis.

(b) Experimental "Auto-Allergic" Anti-GBM Glomerulonephritis

Steblay (1962, 1963 a) produced severe proliferative (crescentic) glome rulonephritis in sheep by the repeated injection of heterologous or homologous GBM in complete Freund's adjuvant (CFA), over a period of 1-3 months. Not only glomerular basement membrane but also heterologous lung basement membrane has the capacity to induce this disease (Rudofsky and Steblay, 1965). Sera from the nephritic sheep are capable of inducing NTN if administered to animals of the same species as the donor of the antigen (Steblay, 1962). Moreover, immunoglobulin from the nephritic sheep or immunoglobulin eluted from their kidneys will fix to the GBM of normal sheep when given intravenously (Lerner and Dixon, 1966 a, b) and is seen as linear staining along the GBM on immunofluorescence. Nephritis may also be transferred from nephritic sheep to normal sheep by a 4-hour cross circulation (Steblay, 1964). Anti-GBM antibody as detected by indirect immunofluorescence or haemagglutination technique may be detected in the circulation of the nephritic animals especially after bilateral nephrectomy.

The pathogenetic agent in this disease is presumably auto-antibody directed against GBM antigenic components shared by the immunising antigen and the sheep glomerulus (Lerner and Dixon, 1966 a, b). The production of auto-allergic disease by these methods establishes that immunological tolerance to self-antigens can be terminated. It has been postulated that tolerance for such auto-antigens resides in T-lymphocytes and that prolonged immunisation schedules induce a new population of non-tolerant T-lymphocytes which co-operate with B-lymphocytes in the production of anti-GBM antibody (Weigle, 1971).

Autoimmune glomerulonephritis due to the production of antibodies to GBM has also been produced by the same immunisation procedure, in rabbits (Unanue, 1966; Unanue and Dixon, 1967) monkeys (Steblay, 1963 b) and guinea-pigs (Unanue and Dixon, 1967; Steblay and Rudofsky, 1971).

Such "auto-allergic" anti-GBM glomerulonephritis is of particular relevance to human Goodpasture's syndrome and some cases of human rapidly progressive glomerulonephritis in which linear deposition of antibody along the GBM is found.

2 : Experimental Immune-Complex Glomerulonephritis

The study of acute and chronic immune-complex disease in experimental animals, induced respectively by the single and repeated administration of non replicating antigens has provided the basis for understanding the pathogenetic mechanisms not only for most cases of human glomerulonephritis but also vasculitis and several systemic "connective tissue" diseases (Cochrane and Koffler, 1973). Studies of

acute and chronic immune-complex nephritis have almost exclusively been confined to rabbits.

(a) Acute Immune-Complex Glomerulonephritis

The single intravenous injection of crude heterologous serum or of a large dose (250 mg/Kg BW) of purified heterologous serum protein (such as bovine serum albumen : BSA) will induce acute arteritis, arthritis and glomerulonephritis in about 60% of rabbits (Rich and Gregory, 1943; Gregory and Rich, 1946; Hawn and Janeway, 1947;Germuth, 1953; Dixon et al, 1958). The disease develops at about 8-14 days after the injection of the protein, during the phase of immune elimination of the antigen and production of circulating soluble immune complexes (Dixon et al, 1958). The disease is self-limiting and resolves once antigen is cleared from the circulation. Host immunoglobulin, foreign protein and complement, distributed in a granular pattern along the GBM may be detected by immunofluorescence and antibody and antigen may be eluted from the kidney. Early in the disease, antigen-antibody deposition may be found in the arterial walls adjacent to the internal elastic lumina.

The principal requirement for the development of acute immune-complex disease is an antibody response sufficient to clear circulating antigen but not so strong that the animal forms only large insoluble complexes of the type formed in antibody excess (Germuth and Rodriguez, 1973). The quantity of antigen bound in the glomeruli of both kidneys at the height of the disease is about 20 μg of BSA (Wilson and Dixon, 1970) and it has been calculated that this corresponds to about 4.4×10^8 molecules of BSA and 1.3×10^9 molecules of antibody per glomerulus

(Wilson and Dixon, 1971).

(b) Chronic Immune-Complex Glomerulonephritis

The production of chronic glomerulonephritis by the repeated injection of foreign serum proteins in rabbits has been studied by several investigators (Longcope, 1913; Vaubel, 1932; Masugi and Sato, 1933; Heptinstall and Germuth, 1957). Detailed studies by Dixon et al (1961) and Germuth and Rodriguez (1973), of rabbits injected daily with heterologous serum proteins have established three broad categories of antibody response and that these responses are directly related to the nephrotoxicity of the complexes generated:

(i) Animals showing a moderate antibody response, develop chronic glomerulonephritis. Such a response provides enough antibody to allow the formation of soluble antigen-antibody complexes in a moderate antigen excess for a large period of each 24-hour interval between injections. There is also some evidence that in these animals, poorly precipitating antibodies are produced and that this is important in determining whether complexes circulate and then deposit in capillary walls (Christian, 1970; Lightfoot, Drusin and Christian, 1970).

(ii) Animals producing a greater antibody response, form complexes in a moderate antibody excess. Such complexes are large and may become trapped in the glomerular mesangium and on the endothelial aspect of the GBM. These complexes may give rise to mild glomerular disease, often focal in type (Germuth and Rodriguez, 1973). A very strong antibody response will lead to no damage.

(iii) Animals producing little or no antibody form complexes in gross antigen excess and these do not cause damage.

Dixon et al (1961) and Wilson and Dixon (1971) have shown that any rabbit can develop chronic glomerulonephritis provided the dose of antigen is adjusted to the antibody response such that the antigen injected daily removes antibody from the circulation and remains temporarily in excess. If the animal does produce large amounts of antibody and the antigen dose is adjusted accordingly, glomerulonephritis appears earlier and is more severe (Wilson and Dixon, 1971).

Immunofluorescence reveals coarse and often confluent granular deposits of antigen, immunoglobulin and C3 along the epithelial aspect of the GBM. These deposits have been confirmed on electronmicroscopy. In mild disease states, deposits may be primarily within the mesangium. The quantity of antigen within the two kidneys is often as much as 800 ug of BSA (Wilson and Dixon, 1971).

Considerable variation in the severity of the glomerulonephritis is found,depending on the antibody response and on whether the antigen dose is varied according to the antibody response. Severity ranges from a mild focal or diffuse endothelial and mesangial proliferative disease to a severe crescentic glomerulonephritis. Varying degrees of basement membrane thickening or reduplications may be seen and polymorphonuclear leucocyte infiltration is common (Dixon et al, 1961).

(c) Heymann (Kidney in Adjuvant) Nephritis

Chronic glomerulonephritis can be induced in rats by the repeated intraperitoneal injection of homologous or heterologous kidney extract

in complete Freund's adjuvant (Heymann et al, 1959). The disease is membranous in type with minimal proliferation or exudation (Heymann et al, 1962, 1963). Immunofluorescence demonstrates host immunoglobulin and complement in discrete beads along the GBM (Heymann et al, 1963; Dixon, Unanue and Watson, 1965) and electronmicroscopy shows electron dense deposits on the epithelial side of the GBM (Hess, Ashworth and Ziff, 1962).

There is strong evidence, particularly derived from the findings on immunofluorescence and electronmicroscopy that this disease is due to the deposition of immune complexes (Unanue and Dixon, 1967) and the responsible antigen has been extracted from the brush border of the proximal convoluted tubules (Edgington, Glassock and Dixon, 1968). It is thought that in normal rats this antigen is found in the circulation. By some mechanism the injected antigen terminates a tolerant state to the tubular antigen and antibodies to the antigen are produced. Naruse et al (1973) have reported tubular antigen in the glomerulus of cases of human extramembranous glomerulonephritis, but these findings have not been confirmed by other investigators (Whitworth and Cameron, 1975 : Personal Communication).

Mechanisms Inducing Immune Complex Deposition In the Kidney

The passive induction of immune-complex disease by either injection of preformed immune complexes or the infusion of antibody into animals with antigens present in the circulation, has in general resulted in only relatively mild and often poorly reproducible lesions (Germuth and Pollack, 1958; McCluskey et al, 1960; Benacerraf et al, 1960; Fish et al, 1966; Cochrane and Weigle, 1973). It has since been

established that for immune complexes to localise in capillaries and induce injury two principal requirements must be fulfilled :

(i) The circulating complexes must be greater than 19S in size (Cochrane and Hawkins, 1968; Germuth and Rodriguez, 1973).

(ii) Capillary permeability must be increased (Henson and Cochrane, 1971). In 1968 Knicker and Cochrane demonstrated increased capillary permeability at the time of acute immune-complex disease in rabbits and that treatment with antagonists of vasoactive amines inhibited complex localisation in glomeruli (Knicker, 1968). It has since been established in rabbits and possibly man (Henson, 1969 a ; Benveniste and Henson, 1971) that the vasoactive amines are released from platelets by a soluble intermediary called platelet-activating factor (PAF). PAF is released in the presence of the antigen from circulating sensitised basophils which have adherent IgE antibody. Complement-dependent mechanisms of release of vasoactive amines from the platelets may also play a role (Cochrane and Koffler, 1973). Recent work has also raised the possibility that immune complex deposition in the kidney may be a direct consequence of C3 receptors present in the glomerulus (Gelfand et al, 1975). The precise localisation of these receptors has not as yet been determined. Once immune complexes are deposited in the vessel wall and injury commences, increase in capillary permeability occurs and immune complex deposition is augmented (Wilson and Dixon, 1971).

Perhaps of equal importance to the factors responsible for the deposition of immune complexes in the tissues are factors responsible

for their removal once deposited. There is some evidence that the glomerular mesangial cells are responsible for the removal of immune complexes from the glomeruli and that immune complexes accumulate along the GBM only when the capacity of the mesangium to remove them is exceeded (Mauer et al, 1972). The mechanism by which the mesangium removes complexes is poorly understood.

Polymorphonuclear leucocytes and macrophages may be important in the removal of complexes from the circulation and possibly also from sites of complex deposition. Both these cells have receptors for immunoglobulin and when immunoglobulins are complexed to antigens, binding to leucocytes and reticulo-endothelial cells is greatly enhanced (Uhr, 1965). Henson (1969 b) has shown that C3 is required for binding IgM-antigen complexes to macrophages (monocytes) and polymorphonuclear leucocytes.

(d) Spontaneous Immune-Complex Glomerulonephritis in Animals

The study of spontaneous immune-complex glomerulonephritis in animals has helped to elucidate various factors that lead to a state of chronic generation of circulating immune complexes and hence glomerulonephritis.

Perhaps the best known spontaneous chronic immune-complex disease in animals is the SLE-like illness of the NZB/W mouse. These animals have a propensity to produce antibodies to nuclear material and generate immune complexes, particularly DNA-anti DNA complexes. Granular deposition of immunoglobulin and C3 along the GBM can be seen (Aarons, 1964), DNA has been demonstrated in the same pattern of

distribution (Lambert and Dixon, 1968) and anti-DNA antibody has been eluted from the kidney (Lambert and Dixon, 1968). Evidence suggests that this disease may be virus-induced: Mellors (1965) directed attention to the persistence of the wild type Gross leukaemia virus in NZB/W mice. Lerner et al (1974) have suggested a second virus is involved, a C-type RNA tumour virus named the Scripps leukaemia virus and Dixon et al (1971) have shown that Gross leukaemia virus will absorb a significant proportion of antibody eluted from the kidneys of NZW/B mice. The excessive antibody response to antigens seen in these animals and certain irregularities in cellular immunity have suggested a deficiency of suppressor T-lymphocyte function (Cantor et al, 1970; Leventhal and Talal, 1970). Bach, Dardenne and Salomon (1973) have demonstrated degeneration of thymus function early in the life of the animal. Whether a genetic abnormality underlies both the immunity deficiency and virus infection or whether a vertically transmitted chronic viral infection induces the immunity deficiency is unknown.

The study of the glomerulonephritis that occurs in Aleutian disease in mink (Hartsough and Gorham, 1956; Henson et al, 1966) has demonstrated another mechanism of chronic generation of immune complexes. In these animals which are persistently infected with Aleutian disease virus, the antibody formed in response to the infection, although large in quantity, is unable to neutralise the virus and thus is unable to clear the virus from the animal. This state of affairs leads to the generation of large amounts of virus-antibody immune complexes which induce chronic glomerulonephritis. A similar mechanism probably operates

in lactic dehydrogenase virus infection of mice (Cochrane and Koffler, 1973).

In chronic lymphocytic choriomeningitis infection in mice, animals in whom a carrier state is induced by neonatal infection with the virus, produce insufficient antibody to eradicate the virus but sufficient to form circulating immune complexes which in turn induce a chronic immune-complex glomerulonephritis (Oldstone and Dixon, 1968, 1969).

PATHOGENESIS OF HUMAN GLOMERULONEPHRITIS

Both known pathogenetic mechanisms of glomerulonephritis (anti-GBM antibody and deposition of immune complexes) may produce either mild, slowly progressive or rapidly progressive disease in humans.

1 : Human Anti-GBM Glomerulonephritis

Approximately 5% of cases of human glomerulonephritis are due to anti-glomerular basement membrane antibody (Dixon, 1968; McCluskey, 1971).

(a) Goodpasture's Syndrome

It has been clearly established that anti-GBM antibody is responsible for Goodpasture's syndrome, a disease manifested by a proliferative glomerulonephritis and pulmonary haemorrhage. Linear deposition of immunoglobulin along the GBM of the kidney is seen, antibody to GBM can be eluted from the kidney (Lerner, Glassock and Dixon, 1967) and anti-GBM antibody can be demonstrated in the patients' serum

especially after bilateral nephrectomy. Antibody eluted from the kidney when injected into normal squirrel monkeys, will fix along the GBM and induce a severe proliferative glomerulonephritis (Lerner et al, 1967).

The cause of the lung haemorrhage is uncertain. In some but not all patients antibody can be demonstrated to be fixed along the lung basement membranes. In one case antibody eluted from the lungs induced a severe crescentic glomerulonephritis when injected into a monkey (Koffler et al, 1969).

More recently removal of circulating anti-GBM antibody in patients with Goodpasture's syndrome, by repeated plasmapheresces,has been shown to be associated with recovery from both the renal and pulmonary manifestations of the disease (Lockwood et al, 1975).

The initial stimulus to the production of autoantibodies against GBM is unknown. Either endogenous antigens or exogenous antigens cross reacting with GBM may be the causative agent. The finding of GBM antigens in the urine of normal animals and man, and the nephritogenicity of these antigens when injected into the animal from which they came, suggest that such urinary antigens may be the immunogens in human anti-GBM disease (Dixon, 1968). It is possible that primary damage to pulmonary GBM may induce antibody formation against GBM. In this latter context reports of Goodpasture's syndrome following exposure to hydrocarbon solvents may be relevant (Beirne and Brennan, 1972).

(b) Anti-GBM Glomerulonephritis Without Lung Disease

In a proportion of patients with rapidly progressive glomerulonephritis, linear deposition of immunoglobulin along the GBM may be

found (Wilson and Dixon, 1973; Sissons et al, 1974). Renal elution studies in these patients have shown that the eluted antibody has less cross-reactivity against lung basement membrane than in Goodpasture's syndrome (McPhaul and Dixon, 1970).

2 : Human Immune-Complex Glomerulonephritis

Granular deposition of immunoglobulins along the GBM or in the mesangium and often accompanied by complement deposition is considered the hallmark of immune-complex glomerulonephritis. Although glomerular immune-complex deposition is thought to account for the majority of cases of human glomerulonephritis (Dixon, 1968; McCluskey, 1971; Berger, Yaneva and Hinglais, 1971) the responsible antigen has been identified in only a minority of cases. In other situations, although the antigen has not been positively identified, strong inferential evidence suggests the identity of the antigen. In the remainder, the diagnosis of chronic immune-complex glomerulonephritis rests on the findings on immunofluorescence. The antigen may be endogenous such as DNA, thyroglobulin and tumour antigens, or exogenous such as infectious agents, foreign proteins or drugs.

(a) Glomerulonephritis of Bacterial Endocarditis

It has long been recognised that glomerulonephritis may occur in patients with bacterial endocarditis (Löhlein, 1910). Granular deposits of immunoglobulin and complement are found in the glomeruli (Morel-Maroger et al, 1972). Other findings suggesting an immune-complex basis to this disease include activation of complement via the classical

pathway (Boulton-Jones et al, 1974), the elution from the kidney of antigens from the infective organism (Gutman et al, 1972) and the detection of cryoglobulinaemia and rheumatoid factor in the serum (Williams and Kunkel, 1962). Similar evidence of immune-complex disease exists in children with glomerulonephritis associated with infected ventriculo-atrial shunts (Stickler et al, 1968).

(b) Poststreptococcal Glomerulonephritis

Evidence for an immune-complex disease includes hypocomplementaemia, granular deposits of immunoglobulin and C3 in the kidney, cryoglobulinaemia (Adam, Morel-Maroger and Richet, 1973) and the demonstration of streptococcal antigens in the kidney early in the disease (Treser et al, 1969).

(c) Immune-Complex Glomerulonephritis Associated With Virus Infection

Cases of glomerulonephritis with granular deposits of immunoglobulin in the kidney have been reported in infectious mononucleosis, chicken pox, mumps and hepatitis. Occasionally Australia-antigen and immunoglobulin have been identified in kidneys (Combes et al, 1971).

(d) Glomerulonephritis Of Systemic Lupus Erythematosus

Immune-complexes are strongly implicated in this disease; granular immunoglobulin and complement deposits in the kidney, elution of anti-DNA antibody from the kidney (Koffler, Schur and Kunkel, 1967), cryglobulinaemia (Adam et al, 1973) and hypocomplementaemia are well described.

(e) Malarial Nephropathy

The evidence for an immune-complex aetiology to this disease rests upon the immunofluorescence findings, the identification of plasmodium malariae antigen in the glomerular deposits (Ward and Kibukamusoke, 1969) and its reduction in frequency when malaria is eradicated (Kibukamusoke, 1973). However, unlike other infective causes of immune-complex glomerulonephritis the disease in an individual patient is unaffected by eradication of the parasite.

(f) Tumour Associated Immune-Complex Glomerulonephritis

In patients with reticuloses who develop the nephrotic syndrome, evidence for an immune-complex aetiology of the renal disease is usually lacking ; antibody and complement are not found in the kidney (Froom et al, 1973; Carpenter and Weiss, 1973). However, in patients with carcinoma (especially of the bronchus) who develop the nephrotic syndrome, intramembranous deposits of immunoglobulin in the kidney are often seen. In one of these latter patients antibody eluted from the kidney reacted with the tumour cells (Loughridge and Lewis, 1971).

(g) Mesangial IgA Disease And Henoch-Schönlein Nephritis

The finding of C3 and properdin deposition in conjunction with the IgA in these diseases (Evans et al, 1973) suggests an immune-complex disease. The association of the episodes of haematuria with pharyngeal infections, and the finding of IgA have led to the speculation that the glomerulonephritis is a virus-induced immune-complex disease.

3 : Glomerulonephritis In Which There Is Little Or No Evidence For An Anti-GBM Or Immune-Complex Pathogenesis

In up to 30% of cases of glomerulonephritis neither immunoglobulin nor complement is found in the glomeruli(eg "minimal change nephrotic syndrome", and some cases of rapidly progressive glomerulonephritis). In others, only complement may be detected (eg "dense deposit" variety of membranoproliferative glomerulonephritis).

Suggested pathogenetic mechanisms of glomerulonephritis in which antibody is not found include :

(i) Immune-complex disease in which the complexes have been removed by the time of diagnosis of the disease.

(ii) Cell mediated immunity. Evidence for cell mediated reactivity against GBM-antigens has been found in many patients with proliferative glomerulonephritis (Rocklin, Lewis and David, 1970; Zabriskie et al, 1970; Dardenne, Zabriskie and Bach, 1972; Macanovic, Evans and Peters, 1972). However, this may simply reflect glomerular damage by other mechanisms.

(iii) Non-immunological mechanisms such as toxins. There is no evidence at all to support this concept, except that the kidney is a major site of excretion of toxic substances.

(iv) Persistent complement activation. The association of persistent hypocomplementaemia with membranoproliferative glomerulonephritis

was recognised by West and colleagues in 1965. The hypocomplementaemia is probably induced by activation of the alternative pathway of C3 activation, by a circulating factor called C3 nephritic factor (C3NeF) (Spitzer et al, 1969; Peters et al, 1972). In patients with partial lipodystrophy such C3 activation with persistent hypocomplementaemia has been shown to precede the development of the glomerulonephritis (Peters et al, 1973; Peters and Williams, 1974). One possible conclusion from these observations is that persistent C activation induces the glomerulonephritis and is supported by the demonstration of C3 receptors in human glomeruli (Gelfand et al, 1975). However, in experiments in which persistent C activation was induced in rabbits with repeated injections of inulin (Verroust, 1972 : Personal Communication) and in B-depleted mice with repeated injections of cobra venom factor (Simpson, 1975 : Personal Communication), glomerular disease could not be induced. A more likely explanation for glomerulonephritis in patients with hypocomplementaemia due to persistent C activation is that increased susceptibility to infection due to complement deficiency is responsible for an immune-complex nephritis.

Immunity Deficiency And The Aetiology Of Immune-Complex Glomerulonephritis

The concept that patients may develop immune-complex glomerulonephritis because of immunity deficiency has largely originated from the recognition of the association between immune-complex disease and complement deficiency. Such immune deficiency would not be so severe

that overt infections result but nevertheless would allow persistence of an infectious organism or infectious organism-derived antigens within the body and hence predispose to immune-complex disease. Failure of immunological defenses might arise in several ways (Peters and Lachmann, 1974).

(a) Low-Affinity Antibodies

The production of low-affinity antibody would be poor at effecting immune elimination of a virus, allowing its replication in the presence of antibody and leading to immune-complex disease. This mechanism probably underlies lymphocytic-choriomeningitis virus glomerulonephritis in mice.(Soothill and Steward, 1971; Alper et al, 1972)

(b) Antibodies To "Wrong Antigens"

Situations where antibody is largely directed against antigens not conferring immunity could result in immune-complex disease.

(c) Complement Deficiency

Patients with either hereditary isolated complement component deficiencies or hypocomplementaemia due to chronic complement activation, have a high incidence of immune-complex diseases especially SLE-like illness and glomerulonephritis (Day et al, 1973; Mancado et al, 1971). Immune adherence, a consequence of complement activation is an important mechanism of removal of micro-organisms from the body.

(d) Inability To Mount A Specific T-Cell Response

In mice and guinea-pigs the capacity to mount a T-cell response to certain antigens is controlled genetically by immune-response (IR) genes

(McDevitt and Benacerraf, 1969). The possibility that a particular genetic soil predisposes to persistent virus infection is a real one.

(e) General T-Cell Deficiency

Normal T lymphocyte responses are required for the eradication of certain virus infections and syndromes of general T-cell deficiency are associated with a tendency to prolonged and unusual virus infections (Fulginitti, 1968).

(f) "Inaccessible" Infection

The persistence of an infectious organism within the body may be explained on mechanical reasons (eg poor blood supply, as is postulated in subacute bacterial endocarditis).

MEDIATION OF INJURY IN GLOMERULONEPHRITIS

Once an immune reaction has occurred in the kidney, be it the deposition of immune complexes or interaction of anti-GBM antibody with GBM or other immunological events as yet undefined, injury to the glomerulus results through activation of host factors by the immune reaction. In the heterologous phase of nephrotoxic nephritis the sequence of events leading to glomerular injury has been quite well defined. However, in immune-complex disease in animals and in glomerulonephritis in man, only limited information is available on the mediation of injury. Mediators that have been studied are particularly those which may be identified in the glomeruli of nephritic kidneys ; such as complement, polymorphonuclear leucocytes, fibrin-fibrinogen, and platelets.

The investigation of mediation of injury has been aided by :

(a) the identification of a particular mediator in the glomerulus

(b) depletion of the mediator from the system

(c) measurement in the urine of products of the mediator (eg lysosomal enzymes from polymorphonuclear leucocytes and fibrin degradation products)

(d) quantitative assessment of injury in the glomerulus (eg by measuring glomerular filtration rate or proteinuria).

1 : Complement

The activation of the complement cascade by antibody-antigen interaction is an important step in the mediation of injury. When complement is activated by either the classical or alternative pathway, biologically active products of several of the complement components are generated. These products are capable of inducing tissue injury by various mechanisms :

(a) C3b, generated from the "bulk" component of complement, C3, is responsible for "immune adherence", the process by which polymorphonuclear leucocytes (PMN), macrophages and in some species platelets adhere to the site of complement fixation

(b) C5a and C3a are anaphylatoxins and thus have the capacity to release mediators from mast cells (Silva, Eisele and Lepow, 1967)

(c) C5a is also a potent chemotactic factor (Snyderman and Mergen-

hagen, 1972) as is the trimolecular complex $C\overline{567}$ (Ward, Cochrane and Müller-Eberhard, 1966). Late acting complement components C6-C9 appear to be of little or no importance in mediation of allergic glomerular damage.

The role of complement in glomerulonephritis has been studied : (a) by depletion of complement components with either aggregated immunoglobulin or zymozan or cobra venom factor (CVF) and (b) by examining injury induced by antibody that does not fix complement. Aggregated immunoglobulin depletes C1 and C4 and to a lesser extent C2 and C3 (Ward and Cochrane, 1965). The anti-complementary activity of cobra venom was first described by Flexner and Noguchi (1903). In the presence of C3 proactivator convertase (C3 PAse or Factor $\overline{D}$) and Mg^{++}, CVF interacts with the C3 proactivator (C3 PA or Factor $\overline{B}$) of the alternative pathway. The final "CVF-C3 Activator" complex splits C3 (Müller-Eberhard, 1974). Whereas aggregated immunoglobulin or zymozan will deplete C components for only 12-24 hours, CVF acts for 5-6 days. Repeated doses of CVF cannot be used because of the invariable generation of antibodies to CVF.

Rats decomplemented by aggregated immunoglobulin or zymozan (Kurtz and Donnell, 1962; Hammer and Dixon, 1963; Unanue and Dixon, 1964; Cochrane, Unanue and Dixon, 1965) and rabbits decomplemented with CVF (Cochrane, Müller-Eberhard and Aikin, 1970) develop only mild proteinuria and show no glomerular PMN exudation in the heterologous phase of NTN. By contrast, although the necrotising arteritis of acute serum sickness is largely prevented by decomplementation with CVF, the glomerulonephritis is not (Henson and Cochrane, 1971). In the

glomerular lesions of acute serum sickness, PMN infiltration is not usually prominent in contrast to the vasculitis in which PMN are abundant.

Digestion of nephrotoxic globulin with pepsin results in a divalent fragment, $F(ab')_2$, which retains antigen binding capacity but has no complement fixing capacity. When $F(ab')_2$ nephrotoxic antibody is injected into rabbits only mild proteinuria results (Baxter and Small, 1963). Similarly, Henson (1971 a) demonstrated that the $\gamma 2$ fraction of sheep antibody to GBM (the non complement fixing fraction) induces considerably less proteinuria than the $\gamma 1$ fraction (the complement fixing fraction).

However, avian antibody to GBM is capable of inducing proteinuria in the heterologous phase despite being a non complement fixing antibody. Simpson et al (1975) have shown that in the heterologous phase of NTN in guinea-pigs the induction of severe proteinuria does not require complement or PMN. Moreover severe proteinuria is also induced with the $F(ab')_2$ portion of the nephrotoxic antibody. In this latter situation it seems that antibody alone can mediate injury.

Thus it can be seen that in the heterologous phase of NTN the induction of injury may be complement-dependent or non complement-dependent, depending on the species in which the NTS is produced and the species in which the disease is induced. The mechanism by which complement induces injury is dependent on the consequent infiltration by PMN (See "Mediation of Injury by PMN").

The role of complement in the autologous phase of injury in NTN has not been previously studied, but will be considered in Chapter 8 of

of this thesis.

In humans the evidence for a role of complement in mediation of glomerular injury rests upon the frequent demonstration of complement in the glomeruli. The possibility that injury may result from non complement-dependent mechanisms is suggested by the finding that about 25% of patients with Goodpasture's syndrome have no detectable C3 in the glomeruli (Wilson and Dixon, 1973; Sissons et al, 1974).

2 : Polymorphonuclear Leucocytes

Polymorphonuclear leucocytes (PMN) accumulate at the sites of the antigen-antibody reaction in various types of allergic tissue injury and lead to severe tissue damage, fibrin deposition and scarring (Cochrane, 1968). They are prominent in the vasculitis of the Arthus reaction, and in the necrotising arteritis of acute serum sickness. Extensive glomerular infiltration by PMN is seen in the heterologous phase of NTN in rabbits and rats. In the autologous phase less obvious PMN accumulation in the glomeruli occurs (Ehrich, Forman and Seifter, 1972). Similar infiltration may be seen in experimental chronic immune-complex glomerulonephritis. Varying degrees of PMN accumulation occur in human poststreptococcal, Henoch-Schonlein, rapidly progressive, and membranoproliferative glomerulonephritis as well as in the proliferative nephritis of Goodpasture's syndrome, Systemic Lupus Erythematosus and polyarteritis (Heptinstall, 1974).

In the heterologous phase of NTN the PMN are seen to gain intimate access to the antibody and complement along the basement membrane, stripping off the endothelial cytoplasm from the GBM in the process

(Cochrane et al, 1965). Similar close apposition of PMN with the GBM, often in association with distortion, thinning and breaks in the GBM has been noted in electronmicroscopical studies of human acute glomerulonephritis (Burkholder, 1969; Strunk, Hammond and Benditt, 1964; Morita, Wenzl and Kimmelsteil, 1971), Goodpasture's syndrome (Duncan et al, 1965; Germuth et al, 1972), membranoproliferative glomerulonephritis and rapidly progressive glomerulonephritis (Clarkson and Seymour, 1975 : Personal Communication).

PMN are the source of numerous potentially damaging enzymes and other proteins, particularly contained within their lysosomal granules (Cohn and Hirsh, 1960; Weissman, 1967). Although the usual role of these enzymes is intracellular digestion they may be actively released from the PMN by immunological stimuli (Henson, 1971 b; Hawkins, 1972; Weissman, Zurier and Hoffstein, 1972). This release is through a direct degranulation to the outside of the cell. It is postulated that the external cell membrane is stimulated by adherence to the fixed antibody, such that granules fuse with it and discharge to the outside as if to a phagocytic vacuole. Release of lysosomal contents is dependent on intracellular cyclic-AMP concentrations (Weissman et al, 1972). Substances raising cyclic-AMP concentrations such as protoglandins E1 and theophylline inhibit lysosomal release. Lysosomal enzymes produce tissue injury either directly by proteolytic digestion of connective tissue elements (Hawkins and Cochrane, 1968;Cochrane and Aiken, 1966) or indirectly by acting as mediators in the release of other injurious agents such as vasoactive amines from mast cells or platelets (Seegers and Janoff, 1966; Henson, 1970;Henson, 1972). PMN have also been shown to release procoagulant material (Rapoport and Hiort, 1967). Kociba, Loeb and Wall (1972)

demonstrated this material in secretion from peritoneal PMN and that it has the characteristics of tissue thromboplastin.

The mechanism by which PMN are attracted to the glomeruli has in general been thought to depend on complement activity. Ward and Cochrane (1965) and Henson and Cochrane (1971) have shown that glomerular PMN accumulation does not occur in complement depleted animals during the heterologous phase of NTN. Whether PMN accumulation is the result of liberation of chemotactic factors (C3a, C5a, $\overline{C567}$) or the result of binding of passing PMN to fixed C3 (the process of immune adherence), is not yet clear.

The role of the PMN in the mediation of injury in the heterologous phase of NTN has been well established. It has been shown that the removal of circulating PMN by either nitrogen mustard or specific anti-polymorph serum (APS) will markedly reduce proteinuria in the heterologous phase to levels similar to those obtained by decomplementation (Cochrane, Unanue and Dixon, 1965; Hawkins and Cochrane, 1968; Henson, 1971 a). In normal rabbit urine two basement membrane antigens appear in low concentration. During the PMN attack these normally excreted GBM antigens appear in the urine in much greater concentration and in addition, unique GBM fragments are also found (Hawkins and Cochrane, 1968). Associated with GBM fragment excretion in the urine is the excretion of PMN catheptic enzymes. That the GBM has been extensively damaged is shown by the non selective character of the proteinuria. In PMN depleted animals, GBM fragments in the urine are not increased, PMN enzymes are not found in the urine and proteinuria is highly selective.

In acute immune-complex glomerulonephritis proteinuria is not reduced by PMN depletion. This is in keeping with the lack of protection from complement depletion and that PMN are not prominent in the glomeruli in this type of nephritis (Henson and Cochrane, 1971).

The role of PMN in the autologous phase of NTN has not previously been investigated. Experiments relating to this are described in Chapter 7 of this thesis.

3 : Fibrin Deposition In The Mediation Of Glomerular Injury

The suggestion that coagulation might be important in the mediation of glomerular injury first came from observations that in some cases of rapidly progressive glomerulonephritis in man and experimental animals, material with the staining properties of fibrin could be found in the glomeruli. Immunofluorescence has confirmed the findings and electron-microscopy has revealed a fibrillary structure of the deposits with the periodicity of fibrin. Subsequently, fibrin deposition has been described in many other types of human glomerulonephritis including membrano-proliferative glomerulonephritis, progressive focal glomerulonephritis, lupus glomerulonephritis, and Henoch-Schönlein purpura (Morel-Maroger, Leathem, Richet, 1972; Wilson and Dixon, 1974). Strictly speaking it is often not possible to accurately determine whether this material is fibrinogen or breakdown products of fibrinogen and fibrin and the term fibrinogen-related antigens has therefore been used.

Glomerular fibrin deposition may be found mainly ; (a) within capillaries (b) within the mesangium and endothelial cells and (c) outside the glomerular tufts in Bowman's space. Proliferative glomerulonephritis

is particularly associated with the latter two patterns of fibrin deposition. The swelling and proliferation of endothelial and mesangial cells are mainly the consequence of phagocytosis of materials formed during the clotting process (Vassalli, Simon and Rouiller, 1963). Fibrin deposition within Bowman's space is a feature of extracapillary cell proliferation with crescent formation. Crescent formation has usually been explained on the basis of extracapillary cell proliferation in response to fibrin deposition in Bowman's space.

The role of fibrin in the mediation of glomerular injury has been further highlighted by the demonstration of fibrin-fibrinogen breakdown products in the serum and urine of patients with proliferative glomerulonephritis and the correlation of severity and activity of the disease with the urinary levels of breakdown products (Clarkson et al, 1971).

The mechanisms by which glomerular fibrin deposition occurs are not well elucidated. Possible mechanisms include the release of procoagulant factors from platelets by antigen-antibody reaction, the activation of Hagerman factor XII by immune complexes, activation of coagulation by damaged GBM and the release of tissue thromboplastin material from PMN (Wardle, 1974; Kociba, Loeb and Wall, 1972). Failure of removal of deposited fibrin either through inhibition of fibrinolysis or reticuloendothelial dysfunction may be another factor (Wardle, 1974).

The importance of fibrin in the mediation of experimental glomerular injury has mainly been studied by observing the effects of anticoagulation with heparin or warfarin or defibrination with ancrod. Heparin inhibits

coagulation by its inhibitory effect on clotting factors II, X (Biggs and Denson, 1972), IX, and XI (Damus, Hicks and Rosenberg, 1973), and warfarin by reducing synthesis of factors II, VII, IX, and X in the liver (Biggs, 1972). Ancrod (Arvin) is a purified coagulant enzyme which can be isolated from the crude venom of the Malayan Pit Viper (Agkistrodon Rhodostoma) (Esnouf and Tunnah, 1967). Ancrod acts directly on fibrinogen, splitting off peptide A, and transforming the fibrinogen molecule into a defective monomer which is however capable of polymerisation and gel formation. In vivo, microclots are formed which are trapped in capillaries and undergo rapid fibrinolysis (Regoeczi, Gergely and MacFarlane, 1966). By this mechanism infusion of ancrod causes a more or less complete disappearance of fibrinogen from the circulation and therein lies its anticoagulant capacity.

Kleinerman (1954) administered massive doses of heparin (3,000-4,000 u/Kg/day) before and during the autologous phase of NTN in rabbits. He was able to show considerable protection from crescent formation and renal failure in heparinised animals. However, proteinuria was unaffected and anticoagulation had no effect if started after glomerular injury had commenced. Vassalli and McCluskey (1964) were able to induce similar protection in NTN in rabbits by anticoagulation with warfarin. They also were unable to effect proteinuria. Halpern et al (1965) confirmed the protection from heparin in massive doses given before the onset of the autologous phase of NTN in rabbits and were also unable to show protection when heparin was given after injury. Heparin has been shown to have no protective effect in experimental nephritis in which glomerular fibrin deposition is not found (Baliah and Drummond,

1972). This later finding suggests that the protective effect of heparin is specifically related to its anticoagulant action and not its anti-inflammatory and anti-complementary properties. Defibrination of the blood with ancrod has been shown to provide quite remarkable protection from glomerular fibrin deposition, crescent formation and renal failure in NTN in rabbits (Naish et al, 1972). Anticoagulation with heparin or ancrod has not been studied in experimental crescentic glomerulonephritis due to chronic immune-complex disease.

Glomerular fibrin deposition probably induces renal damage by a number of mechanisms. These include physical obstruction in the glomerular capillary wall and in Bowman's space and the induction of extracapillary cell proliferation which then in turn "strangles" the glomerulus.

Summary Of The Known Pathways of Mediation Of Glomerular Injury In Experimental Glomerulonephritis

Complement-Dependent Polymorph-Mediated Injury has been well characterised in the heterologous phase of NTN in rabbits and rats when complement fixing nephrotoxic antibody is used.

Injury Independent Of Complement And Polymorphs operates in the heterologous phase of NTN in the guinea-pig, in the same disease in rabbits and in rats when non complement fixing avian nephrotoxic antibody is used and probably in the renal injury of acute serum sickness.

Injury Dependent on Glomerular Fibrin Deposition has been shown to occur in the crescentic glomerulonephritis of NTN. The mediation of the

glomerular fibrin deposition has not been elucidated.

PART II

MATERIALS AND METHODS

Chapter 3

MATERIALS AND METHODS

ANIMALS

Rabbits

Partially inbred male New Zealand White (NZW) rabbits (Froxfield Rabbit Company, Froxfield, Hants., UK) weighing between 2.0 and 2.5 Kg were used for studies of nephrotoxic nephritis and bovine serum albumen induced-chronic immune-complex nephritis and for the production of various antisera.

Rabbits congenitally deficient in the sixth component of complement (C6) (Cambridge strain) and weighing between 2.0 and 3.0 Kg were used for studies of nephrotoxic nephritis in C6-deficient rabbits. The animals were supplied by Professor P.J. Lachmann and the isolated deficiency of C6 had been confirmed in all animals (Lachmann, 1970).

All rabbits were fed on a normal water and pellet diet. They were kept in metabolic cages when 24-hour urine collections were required. Operative procedures such as renal biopsy and harvest of peritoneal polymorphonuclear leucocytes were performed under anaesthesia, 30 mg/Kg body weight (BW) of sodium pentobarbitone (Nembutal - Abbott) being given intravenously (IV). Intravenous injections were given through the marginal ear vein. After obtaining maximal dilatation of the ear vein by placing one drop of xylene on the ear, rabbits were bled by partly cutting the marginal ear vein with a scalpel blade. Random urine samples were obtained by urethral catheterisation with an infant nasogastric feeding tube. Twenty-four hour urine samples were collected by the

following method : The bladder was emptied at zero hour by urethral catheterisation and this specimen discarded. The animal was then placed in a metabolic cage, given free water and food, and all urine passed in the next 24 hours, including that obtained by urethral catheterisation at 24 hours, was collected.

Rabbits were killed either by a lethal dose (90 mg/Kg BW) of Nembutal given IV, or by anaesthesia and then exsanguination by cardiac puncture.

Sheep

Sheep supplied by and housed at Northumberland Hall Veterinary College, Potters Bar, Herts., UK were used for the production of anti-rabbit glomerular basement membrane antiserum.

Goats

British Saanen goats, supplied by and housed at the National Institute for Research into Dairying, Shinfield, UK, were used for the production of anti-rabbit polymorphonuclear leucocyte antiserum.

Guinea-pigs

Outbred Duncan-Hartley guinea-pigs were used to raise anti-rabbit fibrin-fibrinogen antiserum.

COLLECTION AND STORAGE OF SERUM SAMPLES

After the bleeding of an animal into a glass container, the blood was allowed to clot at room temperature for 2 hours and then centrifuged at 2000 RPM for 10 minutes, the serum then being removed. As a preservative 0.01% sodium azide was added to any serum sample that was not to be analysed immediately and the serum was then stored at -20°C until used.

MEASUREMENT OF SERUM CREATININE AND UREA CONCENTRATIONS

These were measured by the autoanalyser technique in the Biochemistry Department of the Artificial Kidney Unit of Hammersmith Hospital.

MEASUREMENT OF SERUM C3 CONCENTRATIONS

Serum C3 was measured by radial immunodiffusion as described in "Radial Immunodiffusion".

BLOOD LEUCOCYTE COUNTS AND DIFFERENTIAL COUNTS

Total leucocyte counts were measured electronically by the Coulter Counter method (Akeroyd et al, 1959). Differential leucocyte counts were made on a May-Grunwald-Giemsa stained smear.

BLOOD PLATELET COUNTS

Platelet counts were measured electronically by the Coulter Counter method (Eastham, 1967).

ESTIMATION OF PROTEIN CONCENTRATION BY THE FOLIN PHENOL REAGENT (Lowry et al, 1951)

Aliquots of 50, 100 and 200 microlitres of the test solution were made up to 1.0 ml with distilled water. For standards, 20, 50, 100, 150 and 200 micrograms of BSA were each dissolved in 1.0 ml of distilled water.

To each sample 3.0 ml of Folin A solution (1.0 ml 1% copper sulphate, 1.0 ml 2% sodium potassium tartrate, 100 ml 2% sodium carbonate in 0.1 N sodium hydroxide) were added, and the mixture allowed to stand at room temperature for 10 minutes. Then 0.3 ml of Folin B solution (Folin and Ciocalteu's phenol reagent diluted 1 in 2 in distilled water) was added and the mixture left at room temperature for a further 30 minutes.

Optical density of the samples was read on an SP 505 spectrophotometer at a wavelength of 750 mμ and the values for the standards plotted, and the protein concentration of the test solution read from the curve.

ESTIMATION OF PROTEIN CONCENTRATION IN URINE BY THE BIURET TECHNIQUE

Urine samples were centrifuges at 1,600 RPM for 10 minutes to remove insoluble material. In 10 ml test tubes, 1 ml of 20% Trichloracetic acid (TCA) was added to 1 ml of each urine sample and to 1ml of standard protein solutions (BSA solutions of concentrations 40, 20, 10, 5, 1, 0.5 and 0.1 mg/ml). After mixing,the samples were centrifuged at 3,000 RPM for 20 minutes. The supernatants were thoroughly drained

by inverting the tubes for 30 minutes. The protein precipitate was completely dissolved in 0.5 ml of N sodium hydroxide, and after adding 2.5 ml of distilled water and 5 ml of Biuret reagent the solution was mixed and left to stand for 30 minutes. The optical densities for the samples were read on an SP 505 spectrophotometer at a wavelength of 750 mμ, a solution of 3 ml of distilled water and 5 ml of Biuret reagent being used for zero optical density. The values for the standards were plotted and the protein concentrations of the urine samples read from the line.

DOUBLE IMMUNODIFFUSION

In this technique, developed by Ouchterlony (1953; 1958), 1.5% agarose (BDH Biochemicals, UK) in saline was melted, cooled to about 60°C and then poured on to a glass plate and allowed to set. A central well of 5 mm diameter and surrounding wells of 3, 4, 5, 6, 8 and 9 mm were cut, the periphery of the central well being 4 mm distant from the periphery of each surrounding well. The central well was filled with the required antiserum and the surrounding wells with the antigen containing reagent. Diffusion and development took place in a humid chamber at constant room temperature, the plate being examined at 24, 48 and 72 hours for the presence of precipitin lines.

RADIAL IMMUNODIFFUSION

This technique as described by Mancini, Carbonara and Heremans (1965) was used to measure serum concentrations of rabbit C3 and anti-BSA antibodies. Ten ml of 1.5% agarose containing either 0.4 ml of goat

anti-rabbit C3 antiserum, or 0.25 mg of BSA, were placed on to a glass slide 3.25 cm x 3.25 cm. Wells of either 2 mm (C3 plates) or 3 mm (anti-BSA plates) diameter were cut and filled with 5 or 10 microlitres respectively of the serum sample. A range of normal values was provided by measuring on the same plate, either a pooled sample of normal rabbit serum (for C3 estimation) or a sample of anti-BSA antiserum of known antibody concentration (for anti-BSA estimation), undiluted and at dilutions of 75%, 50%, 25%, 10% and 5%. After filling the wells the plates were left in a humid environment at room temperature and precipitation allowed to occur. The diameter of the precipitin rings was read with a graduated magnifying glass, and the values for the control serum and its dilutions were plotted against the dilution percentage, the latter values being on a logarithmic scale. The concentration of C3 or anti-BSA in the test serum was read from this line, and expressed as a percentage of the normal serum pool (C3) or as mg/ml of antibody (anti-BSA). All measurements were made in duplicate and the mean value used as the final result.

PREPARATION OF RABBIT GLOMERULAR BASEMENT MEMBRANE (GBM)

(a) Isolation of Glomeruli

The isolation procedure was based on the method of Krakower and Greenspon (1951) as modified by Spiro (1967 a) and Westberg and Michael (1970). Kidneys were removed from normal rabbits, stripped of their capsule and fat and stored at -40°C. Thirty frozen kidneys were almost

completely thawed in a plastic bag immersed in water (22-24°C). The medulla was dissected from the cortex and discarded. The cortex was then minced in a household meat mincer.

Minced cortex was transferred to a 100-mesh (pore size 150 microns) stainless steel sieve (Endecotts - Test Sieves - Ltd.), and forced through the sieve holes with the bottom of a glass beaker. The sieve was repeatedly brushed with a wire brush and washed with cold 0.85% sodium chloride solution, to clear obstructed pores. Material was forced through until only pale tissue (connective tissue and medulla) was left on the sieve. The sieved suspension was then poured through a 60-mesh sieve (pore size 250 microns) which retained larger tissue fragments such as tubules that had passed through the 100-mesh sieve end on. The sieved suspension was finally passed through a 250-mesh sieve (pore size 63 microns). The material retained by this latter sieve contained the glomeruli. The retained glomeruli were repeatedly washed with cold 0.85% sodium chloride until nothing but glomeruli were seen, when a sample was examined by phase contrast microscopy. The glomeruli were washed from the sieve into a beaker, with streams of cold 0.85% sodium chloride and then transferred to 50 ml plastic centrifuge tubes. The suspension was centrifuged at 1,500 RPM for 10 minutes. The sediment was washed four times in distilled water with centrifugations at 2,800 RPM for 10 minutes. The sediment was again examined by phase contrast microscopy to confirm the purity of the glomeruli.

(b) Preparation Of Particulate GBM

The glomerular sediment was suspended in ice-cold 1 M sodium

chloride of volume twenty times the sediment. The glomeruli were sonicated in 30 ml portions in 50 ml beakers immersed in an ice bath, using an MSE Ultrasonicator Disintegrator Model 150 W, with a 9 mm stainless steel probe. Bursts of sonication of 30 seconds were given with 1-minute periods of cooling of the probe in ice water between bursts. After a total sonication time of 10 minutes, and allowing several minutes for the glomeruli to settle, the suspension was examined under the phase contrast microscope to see if all glomeruli had been disrupted. Sonication was repeated until all glomeruli had been disrupted.

The suspension was finally passed through a 250-mesh sieve to remove non-disrupted glomeruli and tubule fragments. The filtrate was transferred to 50 ml tubes and centrifuged at 3,000 RPM for 15 minutes. The sediment was suspended in 1 M sodium chloride and washed and centrifuged three times. The sediment was then suspended in 50 ml of distilled water, washed and centrifuged four times at 3,000 RPM for 5 minutes. The sediment was finally suspended in 4 ml of distilled water, lypholysed and stored at -70^{o}C until used as particulate GBM or solubilised with collagenase.

(c) Preparation Of Soluble GBM

Particulate GBM was solubilised by proteolytic enzyme (Collagenase - Sigma Chemical Company, St. Louis, USA) by the method of Spiro (1967 b) with the modification that the incubation with the enzyme was prolonged to 5 days and not 3. The digestion was performed in 0.01 M Tris acetate-buffer (pH 7.4), in the presence of 0.005 M calcium

acetate at 37°C. The particulate GBM was suspended in tris buffer (25 mg/ml). Collagenase was added initially in a quantity of 0.7 mg per 100 mg of GBM. At 24, 48, 72 and 96 hours, further enzyme in quantities of 35 mg, 10 mg, 10 mg, 10 mg per 100 mg of GBM respectively was added. A crystal of thymol was added at the beginning of the incubation to prevent bacterial growth. During the period of incubation the mixture was constantly stirred. On the sixth day the suspension was centrifuged at 2,000 RPM for 30 minutes. The supernatant containing the soluble GBM was removed, heated at 60°C for 30 minutes and stored at -20°C until used. The concentration of soluble GBM in the supernatant was determined by the folin phenol reagent (Lowry et al, 1951).

PREPARATION, PURIFICATION, AND EVALUATION OF SHEEP ANTI-RABBIT GBM ANTISERUM (NEPHROTOXIC SERUM)

(a) Preparation Of Nephrotoxic Serum (NTS)

Sheep were immunised with rabbit GBM by the method of Steblay (1962). Twenty-five mg of particulate rabbit GBM were incorporated into Freund's complete adjuvant (CFA - Difco Laboratories, USA) and injected intramuscularly into a sheep every two weeks. Blood samples were taken from the sheep at weekly intervals for estimation of the anti-rabbit GBM antibody titre (see "Haemagglutination assay of anti-rabbit GBM antibody"). After a total of ten injections of GBM, a titre of $\frac{1}{4 \times 10^6}$ of antibody to rabbit GBM was obtained (Fig. 3.1). The sheep was bled out and killed. At death the animal's serum creatinine had risen to 3.7 mg/100 ml and its kidneys showed a severe crescentic glomerulonephritis with extensive fibrin deposition and C3 and sheep

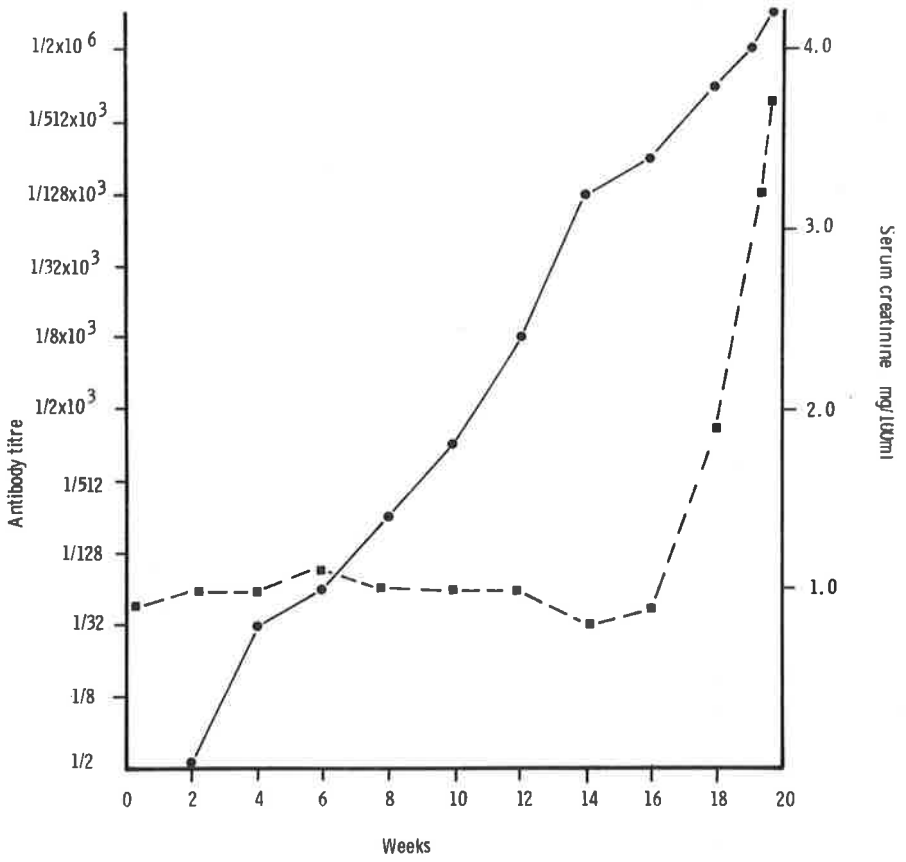

Fig. 3.1. Anti-rabbit GBM antibody titres (●) and serum creatinine concentrations (■) in a sheep repeatedly injected with rabbit GBM in FCA.

immunoglobulin were found deposited in a linear fashion along the GBM. The serum obtained after standing the clotted blood for 2 hours at room temperature was stored in 25 ml aliquots at -40°C, with the addition of 0.01% sodium azide as a preservative.

The crude NTS contained a titre of 1/512 of agglutinating antibody to rabbit erythrocytes and showed a single precipitin line against rabbit serum on double diffusion in gel. After decomplementing the NTS by heating at 56°C for 30 minutes, the NTS was absorbed against normal rabbit erythrocytes (4 : 1 V/V) and optimal proportions of normal rabbit serum (15 : 1 V/V). After absorption, antibodies to rabbit erythrocytes and rabbit serum were no longer detected in the NTS and the titre of anti-GBM antibody remained unchanged.

(b) Preparation Of Nephrotoxic Globulin (NTG)

NTG was prepared from the NTS by 50% saturated ammonium sulphate precipitation (Heide and Schwick, 1973). An equal volume of saturated ammonium sulphate solution was slowly added to the serum, with continual mixing. Complete flocculation of the globulin was obtained by leaving the mixture at 4°C for 30 minutes. The globulin was precipitated by centrifugation at 2,000 RPM for 10 minutes and was then washed once in 50% saturated ammonium sulphate solution. The precipitate was redissolved in distilled water and the ammonium sulphate precipitation and washing process was repeated twice. The precipitate was finally dissolved in a small volume of phosphate buffered saline (PBS) (pH 7.3). The residual ammonium sulphate was removed by dialysis of the globulin solution in cellophane tubing (Visking - Scientific Instrument Centre, London) against PBS for 48 hours at 4°C with constant stirring and

frequent changes of the buffer. The NTG was restored to the original volume of NTS by addition of PBS containing 0.01% sodium azide. The protein concentration of the NTG solution, as determined by the folin phenol reagent (Lowry et al, 1951), was 27 mg/ml.

(c) Evaluation Of The NTG

Three normal NZW rabbits were injected IV with 1 ml of NTG/Kg BW. The animals were killed one hour later and the kidneys were examined by direct immunofluorescence, staining with fluorescein isothiocyanate (FITC) labelled anti-sheep immunoglobulin antiserum. Heavy deposition of sheep immunoglobulin in a smooth linear fashion along the GBM was found in all three animals. Immunoglobulin was not detected along tubular basement membrane (TBM).

PREPARATION, PURIFICATION AND EVALUATION OF GOAT ANTI-RABBIT POLYMORPHONUCLEAR LEUCOCYTE ANTISERUM (ANTIPOLYMORPH SERUM)

(a) Preparation of Anti-Polymorph Serum (APS)

A modification of the method of Kniker and Cochrane (1965) was used. Polymorphonuclear leucocytes (PMN) were obtained from the peritoneal cavity of rabbits by the method of Cohn and Hirsch (1960). Rabbits were injected intraperitoneally with 200 ml of 0.1% rabbit glycogen (BDH Biochemicals, UK) in sterile saline. Four hours later 100 ml of 0.9% sodium chloride (containing 500 u heparin) were introduced into the peritoneal cavity and the exudate was withdrawn through a peritoneal canula by gravity drainage. Each exudate contained at least

10×10^8 PMN and May-Grunwald-Giemsa stained smears showed that 99% or more of the exudate cells were PMN. Exudates contaminated with large numbers of erythrocytes were discarded. The cells were centrifuged at 1,800 RPM for 10 minutes and washed three times in 0.9% sodium chloride. The final suspension of PMN was adjusted to 1×10^8 cells/ml in 0.9% sodium chloride and incorporated into FCA. Approximately 5×10^8 cells were injected subcutaneously into the neck of a goat on each of five occasions, at two-weekly intervals. The titre of anti-rabbit PMN antibodies was determined by a PMN agglutination test in which 0.025 ml of a 1% suspension of rabbit peritoneal PMN were added to 0.05 ml of doubling dilutions of antiserum in microhaemagglutination plates. At the end of the immunisation course the animal was bled three times at weekly intervals. The blood was clotted at room temperature for 2 hours and the serum separated. The pooled serum from the three bleeds (600 ml) contained a titre of anti-PMN antibody of $\frac{1}{2 \times 10^6}$

(b) Purification Of APS

To remove contaminating antibodies, particularly to rabbit lymphocytes, platelets, erythrocytes and plasma, the APS (after decomplementation by heating at 56°C for 30 minutes) was extensively absorbed against these antigens in the following way. Normal rabbits were injected IV with 1.75 mg/Kg BW of mechlorethamine hydrochloride (nitrogen mustard - Boots Ltd, UK) to deplete the animals of PMN. On the third day after injection the animals were bled out, the blood being collected in siliconised tubes containing 2% disodium EDTA (9 : 1 V/V). On differential and

total white cell counting PMN counts were less than 200/mm^3 whilst lymphocyte and platelet counts were near normal (mean cell count of 3250/mm^3 and 220 x 10^3/mm^3 respectively). The blood was centrifuged at 1,600 RPM and the plasma removed, leaving the buffy coat with the erythrocytes. The erythrocyte buffy coat fraction was washed three times in PBS (pH 7.3). Peritoneal, mediastinal and cervical lymph nodes were removed from the rabbits, cut into small pieces with scissors, and a single cell lymphocyte suspension was prepared by gentle disruption of the lymph node fragments in a Potter-Elvehjen grinder. The cells were washed twice in PBS. The APS was successively absorbed against 1/5 volume of packed erythrocytes (containing mononuclear leucocytes and platelets), 1/10 volume of rabbit plasma. After absorption, titres of erythrocyte agglutinating antibody and lymphocyte agglutinating antibody in the APS were less than 1/8. The titre of anti-PMN antibody after absorption was $\frac{1}{1 \times 10^6}$

The globulin fraction (APG) of the absorbed APS was prepared by 50% saturated ammonium sulphate precipitation, as described in purification of NTS. The APG was reconstituted with PBS (containing 0.01% sodium azide) to the original volume of the APS.

(c) Evaluation Of The APG

Eight normal rabbits were injected IV daily for 5 days with either 1, 3, 5 or 7 ml of NTG. Peripheral blood white cell counts, differential white cell counts, and platelet counts were determined just before each injection, and at 1, 6, 12 and 15 hours after each injection. Two animals given 5 and 7 ml of NTG were killed on Day 5 and their kidneys removed

for histology and immunofluorescence examination. Serum creatinine and C3 concentrations and urine protein concentrations were determined daily in all animals.

It was found that doses of 1 and 3 ml of APG per day, although inducing immediate neutropoenia to counts of 0-100 cells/mm^3, failed to maintain a neutropoenia of less than 400 cells/mm^3 for longer than 12 hours. Doses of 5 and 7 ml of APG per day maintained PMN counts at less than 400 cells/mm^3 for the complete 24 hour period between doses. These doses of APG did not reduce peripheral blood mononuclear cell (lymphocytes and monocytes) counts and platelet counts by more than 15%. No change in renal function, as assessed by serum creatinine concentration, was evident during the 5 days of NTG administration. Serum C3 concentrations were similarly not altered. Light microscopy of the kidneys removed on Day 5 showed no abnormality and immunofluorescence examination revealed no glomerular deposition of rabbit immunoglobulin, C3 or fibrin.

The IV administration of APG was associated with mild vasoconstriction, as manifested by cold ears. This was evident only on the first and second days of APG administration and lasted only 3-4 hours after the administration. The vasoconstriction probably resulted from the intravascular release of vasoactive substances from destroyed PMN before persistent PMN depletion was attained.

PREPARATION AND PURIFICATION OF GOAT ANTI-RABBIT C3 ANTISERUM

The third component of complement (C3) was isolated from rabbit serum with zymosan. Zymosan (Sigma, UK) (2.5 mg/ml of serum) was

added to normal rabbit serum and the mixture was incubated at 37°C for 30 minutes. The mixture was then centrifuged at 2,000 RPM and the zymosan-C3 precipitate was washed once in PBS and twelve times in 2M sodium chloride to remove attached immunoglobulin. The precipitate from 3 ml of serum was used for each immunisation injection. The goat was given 2 intramuscular injections of zymosan-C3, the first in CFA and the second (2 weeks later) in incomplete Freund's adjuvant (IFA). One week later the animal was bled.

Two precipitin arcs were found on double diffusion in gel (Ouchterlony) when the antiserum was tested against normal rabbit serum. On immunoelectrophoresis (IEP) the antiserum was shown to have a small quantity of anti-rabbit IgG antibody as well as the anti-rabbit C3 antibody. The anti-IgG antibody was absorbed out by adding 1/10 volume of rabbit serum from an animal decomplemented with cobra venom factor (CVF) 24 hours previously. After absorption the antiserum showed only one precipitin line against normal rabbit serum on Ouchterlony, and IEP showed only anti-rabbit C3 activity.

GUINEA-PIG ANTI-RABBIT FIBRIN-FIBRINOGEN ANTISERUM

Fibrinogen was prepared from rabbit plasma by the following method. Briefly, barium sulphate (5 G/100 ml of plasma) was added twice to the plasma to remove prothrombin and the fibrinogen was then precipitated with 25% saturated ammonium sulphate. The final fibrinogen precipitate was dissolved in 0.3 M potassium chloride and dialysed in cellophane tubing against PBS for 24 hours. The fibrinogen solution was centrifuged to remove insoluble material. The concentration of

fibrinogen was determined by the folin phenol reagent (Lowry et al, 1951).

Each guinea-pig was injected intramuscularly with 5 mg of fibrinogen solution dispersed in an equal volume of FCA on each of two occasions at a 2-week interval. Two weeks after the second injection the animals were bled out and the serum collected. The specificity of the antiserum was tested by double diffusion in gel (Ouchterlony) against both normal rabbit serum and plasma. A single precipitin line was obtained against plasma but no line was seen against serum.

RABBIT ANTI-BOVINE SERUM ALBUMEN ANTISERUM

Each rabbit was injected intramuscularly with 10 mg of bovine serum albumen (BSA - Armour, UK) in FCA, followed 2 weeks later by 10 mg of BSA in IFA. One week later the animals were bled out and the serum obtained. The concentration of anti-BSA antibody was determined by the following method. The approximate equivalence ratio of BSA to anti-BSA was determined by double diffusion in gel (Ouchterlony). To determine the exact equivalence ratio, decreasing amounts of ^{125}I-labelled BSA were added to constant amounts of the anti-BSA antiserum in micro-titre tubes. The antiserum -BSA mixtures were left at room temperature for 1 hour and at 4^{o}C for 12 hours. The tubes were then spun at 2,000 RPM for 15 minutes. The point of BSA - anti-BSA equivalence was taken as the first tube in which BSA could no longer be found in the supernatant (in latter tubes free antibody was found in the supernatant). The protein concentration of the precipitate in this tube was determined by the folin phenol reagent, and the amount of antibody determined by

deduction of the known amount of BSA that had been added.

ESTIMATION OF SHEEP ANTI-RABBIT GBM ANTIBODY TITRE BY PASSIVE HAEMAGGLUTINATION

Passive haemagglutination tests were carried out in microtitre plates (Flow Laboratories, UK) and the other equipment was from standard microhaemagglutination kit (Flow Laboratories).

Soluble rabbit GBM was coupled to human group 0 Rh+ erythrocytes with gluteraldehyde using the technique of Avrameas (Avrameas, 1969; Avrameas, Taudou and Chuilon, 1969) as modified by Macanovic (1973). Glutaraldehyde was obtained as 26% aqueous solution from Tubb Laboratories, and was used without further purification. Human group 0 Rh+ blood was collected in acid citrate dextrose solution (20 ml/4ml) and mixed well. The erythrocytes were washed three times with N-saline. To 1.25 ml of soluble GBM in saline (2 mg/ml conc.), 0.05 ml of well packed erythrocyte was added. The suspension was incubated at room temperature for 5 minutes and then while being gently stirred, 0.25 ml of 2.5% glutaraldehyde solution was added. The stirring was continued for one hour at room temperature and the suspension was then centrifuged at 1,200 RPM for 10 minutes. The packed sensitised cells were washed twice in 0.9% sodium chloride and finally in a stabilising serum, 1% rabbit serum saline (Fulthorpe, 1957; Gill, 1964) (the rabbit serum had been previously decomplemented and absorbed against human group 0 Rh+ erythrocytes). A 1% suspension of sensitised erythrocytes was prepared by resuspending the cells in 5 ml of 1% rabbit serum saline (1% RSS).

Sera to be tested for anti-rabbit GBM antibody were decomplemented by heating at 56°C for 30 minutes and absorbed with normal human group 0 Rh+ erythrocytes (3 : 1 V/V). Serial dilutions of serum (in 1% RSS) were placed in the wells of a microtitre plate (Takatsy, 1955). To 0.05 ml of the serum (and various dilutions of it), 0.025 ml of 1% suspension of sensitised erythrocytes was added. The plate was sealed, gently aggitated for 5 minutes and then left to stand at room temperature. The haemagglutination titre was read at 6 and 24 hours. A smooth matt of the cells at the bottom of the wells was designated as positive and a "button" of cells in the centre of the wells as negative. The end point of the antibody titre was taken to be the highest dilution of serum causing complete agglutination of cells, and the titre of the serum was expressed as the reciprocal of the initial dilution of the serum.

Controls consisted of : (1)sensitised cells and normal sheep serum (2) sensitised cells and 1% RSS, and (3) sensitised cells and normal rabbit serum.

ESTIMATION OF RABBIT ANTI-SHEEP IMMUNOGLOBULIN ANTIBODY TITRE BY HAEMAGGLUTINATION

Human group 0 Rh+ erythrocytes were prepared as described above (passive haemagglutination test for anti-GBM antibody) and coated with sheep immunoglobulin in the following way : The highest dilution of a sheep antiserum to human erythrocytes that would agglutinate human erythrocytes was determined. Equal volumes of a four-fold dilution of this highest dilution of antiserum and 2% human group 0 erythrocytes were mixed and incubated at room temperature for 30 minutes. The suspension

was centrifuged at 1,200 RPM for 15 minutes and the sensitised erythrocytes washed twice in 0.9% sodium chloride and once in 1% RSS. A 1% suspension of sensitised erythrocytes was prepared in 1% RSS.

Haemagglutination assay for anti-sheep immunoglobulin antibody was performed by adding the sensitised erythrocytes to serial dilutions of the serum to be tested, in the same way as described in the assay of anti-GBM antibody.

TESTS OF BLOOD COAGULATION

Whole Blood Clotting Time

The method of Lee and White was used. Animals were bled into a plastic syringe and the blood was immediately transferred to glass clotting tubes kept in a water-bath at 37°C. The tubes were tilted every 15 seconds and the time of clotting noted. All clotting time estimations were performed in duplicate.

Thrombin Clotting Time

Blood was collected into EDTA (sequestrene) containers and the plasma was separated by centrifugation at 2,000 RPM. 0.2 ml of the test or normal plasma were placed in standard clotting tubes in a water-bath at 37°C, and allowed to warm for 1-3 minutes. 0.1 ml of ice-cold thrombin solution (bovine thrombin in 1/40 M calcium chloride) was then added and the tubes were repeatedly tilted to determine the time of clotting. Thrombin clotting time for normal plasma was between 11 and 15 seconds. All estimations were performed in duplicate.

Clot Quality Test For Adequacy Of Defibrination

This test, as described by Reid, Chan and Thean (1963), involves the observation of the volume of extruded cell deposit and the size of the remaining clot after contraction of the clot has occurred. The quality of the clot is a function of the blood fibrinogen level. Five grades of clot quality are described, depending on the percentage of the original blood volume occupied by the cell deposit.

RADIOLABELLING OF PROTEINS WITH EITHER ^{125}I OR ^{131}I

A modification of the method described by McConahey and Dixon (1966) was used. This technique depends on the capacity of chloramine-T, a mild oxidising agent to link certain anions, such as iodine, to the tyrosine residues to protein molecules. Chloramine-T powder was stored in aliquots in glass-vials wrapped in silver foil to avoid damage from prolonged light exposure. A fresh solution of chloramine-T of 2.5 mg/ml in distilled water was prepared prior to each iodination. A solution of sodium metabisulphite was prepared in the same concentration. The isotopes were of iodine (either ^{125}I or ^{131}I) in the form of sodium iodide, buffered in sodium hydroxide to pH 8.6 (Obtained from Amersham, London). Protein to be labelled was of known concentration (preferably greater than 1 mg/ml) and was dialysed in cellophane tubing against PBS (pH 7.5) at 4°C for 12 hours prior to iodination. The radioisotope (10 µl/millicurie) was added to the protein solution, with a micro-capillary tube (Drummond) and gently shaken for 10 seconds. Chloramine-T was introduced (15 µg/mg of protein) and mixed gently but continuously for 60 seconds, at which time sodium metabisulphate was added

(30 µg/mg of protein) to neutralise the chloramine-T. Exposure to chloramine-T was limited to 60 seconds to minimise protein molecular damage. The efficiency of labelling was determined by comparing free to protein bound radioiodine counts using the trichloroacetic acid (TCA) protein precipitation method (see below). Free iodine was removed by dialysis in cellophane tubing against PBS for at least 48 hours at 4°C with constant stirring and frequent changes of buffer. The percentage of free iodine remaining was determined by TCA precipitation of a sample of the iodinated protein solution. Dialysis was continued until the percentage of free to bound iodine was less than 1%.

MEASUREMENT OF ^{125}I OR ^{131}I RADIOACTIVITY IN SERUM SAMPLES

Radioactivity was measured in a Packard Gamma Counter. Energy emission for each isotope was recorded in different channels so that both isotopes could be measured at the one time. There was a crossover of about 15% of ^{131}I counts into the ^{125}I channel, so each time both isotopes were being measured together the actual percentage crossover was determined and the ^{125}I counts adjusted accordingly.

2 ml of serum were counted for 1-10 minutes depending on the quantity of gamma emission. Free iodine activity (non-protein bound) was determined by the TCA protein precipitation method : The protein in the 2 ml serum sample was precipitated by the addition of 2 ml of 20% TCA. After centrifugation at 3,000 RPM for 10 minutes the

supernatant was removed and passed through non-absorbent nylon wool to remove residual fragments of precipitated protein. A 2ml sample of the supernatant (containing free iodine) was counted.

PROCEDURE USED FOR DETERMINING THE QUANTITY OF NEPHROTOXIC ANTIBODY (NTAb) FIXED IN THE KIDNEY

Rabbits to be injected with radiolabelled NTG were housed in metabolic cages and given drinking water containing potassium iodide at a concentration of 0.02 mg/100 ml and sodium chloride (0.45 mg/100 ml) to minimise uptake of radioiodine by the renal tubular cells.

Normal sheep globulin was labelled with ^{131}I and added to nephrotoxic globulin labelled with ^{125}I in an activity ratio of 1:10. The specific quantity of labelled NTG (with the labelled normal sheep globulin) was given IV to the rabbit. The total dose of radioiodine given to each animal was about 1 μCi. At 24 hours the animal was exsanguinated under anaesthesia and the kidneys were perfused in situ with 50 ml of warm saline. The kidneys were removed and after cutting one kidney into small portions the gamma emission of both isotopes in this kidney was measured by counting all portions in a Packard Gamma Counter. The ratio of ^{125}I to ^{131}I counts in the serum was determined by counting 2 ml of the serum obtained at death. The normal sheep globulin labelled with ^{131}I represented the trapped but unbound NTG. The amount of NTAb bound in the kidney was calculated using the formula:

ug of NTAb bound in one kidney =

$$\frac{{}^{125}\text{I Kidney Counts} - \left\{\frac{{}^{125}\text{I Counts per ml of serum}}{{}^{131}\text{I Counts per ml of serum}}\right\} \times {}^{131}\text{I Kidney Counts}}{{}^{125}\text{I Counts per ug of NTG injected}}$$

^{125}I-LABELLED BSA CLEARANCE STUDIES

In the evaluation of the anti-BSA antibody response in rabbits being repeatedly injected IV with BSA to induce chronic immune-complex glomerulonephritis (see Chapter 5) ^{125}I-labelled BSA clearance studies were performed.

Instead of the daily dose of unlabelled BSA the same dose of ^{125}I-labelled BSA was given to the animals IV. Control animals were normal rabbits given 15 mg of ^{125}I-labelled BSA containing the same radioactivity as that given to the test animals. The rabbits were bled at 1, 6, 12 and 24 hours after the injection and the percentage of BSA remaining in the circulation determined. The counts reflecting 100% circulating BSA were calculated by bleeding the control animals 5 minutes after the ^{125}I-BSA injection and using this value for 100% in the test animals (after adjustment for weight).

The quantity of BSA in the circulation complexed to anti-BSA was determined by the Farr technique (Farr, 1958). Two millilitres of serum were counted and then the globulin in the serum was precipitated with 50% saturated ammonium sulphate. The precipitate (containing complexed BSA) was washed once in 50% saturated ammonium sulphate and then

counted.

Clearance of BSA from the circulation was said to have occurred when less than 1% of the injected dose of BSA remained in the circulation.

PURIFICATION OF COBRA VENOM FACTOR

Pure cobra venom factor (CVF) was prepared from crude venom extract in powder form (Sigma, USA) by the method of Ballow and Cochrane, (1969). Two grams of extract were dissolved in 100 ml of 0.01M phosphate 0.01% azide buffer (pH 7.5; conductivity 1.8 mmho/cm^3), dialysed against the same buffer for 3 days at 4^{o}C and then fractionated in DEAE-52 cellulose to remove neurotoxins, these being eluted in the breakthrough peak with 0.01M phosphate buffer. The fractions containing neurotoxins were immediately detoxified by autoclaving. The active CVF was eluted in two very close peaks, towards the end of a saline gradient rising to 0.5M in three column volumes. Each fraction of these two peaks was tested for anticomplementary activity by determining its capacity to inhibit red cell lysis. 0.1 ml of each fraction was added to 0.1 ml of 1/10 human serum (diluted in CFT) and the mixture was then incubated at 37^{o}C for 30 minutes. 0.2 ml of 1% sensitised sheep erythrocytes (EA) was then added and incubation continued for a further 30 minutes. Each tube was then examined visually for inhibition of red cell lysis and fractions showing more than 50% lysis inhibition were pooled and concentrated to 30 ml by positive pressure ultrafiltration. Further purification of the CVF to remove phospholipase was performed on G-200 sephadex, equilibrated with 0.01M phosphate, 0.01% azide, 0.05M NaCl (pH 7.2 ; conductivity 45 mmho/cm^3). Two

protein peaks appeared early in this fractionation and the first of these contained the CVF. These fractions were also assayed for anti-complementary activity by inhibition of red cell lysis and the active fractions were pooled and concentrated by Amicon ultra filtration . The anticomplementary activity of the final preparation was quantitated by serial dilution of the CVF, the degree of dilution which maintained greater than 50% inhibition of lysis representing the number of cobra factor units per ml.

FLUORESCEIN CONJUGATION OF ANTISERA

Prior to conjugation the specificity of an antiserum was assessed by the double immunodiffusion in gel and/or IEP and impure antisera were absorbed out against the appropriate antigens (see "Production of individual antisera"). Antisera were conjugated to fluoroisothiocyanate (FITC) by the method of Wood, Thompson and Goldstein (1965).

The globulin fraction of an antiserum was precipitated with 18% sodium sulphate, the mixture being stirred continuously for 3 hours to allow complete flocculation of the globulin. After centrifugation the precipitated globulin was washed three times in 18% sodium sulphate. The globulin was finally resuspended in a minimal volume of 0.9% sodium chloride and dialysed in cellophane tubing for 24 hours at 4^{o}C against 0.9% sodium chloride. The protein concentration of the globulin solution was determined by the folin phenol reagent and the protein concentration of the solution adjusted to 25 mg/ml by addition of 0.9% sodium chloride.

A solution of FITC (FITC Monomer 1 - BDH Chemicals, Ltd., UK)

at 2 mg/ml in 0.2 M Na_2HPO_4, Na_3PO_4 (pH 9.3) buffer (Buffer A) was prepared just prior to conjugation. 1 mg of FITC solution and 2 ml of buffer A were added slowly to each 100 mg of globulin to be conjugated, care being taken that the pH did not fall below 9.0. The solution was continuously stirred for one hour. The pH of the solution was then reduced to 7.6 with 0.2 M NaH_2PO_4.

Fractionation of the FITC-globulin was performed by Sephadex G-25 gel filtration and diethylaminoethyl (DEAE) cellulose anion exchange chromatography. A 50 ml Sephadex G-25 chromatography column was prepared as described by Fahey and Terry (1973) and equilibrated with 0.01 M phosphate buffer (pH 7.6). To the column a volume of 10 ml of FITC-globulin solution was added. The column was eluted with 0.01M phosphate buffer (pH 7.6). The eluted fractions comprising the first protein peak were kept whilst the second protein peak which contained unbound FITC was discarded.

A 50 ml DEAE-cellulose chromatography column was prepared as described by Fahey and Terry (1973) and equilibrated with 0.01 M phosphate buffer at pH 7.6. The FITC-globulin sample was placed on the column and eluted successively with 50 ml of the following 3 buffers:

(1) 0.01 M phosphate buffer (pH 7.6)

(2) 0.01 M phosphate buffer (pH 7.6) containing 0.2% NaCl

(3) 0.01 M phosphate buffer (pH 7.6) containing 0.9% NaCl.

The eluates from the first and second buffers were discarded. The fractions contained in the concentrated protein peak of the third buffer elution, containing the high concentrations of purified FITC-

labelled immunoglobulin, were kept.

Each conjugate was tested for specificity and potency of staining on direct immunofluorescence. They were tested on tissue known to contain or to not contain the antigen against which the antiserum had been raised. Non specific tissue staining could usually be prevented by dilution of the conjugate before application to the tissue. The conjugates were absorbed before use with pig liver powder (Wellcome Research Laboratories, UK), using 1/5 powder to volume of stain.

Antisera conjugated to FITC by this method included guinea-pig anti-rabbit fibrin-fibrinogen, goat anti-rabbit C3, and the rabbit anti-BSA. FITC conjugated sheep anti-rabbit immunoglobulin and rabbit anti-sheep immunoglobulin were obtained commercially (Wellcome Research Laboratories, UK) and specificity of staining was confirmed by double immunodiffusion in gels (Ouchterlony), IEP, and by direct immunofluorescence.

EXAMINATION OF RENAL TISSUE BY DIRECT IMMUNOFLUORESCENCE

(a) Preparation And Staining Of The Tissue

Renal biopsy specimens of portions of kidney taken immediately at the death of an animal were embedded in an inert embedding medium (Tissue - Tek OCT Compound : Ames Company, UK) contained on a small piece of cork. The tissue was snap frozen by gently immersing the specimen in liquid nitrogen and stored at -70°C until examined. Sections of tissue of 4 μ in thickness were cut in a cryostat (Slee,

London) and transferred to acid-washed slides kept at room temperature. The sections were dried thoroughly by air and fixed by immersion in dry acetone ((prepared by adding aluminium sodium silicate molecular sieve type 4A (BDH Chemicals Ltd., UK) to acetone)) for 15 minutes. The sections were washed three times for 10 minutes in PBS (pH 7.3) with continual stirring of the buffer. The respective fluoresceinated antisera (stains) were applied to the sections in a quantity sufficient to cover the sections and the sections were left in a wetbox at room temperature for one hour. The non-bound stain was then removed by washing the sections three times for 10 minutes in PBS with continual stirring. The sections were dried by air and mounted in 95% glycerol, 5% PBS and covered with a coverslip.

(b) Examination Of Stained Tissue Under The Immunofluorescence Microscope

Stained sections were examined with a Leitz Orthoplan microscope using a high pressure mercury light source (HBO - 200), K-500 FITC interference filter, BG-38 red suppression filter and a K-510 or K-530 barrier filter. Water immersion objectives of magnification 25x and 50x were used. The sites and pattern of deposition of immunoglobulin, C3, BSA and fibrin-fibrinogen were determined.

Assessment of quantity of deposition was limited to absent, mild, moderate, and heavy except for fibrin-fibrinogen for which a scoring system for quantitating of deposition was devised.

ASSESSMENT OF GLOMERULAR FIBRIN-FIBRINOGEN DEPOSITION BY IMMUNOFLUORESCENCE

The degree of glomerular fibrin-fibrinogen in the kidney from rabbits with nephrotoxic nephritis and BSA-induced chronic immune-complex nephritis wassemi-quantitatively assessed by immunofluorescence. Sections of kidney were stained with FITC-conjugated guinea-pig anti-rabbit fibrin-fibrinogen antiserum and the extent of staining in each glomerulus was scored from 0-3 as follows : A score or grade of 0 represented no fibrin, and scores or grades of 1, 2 and 3 represented fibrin deposition involving $< \frac{1}{3}$, $\frac{1}{3}$-$\frac{2}{3}$ and $> \frac{2}{3}$ respectively, of the the surface area of glomerular tuft and crescent and Bowman's space. At least 30 glomeruli per animal were assessed in this way and the result for each animal was expressed either as a mean fibrin score per glomerulus per animal (maximum possible score = 3) or as per cent incidence of each grade of fibrin deposition for the animal.

HISTOLOGY

Renal biopsy specimens or renal tissue taken at the death of an animal were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 4 μ. Sections were stained with haematoxylin and eosin (H and E), periodic acid-schiff (PAS) and Martius Scarlet and Blue (MSB - Lendrum et al, 1962).

ASSESSMENT OF GLOMERULAR EXTRACAPILLARY CELL PROLIFERATION BY LIGHT MICROSCOPY

The extent of extracapillary cell proliferation (crescent formation)

in rabbits with crescentic glomerulonephritis was semi-quantitatively assessed by light microscopy. Each glomerulus was scored from 0-3 as follows : A score or grade of 0 represented no crescent formation ; scores or grades of 1, 2 and 3 represented the involvement of $< \frac{1}{3}$, $\frac{1}{3}$-$\frac{2}{3}$ and $> \frac{2}{3}$ respectively, of the circumference of the glomerulus by proliferating extracapillary cells. At least 50 glomeruli per animal were assessed in this way and the result for each animal expressed either as a mean score per glomerulus per animal (maximum possible score = 3) or as per cent incidence of each grade of crescent formation for the animal.

PHARMACOLOGICAL AGENTS

Heparin

Sodium heparin (Paines and Byrne, Ltd., UK) was used for anticoagulation of rabbits with NTN.

Mechlorethamine Hydrochloride Or Nitrogen Mustard

(Mustine Hydrochloride - Boots Ltd., UK) was used for depletion of circulating polymorphonuclear leucocytes.

Ancrod (Arvin)

The purified procoagulant fraction of the venom of the Malayan Pit Viper was donated by Twyford Pharmaceutical Services Ltd., London and was used for defibrination of rabbits.

Penicillin

Sodium benzylpenicillin (Crystapen - Glaxo Laboratories Ltd., UK).

Streptomycin

Streptomycin sulphate (Glaxo Laboratories Ltd., UK).

STATISTICAL ANALYSIS OF RESULTS

Linear Regression Analysis (Snedecor, 1956) was used for the comparison of dose of NTG given to animals to quantity of NTAb fixed in the kidneys, and comparison of quantity of NTAb fixed to proteinuria.

Wilcoxon Rank Sum Test for Two Samples This method of analysis of unpaired measurements as described by Wilcoxon (1945) and modified by White (1952) for groups of unequal sizes, was used for the statistical analysis of all other results.

PART III

PRODUCTION AND EVALUATION OF TWO MODELS OF EXPERIMENTAL GLOMERULONEPHRITIS

Chapter 4

NEPHROTOXIC NEPHRITIS

Introduction

In this chapter I have described experiments designed to characterise and quantitate the two phases of nephritis in rabbits following the administration of our preparation of nephrotoxic globulin (NTG). The principal facets examined were :

(a) the relationship of heterologous phase proteinuria to quantity of nephrotoxic antibody fixed in the kidney

(b) proteinuria in the autologous phase

(c) serum C3 concentrations in both phases

(d) impairment in renal function

(e) histopathology of the kidney

(f) immunopathology of the kidney in both phases

I have also described the production and characterisation of a "telescoped" model of NTN in rabbits.

EXPERIMENTAL PROTOCOLS

1 : THE HETEROLOGOUS PHASE OF THE STANDARD MODEL OF NTN

Six groups of rabbits were injected intravenously (IV) with different doses of ^{125}I-labelled NTG (containing ^{131}I-labelled normal sheep globulin as described in Methods). The doses of NTG used were :

Group A (6 animals)	:	0.5 ml (13.5 mg)/Kg BW
Group B (6 animals)	:	0.75 ml (20 mg)/Kg BW
Group C (4 animals)	:	1.0 ml (27 mg)/Kg BW
Group D (4 animals)	:	1.5 ml (40 mg)/Kg BW
Group E (4 animals)	:	2.0 ml (52 mg)/Kg BW
Group F (6 animals)	:	2.0 ml (52 mg)/Kg BW

In Group F all animals were depleted of circulating polymorphonuclear leucocytes (PMN) by the IV injection of 1.25 mg/Kg BW of nitrogen mustard 3 days before the injection of NTG.

The animals were placed in metabolic cages and 24-hour urine collections for protein estimation were obtained before and after the injection of NTG. Blood was collected at zero hour and 24 hours for serum C3 concentration. At 24 hours the animals were killed by exsanguination under anaesthesia and the kidneys were removed for : histology ; immunofluorescence examination for sheep immunoglobulin, rabbit immunoglobulin, rabbit C3 and rabbit fibrin ; and estimation of fixed nephrotoxic antibody (NTAb).

2 : THE AUTOLOGOUS PHASE OF THE STANDARD MODEL OF NTN

Three groups of rabbits, each containing six animals, were injected IV with either 0.75 ml, 1.0 ml or 1.5 ml of NTG/Kg BW (Day 0). Urine samples were collected each day by urethral catheterisation and protein concentration measured. The animals were bled daily for measurement of serum C3, creatinine and urea concentrations. All rabbits were killed on Day 12 and the kidneys were removed for histology and for immunofluorescence examination using the same antisera as in protocol 1. The extent of glomerular fibrin deposition and crescent formation was assessed as described in Methods.

3 : "TELESCOPED" MODEL OF NTN

Six rabbits were immunised intramuscularly with 10 mg of normal sheep globulin preparation in FCA (Day -7).

Seven days later (Day 0) each animal was injected IV with 1.0 ml of NTG/Kg BW. Urine samples were obtained daily by urethral catheterisation for protein measurement. Serum C3 and creatinine concentrations and the titres of rabbit anti-sheep immunoglobulin antibody were measured daily. The animals were killed on Day 5, the kidneys being removed for histology and immunofluorescence as in protocol 2.

RESULTS

PROTOCOL 1 - THE HETEROLOGOUS PHASE

(a) The Relationship Of Dose Of NTG To Antibody Fixed In The Kidney

As shown in Fig. 4.1 there was a linear relationship between dose of NTG administered and the quantity of antibody fixed in the kidneys over the limited range of doses used. This relationship was highly significant (correlation coefficient 0.978 ; slope 0.21 ; $p<0.01$). Over all the dose ranges, a mean of 0.94% (range 0.61-1.21%) of administered globulin was fixed in the two kidneys.

(b) The Relationship Of Antibody Fixed In The Kidney To Proteinuria (Fig. 4.2)

Only very mild proteinuria was induced when less than 300 µg of NTAb were fixed in each kidney. When fixed NTAb exceeded 300 µg/kidney marked rises in urine protein excretion were seen with only small increases of fixed antibody.

(c) Serum C3 Concentrations (Fig. 4.3)

A reduction of less than 10% in serum C3 concentrations at 24 hours was seen in animals receiving 0.5 or 0.75 ml of NTG/Kg BW. However, with progressively larger doses of NTG, the fall in serum C3 increased progressively such that in animals given 2.0 ml of NTG/Kg BW the serum C3 concentration at 24 hours had fallen to a mean of 33% (range 26-42%). The fall in C3 concentration was significantly greater in animals given 1.5 or 2.0 ml/Kg BW than in animals given 0.5 or 0.75 ml/Kg BW ($P<0.01$).

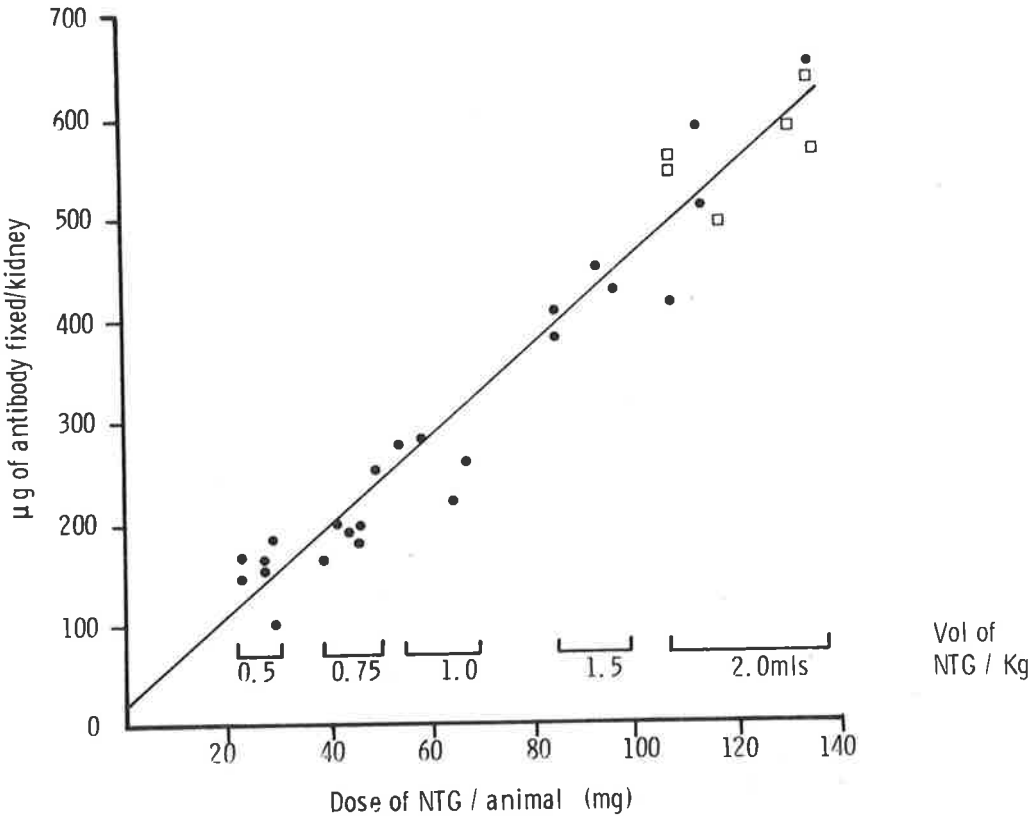

Fig. 4.1 The relationship in NTN between quantity of NTG administered and quantity of NTAb fixed in the kidney. Normal rabbits (●) and rabbits depleted of circulating PMN by nitrogen mustard (□).

Correlation coefficient for all animals, 0.978; slope 0.21; $P < 0.01$

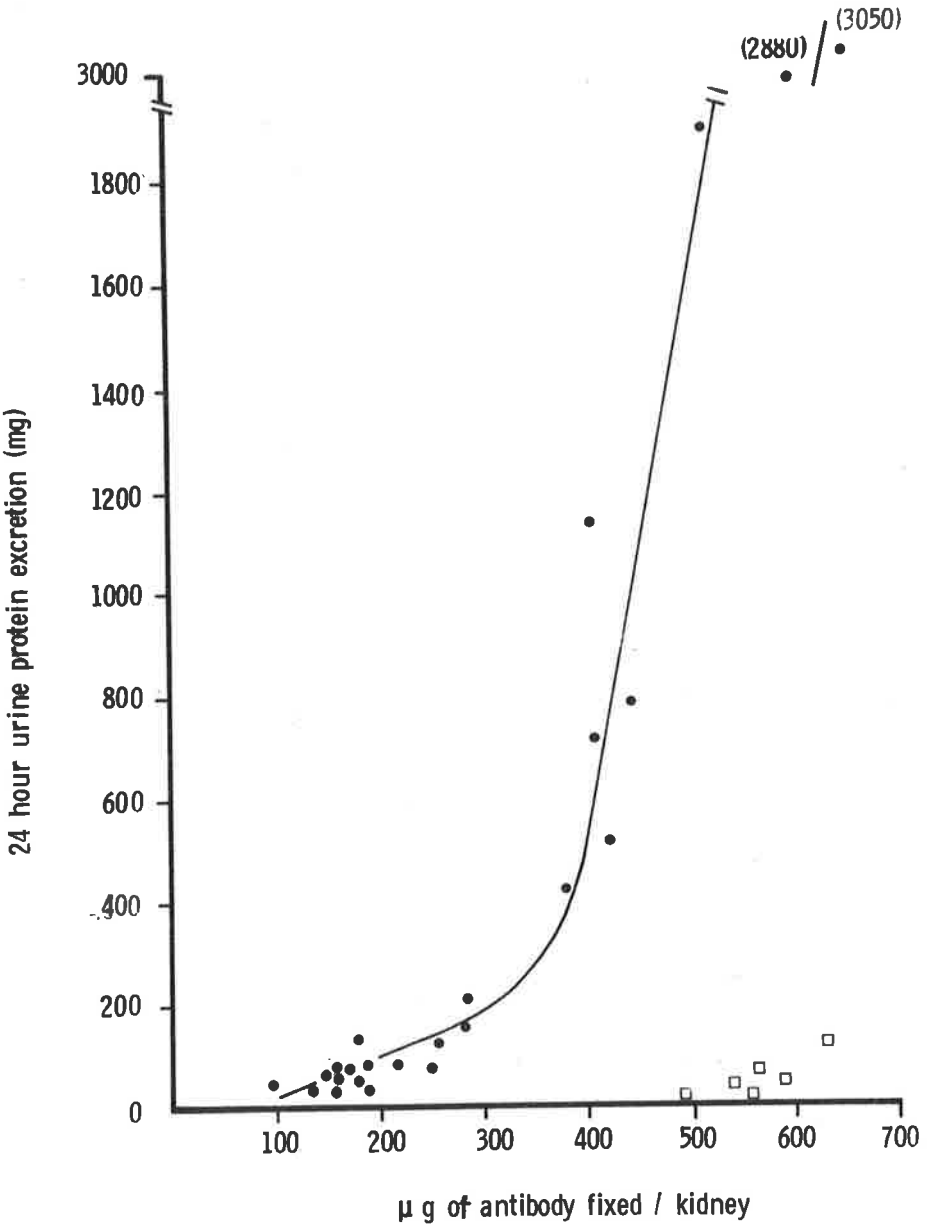

Fig. 4.2. The dose-response relationship between proteinuria and antibody fixed per kidney in the heterologous phase of NTN. Normal rabbits (●) and rabbits depleted of circulating PMN by nitrogen mustard (□).

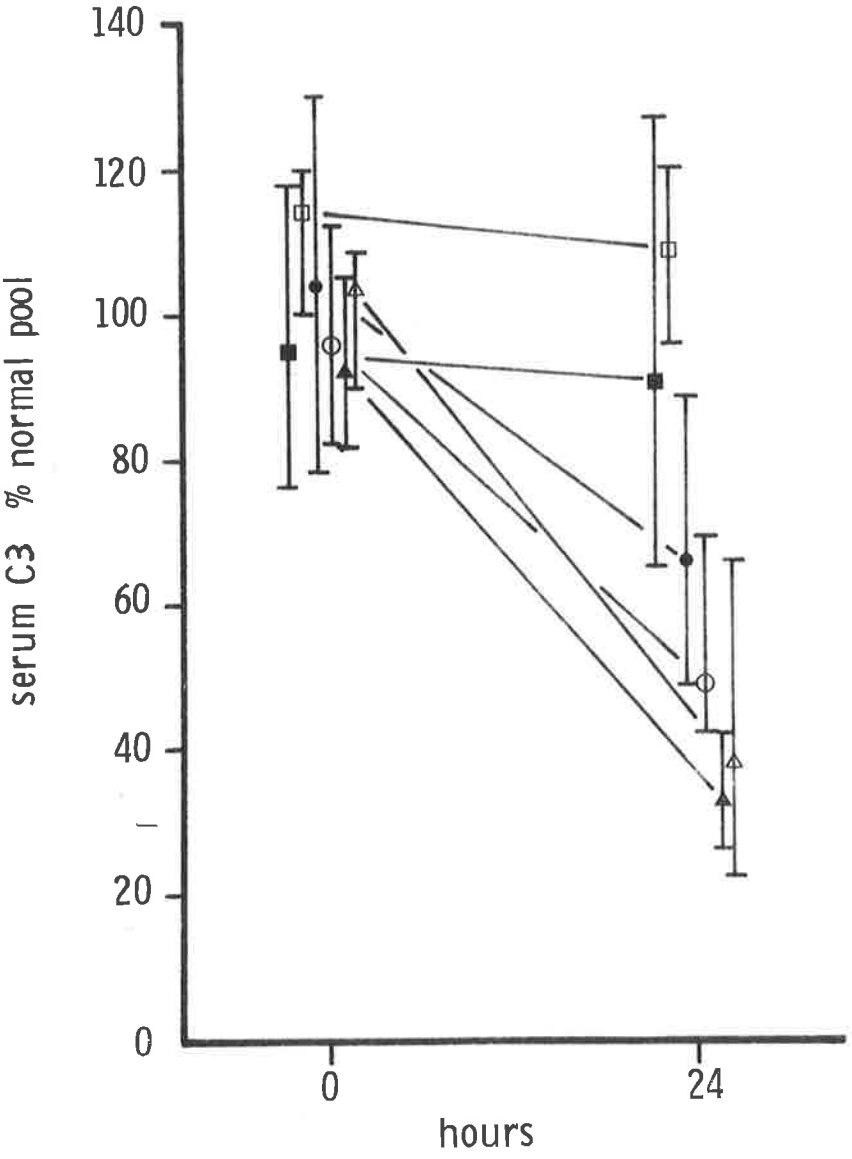

Fig. 4.3 Mean serum C3 concentrations (I range) in the heterologous phase of NTN. Six groups of animals given different doses of NTG :

Group A (■) 0.5 ml/Kg
Group B (□) 0.75 ml/Kg
Group C (●) 1.0 ml/Kg
Group D (○) 1.5 ml/Kg
Group E (▲) 2.0 ml/Kg
Group F (△) 2.0 ml/Kg + nitrogen mustard

Group	n	Dose of NTG ml/Kg BW	Mean number of PMN per glomerulus (range)
A	6	0.5	2.2 (0.4 - 3.8)
B	6	0.75	1.3 (0.5 - 2.1)
C	4	1.0	5.6 (1.9 - 9.6)
D	4	1.5	8.7 (5.4 -15.2)
E	4	2.0	15.8 (7.8 -39.4)
F	6	2.0 + HN_2	0.4 (0 - 0.7)

Table 4.1 Mean number of polymorphonuclear leucocytes (PMN) per glomerulus at 24 hours, in rabbits injected with various doses of NTG. (HN_2 - Nitrogen Mustard)

(d) Immunofluorescence (Fig. 4.4)

Linear deposition of sheep immunoglobulin and rabbit C3 along the GBM was found in all animals. No difference in the intensity of staining could be detected between the groups of animals. In no specimen was rabbit immunoglobulin or fibrin detected. Neither immunoglobulin nor C3 was found along the tubular basement membrane.

(e) Histology (Fig. 4.5)

The only obvious change on light microscopy of the kidneys was a PMN infiltration of the glomeruli in animals given the larger doses of NTG. Glomerular PMN were inconspicuous in animals given doses of 0.5 and 0.75 ml of NTG/Kg but increased to a mean of 15.8 cells/glomerulus in animals given 2 ml/Kg BW (Table 4.1).

(f) Animals Depleted Of Circulating PMN

Although the quantities of antibody fixed in the kidneys of animals depleted of PMN (Group F) were similar to the quantities in animals given the same dose of NTG but not depleted of PMN, only very mild proteinuria was induced in PMN-depleted animals (Fig. 4.2) and virtually no PMN were detected in the glomeruli (Table 4.1). However, serum C3 concentrations (Fig. 4.3) and immunofluorescence findings were the same in PMN-depleted animals as in non-depleted animals.

PROTOCOL 2 - THE AUTOLOGOUS PHASE

(a) Renal Function (Figs. 4.6 and 4.7 ; Tables 4.2 and 4.3)

Only mild elevation of serum urea and creatinine concentrations

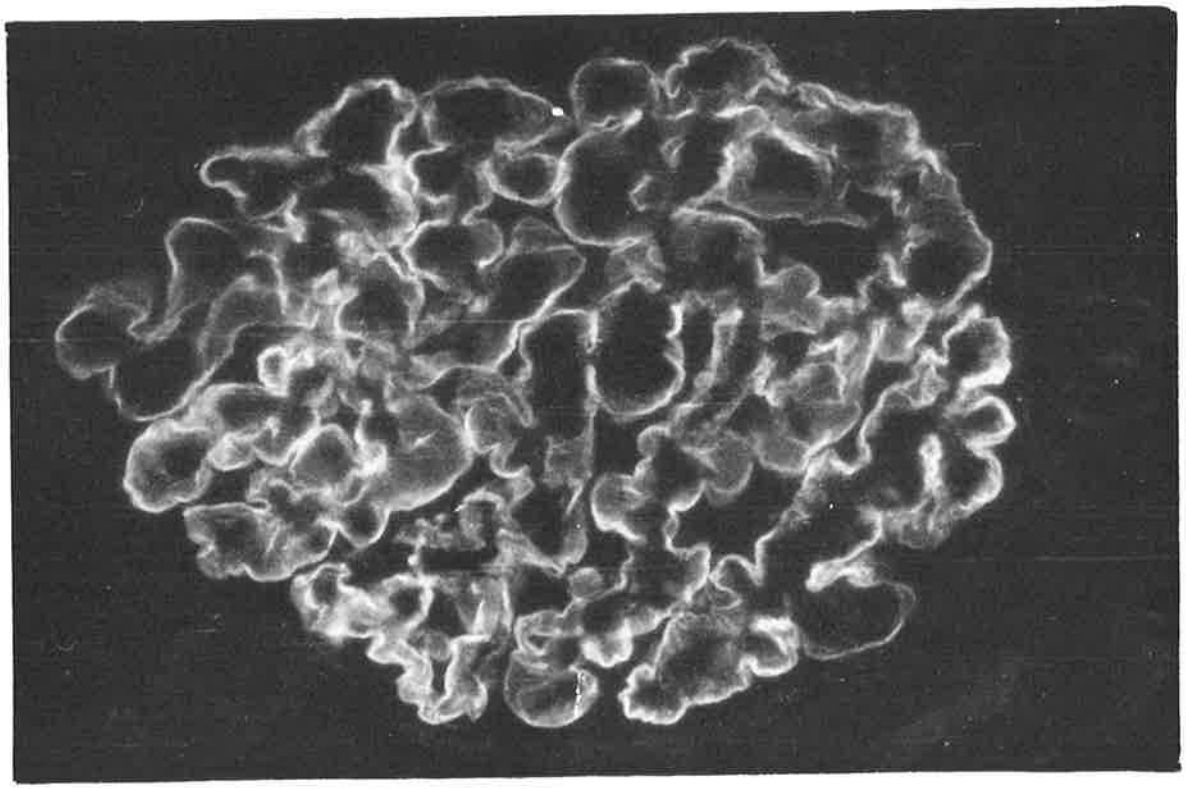

Fig. 4.4 Linear deposition of sheep immunoglobulin along the GBM of a rabbit during the heterologous phase of NTN. (FITC-conjugated rabbit anti-sheep immunoglobulin. Magnification x 600)

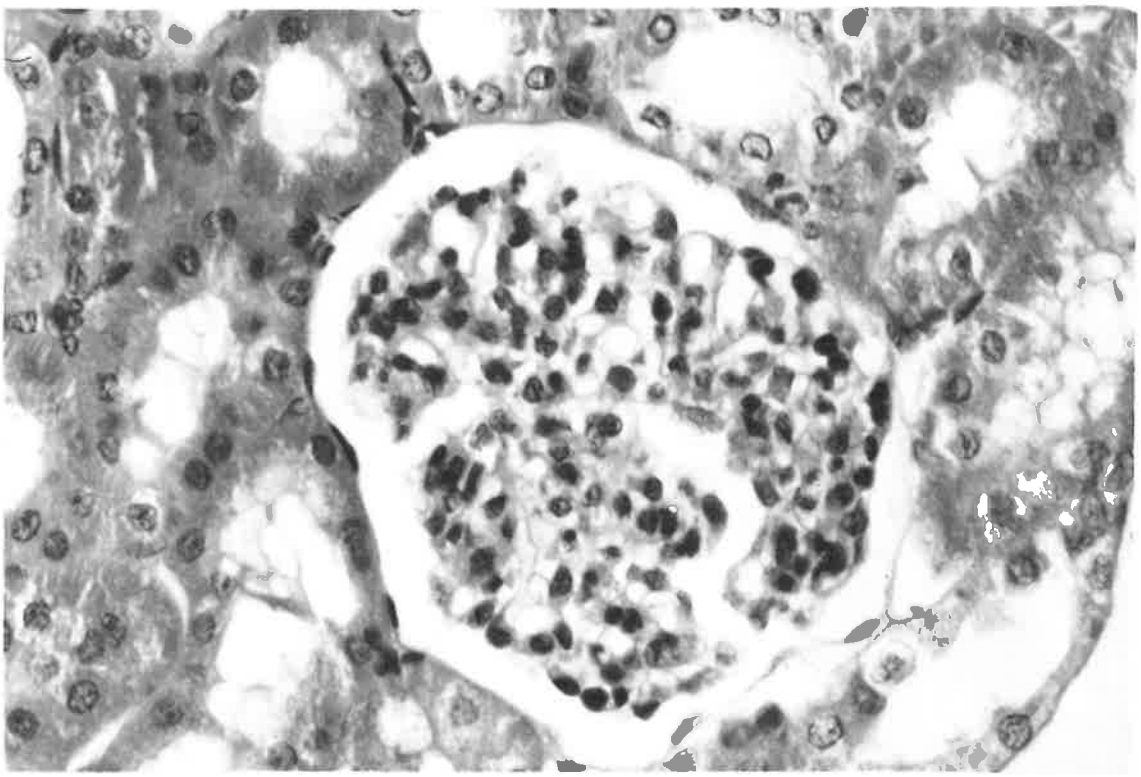

Fig. 4.5 Infiltration of a glomerulus by polymorphonuclear leucocytes during the heterologous phase of NTN in the rabbit. (H & E x 600)

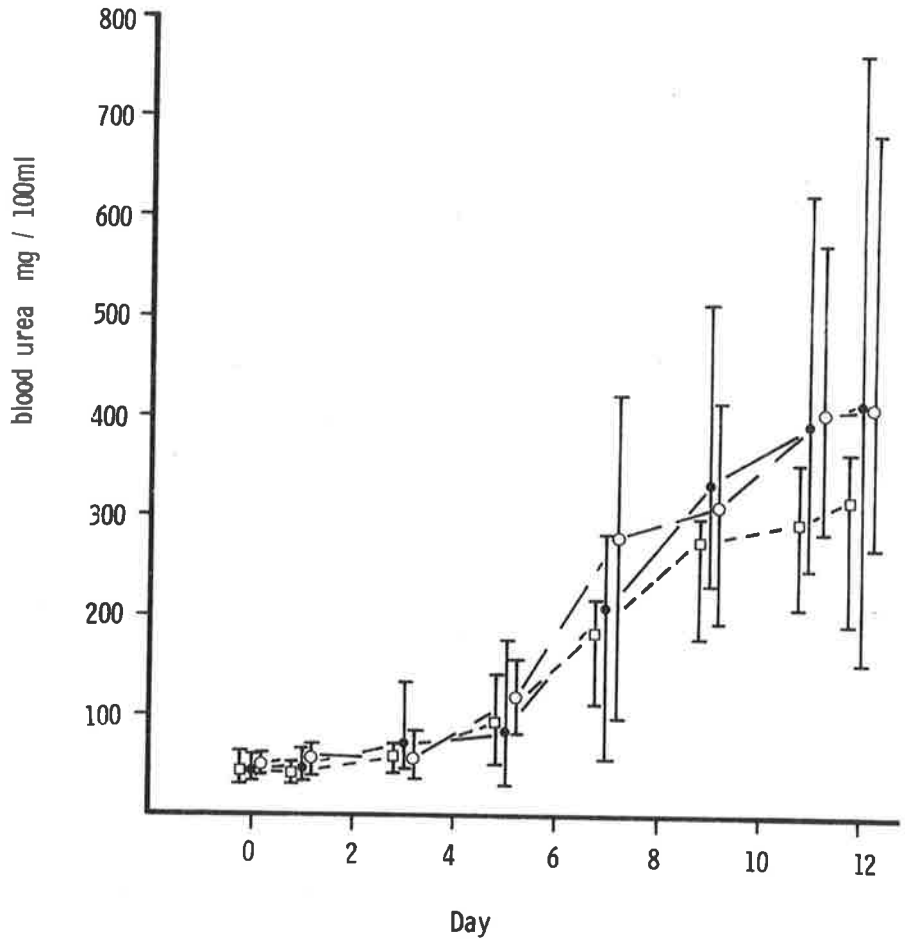

Fig. 4.6 Mean blood urea concentrations (range I) in the standard model of NTN. Three groups of animals given different doses of NTG :

Group A	(□)	0.75 ml/Kg
Group B	(●)	1.0 ml/Kg
Group C	(○)	1.5 ml/Kg

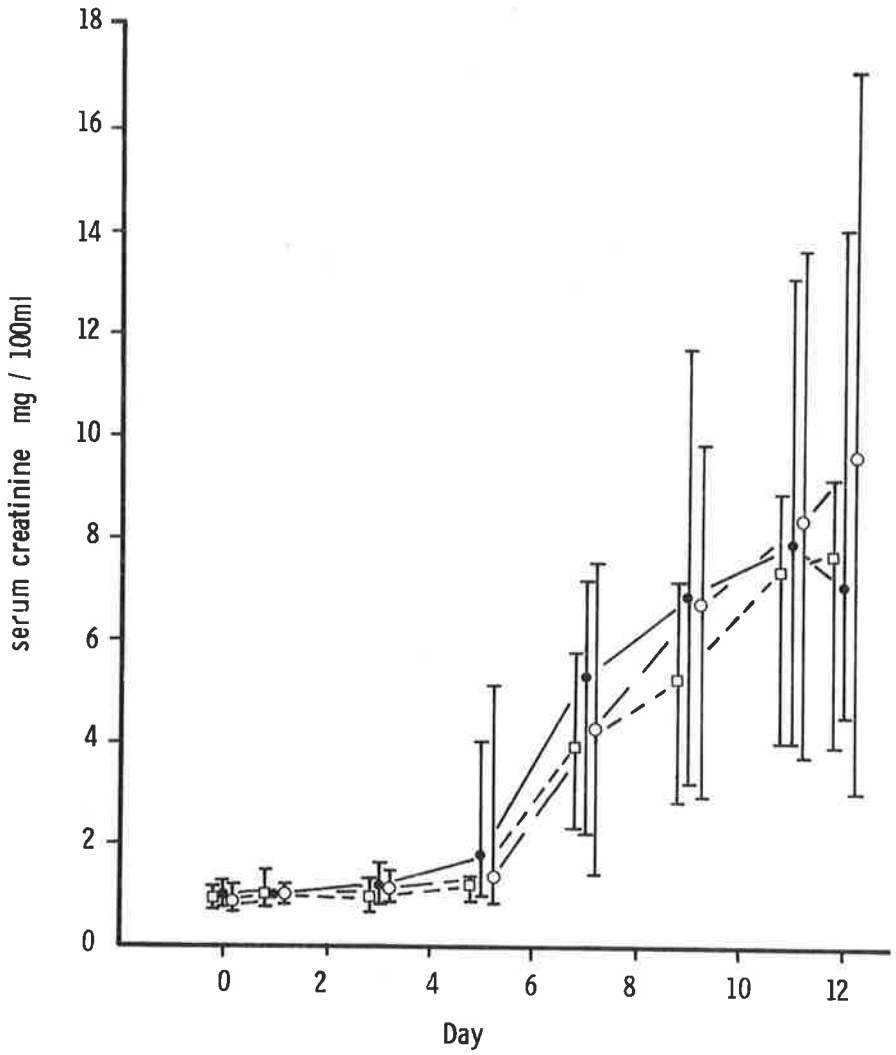

Fig. 4.7 Mean serum creatinine concentrations (range I) in the standard model of NTN. Three groups of animals given different doses of NTG :

Group A (□) 0.75 ml/Kg
Group B (●) 1.0 ml/Kg
Group C (○) 1.5 ml/Kg

ANIMAL GROUP	DOSE OF NTG ml/ Kg	n	SERUM UREA CONCENTRATION mg/100 ml : Mean (Range)							
			Day 0	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 12
A	0.75	6	46 (31-64)	42 (34-50)	56 (41-68)	92 (50-142)	185 (110-215)	268 (176-292)	296 (204-350)	315 (188-360)
B	1.0	6	45 (36-57)	49 (33-65)	73 (45-130)	82 (29-174)	206 (53-278)	332 (230-510)	392 (240-620)	411 (153-760)
C	1.5	6	49 (44-57)	56 (42-68)	52 (36-86)	116 (82-160)	286 (96-420)	311 (192-412)	400 (280-562)	412 (264-680)

Table 4.2 Serum urea concentrations in the standard model of NTN. Three groups of animals given different doses of NTG.

ANIMAL GROUP	DOSE OF NTG ml/Kg	n	SERUM CREATININE CONCENTRATION mg/100 ml : Mean (Range)							
			DAY 0	DAY 1	DAY 3	DAY 5	DAY 7	DAY 9	DAY 11	DAY 12
A	0.75	6	0.95 (0.8-1.1)	1.0 (0.8-1.4)	0.92 (0.7-1.3)	1.2 (0.9-1.3)	3.9 (2.3-5.8)	5.24 (2.8-7.1)	7.4 (4.0-8.8)	7.6 (3.9-9.1)
B	1.0	6	1.06 (0.8-1.2)	1.1 (0.8-1.4)	1.3 (0.9-1.6)	1.76 (1.0-4.0)	5.32 (2.2-7.2)	6.9 (3.1-11.7)	7.92 (4.0-13.1)	7.02 (4.5-14.0)
C	1.5	6	0.9 (0.7-1.1)	1.0 (0.8-1.1)	1.1 (0.9-1.5)	1.45 (0.8-5.1)	4.3 (1.4-7.5)	6.8 (2.9-9.8)	8.4 (3.7-13.6)	9.52 (3.0-17.1)

Table 4.3. Serum Creatinine Concentrations in the Standard Model of NTN. Three Groups of Animals Given Different Doses of NTG.

occurred before Day 5 of the disease. Thereafter there was a rapid deterioration in renal function in all the animals in each of the three groups, such that the mean serum creatinines for the three groups on Day 12 were in the range 7.0-8.5 mg/100 ml. At no stage was there a significant difference in either serum urea or creatinine concentrations between the groups despite the different doses of NTG used to induce the disease. Good correlation was seen between serum urea and serum creatinine concentrations as indication of impairment of renal function.

(b) Proteinuria (Table 4.4)

As shown in the results of protocol 1, proteinuria in the heterologous phase was mild in animals receiving 0.75 ml of NTG/Kg BW and moderately heavy in animals receiving 1.5 ml/Kg BW. Proteinuria disappeared in most animals given 0.75 or 1.0 ml of NTG/Kg BW by Day 2 but mild proteinuria persisted in animals given 1.5 ml NTG until the onset of heavy proteinuria in the autologous phase. Heavy proteinuria developed in all animals as from Day 5 and maximum urine protein concentrations were seen on Days 6-9. Thereafter urine protein concentrations fell in concordance with the extensive deterioration in renal function. There was no significant difference on any day in the autologous phase in urine protein concentrations between the groups of animals ($P > 0.1$ for all estimations).

(c) Serum C3 Concentrations (Fig. 4.8 ; Table 4.5)

As had been noted in the results of protocol 1, the fall in serum C3 concentration in the heterologous phase was proportional to the dose of NTG administered. By Day 3 C3 concentrations had returned to, or

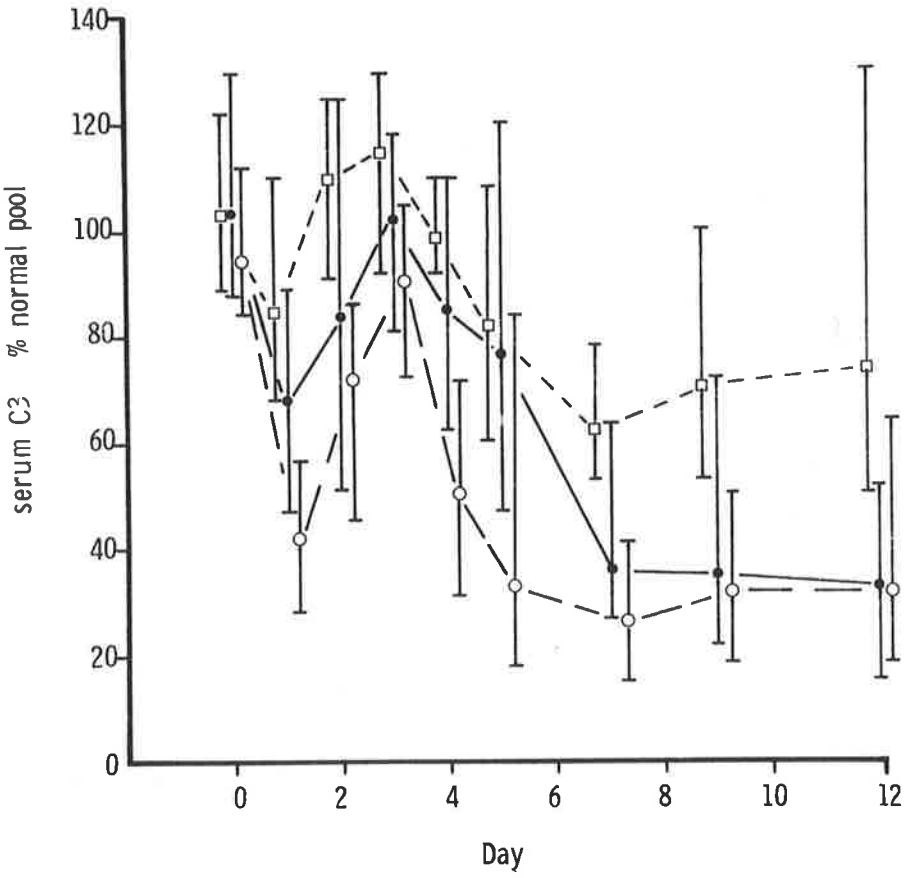

Fig. 4.8 Mean serum C3 concentrations (range I) in the standard model of NTN. Three groups of animals given different doses of NTG :

Group A	(□)	0.75 ml/Kg
Group B	(●)	1.0 ml/Kg
Group C	(o)	1.5 ml/Kg

ANIMAL GROUP	DOSE OF NTG ml/Kg	n	URINE PROTEIN CONCENTRATION mg / ml : Mean (Range)							
			DAY 1	DAY 2	DAY 4	DAY 5	DAY 6	DAY 8	DAY 10	DAY 12
A	0.75	6	0.6 (0.3-0.9)	< 0.3	0.6 (0.5-0.9)	2.5 (0.9-4.2)	12.9 (3.8-20.1)	16.2 (9.2- 32)	10.2 (5.2-18.1)	5.9 (4.9-6.5)
B	1.0	6	0.9 (0.3-2.0)	0.5 (0.3-1.8)	0.8 (0.4-1.3)	5.4 (2.1-11.6)	33.2 (15.1- 59)	31.2 (6.8- 85)	13.1 (4.0- 27)	9.2 (3.7-21.7)
C	1.5	6	10.2 (6.2-16.2)	3.1 (0.7-5.3)	0.9 (0.4-1.9)	3.6 (1.1 - 7.5)	23.2 (5.2- 35)	20.5 (3.5- 29)	10.5 (4.1 - 28)	7.1 (3.2 -12.5)

Table 4.4 Urine Protein Concentrations in the Standard Model of NTN. Three Groups of Animals Given Different Doses of NTG. All Animals Excreted Less Than 0.3 mg of Protein/ml of Urine, Before Receiving NTG.

ANIMAL GROUP	DOSE OF NTG ml/Kg	n	SERUM C3 CONCENTRATION % NORMAL POOL : Mean (Range)								
			DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 7	DAY 9	DAY 12
A	0.75	6	103 (89-122)	90 (68-110)	110 (91-125)	115 (92-130)	99 (92-110)	82 (60-108)	62 (53-78)	70 (53-100)	73 (50-130)
B	1.0	6	103 (88-130)	68 (47-89)	84 (51-125)	102 (81-118)	85 (62-110)	77 (47-120)	36* (27-63)	35* (21-72)	33*** (15-51)
C	1.5	6	94 (84-112)	41***Δ (28-56)	72*** (45-86)	90 (72-105)	50***Δ (31-105)	33***ΔΔΔ (18-84)	26*** (15-41)	32*** (18-50)	32*** (18-64)

Table 4.5 Serum C3 Concentrations in the Standard Model of NTN. Three Groups of Animals given different doses of NTG.

*P< 0.05 ***P< 0.01 : in respect to Group A

ΔP< 0.05 ΔΔΔP< 0.01 : in respect to Group B

risen above, the concentrations on Day 0. After Day 3 a rapid, sustained fall in C3 concentration was seen in all groups although this fall wassignificantly greater on Days 7, 9 and 12 in animals given 1.5 ml NTG/Kg BW compared to those given 0.75 ml/Kg BW ($P<0.01$ on each day).

(d) Immunofluorescence

(i) Immunoglobulin and C3 (Fig. 4.9) Linear deposition of sheep and rabbit immunoglobulin and rabbit C3 along the GBM was seen in all animals. No difference in staining could be detected between the three groups of animals. Neither immunoglobulin nor C3 was found along tubular basement membrane, or along the basement membrane of the parietal layer of Bowman's capsule.

(ii) Fibrin (Figs. 4.10-4.12) Extensive glomerular deposition of fibrin in all animals was found. This deposition was principally within Bowman's space and crescents but was also found within the glomerular tufts. There was no difference in the extent of glomerular fibrin deposition between the groups of animals, as assessed either by the mean fibrin score per glomerulus per animal or the per cent incidence of each grade of fibrin deposited per animal (Table 4.6a).

(e) Histology (Figs. 4.13-4.16)

In most animals a severe crescentic glomerulonephritis developed such that in all the three groups a mean of 41% of glomeruli showed crescents covering greater than two thirds of the circumference of the

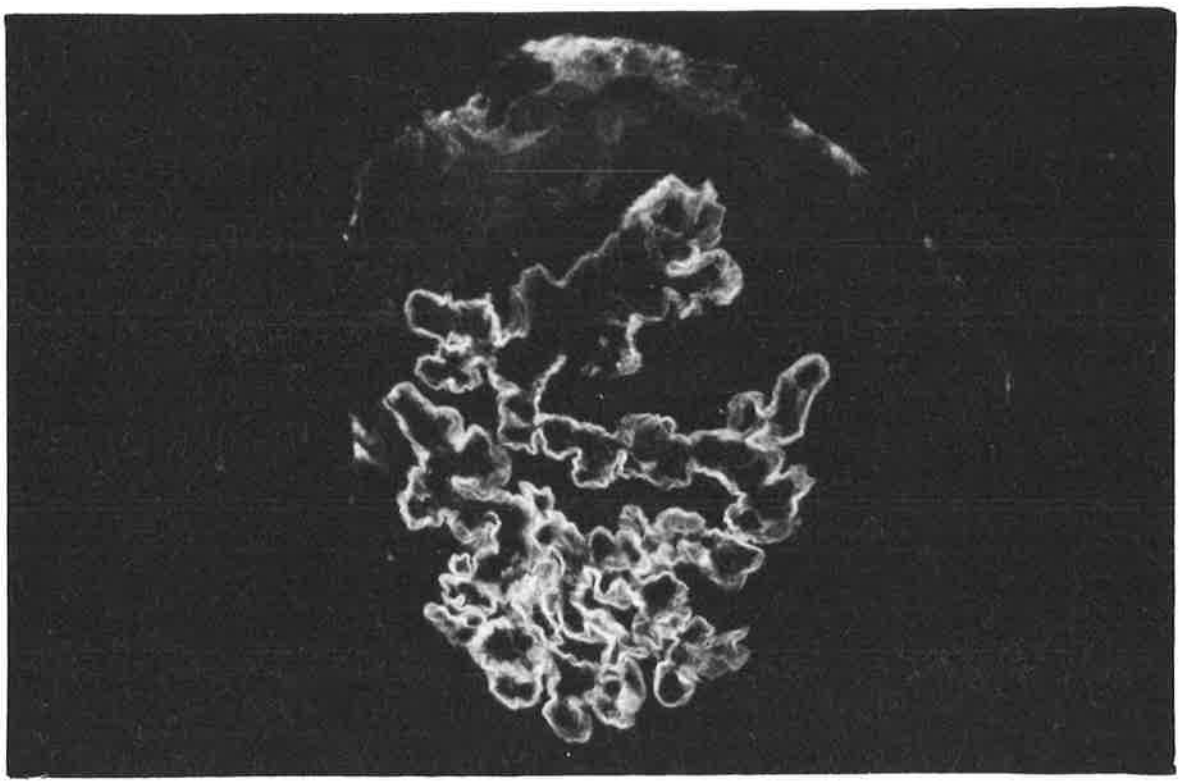

Fig. 4.9 Linear deposition of rabbit immunoglobulin along the GBM of a rabbit during the autologous phase of NTN. The glomerular tuft is distorted by a large surrounding crescent.
(FITC-conjugated sheep anti-rabbit immunoglobulin. Magnification x 600)

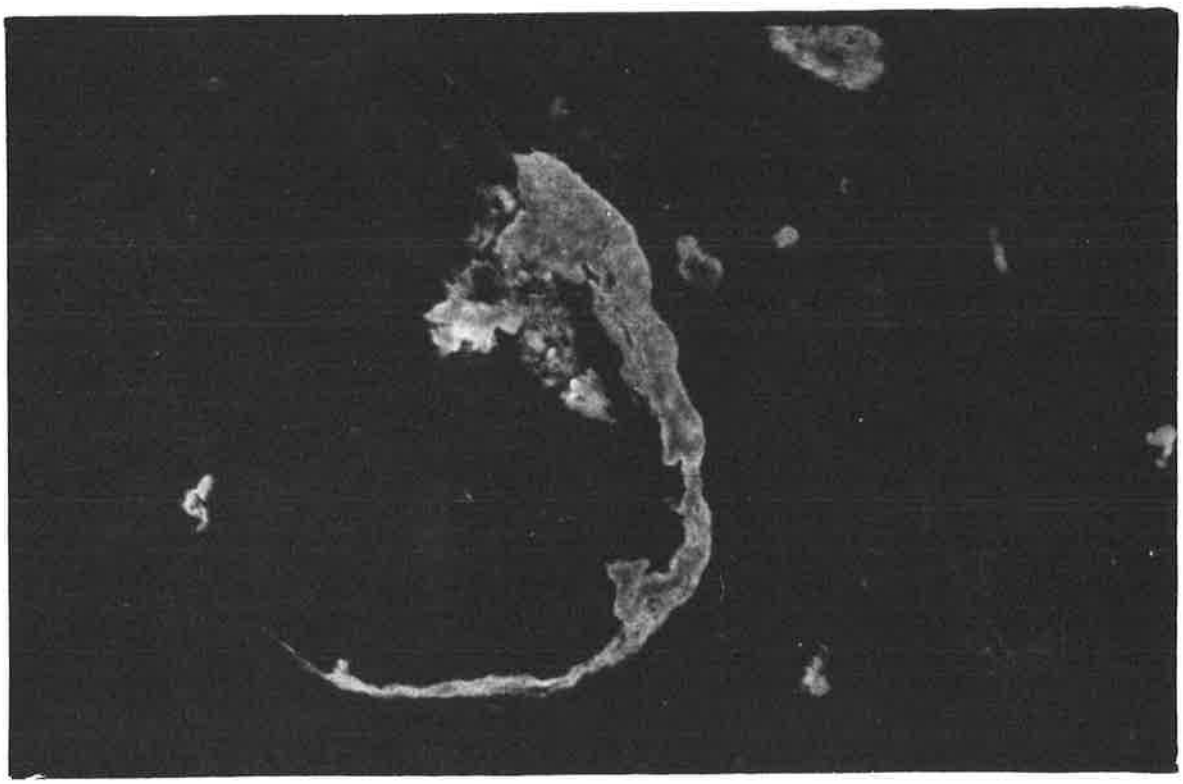

Fig. 4.10 Glomerular fibrin deposition (Grade 1) within Bowman's space and crescent in the autologous phase of NTN in the rabbit.

(FITC-conjugated guinea-pig anti-rabbit fibrin-fibrinogen. Magnification x 600)

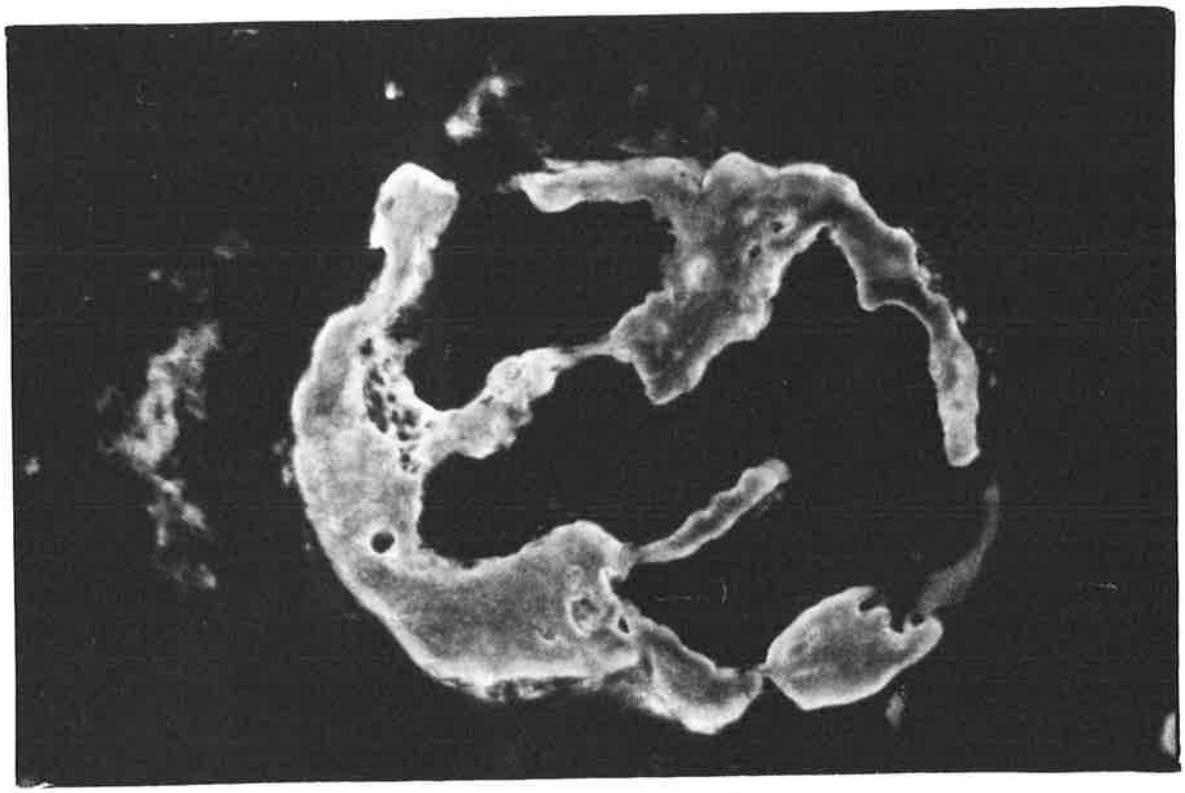

Fig. 4.11 Glomerular fibrin deposition (Grade 2) within Bowman's space and crescent in the autologous phase of NTN in the rabbit.
(Magnification x 600)

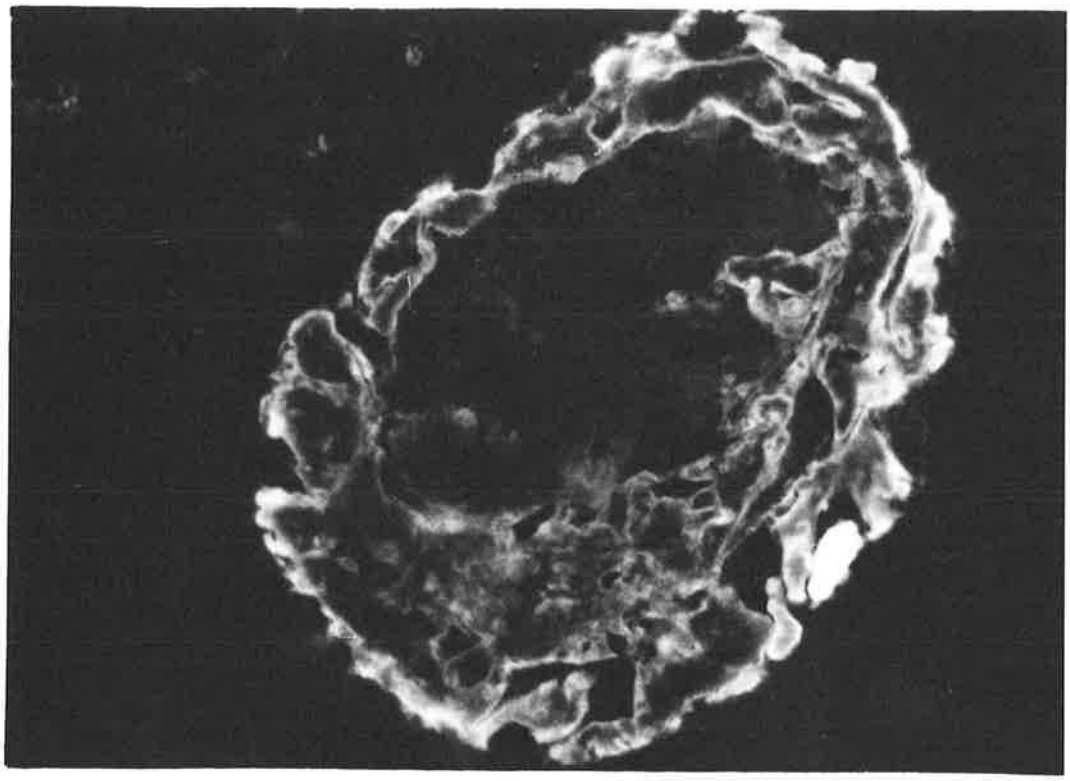

Fig. 4.12 Very extensive glomerular fibrin deposition (Grade 3) in the autologous phase of NTN in the rabbit. (Magnification x 600)

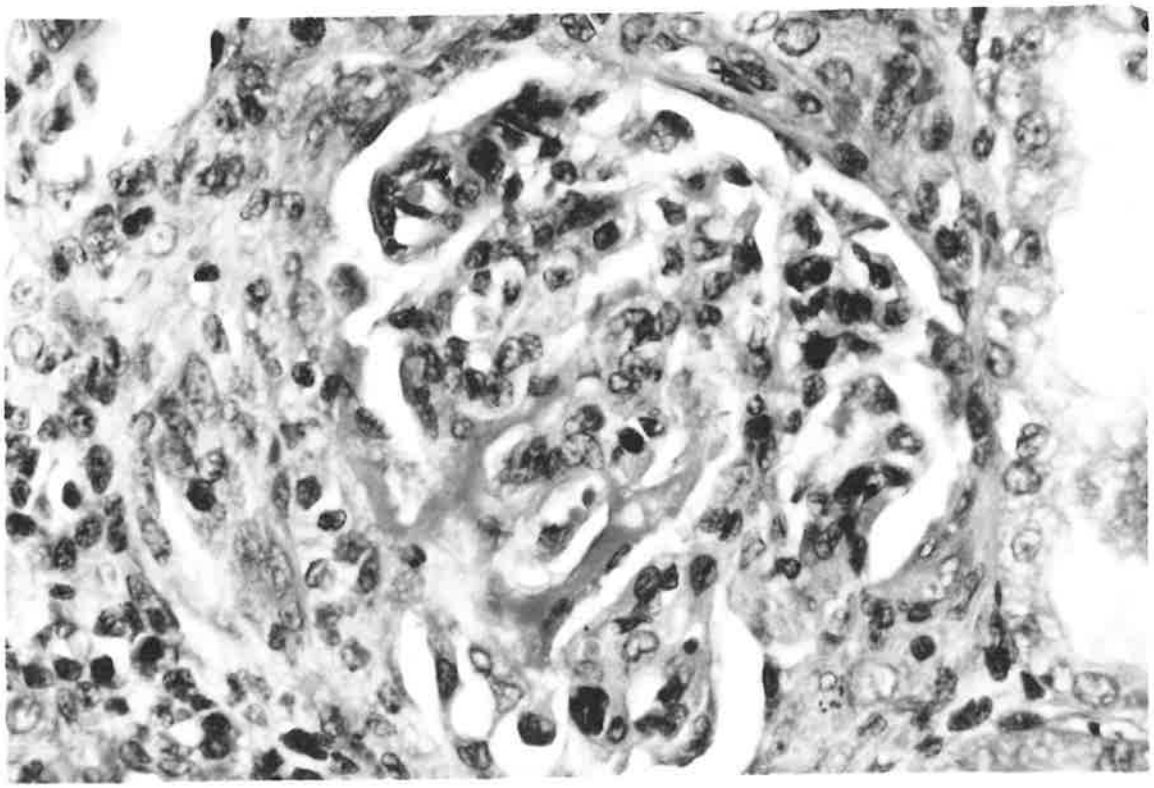

Fig. 4.13 The autologous phase of NTN in the rabbit (Day 12). A glomerulus showing extracapillary cell proliferation with crescent formation (Grade 1), proteinaceous exudation (fibrin) within Bowman's space and infiltration of the glomerular tuft by polymorphonuclear leucocytes.

(H & E x 600)

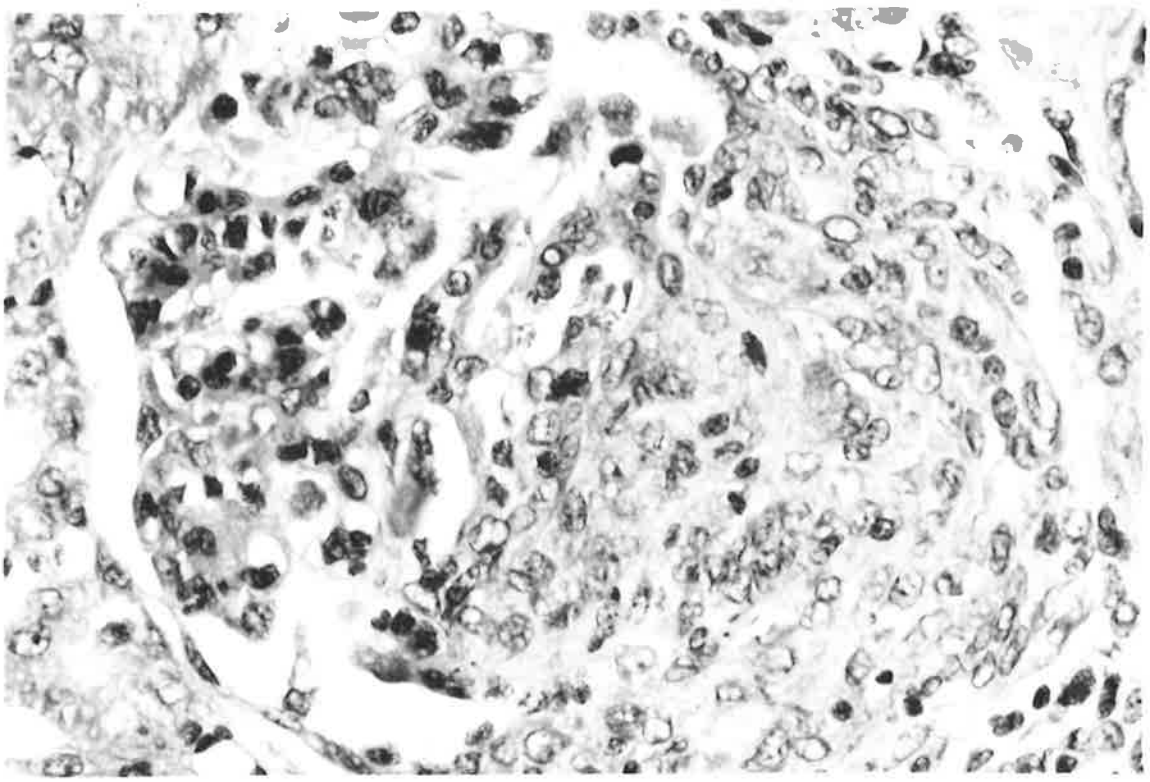

Fig. 4.14 The autologous phase of NTN in the rabbit (Day 12). A glomerulus showing more extensive extracapillary proliferation. (Grade 2 crescent). (H & E x 600)

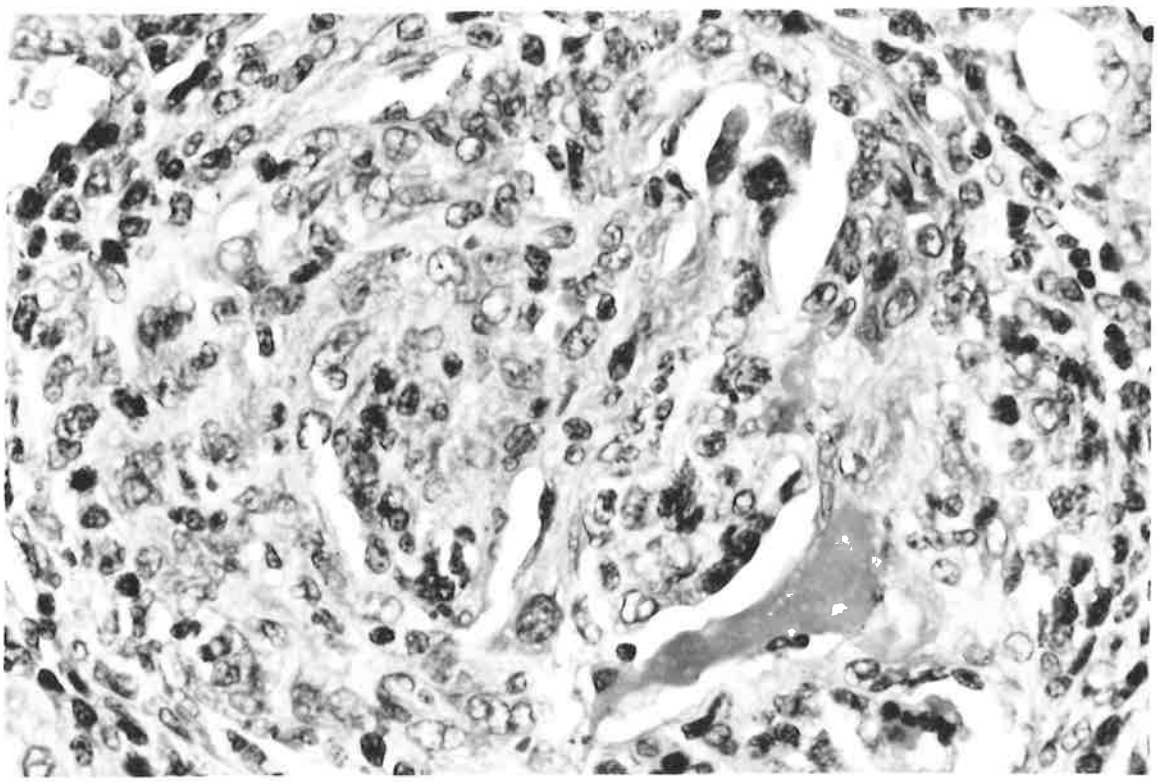

Fig. 4.15 The autologous phase of NTN in the rabbit (Day 12). Total replacement of a glomerular tuft by proliferating extracapillary cells (Grade 3 crescent). A heavy infiltration of polymorphonuclear leucocytes is also present. (H & E x 600)

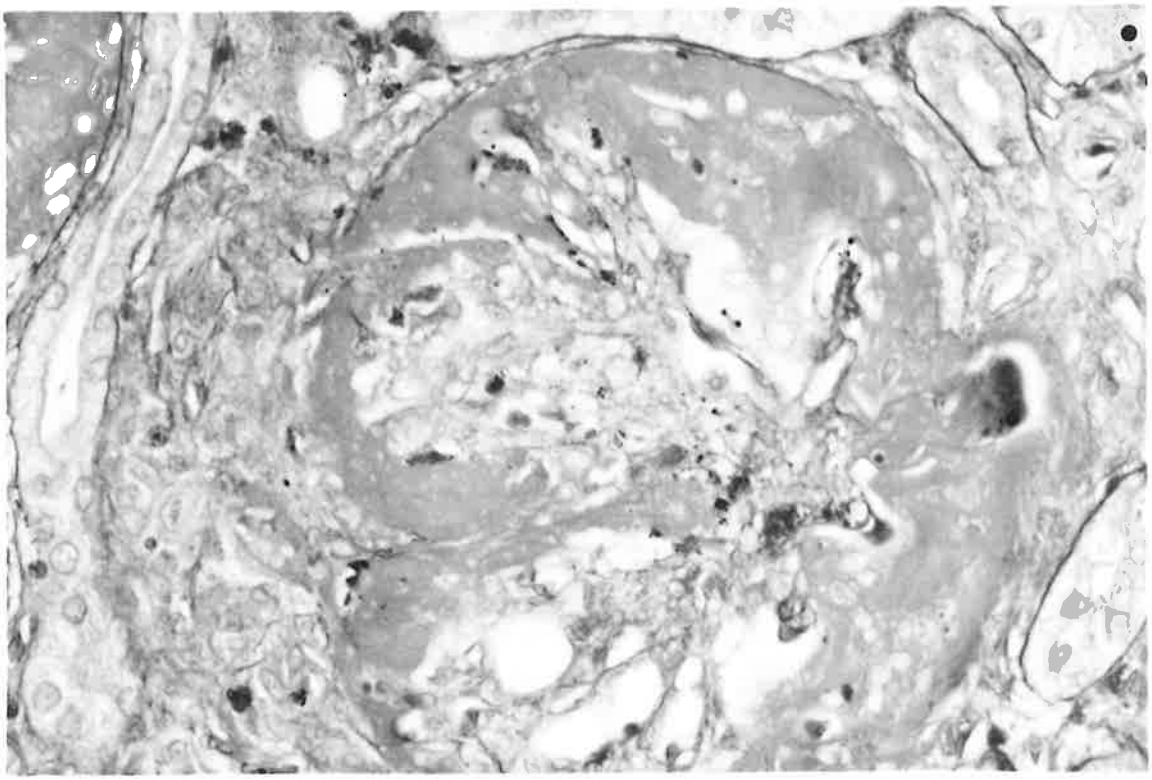

Fig. 4.16 The autologous phase of NTN in the rabbit (Day 12). Extensive glomerular necrosis and proteinaceous exudation (fibrin). (PAS x 600)

ANIMAL GROUP	DOSE OF NTG ml/Kg	n	PER CENT INCIDENCE OF EACH GRADE OF FIBRIN DEPOSITION : Mean (Range)				MEAN FIBRIN SCORE (Range)
			GRADE 0	GRADE 1	GRADE 2	GRADE 3	
A	0.75	6	1.3 (0-5)	25.0 (15-35)	47.5 (35-60)	26.2 (20-35)	1.92 (2.1 -1.85)
B	1.0	6	0	27.0 (10-60)	56.0 (30-70)	17.0 (0-40)	1.90 (1.5-2.3)
C	1.5	6	15.0 (0-30)	26.6 (10-50)	31.4 (30-35)	27.0 (0-55)	1.68 (1.2-2.45)

Table 4.6 (a). Intraglomerular Fibrin Deposition on Day 12 in the Standard Model of NTN. Three Groups of Animals given Different Doses of NTG.

glomerulus. Extensive proteinaceous exudation was seen in Bowman's space and in the crescents corresponding to the fibrin seen on immunfluorescence. PAS stain showed extensive distortion and occasionally fragmentation of the GBM. GBM thickening was not seen. An impression of mild endothelial and mesangial cell proliferation was gained but this was often difficult to assess when the glomerular tuft was distorted and compressed by crescents. PMN were prominent, both within the glomerular tufts and within crescents. This infiltration was focal and showed striking variation from glomerulus to glomerulus. Thus 14% of glomerular tufts contained 0-1 PMN. 63% contained 2-5 PMN and 23% contained 5-36 PMN. Interstitial infiltration by PMN was also prominent in some animals, but this appeared to bear little relationship to the extent of glomerular tuft infiltration.

When extracapillary cell proliferation was quantitated by either the mean crescent score per animal or the per cent incidence of each grade of extracapillary cell proliferation per animal, no difference was found between the three groups of animals (Table 4.6b).

PROTOCOL 3 - "TELESCOPED" MODEL OF NTN

(a) Rabbit Anti-Sheep Immunoglobulin Antibody Titres (Fig. 4.17)

Seven days after the immunisation of the rabbits with normal sheep immunoglobulin, anti-sheep immunoglobulin antibody titres had risen to a mean of 1/4096. Antibody became undetectable immediately after the administration of the NTG but titres progressively rose again to a mean titre of 1/1024 on Day 5.

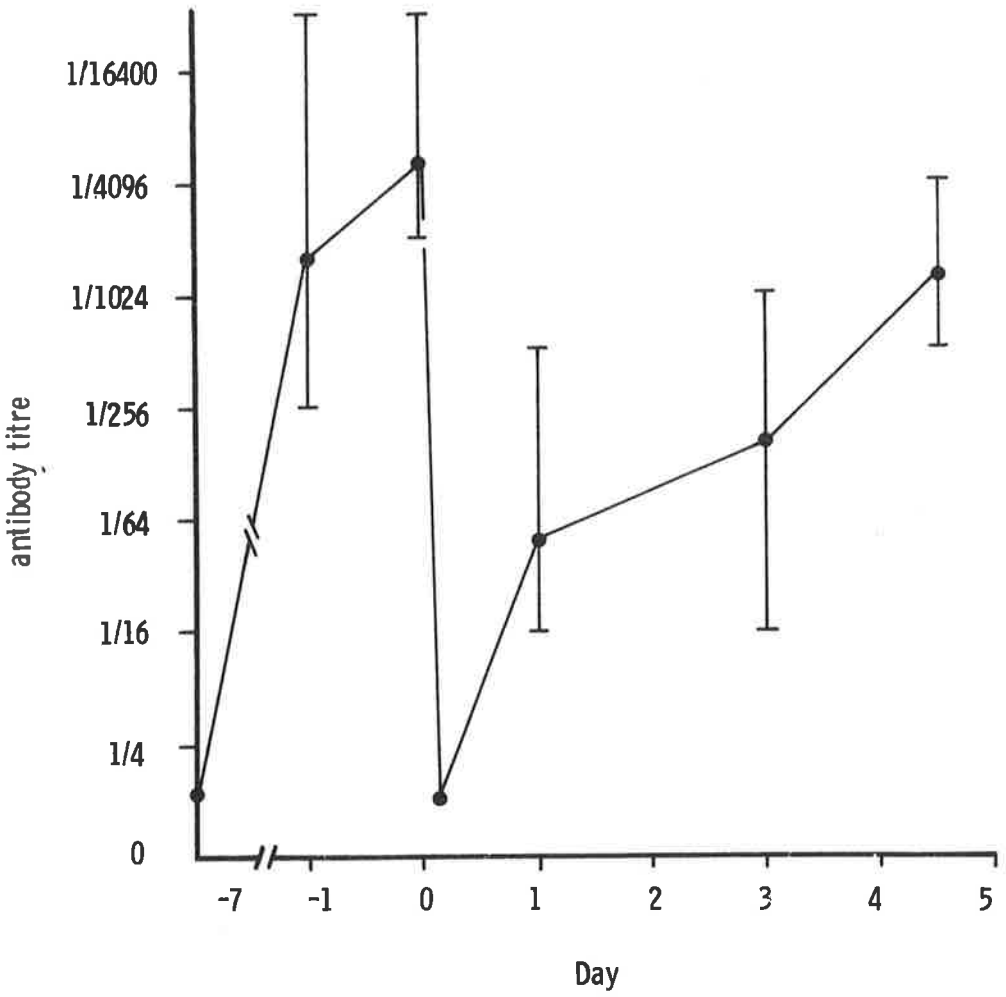

Fig. 4.17 Mean anti-sheep immunoglobulin antibody titres (range I) in the "telescoped" model of nephrotoxic nephritis.

ANIMAL GROUP	DOSE OF NTG ml/Kg	n	PER CENT INCIDENCE OF EACH GRADE OF EXTRA-CAPILLARY CELL PROLIFERATION : Mean (Range)				MEAN CRESCENT SCORE (Range)
			GRADE 0	GRADE 1	GRADE 2	GRADE 3	
A	0.75	6	6.5 (0 - 10)	17.5 (8 - 42)	41.0 (30 - 48)	35.0 (10 - 50)	2.14 (0.92-2.72)
B	1.0	6	15.4 (0 - 70)	11.0 (6 - 28)	25.6 (14 - 42)	48.0 (8 - 76)	2.07 (0.62-2.68)
C	1.5	6	12.0 (0 - 32)	22.7 (12 - 40)	29.3 (12 - 40)	36.0 (12 - 60)	1.89 (1.16-2.36)

Table 4.6(b). Extracapillary Cell Proliferation on Day 12 in the Standard Model of NTN. Three Groups of Animals given Different Doses of NTG.

(b) Renal Function (Fig. 4.18)

Rapid and progressive impairment in renal function was seen as from Day 1. The mean serum creatinine on Day 5 was 5.8 mg/100 ml (range 2.4-10.6).

(c) Proteinuria (Table 4.7)

Heavy proteinuria was found on every day after the injection of NTG.

(d) Serum C3 Concentrations (Fig. 4.19)

A sustained fall in C3 was seen in all animals immediately after the injection of NTG.

(e) Immunofluorescence

(i) Immunoglobulin and C3 Linear deposition of sheep and rabbit immunoglobulin and rabbit C3, along the GBM, was seen in all animals.

(ii) Fibrin Glomerular fibrin deposition was extensive and comparable to that seen on Day 12 in the standard model of NTN (Table 4.8).

(f) Histology (Table 4.9)

Extracapillary cell proliferation with crescent formation was extensive although not as severe as on Day 12 in the standard model of NTN. Glomerular infiltration by PMN was similarly prominent.

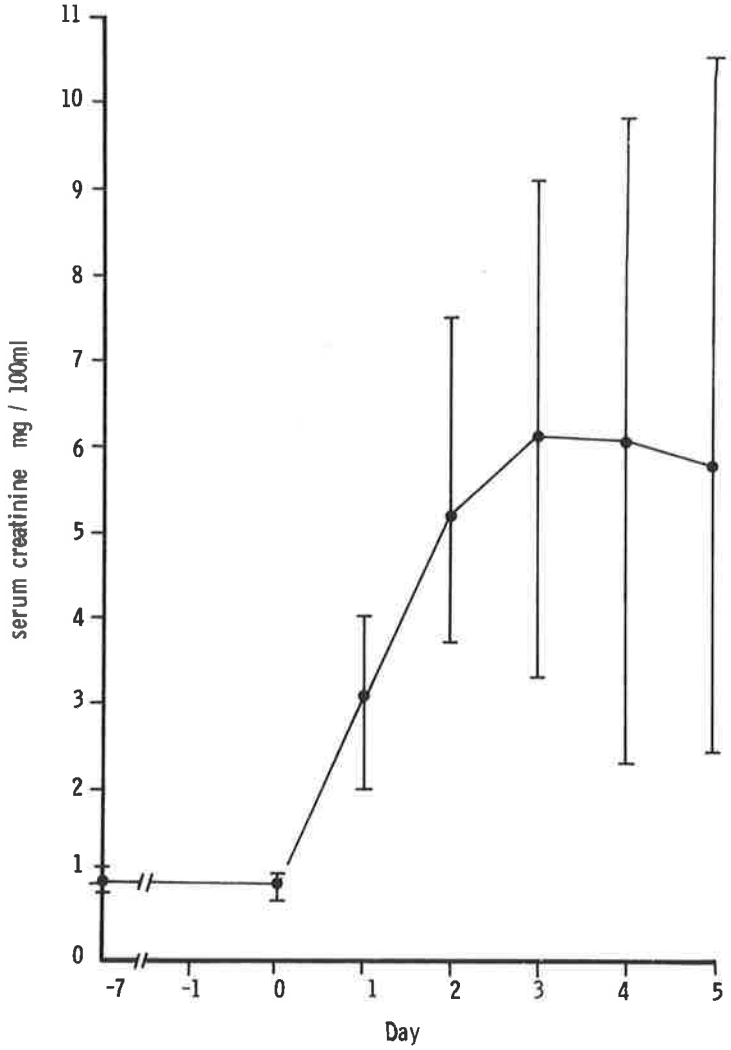

Fig. 4.18 Mean serum creatinine concentrations (range I) in the "telescoped" model of NTN.

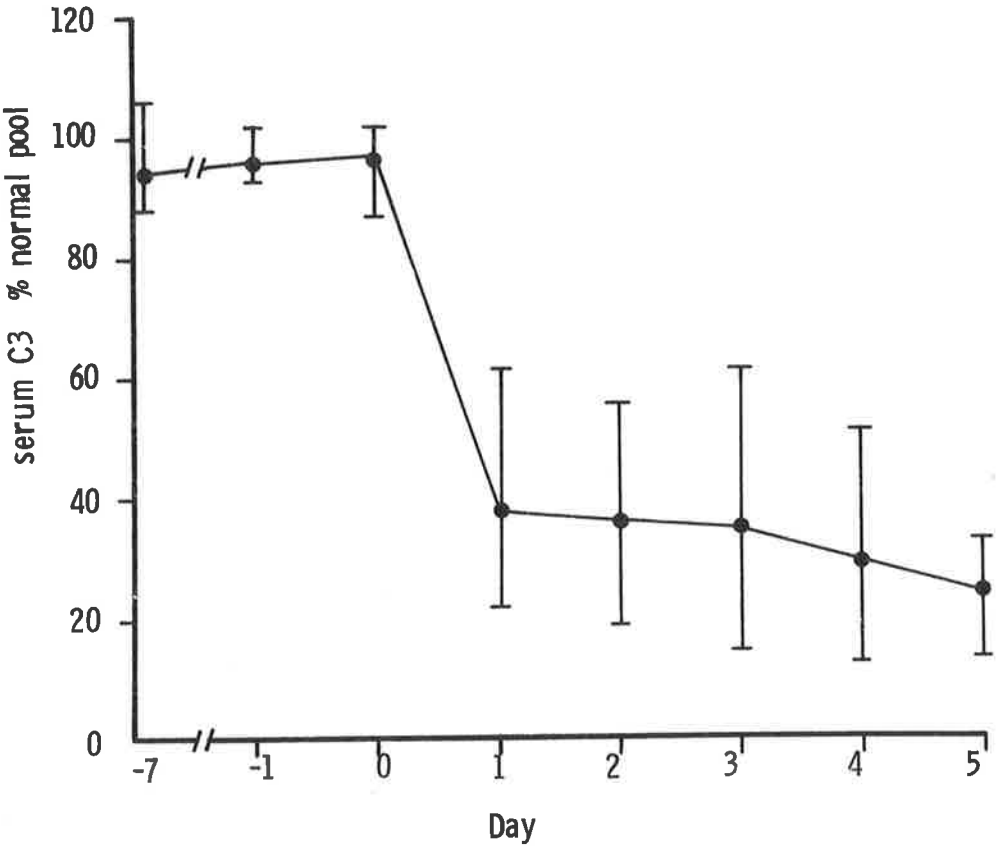

Fig. 4.19 Mean serum C3 concentrations (range I) in the "telescoped" model of NTN.

URINE PROTEIN CONCENTRATION mg/ml : Mean (Range)					
DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
<0.3	28.6 (11.3- 40)	9.6 (5.9-18.3)	11.7 (4.1-20.1)	9.0 (3.4-14.6)	10.1 (5.1-16.2)

Table 4.7 Urine Protein Concentrations in the "Telescoped" Model of NTN.

PER CENT INCIDENCE OF EACH GRADE OF FIBRIN DEPOSITION : Mean (Range)				MEAN FIBRIN SCORE (Range)
GRADE 0	GRADE 1	GRADE 2	GRADE 3	
10.0 (0 - 20)	20.0 (5 - 50)	35.0 (20 - 50)	35.0 (10 - 45)	1.80 (1.2 - 2.3)

Table 4.8 Intraglomerular Fibrin Deposition in the "Telescoped" Model of NTN.

PER CENT INCIDENCE OF EACH GRADE OF EXTRACAPILLARY CELL PROLIFERATION : Mean (Range)				MEAN CRESCENT SCORE (Range)
GRADE 0	GRADE 1	GRADE 2	GRADE 3	
29.3 (7 - 73)	23.3 (3 - 32)	23.4 (10 - 36)	24.0 (0 - 46)	1.02 (0.43 - 2.06)

Table 4.9 Extracapillary Cell Proliferation in the "Telescoped" Model of NTN.

SUMMARY OF RESULTS AND DISCUSSION

1 : THE HETEROLOGOUS PHASE OF THE STANDARD MODEL OF NTN

In these experiments I have shown :-

(a) The quantity of NTAb fixed in the kidney was proportional to the dose of NTG administered, over the limited range of doses used.

(b) A critical quantity of 300-400 µg of our preparation of NTAb had to be fixed in each kidney before the serum C3 concentration fell, PMN accumulated in the glomeruli and heavy proteinuria developed.

(c) The prior depletion of circulating PMN resulted in marked protection from glomerular PMN infiltration and proteinuria whilst the quantity of NTAb fixed in the kidney and fall in serum C3 concentration were similar to those values in animals not depleted of PMN and given the same dose of NTG.

These findings confirm those of Henson (1971 a) who showed that a critical quantity of fixed antibody was required before proteinuria developed and that thereafter small increases in fixed antibody induced marked increases in proteinuria. They also confirm previous findings that the system of injury in the heterologous phase of NTN in rabbits injected with complement fixing antibody is PMN-dependent (Cochrane, Unanue and Dixon, 1965; Hawkins and Cochrane, 1968; Henson, 1971 a). Other workers have also shown that the injury is complement dependent in that

complement activation is required for the PMN infiltration (Kurtz and Donnell, 1962; Hammer and Dixon, 1963; Cochrane, Müller-Eberhard and Aikin, 1970). I have not performed experiments to confirm this.

2 : THE AUTOLOGOUS PHASE OF THE CONVENTIONAL MODEL OF NTN

The experiments of protocol 2 have shown that the injection of 0.75-1.5 ml/Kg BW of our NTG preparation induced within twelve days a severe crescentic glomerulonephritis with a corresponding severe impairment of renal function. The kidneys showed linear deposition of sheep and rabbit immunoglobulin and C3 along the GBM and glomerular fibrin deposition was extensive. Glomerular PMN accumulation was observed although it was not as extensive as in the heterologous phase of injury.

Although different doses of NTG induced significantly different degrees of proteinuria and falls in serum C3 concentration in the heterologous phase, no differences could be detected in proteinuria, renal function, glomerular fibrin deposition or crescent formation in the autologous phase between the groups given these different doses of NTG.

It can be seen that this model of NTN in rabbits has provided an excellent reproducible model of severe crescentic glomerulonephritis. In many respects, human rapidly progressive glomerulonephritis resembles this model of NTN ; glomerular fibrin deposition and crescent formation is extensive, distortions and even breaks in GBM may be seen

(Morita, Suzuki and Churg, 1973) and PMN leucocytes may be present in glomeruli in small numbers or in great profusion (Heptinstall, 1974). A significant number of cases of human rapidly progressive glomerulonephritis show a linear deposition of antibody along the GBM although a granular pattern of immunoglobulin deposition suggesting immune complex deposition is probably more common (Lerner et al, 1967; Lewis et al, 1971; Morel-Maroger et al, 1972).

3 : "TELESCOPED" MODEL OF NTN

It has been shown that the immunisation of rabbits with normal sheep immunoglobulin 7 days before the administration of NTG resulted in a severe crescentic glomerulonephritis, developing within 5 days. Because circulating rabbit antibody to sheep immunoglobulin was present before the injection of NTG, the heterologous and autologous phases of injury were indistinguishable.

The advantage of a model of NTN in which severe injury develops within 5 days of the injection of NTG is that certain procedures in the investigation of mediation of injury, such as decomplementation with cobra venom factor, may be initiated prior to the injection of NTG. This advantage will be further elucidated in Part IV of this thesis.

Chapter 5

BOVINE SERUM ALBUMEN-INDUCED CHRONIC IMMUNE-COMPLEX NEPHRITIS

Introduction

The repeated daily IV administration of bovine serum albumen (BSA) to rabbits will result in repeated deposition of immune complexes of BSA-anti-BSA in the kidney with resultant chronic glomerulonephritis (see Introduction). Whether an animal will develop glomerulonephritis, and the severity of the nephritis that does develop, depends on the following factors: the antibody response of the animal, the dose of antigen used and whether the dose is varied according to antibody response, and the period of administration of the antigen (Dixon et al, 1961; Germuth et al, 1967). The wide variation in the severity of the glomerulonephritis, from mild mesangial proliferative disease to a severe crescentic nephritis, makes the model somewhat unsuitable for use in evaluation of various therapeutic measures or investigation of mechanisms of glomerular injury. On the other hand, as the glomerulonephritis is due to immune complex deposition the model is of particular relevance to human chronic glomerulonephritis.

In this chapter I have described the production of a model of BSA-induced chronic immune-complex glomerulonephritis in which a high proportion of animals developed a severe crescentic glomerulonephritis. This was achieved by immunising the animals with BSA prior to the daily IV BSA administration and varying the daily dose of BSA according to antibody response. The reason for immunisation with BSA in adjuvant was to induce maximum antibody production in all animals.

EXPERIMENTAL PROTOCOL

Twenty rabbits were immunised intramuscularly with 10 mg of BSA in FCA 5 days before starting the daily IV BSA administration.

As from Day 0, each animal was given a single daily IV injection of 15 mg of BSA in saline until circulating anti-BSA antibody was demonstrated. Thereafter the dose of antigen was adjusted according to the antibody response. Antibody response was monitored by two methods :

1 : The measurement twice a week of circulating antibody by radial immunodiffusion in gel (Mancini technique - see Methods), at 1 hour, 6 hours, 12 hours and 24 hours after the previous dose of BSA.

2 : A ^{125}I-labelled BSA clearance study (as described in Methods) performed once a week.

The objec t was to keep the animals in circulating antigen excess for 8-18 hours after each dose of BSA.

The dose of BSA was raised by 5 mg for every 500 ug of circulating anti-BSA antibody present in 1 ml of serum 24 hours after the last dose of BSA.

Urine samples for protein concentration and serum samples for C3 and creatinine concentrations were obtained twice weekly.

A renal biopsy was performed on each surviving animal on Day 35. Surviving animals were killed on Day 54 and the kidneys were removed for histology and for immunofluorescence examination for BSA, rabbit immunoglobulin and C3.

RESULTS

(a) Deaths

Thirteen rabbits died ; three from diseases unrelated to BSA administration (middle ear infection in one and infective diarrhoea in two), six from anaphylaxis on Days 12, 18, 22 and 23, and four from renal failure on Days 41, 44, 48 and 50.

(b) Anaphylaxis

Mild or moderate dyspnoea immediately following an IV dose of BSA was seen in most animals at some stage. Fatal anaphylaxis was largely prevented by slow administration of the BSA, increasing the daily dose of BSA according to antibody response and giving large doses of BSA in divided doses over a period of one hour. Six animals died in the first 28 days from anaphylaxis because insufficient attention had been given to these preventative measures. Antibody response in animals dying from anaphylaxis was no greater than in those in which anaphylaxis did not occur.

(c) Clearance of ^{125}I-labelled BSA

The rates of clearance of BSA from the circulation were grouped as follows : (Fig. 5.1)

Group A - antigen cleared within 1 hour of BSA administration
Group B - antigen cleared between 1 and 6 hours
Group C - antigen cleared between 6 and 12 hours
Group D - antigen cleared between 12 and 24 hours
Group E - antigen still present in the circulation at 24 hours

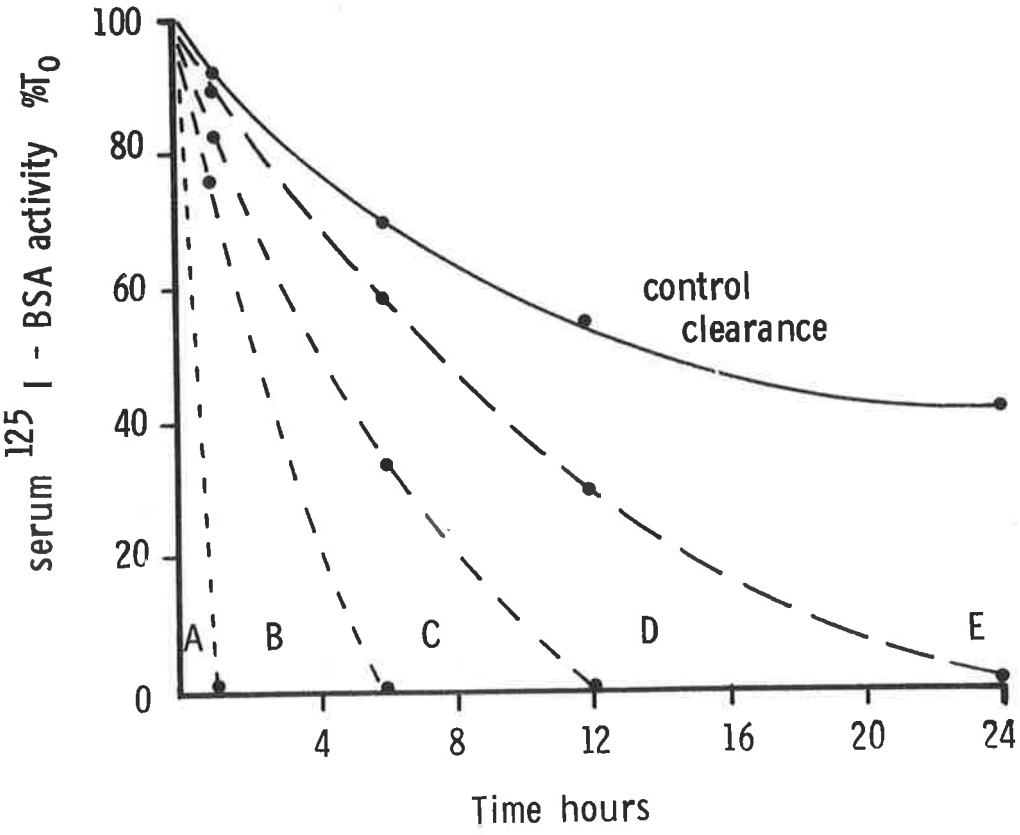

Fig. 5.1 Clearance of radiolabelled BSA from the circulation in animals repeatedly injected IV with BSA. Five groups of animals defined, according to the rate of clearance of BSA :

Group A - BSA cleared within 1 hour of administration

Group B - BSA cleared between 1 and 6 hours

Group C - BSA cleared between 6 and 12 hours

Group D - BSA cleared between 12 and 24 hours

Group E - BSA still present in the circulation at 24 hours

It was found that animals could be readily switched from one group to another by increasing or decreasing the daily dose of antigen.

The greatest concentrations of circulating immune complexes of BSA - anti-BSA were found in groups C and D. Although the concentrations at 6 hours in Group D were half those in Group C, the period during which complexes were circulating was probably twice as long in Group D as in Group C. Circulating immune complexes were not found in Groups A or E at any stage and in Group B small concentrations only were detected at 1 hour. One of these clearance studies, performed on Day 14 is given in Table 5.1. This table also shows that there is an excellent correlation between the disappearance of antigen from the circulation as measured by ^{125}I-BSA clearance and the appearance of circulating anti-BSA antibody in the serum.

(d) Anti-BSA Antibody Response And Daily Dose of BSA (Fig. 5.2)

All animals produced anti-BSA antibody. The antibody response, as measured by the dose of antigen required to maintain antigen excess for 8-18 hours of each 24-hour period was maximal from Days 21-42 but thereafter declined rapidly. During this 21 day period of maximum antibody response the lowest daily dose of BSA administered to an animal was 30 mg, and the highest 260 mg.

(e) Proteinuria

Proteinuria was detected as early as Day 6 in four animals and was present in all the others by Day 21. Once present, proteinuria was persistent in all animals. Heavy proteinuria (in excess of 20 mg/ml) was

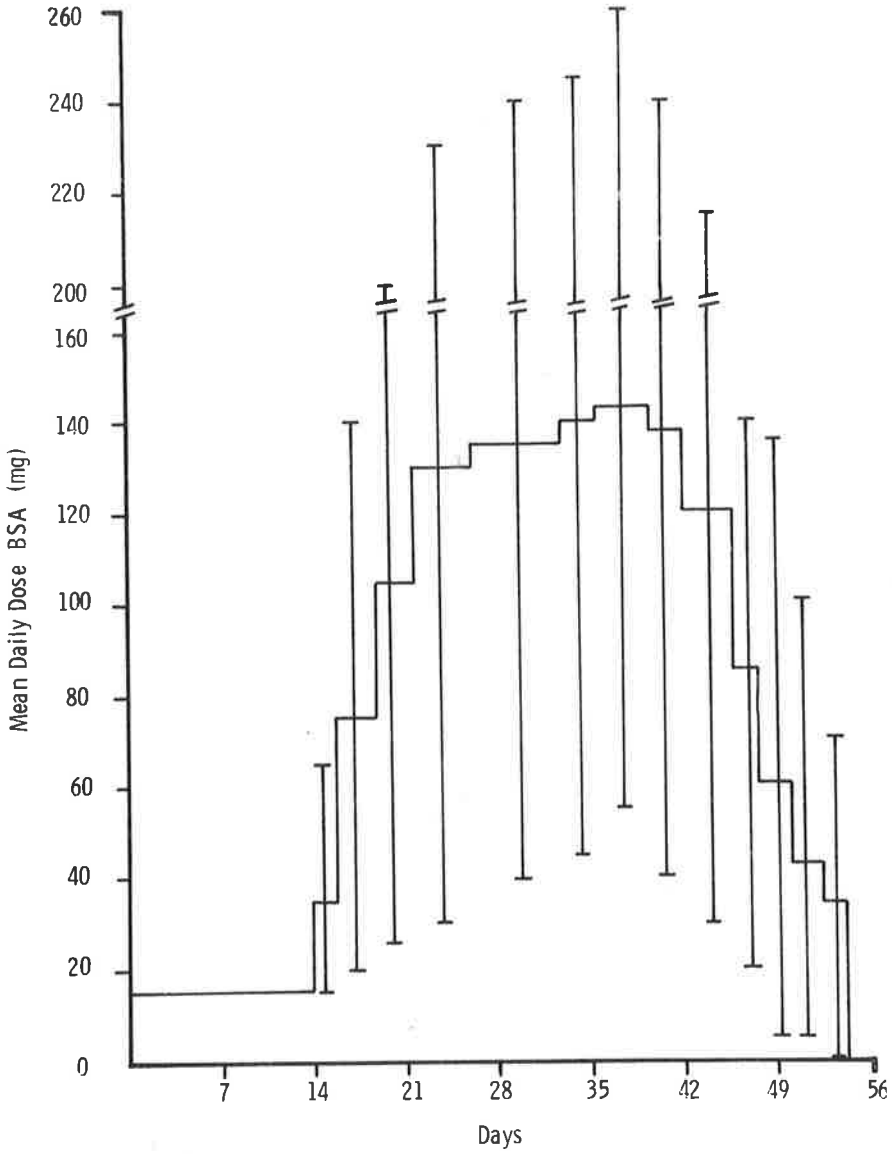

Fig. 5.2 Mean daily dose of BSA (range I) in rabbits with BSA-induced chronic immune-complex glomerulonephritis.

ANIMAL GROUP	n	Mean per cent of injected ^{125}I-BSA in the circulation (Mean per cent of injected ^{125}I-BSA complexed to globulin)				Presence of detectable serum antibody to BSA - number of animals			
		1 HR	6 HR	12 HR	24 HR	1 HR	6 HR	12 HR	24 HR
A	2	1 (0)	1 (0)	1 (0)	1 (0)	1	2	2	2
B	3	37 (0)	1 (0)	1 (0)	1 (0)	0	3	3	3
C	7	80 (1.1)	17 (5.9)	1 (0)	1 (0)	0	0	7	7
D	5	86 (0)	46 (2.8)	15 (3.5)	1 (0)	0	0	0	5
E	1	90 (0)	64 (0)	42 (0)	21 (0)	0	0	0	0
Controls	2	93 (0)	70 (0)	55 (0)	42 (0)	0	0	0	0

Table 5.1 ^{125}I-BSA Clearance Study on Day 14 in Rabbits receiving daily IV doses of BSA. Controls had not previously received BSA.

seen in 65% of animals at some stage of the disease, moderate proteinuria (11-20 mg/ml) in 35% and mild proteinuria (2-10 mg/ml) in the remaining 10%.

Proteinuria developed earlier and was heavier in animals in which BSA was consistently cleared from the circulation between 6 and 24 hours and antibody to BSA was first detected in the serum between 6 and 24 hours (Table 5.2).

(f) Serum C3 Concentrations (Fig. 5.3)

A progressive fall in the serum C3 concentration was observed from Day 15, and concentrations reached a minimum mean of 43.2% (range 19-54%) on Day 35. On Day 54 the mean concentration was 54.1% (range 28-84%).

(g) Renal Function (Fig. 5.4 ; Table 5.3)

Only mild impairment in renal function was seen before Day 28 of the disease. After Day 35, renal function deteriorated rapidly in most animals such that four animals died from renal failure. Again, animals in which antigen was consistently cleared from the circulation by between 6-24 hours and free antibody appeared in the circulation, developed renal failure more rapidly (Table 5.2).

(h) Immunofluorescence

(i) Immunoglobulin, BSA and C3

Animals dying before Day 35 (Fig. 5.5)

Mild to moderate deposits of BSA, rabbit immunoglobulin and

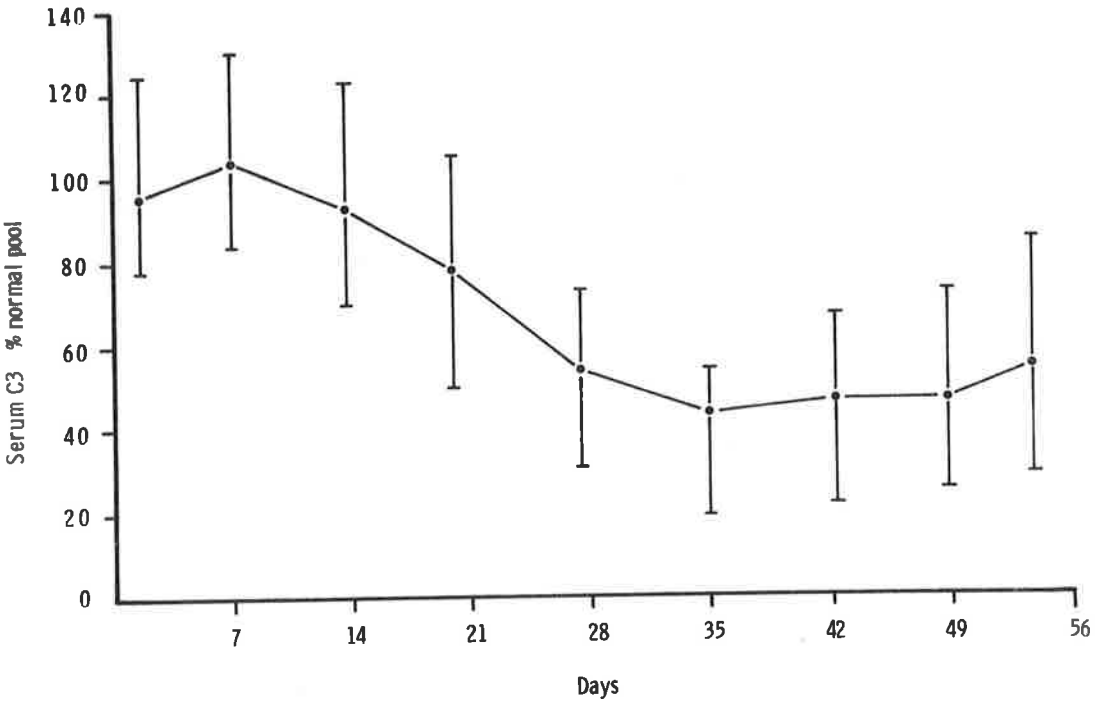

Fig. 5.3 Mean serum C3 concentrations (range I) in rabbits with BSA-induced chronic immune-complex glomerulonephritis.

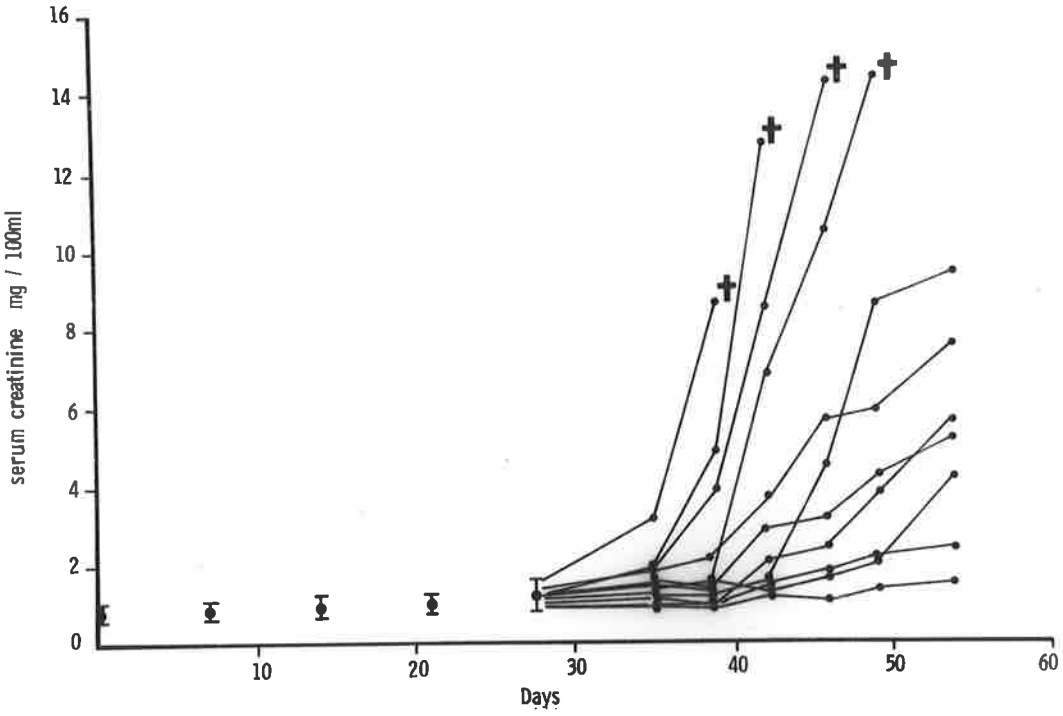

Fig. 5.4 Serum creatinine concentrations in rabbits with BSA-induced chronic immune-complex glomerulonephritis. († Died)

FREQUENCY OF CLEARANCE OF CIRCULATING BSA BY BETWEEN 6 AND 24 HOURS	RABBIT NUMBER	DAY PROTEINURIA FIRST DETECTED (Mean)	MAXIMUM URINE PROTEIN CONCENTRATION mg/ml (Mean)	SERUM CREATININE ON DAY 54 OR AT DEATH mg/100 ml (Mean)
CONSISTENTLY	1	9	18	9.4
	3	6	65	14.3
	11	14 (10.4)	34 (40.2)	12.8 (10.5)
	16	14	58	7.5
	18	9	26	8.6
OFTEN	13	21	45	5.6
	14	14 (14.6)	14 (25.4)	5.1 (8.1)
	17	9	18	14.2
OCCASIONALLY	9	14 (17.5)	4.5 (8.3)	4.3 (3.3)
	5	21	12	2.3
NEVER	10	21	2.8	1.5

Table 5.2 BSA-Induced Chronic Immune-Complex Glomerulonephritis. Animals surviving longer than 35 days. The relationship of consistency of clearance of circulating BSA by between 6 and 24 hours, to the time of appearance of Proteinuria, Degree of Proteinuria, and Serum Creatinine at death.

Rabbit Number	Serum Creatinine Concentration mg/100 ml							
	Day 14	Day 21	Day 28	Day 35	Day 42	Day 46	Day 49	Day 54
1	0.9	1.1	1.1	1.2	1.6	4.5	8.6	9.4
2	† anaphylaxis							
3	0.9	0.9	1.2	1.6	6.8	10.5	14.3 †	renal failure
4	0.9	1.0	† anaphylaxis					
5	0.7	0.8	1.0	1.1	1.5	1.7	2.1	2.3
6	1.0	0.9	† infective diarrhoea					
7	0.7	0.9	† anaphylaxis					
8	0.9	† anaphylaxis						
9	1.1	1.0	1.2	1.4	1.3	1.6	2.0	4.3
10	1.0	0.9	0.9	0.9	1.1	1.0	1.3	1.5
11	1.1	1.1	1.2	2.9	12.8	† renal failure		
12	1.2	1.0	† anaphylaxis					
13	0.9	1.0	1.0	0.9	2.1	2.4	3.8	5.6
14	0.9	1.2	1.1	1.5	2.9	3.1	4.2	5.1
15	† middle ear infection							
16	0.9	1.0	1.4	1.8	3.6	5.6	5.9	7.5
17	1.0	0.9	1.1	1.9	8.4	14.2	† renal failure	
18	1.1	0.8	1.6	3.1	† renal failure			
19	1.0	1.0	† anaphylaxis					
20	0.9	† infective diarrhoea						
Mean	0.9	0.9	1.2	1.7	4.2	4.9	5.3	5.1

Table 5.3 Serum Creatinine Concentrations in Rabbits with BSA-Induced Chronic Immune-Complex Glomerulonephritis. Mean Concentration prior to starting BSA (Day 0) was 0.7 mg/100 ml (Range 0.6-1.0 mg/100 ml)

(† Died)

rabbit C3 were seen in the mesangium of four rabbits dying from anaphylaxis after Day 18. In addition, mild granular deposits along the GBM were seen in two of these animals.

Biopsies at Day 35 (Fig. 5.6)

Heavy granular deposits of rabbit immunoglobulin, C3 and BSA predominently along the GBM was seen in nine of the eleven animals. In the remaining two animals (numbers 5 and 10) less marked GBM but greater mesangial deposits were observed.

Animals dying after Day 35 or killed on Day 54 (Figs. 5.7 and 5.8)

Heavy granular deposits of rabbit immunoglobulin, C3 and BSA along the GBM were seen in all animals.

(ii) Fibrin

Glomerular fibrin deposition was not seen in any animal before Day 35 or in the biopsy specimens taken on Day 35.

Animals dying after Day 35 or killed on Day 54

Extensive glomerular fibrin deposition particularly within Bowman's space and crescents, but also within the glomerular tuft, was seen in nine of the eleven animals (Table 5.4). This fibrin deposition was similar to that seen in NTN.

(i) Histology

Animals dying before Day 35

Abnormal histological changes were not seen in the kidneys of the three animals dying from infective diarrhoea or middle ear infection

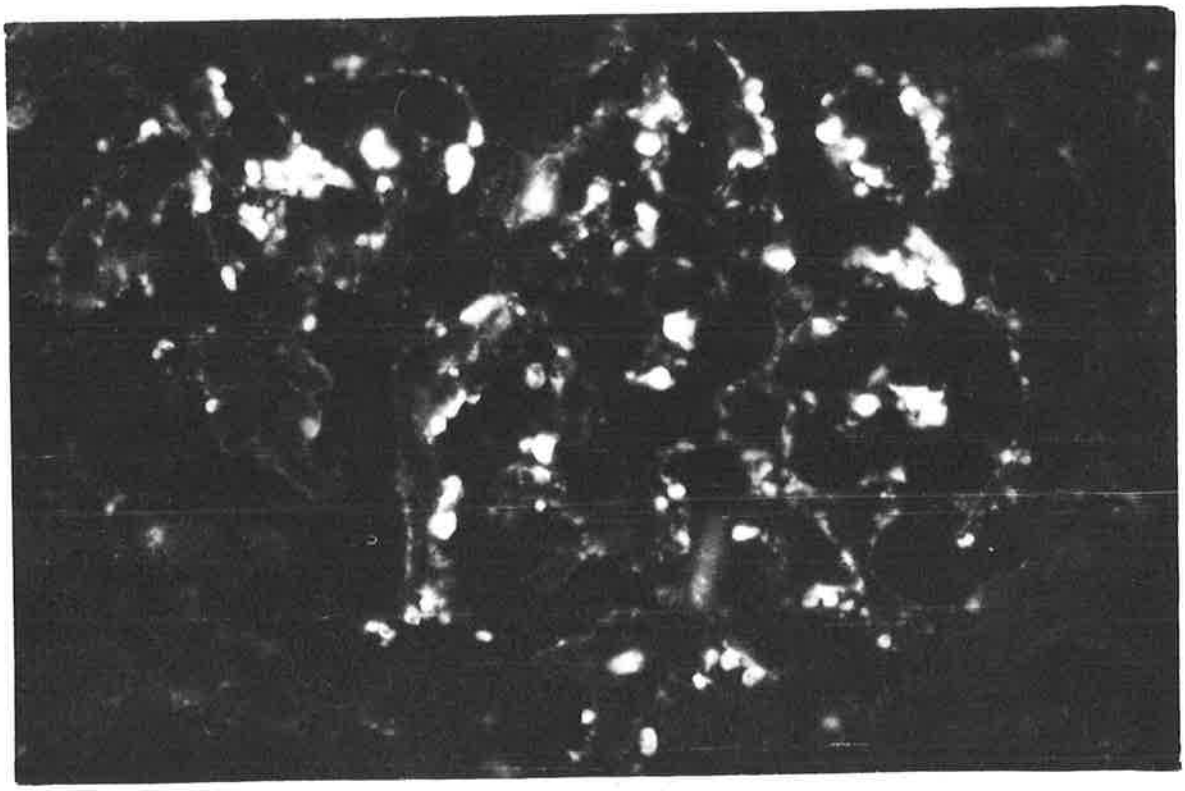

Fig. 5.5 BSA-induced chronic immune-complex glomerulonephritis in the rabbit. Day 22. BSA deposits principally located within the mesangium.
(FITC-conjugated rabbit anti-BSA. Magnification x 600)

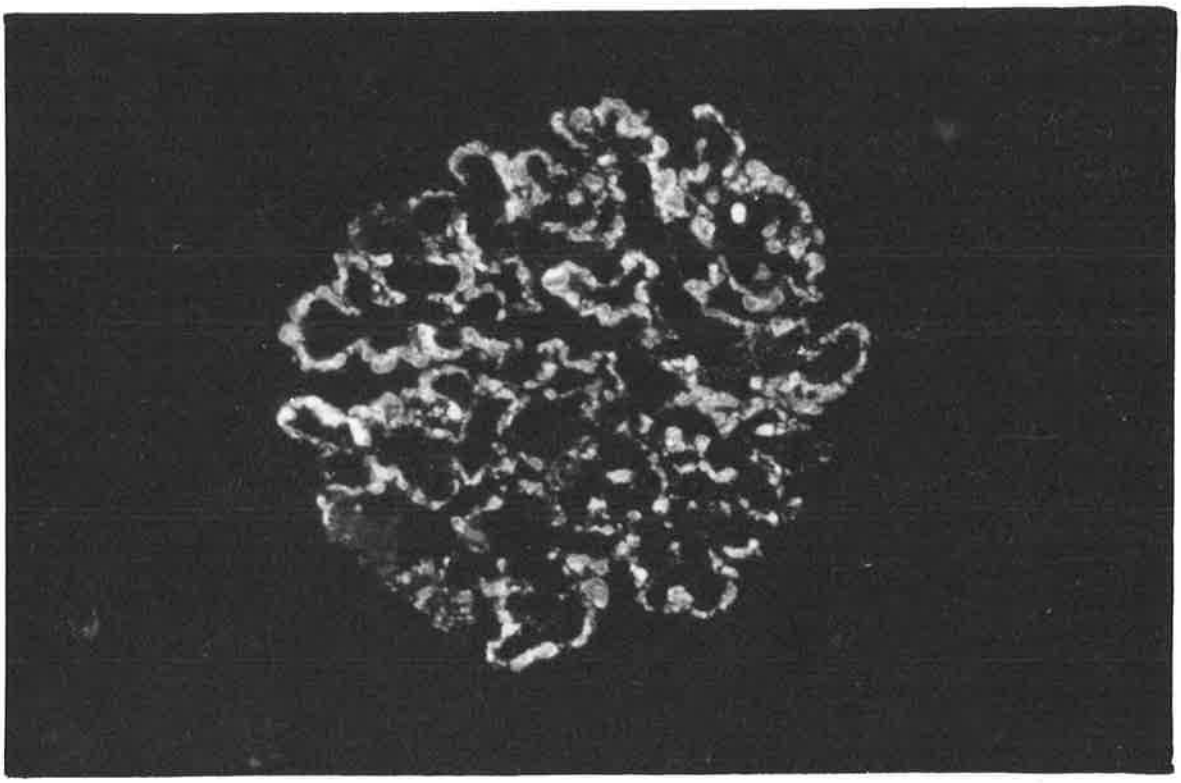

Fig. 5.6 BSA-induced chronic immune-complex glomerulonephritis. Day 35. Heavy granular deposition of BSA along the epithelial aspect of the GBM. (Magnification x 600)

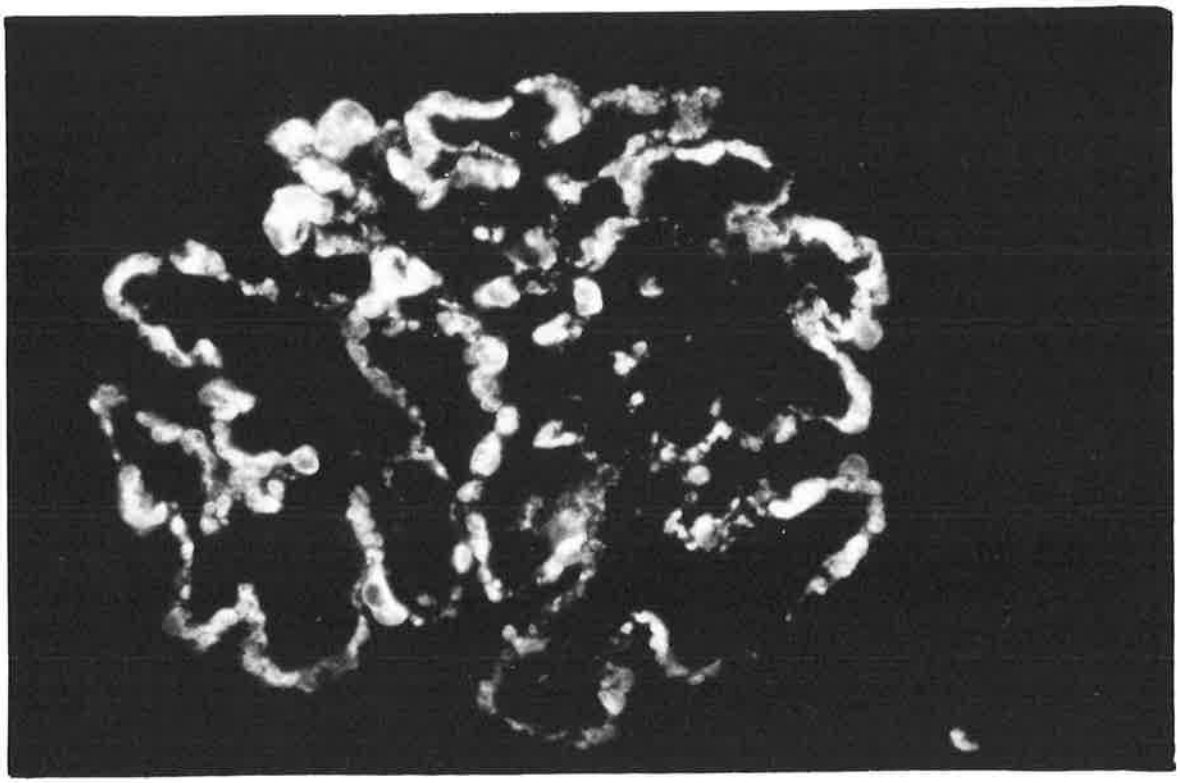

Fig. 5.7 BSA-induced chronic immune-complex glomerulonephritis. Day 54. Heavy granular deposition of rabbit immunoglobulin along the epithelial aspect of the GBM. The capillary loops are distorted by the cellular proliferation. (FITC-conjugated sheep anti-rabbit immunoglobulin. Magnification x 600)

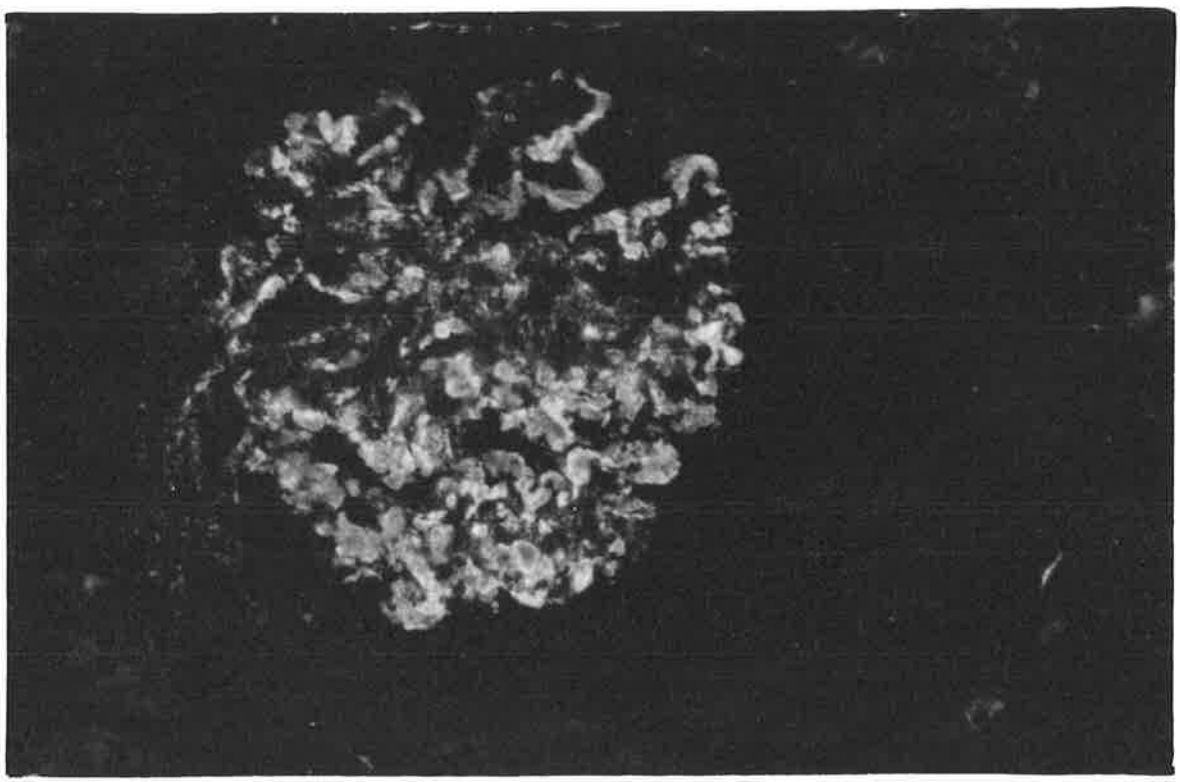

Fig. 5.8 BSA-induced chronic immune-complex glomerulonephritis. Day 54. Glomerular tuft showing BSA deposits along the GBM but distorted by a surrounding crescent.
(Magnification x 200)

RABBIT NUMBER	SERUM CREATININE mg/100 ml	FIBRIN SCORE	CRESCENT SCORE
1	9.4	1.7	2.1
3	14.3	2.3	2.8
5	2.3	0	0
9	4.3	0.7	1.2
10	1.5	0	0
11	12.8	2.1	2.4
13	5.6	0.8	1.3
14	5.1	0.5	1.1
16	7.5	1.2	1.4
17	14.2	1.9	2.4
18	8.6	1.1	1.8
Mean	7.8	1.12	1.50

Table 5.4 Serum Creatinine Concentrations, Fibrin Scores and Crescent Scores on Day 54 or at Death after Day 35, in Rabbits with BSA-Induced Chronic Immune-Complex Glomerulonephritis.

or of the animal who died from anaphylaxis on Day 12. Of the four animals dying from anaphylaxis after Day 18, mild endothelial and mesangial cell proliferative changes were seen, and in two, these changes were associated with PMN infiltration.

Biopsies at Day 35 (Figs. 5.9-5.11)

In the eleven specimens examined, a number of histological changes were seen. All animals showed some degree of diffuse or focal endothelial and mesangial cell proliferation. Infiltration of glomeruli by PMN was common and occasionally very extensive. In the PAS stained sections diffuse thickening of the GBM was seen in four animals and reduplication and splitting of the GBM in three animals. Although adhesion between parietal and visceral layers of epithelial cells were common, extracapillary cell proliferation with crescent formation was not seen.

Animals dying after Day 35 or killed on Day 54 (Fig. 5.12)

Diffuse or focal endothelial and mesangial cell proliferation was seen in all kidneys and infiltration of glomeruli by PMN was prominent. Again, GBM changes of uniform thickening or splitting were commonly seen. Extensive extracapillary cell proliferation with crescent formation was observed in nine of the eleven kidneys (Table 5.4).

SUMMARY OF RESULTS AND DISCUSSION

These experiments have shown that in BSA-induced chronic immune-complex disease in rabbits, a high percentage of animals (82% of those not dying from anaphylaxis or unrelated diseases) developed a severe

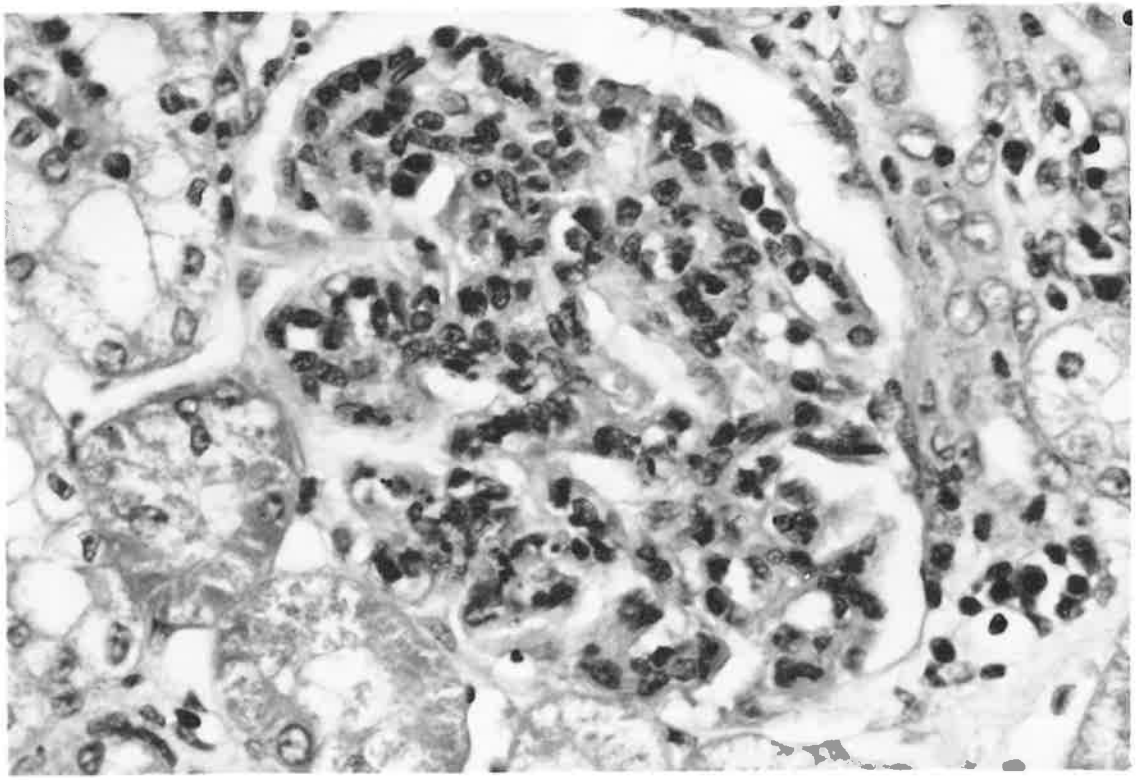

Fig. 5.9 BSA-induced chronic immune-complex glomerulonephritis in rabbits. Renal biopsy specimen taken on Day 35, showing diffuse proliferation of mesangial and endothelial cells and a heavy infiltration of polymorphonuclear leucocytes. (H & E x 600)

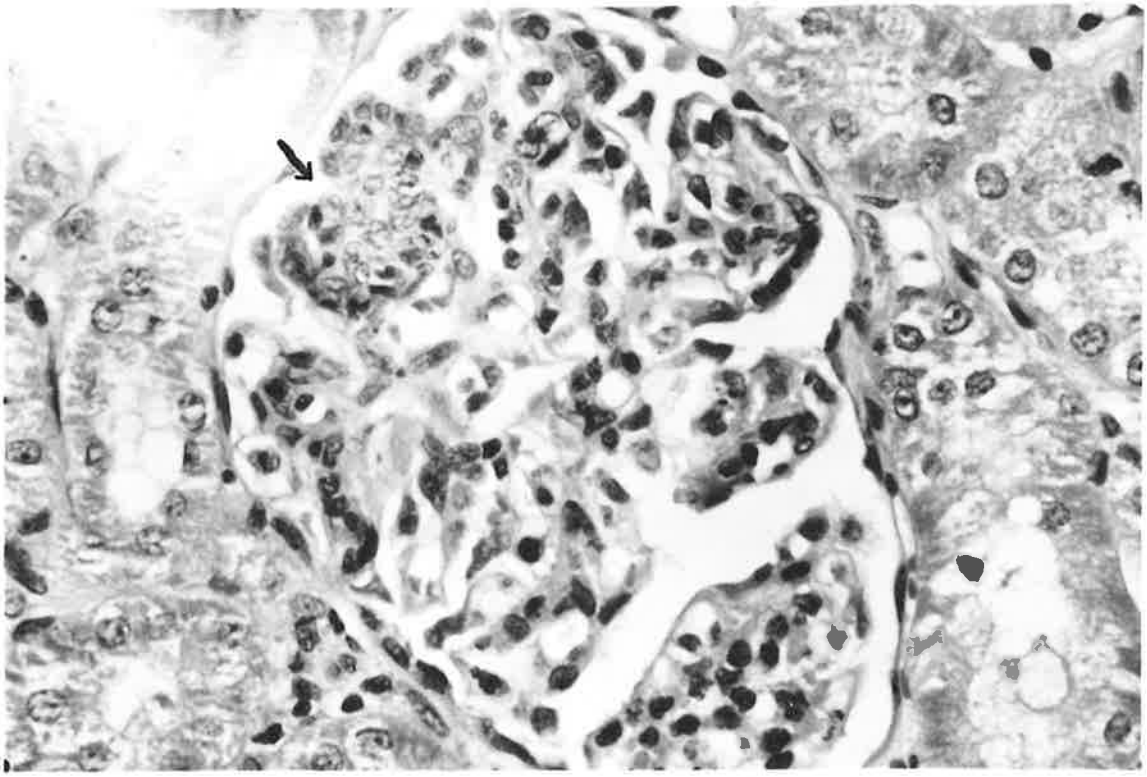

Fig. 5.10 BSA-induced chronic immune-complex glomerulonephritis in rabbits. Renal biopsy specimen taken on Day 35, showing an area of focal mesangial cell proliferation (↑) and diffuse glomerular infiltration by polymorphonuclear leucocytes. (H & E x 600)

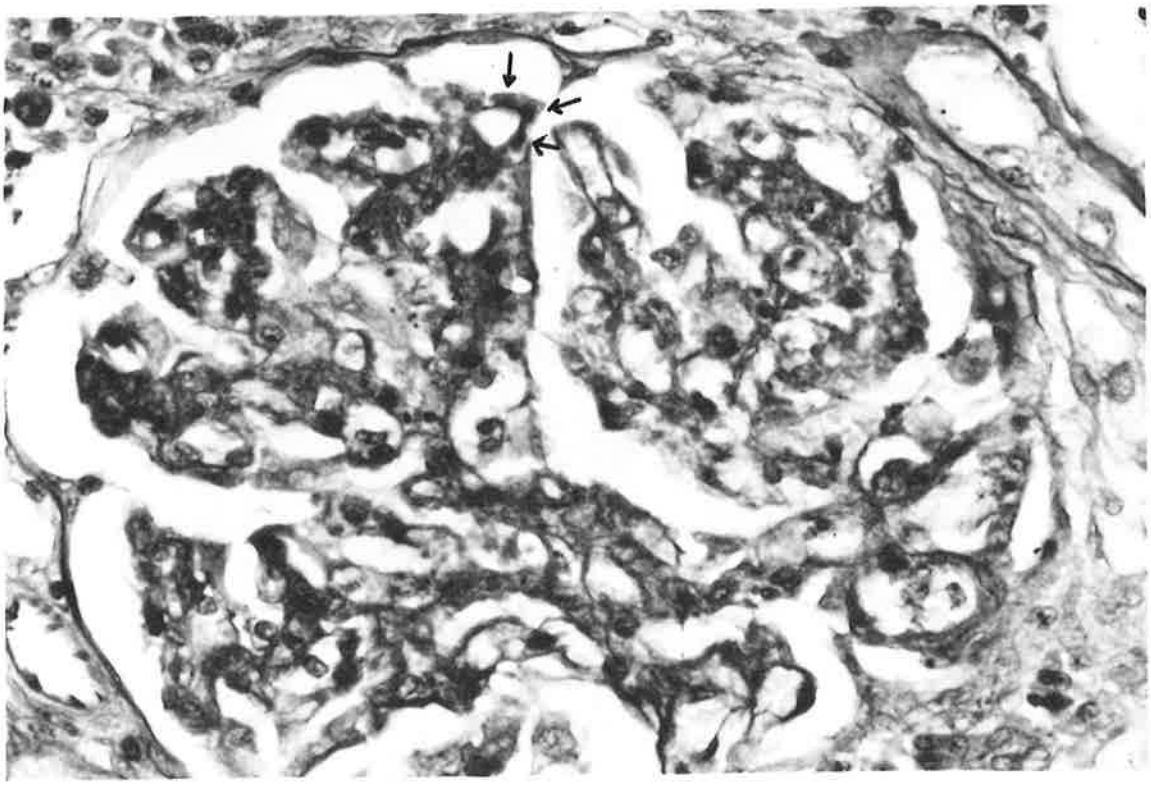

Fig. 5.11 BSA-induced chronic immune-complex glomerulonephritis in rabbits. Renal biopsy specimen taken on Day 35, showing an increase in mesangial matrix, distortion and splitting of the GBM, and deposits on the epithelial aspect of the GBM (↑) (PAS x 600)

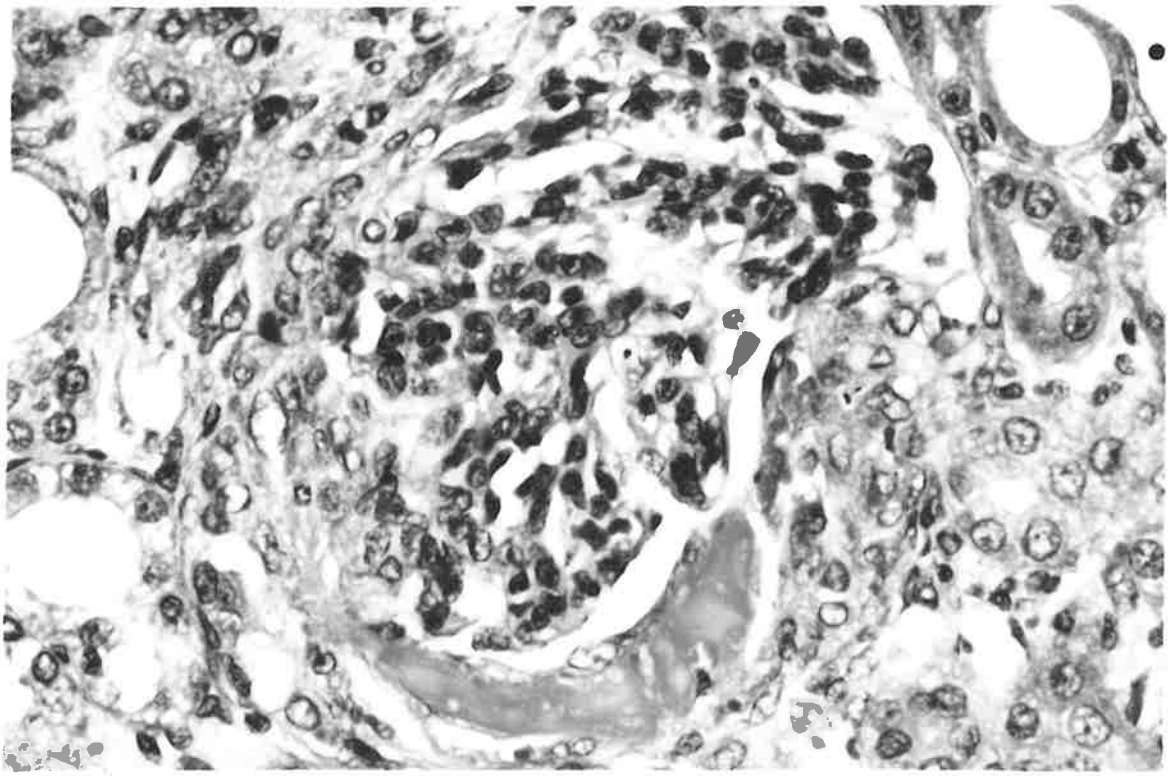

Fig. 5.12 BSA-induced chronic immune-complex glomerulonephritis in rabbits. A glomerulus from an animal dying on Day 44 from renal failure, showing extracapillary cell proliferation, fibrin deposition and diffuse proliferative and exudative change in the residual glomerular tuft.

(H & Ex600)

crescentic glomerulonephritis when :

(a) Anti-BSA antibody response was augmented by immunisation with BSA in adjuvant prior to starting daily IV doses of BSA.

(b) The daily dose of BSA was adjusted to each animal's anti-BSA antibody response, so that the animal was in circulating antigen excess for 6-24 hours of each 24 hour period.

Proteinuria was the first manifestation of glomerulonephritis, developing as early as Day 6. A fall in serum C3 concentrations was in evidence as from Day 15. A progressive rapid deterioration in renal function was not seen until after Day 35. By Day 35, all animals had developed a proliferative glomerulonephritis but crescents and glomerular fibrin deposition were not present. A severe crescentic nephritis in which glomerular fibrin deposition was extensive developed within the next two weeks in nine of the eleven animals.

Immunofluorescence examination of the kidney in the first 14 days demonstrated predominently mesangial deposition of BSA, rabbit immunoglobulin and C3, but by Day 35 granular deposits - predominently along the GBM - were seen. By death or Day 54 this granular deposition was very heavy and often confluent.

In monitoring the dose of antigen so that antigen excess was maintained for 6-24 hours of each 24 hour period, excellent correlation was seen between clearance of circulating antigen as measured by the ^{125}I-BSA clearance studies and the appearance of circulating anti-BSA antibody as measured by radial immunodiffusion in gel (Mancini test).

The simplicity of the Mancini test makes this the method choice in following antibody response and determining the daily dose of BSA.

Animals clearing antigen from the circulation within 6-24 hours after the daily dose of BSA had the largest quantities of circulating BSA - anti-BSA immune complexes, developed proteinuria earlier and developed more severe glomerulonephritis.

In retrospect, the loss of six animals from anaphylaxis was probably partly avoidable. Adrenalin or antihistamines were deliberately not administered to prevent anaphylaxis as these substances are known to affect the deposition of immune complexes in the kidney (Kniker, 1968).

The usual methods of production of BSA-induced chronic immune-complex glomerulonephritis in rabbits involve either a fixed daily dose of BSA or a dose dependent on antibody response. With either procedure only 15-30% of animals develop a severe crescentic glomerulonephritis, for reasons discussed in the Introduction. Moreover, the development of the crescentic nephritis takes 3-5 months. It would seem from my experiments that the augmentation of antibody response by preimmunisation, so that all animals produced antibody and larger doses of BSA could be administered, was responsible for the development of a severe crescentic nephritis in a high proportion of animals and in a short period of time.

With the development of this model of severe crescentic nephritis I have been able to examine the mediation of glomerular injury by fibrin in chronic immune-complex glomerulonephritis (see Chapter 6).

PART IV

MEDIATION OF INJURY IN EXPERIMENTAL CRESCENTIC GLOMERULONEPHRITIS

INTRODUCTION

Experimental crescentic glomerulonephritis may be induced in rabbits by either anti-glomerular basement membrane antibody (nephrotoxic nephritis and "auto-allergic" anti-GBM nephritis) or chronic immune complex deposition (BSA-induced chronic immune-complex glomerulonephritis) (see Part III of these thesis). Whatever method of induction is used, extracapillary cell proliferation with crescent formation is extensive, glomerular fibrin deposition is prominent, polymorphonuclear leucocytes are found with the glomeruli and complement is deposited within glomeruli together with the antibody and antigen.

In this section I have described and discussed experiments used to investigate the mediation of injury in experimental crescentic glomerulonephritis in rabbits. The role of glomerular fibrin deposition has been examined in the crescentic glomerulonephritis of both NTN and BSA-induced chronic immune-complex disease. The role of polymorphonuclear leucocytes and complement activation has been examined in NTN. The experiments have led to, amongst other things, the definition of a system of glomerular injury which is independent of complement but dependent on polymorphonuclear leucocytes and glomerular fibrin deposition.

The findings are of relevance to human cresc entic glomerulonephritis in many types of which polymorphonuclear leucocyte infiltration and glomerular fibrin deposition may be found. These types of human glomerulonephritis include rapidly progressive glomerulonephritis,

the glomerulonephritis of Goodpasture's syndrome, Henoch-Schönlein glomerulonephritis, the severe proliferative glomerulonephritis of systemic lupus erythematosus, progressive focal proliferative glomerulonephritis and occasionally acute exudative glomerulonephritis (Heptinstall, 1974).

Chapter 6

FIBRIN IN EXPERIMENTAL CRESCENTIC GLOMERULONEPHRITIS

Introduction

As discussed in Chapter 2, crescent formation has usually been explained as a reaction to the products of exudation in the capsular space, particularly fibrin-fibrinogen (fibrin) (Heptinstall, 1974). This concept has been supported by the demonstration that the prevention of glomerular fibrin deposition by anticoagulation or defibrination provided protection from crescent formation and renal failure (Kleinerman, 1954; Vassalli and McCluskey, 1964; Halpern et al, 1965; Naish et al, 1972) (see Chapter 2).

These findings have led to the use of anticoagulation in the treatment of human crescentic glomerulonephritis. However, it has not been widely appreciated that the doses of heparin or warfarin needed to provide protection in experimental crescentic nephritis were vastly in excess of those therapeutically practicable in man. Clotting times were often prolonged to 10-15 times normal and in one series (Vassalli and McCluskey, 1964) 30% of animals died from haemorrhage. Although anticoagulation with heparin or warfarin may prevent or substantially reduce intravascular fibrin deposition, the capacity of heparin or warfarin to prevent fibrin deposition outside the circulation, such as in Bowman's space, has not been clarified. I have therefore performed experiments (described in protocol 1) in which the effect of different doses of heparin in NTN in rabbits has been assessed and have compared

the results with those obtained by defibrination with ancrod.

All previous studies of anticoagulation in experimental crescentic glomerulonephritis have been performed in animals with NTN (anti-GBM disease). However, most cases of glomerulonephritis in man are thought to be due to deposition of immune complexes. In protocol 2 I have described experiments in which rabbits developing crescentic nephritis due to BSA-induced chronic immune-complex disease have been defibrinated with ancrod.

In most studies of anticoagulation in NTN, protection has only been found when anticoagulants were started before glomerular fibrin deposition and crescent formation had developed (Kleinerman, 1954; Halpern et al, 1965). In only one study (Borrero et al, 1973) was some degree of protection afforded by anticoagulation after the disease process had started. However, in this latter study (NTN in Hares) the disease process was quite mild and the mean concentration of blood urea nitrogen in control animals did not rise above 49 mg/100 ml. I have therefore undertaken experiments (protocol 3) in which rabbits with NTN have been defibrinated from Day 10 of the disease, at a time when extensive glomerular fibrin deposition has occurred and crescents are developing. In these animals I have also investigated the capacity of the animal to remove fibrin deposits from the glomerulus.

EXPERIMENTAL PROTOCOLS

1 : ANTICOAGULATION WITH HEPARIN AND DEFIBRINATION WITH ANCROD IN NTN

Forty-five rabbits were injected IV with 1 ml of NTG/Kg BW (Day 0). Subsequently twenty-one rabbits received heparin, six ancrod and eighteen control animals received saline.

Heparin Group

In sixteen animals sodium heparin was commenced on Day 4 and given subcutaneously twice daily until the animals were killed on Day 12. Three dosage regimes were used :

Five rabbits were given 300 u/Kg/day
Six rabbits were given 1000 u/Kg/day
Five rabbits were given 2000 u/Kg/day

In a further five animals 2000 u/Kg/day were again given, but the heparin was commenced on Day 1.

Ancrod Group

Ancrod was commenced on Day 1. A dose of 0.5 u/Kg was injected IV, followed an hour later by 1 u/Kg IV. Defibrination was maintained by twice daily IV injections of 1-2 u/Kg depending upon the adequacy of defibrination.

Control Group

Starting on Day 4, 1 ml of saline was injected subcutaneously twice daily.

All animals were bled on alternate days for estimation of serum urea, creatinine, and C3 concentrations. Urine protein concentrations were determined daily on samples obtained by urethral catheterisation. Whole blood clotting times and thrombin clotting times were determined daily at 1, 4, 8 and 12 hours after the morning dose of heparin or saline. Defibrination in ancrod-treated animals was assessed daily by the clot quality test.

All animals were killed on Day 12 and the kidneys removed for histology and immunofluorescence examination for sheep immunoglobulin, rabbit immunoglobulin, rabbit C3 and rabbit fibrin.

2 : DEFIBRINATION IN BSA-INDUCED CHRONIC IMMUNE-COMPLEX GLOMERULONEPHRITIS

The model of BSA-induced chronic immune-complex glomerulonephritis described in Chapter 5 was used.

Thirty rabbits were immunised IM with 10 mg of BSA in FCA. Five days later (Day 0) single daily IV injections of 15 mg of BSA were started. When anti-BSA antibody appeared in the serum, the dose of BSA was adjusted so that free anti-BSA antibody only appeared in the serum between 6-24 hours after the previous dose of BSA. Doses of BSA in excess of 50 mg/day were administered over a 1-2 hour period to minimise anaphylactic reactions.

The animals were bled weekly until renal failure developed and thereafter more frequently, to measure serum creatinine and C3 concentrations. Urine protein concentrations in urine obtained by

urethral catheterisation were measured twice weekly.

On Day 35 the surviving animals were matched so that proteinuria, the mean daily dose of BSA given to each animal and serum creatinine concentrations were similar in two groups, each of ten animals. Renal biopsies were taken on Day 35 from four randomly chosen animals in both groups.

Defibrination in the ancrod group was started on Day 35 and continued until death or cessation of the experiment. The induction of defibrination was as in protocol 1 and maintenance doses depended on the adequacy of defibrination as assessed by the clot quality test. Control animals received the same volume of sterile saline IV.

Surviving animals in both groups were killed on Day 54, the kidneys being removed for histology and immunofluorescence examination for rabbit immunoglobulin, C3, fibrin and BSA.

3 : DEFIBRINATION IN NTN AFTER GLOMERULAR FIBRIN DEPOSITION

Two separate experiments were performed:

(A) Eighteen rabbits were injected IV with 1 ml/Kg of NTG on Day 0. Nine were defibrinated with ancrod from Day 10 of the disease until death or cessation of the experiment on Day 24. The same method of ancrod administration was used as in protocol 2. Nine control animals were given similar doses of saline IV from Day 10. On alternate days, blood was sampled for serum creatinine estimation and urine by urethral

catheterisation, for protein estimation.

Surviving animals were killed on Day 24, kidneys being removed for histology and immunofluorescence examination for rabbit and sheep immunoglobulin and rabbit C3 and fibrin.

(B) Twenty-two rabbits were injected IV with 1 ml/Kg of NTG (Day 0). Fourteen animals were defibrinated with ancrod from Day 10, the remainder receiving saline. Animals from both the defibrinated and control groups were killed on various days after Day 10 and glomerular fibrin deposition assessed.

RESULTS

1 : ANTICOAGULATION WITH HEPARIN AND DEFIBRINATION WITH ANCROD IN NTN

(a) Deaths

One animal in the control group died from renal failure on Day 11. No deaths occurred in treated animals.

(b) Anticoagulation

(i) Animals given 300 u of heparin/Kg/day : at 1, 4 and 8 hours after each dose of heparin the ranges of whole blood clotting times were respectively 2.1-3.2 (mean 2.6), 2.5-3.5 (mean 3.1), and 2.2-3.2 (mean 2.5) times control. Clotting times had fallen to a range 1.5-2.0 (mean 1.8) times control just prior to the next dose of heparin (ie approximately

12 hours).

(ii) Animals given 1000u of heparin/Kg/day maintained whole blood clotting times in the range 4.1-6.2 (mean 5.1) times control at all times. Thrombin clotting times were prolonged to between 30 and 70 seconds (control 9-13 seconds).

(iii) Animals receiving 2000 u of heparin/Kg/day had virtually unclottable blood, with thrombin clotting times varying between 50 and 500 seconds and the whole blood clotting times in the range 15-20 (mean 17.3) times normal at all times.

(iv) Ancrod : at all times and in all animals given ancrod, clot quality was of grade 4 or 5, indicating severe hypo-fibrinogenaemia.

(c) Renal Function (Fig. 6.1 ; Tables 6.1 and 6.2)

Serum urea and creatinine concentrations remained within the normal range in all animals until Day 5, then rose rapidly and progressively in untreated animals and in the groups given 300 and 1000 u of heparin/Kg/day. Animals given 2000u of heparin/Kg/day and those given ancrod, although showing a rise in the serum urea and creatinine from Days 5-9, thereafter showed a progressive fall in these variables. There was no significant difference in the serum urea and creatinine concentrations on Day 12 between the control animals and animals given either 300 or

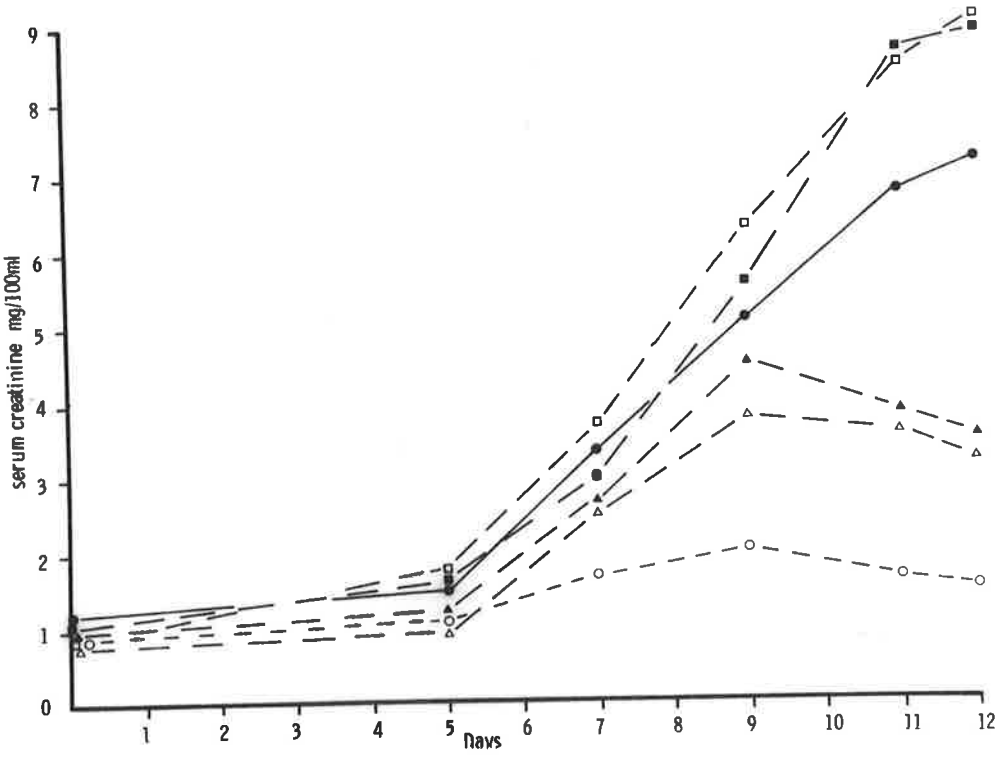

Fig. 6.1 Mean serum creatinine concentrations for control animals (●), animals given 300 u of heparin (■), 1000 u of heparin (□), 2000 u of heparin (▲), 2000 u of heparin as from day 1 (▲) and ancrod (○) with NTN.

ANIMAL GROUP	n	SERUM UREA CONCENTRATION mg/100 ml : Mean (Range)					
		DAY 0	DAY 5	DAY 7	DAY 9	DAY 11	DAY 12
SALINE CONTROLS	18	41 (30-52)	68 (33-228)	142 (80-348)	247 (110-597)	323 (90-600)	362 (120-738)
HEPARIN 300 u	5	41 (38-46)	60 (50-75)	154 (94-207)	250 (132-426)	325 (126-504)	351 (150-540)
HEPARIN 1000 u	6	40 (34-44)	94 (42-228)	188 (49-348)	297 (117-597)	388 (90-600)	460 (115-738)
HEPARIN 2000 u	5	42 (40-44)	52 (43-59)	142 (42-311)	216 (97-341)	188 * (130-220)	156 *** (120-176)
HEPARIN 2000u (Day 1)	5	39 (33-46)	86 (39-148)	146 (65-235)	187 (87-259	209 * (110-276)	152 *** (120-176)
ANCROD	6	42 (34-40)	60 (43-81)	123 (60-155)	109 (48-162)	75 *** Δ (42-136)	68 *** ΔΔΔ (40-116)

Table 6.1 Serum Urea Concentrations in Control, Heparin-Treated and Ancrod-Treated Rabbits with NTN.

***P<0.01 *P<0.05 : in respect to Controls

ΔΔΔ P<0.01 Δ P<0.05 : in respect to Heparin 2000 u Group

ANIMAL GROUP	n	SERUM CREATININE CONCENTRATION mg/100 ml : Mean (Range) DAY 0	DAY 5	DAY 7	DAY 9	DAY 11	DAY 12
SALINE CONTROLS	18	1.1 (0.7-1.2)	1.5 (0.8-4.0)	3.4 (1.1-7.2)	5.1 (1.3-11.7)	6.8 (2.2-13.5)	7.2 (2.5-14.0)
HEPARIN 300 u	5	1.0 (0.8-1.1)	1.6 (1.3-2.0)	3.0 (1.6-4.3)	5.6 (3.3-10.5)	8.6 (4.2-12.0)	8.9 (4.5-10.8)
HEPARIN 1000 u	6	0.9 (0.7-1.0)	1.7 (1.1-3.8)	3.7 (1.5-7.4)	6.3 (1.5-10.6)	8.5 (2.7-12.0)	9.1 (2.5-13.4)
HEPARIN 2000 u	5	0.9 (0.7-1.0)	0.9 (0.9-1.0)	2.5 (1.0-3.9)	3.8 (1.9-5.1)	3.6* (2.8-4.8)	3.2*** (2.6-3.8)
HEPARIN 2000u (Day 1)	5	1.0 (0.8-1.1)	1.2 (0.8-1.6)	2.6 (1.2-4.5)	4.5 (2.1-5.9)	3.8* (2.6-5.2)	3.5*** (2.9-3.9)
ANCROD	6	0.9 (0.7-1.1)	1.0 (0.7-1.2)	1.7 (1.3-2.0)	2.0* (1.6-2.5)	1.6***ΔΔΔ (1.3-2.0)	1.5***ΔΔΔ (1.3-2.0)

Table 6.2 Serum Creatinine Concentrations in Control, Heparin-Treated and Ancrod-Treated Rabbits with NTN.

***P<0.01 *P<0.05 : in respect to Controls

ΔΔΔ P<0.01 : in respect to Heparin 2000 u Group

1000 u of heparin/Kg/day ($P > 0.1$). However, the serum urea and creatinine concentrations on Day 12 in the animals given 2000 u of heparin/Kg/day were significantly less than the concentrations in control animals and animals given 300 and 1000 u of heparin/Kg/day ($P < 0.01$). Similarly the concentrations on Day 12 in animals given ancrod were significantly less than in controls and animals given 300 and 1000 u of heparin/Kg/day ($P < 0.01$), and also significantly less than in animals given 2000 u of heparin/Kg/day ($P < 0.01$). There was no difference at any time in the urea and creatinine concentrations between animals given 2000 u of heparin/Kg/day commencing on Day 1 and animals given the same dose commencing on Day 4.

(d) Proteinuria (Table 6.3)

On no day were urine protein concentrations significantly different between the groups of animals ($P > 0.1$).

(e) Serum C3 Concentrations (Table 6.4)

There was no significant difference at any stage in serum C3 concentrations between the groups of animals ($P > 0.1$).

(f) Immunofluorescence

(i) Immunoglobulin And Complement

Sheep and rabbit immunoglobulin and rabbit C3 were deposited in a smooth linear fashion along the GBM in all animals. No difference in intensity of staining could be detected between the groups of animals.

ANIMAL GROUP	n	URINE PROTEIN CONCENTRATION mg/ml : Mean (Range)					
		DAY 0	DAY 1	DAY 5	DAY 7	DAY 9	DAY 12
SALINE CONTROLS	18	<·3	1.6 (0.3-6.6)	10.6 (1.1-26.2)	17.6 (5- 41)	16.7 (1.5 - 77)	7.2 (1.4-45)
HEPARIN 300 u	5	<·3	0.9 (0.4-2.0)	5.4 (1.2-9.5)	24.9 (7.4 - 27)	26.7 (7.6 - 85)	4.1 (1.0-8.1)
HEPARIN 1000 u	6	<·3	1.93 (0.4-4.0)	8.7 (3.8-16.0)	13.8 (2 - 33)	21.3 (1.4 - 47)	8.1 (2.9-20.7)
HEPARIN 2000 u	5	<·3	1.2 (0.3-2.5)	6.9 (4.1-10.4)	19.8 (3.1- 46)	10.8 (7.7 - 13)	8.8 (4.9 - 11.3)
HEPARIN 2000 u (Day 1)	5	<·3	2.1 (0.5-4.1)	10.9 (2.3-15.9)	21.6 (3.9 - 54)	19.8 (5.6 - 81)	10.2 (3.1-18.6)
ANCROD	6	<·3	1.5 (0.3-2.9)	7.9 (2.8-13.1)	12.8 (6.5-17.1)	16.6 (15 - 19.4)	7.3 (5.6-8.8)

Table 6.3 Urine Protein Concentrations in Control, Heparin-Treated and Ancrod-Treated Animals with NTN

ANIMAL GROUP	n	SERUM C3 CONCENTRATION % NORMAL POOL : Mean (Range)					
		DAY 0	DAY 1	DAY 5	DAY 7	DAY 9	DAY 12
SALINE CONTROLS	18	102 (86-126)	68 (46-87)	66 (47-110)	36 (25-63)	39 (33-68)	54 (25-80)
HEPARIN 300 u	5	95 (80-145)	60 (43-120)	No Result	53 (28-86)	46 (29-100)	48 (30-94)
HEPARIN 1000 u	6	94 (64-105)	67 (48-83)	52 (25-100)	25 (13-52)	32 (13-55)	41 (16-70)
HEPARIN 2000 u	5	105 (93-135)	55 (46-78)	74 (64-86)	43 (40-55)	52 (33-64)	60 (38-86)
HEPARIN 2000 u (Day 1)	5	100 (94-105)	63 (50-75)	51 (36-68)	33 (25-41)	No Result	45 (28-69)
ANCROD	6	94 (89-122)	53 (39-87)	45 (36-56)	50 (32-66)	42 (22-71)	64 (29-84)

Table 6.4 Serum C3 Concentrations in Control, Heparin-Treated and Ancrod-Treated Rabbits with NTN.

(ii) Fibrin (Table 6.5)

No significant difference in the mean fibrin score per glomerulus or the per cent incidence of each grade of glomerular fibrin deposition was observed between controls and groups given 300 and 1000 u of heparin/Kg/day ($P > 0.1$). However, fibrin deposition in animals given 2000 u of heparin/Kg/day was significantly lower than in controls or animals given 300 and 1000 u/Kg/day. In the defibrinated animals the fibrin score was not only significantly lower than for controls but also lower than for animals treated with heparin in the doses 300, 1000 and 2000 u/Kg/day. No differences were observed between the groups in which heparin was started on Day 1 and Day 4.

(g) Histology (Table 6.6)

No significant difference was observed in the degree of extra-capillary cell proliferation between controls and animals given 300 and 1000 u of heparin/Kg/day ($P > 0.1$). However, crescent formation was significantly lower in animals given 2000 u of heparin/Kg/day. In the ancrod-treated group crescent formation was significantly less than in controls and all groups given heparin.

(h) Correlation Between Glomerular Fibrin Deposition, Crescent Formation And Renal Function

As shown in Figure 6.2, a highly significant linear correlation existed between the degree of glomerular fibrin deposition and the degree of crescent formation (correlation coefficient 0.897; slope 0.842 ;

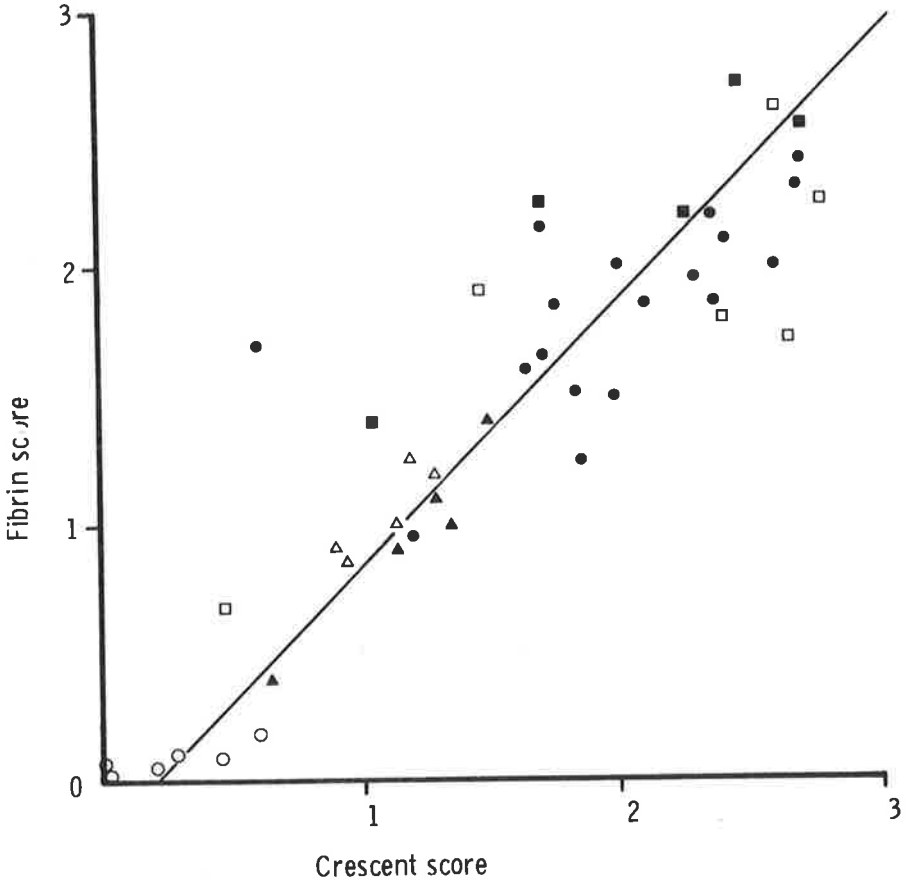

Fig. 6.2 Relationship between the degree of glomerular fibrin deposition and crescent formation in NTN, as assessed on Day 12 of the disease, in control animals (●), animals given 300 u of heparin (■), 1000 u of heparin (□), 2000 u of heparin (Δ), 2000 u of heparin as from Day 1 (▲) and ancrod (O).

Correlation coefficient for all animals 0.897 ; slope 0.842 ; $P < 0.01$

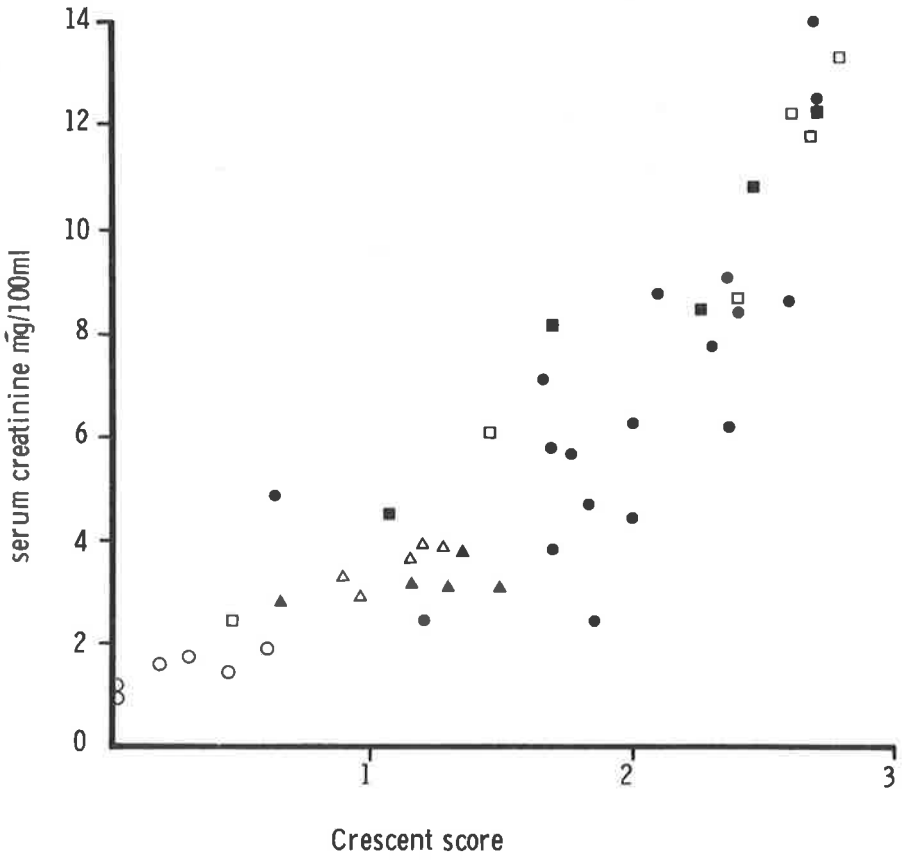

Fig. 6.3 Relationship between the degree of crescent formation and impairment in renal function in NTN, in control animals (●), animals given 300 u of heparin (■), 1000 u of heparin (□), 2000 u of heparin (▲), 2000 u of heparin as from Day 1 (▲) and ancrod (○).

ANIMAL GROUP	n	PER CENT INCIDENCE OF EACH GRADE OF FIBRIN DEPOSITION : Mean (Range) GRADE 0	GRADE 1	GRADE 2	GRADE 3	MEAN FIBRIN SCORE (Range)
SALINE CONTROLS	18	3.5 (0 - 25)	29.7 (10 - 60)	47.4 (20 - 70)	19.4 (0 - 50)	1.81 (0.95-2.3)
HEPARIN 300 u	5	1.0 (0 - 5)	20.0 (0 - 50)	32.0 (10 - 50)	47.0 (15 - 80)	2.20 (1.4 - 2.5)
HEPARIN 1000 u	6	7.5 (0 - 45)	26.6 (10 - 40)	45.9 (15 - 65)	20.0 (0 - 40)	1.80 (0.7 -2.25)
HEPARIN 2000 u	5	31.0 *** (10 - 60)	46.0 ** (25 - 70)	19.0 *** (0 - 35)	4.0 * (0 - 15)	0.97 *** (0.4 - 1.4)
HEPARIN 2000 u (Day 1)	5	34.0 *** (25 - 45)	34.0 (25 - 40)	26.0 *** (20 - 35)	6.0 * (0 - 15)	1.04 *** (0.85-1.2)
ANCROD	6	91.7 *** ΔΔΔ (80-100)	8.3 *** ΔΔΔ (0 - 20)	0 *** Δ	0 ***	0.09 *** ΔΔΔ (0 - 0.2)

Table 6.5 Glomerular Fibrin Deposition on Day 12 in Control, Heparin-Treated and Ancrod-Treated Rabbits with NTN.

*** P<0.01
ΔΔΔ P<0.01
** P<0.02
* P<0.05 : in respect to Controls
Δ P<0.05 : in respect to Heparin 2000 u Group

ANIMAL GROUP	n	PER CENT INCIDENCE OF EACH GRADE OF EXTRA-CAPILLARY CELL PROLIFERATION : Mean (Range)				MEAN CRESCENT SCORE (Range)
		GRADE 0	GRADE 1	GRADE 2	GRADE 3	
SALINE CONTROLS	18	9.5 (0 - 68)	22.7 (0 - 50)	33.5 (14 - 50)	34.3 (10 - 70)	1.99 (0.62 - 2.7)
HEPARIN 300 u	5	8.0 (0 - 25)	20.0 (0 - 45)	33.0 (30 - 35)	39.0 (0 - 70)	2.03 (1.06 - 2.7)
HEPARIN 1000 u	6	15.0 (0 - 64)	12.3 (0 - 30)	25.0 (4 - 40)	47.7 (4 - 72)	2.06 (0.48 -2.78)
HEPARIN 2000 u	5	29.0 *** (20 - 55)	35.0 ** (20 - 55)	19.0 (15 - 25)	17.0 * (0 - 25)	1.20 *** (0.65 - 1.5)
HEPARIN 2000 u (Day 1)	5	28.0 *** (15 - 55)	39.0 * (15 - 50)	22.0 (15 - 30)	9.0 ** (0 - 15)	1.15 *** (0.95 - 1.30)
ANCROD	6	79.2 *** ΔΔΔ (65 - 100)	15.8 (0 - 30)	5.0 *** ΔΔΔ (0 - 15)	0 *** Δ	0.49 *** ΔΔΔ (0.2 - 1.0)

Table 6.6 Extracapillary Cell Proliferation on Day 12 in Control, Heparin-Treated and Ancrod - Treated Rabbits with NTN.

***P<0.01 **P<0.02 *P<0.05 : in respect to Controls

ΔΔΔ P<0.01 ΔP<0.05 : in respect to Heparin 2000u Group

$P < 0.01$). Serum creatinine concentration was also closely related to the extent of crescent formation (Fig. 6.3).

2 : DEFIBRINATION IN BSA-INDUCED CHRONIC IMMUNE-COMPLEX GLOMERULONEPHRITIS

(A) IMMUNOPATHOLOGICAL SEQUENCES UP TO DAY 35 (ie Before Defibrination)

(a) Deaths

Ten animals died before Day 35, five from anaphylaxis and five from causes seemingly unrelated to BSA administration : infective diarrhoea in two ; pneumonia in one ; and two were killed because of middle ear infection.

(b) Histology at Day 35

Diffuse endothelial and mesangial cell proliferation with prominent polymorphonuclear leucocyte infiltration was found in all eight biopsies ; extracapillary cell proliferation was not seen. Immunofluorescence showed coarse granular deposits of BSA, rabbit IgG and C3, predominantly along capillary loops but also within the mesangium. Glomerular fibrin deposition was not seen.

(c) Comparison Of Controls And Animals To Be Defibrinated

Analysis of antibody production, dose of antigen required to maintain antigen excess, proteinuria and renal function were similar between the two groups of animals (Fig. 6.4 ; Table 6.7).

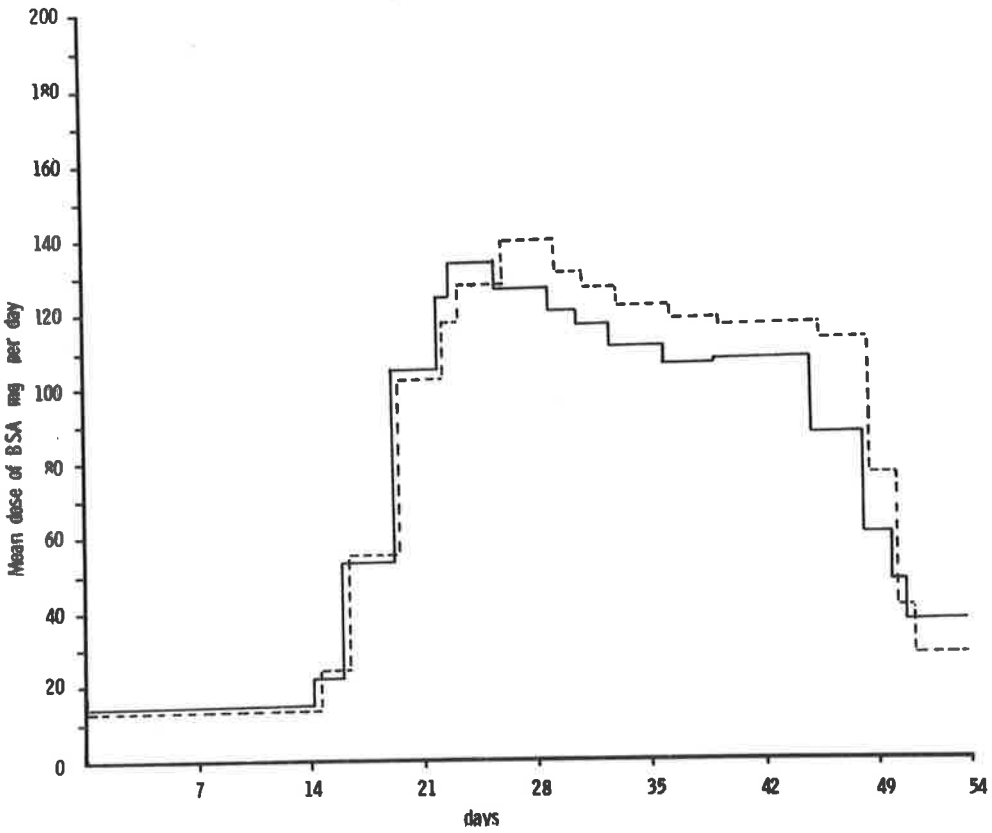

Fig. 6.4 Comparison of the mean daily doses of BSA between the control (——) and ancrod-treated (----) groups of animals with chronic BSA-induced immune-complex glomerulonephritis.

ANIMAL GROUP	n	MEAN DAILY DOSE OF BSA UNTIL DAY 35 mg : Mean(Range)	URINE PROTEIN CONCENTRATION ON DAY 35 mg/ml: Mean (Range)	DAY WHEN PROTEINURIA FIRST DETECTED Mean (Range)	SERUM CREATININE CONCENTRATION ON DAY 35 mg/100 ml : Mean (Range)
Controls	10	74.5 (54 - 89)	6.4 (0.5 - 14.5)	25.2 (21 - 30)	1.5 (0.9 - 2.5)
Group to be defibrinated	10	75.4 (47 - 109) P > 0.1	10.2 (1.8 - 21.1) P > 0.1	24.9 (21 - 30) P > 0.1	1.6 (0.9 - 3.1) P > 0.1

Table 6.7 Chronic BSA-Induced Glomerulonephritis. Comparison of Mean Daily Dose of BSA, Proteinuria and Serum Creatinine on Day 35 and Duration of Proteinuria in Control Animals and Animals to be Defibrinated.

(B) IMMUNOPATHOLOGICAL EVENTS IN DEFIBRINATED AND CONTROL ANIMALS AFTER DAY 35

(a) Renal Function (Fig. 6.5 ; Table 6.8)

(i) Control Group

Three animals developed severe renal failure, dying between Days 43 and 46. One animal died from unknown causes on Day 43. All surviving animals except one showed progressive deterioration in renal function, the mean serum creatinine rising from 1.5 mg/100 ml (range 0.9-2.5 mg/100 ml) on Day 35 to 4.28 mg/ml (range 1.2-6.8 mg/100 ml) on Day 54.

(ii) Defibrinated Groups

Only one of the treated animals died from renal failure, the serum creatinine at death being 7.1 mg/100 ml and in this animal the serum creatinine before defibrination was already 3.1 mg/ 100 ml. Of the nine survivors in the treated group, three showed a reduction in the serum creatinine, and the remainder showed a rise in serum creatinine between Days 35 and 54.

The mean serum creatinine in the treated group was 1.59mg/ 100 ml (range 0.8-3.1 mg/100 ml) on Day 35, rising to 1.91 mg/100 ml (range 1.1-4.2 mg/100 ml) on Day 54 in the nine survivors, an increase which was significantly less than that occurring in the control group ($P < 0.02$). This difference in renal function between treated and control

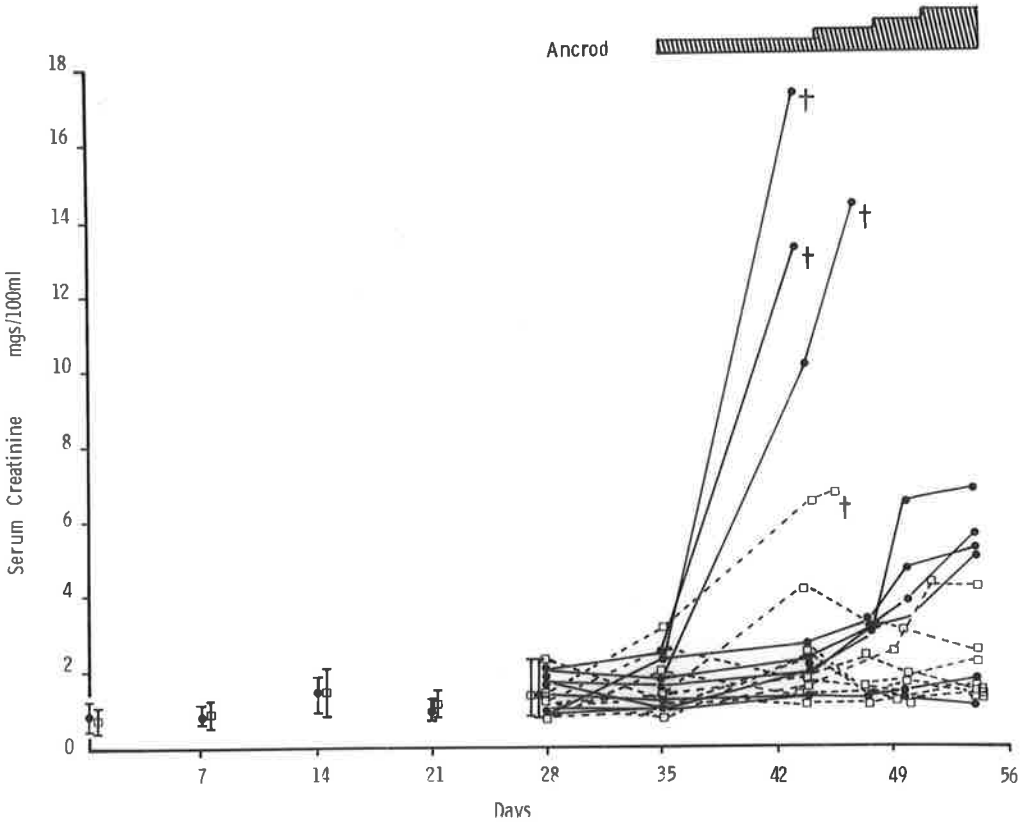

Fig. 6.5 Serum creatinine concentrations in control (●) and ancrod-treated (□) animals with BSA-induced chronic immune-complex glomerulonephritis. († Died)

ANIMAL GROUP		n	MEAN DAILY DOSE OF BSA mg: Mean(Range)	SERUM CREATININE CONCENTRATION mg/100ml : Mean (Range) DAY 35	DAY 44	DAY 47	DAY 50	DAY 54
Controls	All Animals	10	77.3 (31 - 115)	1.5 (0.9-2.5)	5.35 (1.3-17.4)	4.03 (1.3-14.4)	3.47 (1.2-6.5)	4.28 (1.2-6.8)
	Animals receiving 60 mg BSA per day	7	91.0 (64 - 115)	1.69 (0.9-2.5)	7.04 (1.8-17.4)	5.12 (2.0-14.4)	4.55 (3.2-6.5)	5.7 (5.1- 6.8)
Ancrod Treated	All Animals	10	76.9 (46- 119)	1.58 (0.9-3.1)	2.47 (1.1-7.1)	1.76 (1.1-3.1)	1.87* (1.1-4.2)	1.91** (1.1-4.2)
	Animals receiving 60 mg BSA per day	7	87.7 (64 - 119)	1.67 (1.1-3.1)	2.86 (1.3-7.1)	2.01* (1.4-3.1)	2.2** (1.2-4.2)	2.21*** (1.4-4.2)

Table 6.8 Serum Creatinine Concentrations in Control and Ancrod-Treated Rabbits with BSA-Induced Chronic Immune-Complex Glomerulonephritis.

***P< 0.01 **P< 0.02 *P<0.05

animals was even greater if serum creatinine concentrations at either Day 54 or death were compaired (Fig. 6.6).

(b) Proteinuria

Analysis of urine samples obtained by catheterisation showed that proteinuria persisted until death in all animals. No differences were found between treated and control groups (Table 6.8a). However, quantitation of the total 24 hour protein excretion was not practicable.

(c) Serum C3 Concentrations (Table 6.9)

A progressive fall in the serum C3 concentrations was observed to a minimum mean concentration by Day 35 of 40% (range 26-63%) in the treated group and 44% (range 24-49%) in the controls. On no day were there significant differences in the C3 concentrations between control and treated groups ($P > 0.1$ for every measurement).

(d) BSA Dosage And Levels Of Antibody To BSA

Steady antibody production was maintained in both groups of animals until Days 42-49 when a steep fall in antibody levels (and hence dosage of BSA) occurred. No significant difference existed in BSA doses between treated and control groups on any day ($P > 0.1$) (Fig. 6.4).

(e) Immunofluorescence

(i) Immunoglobulin, Complement and BSA

Heavy granular deposition of rabbit IgG, C3 and BSA predominantly along capillary loops was seen in eighteen rabbits, the remaining two showing less marked loop but

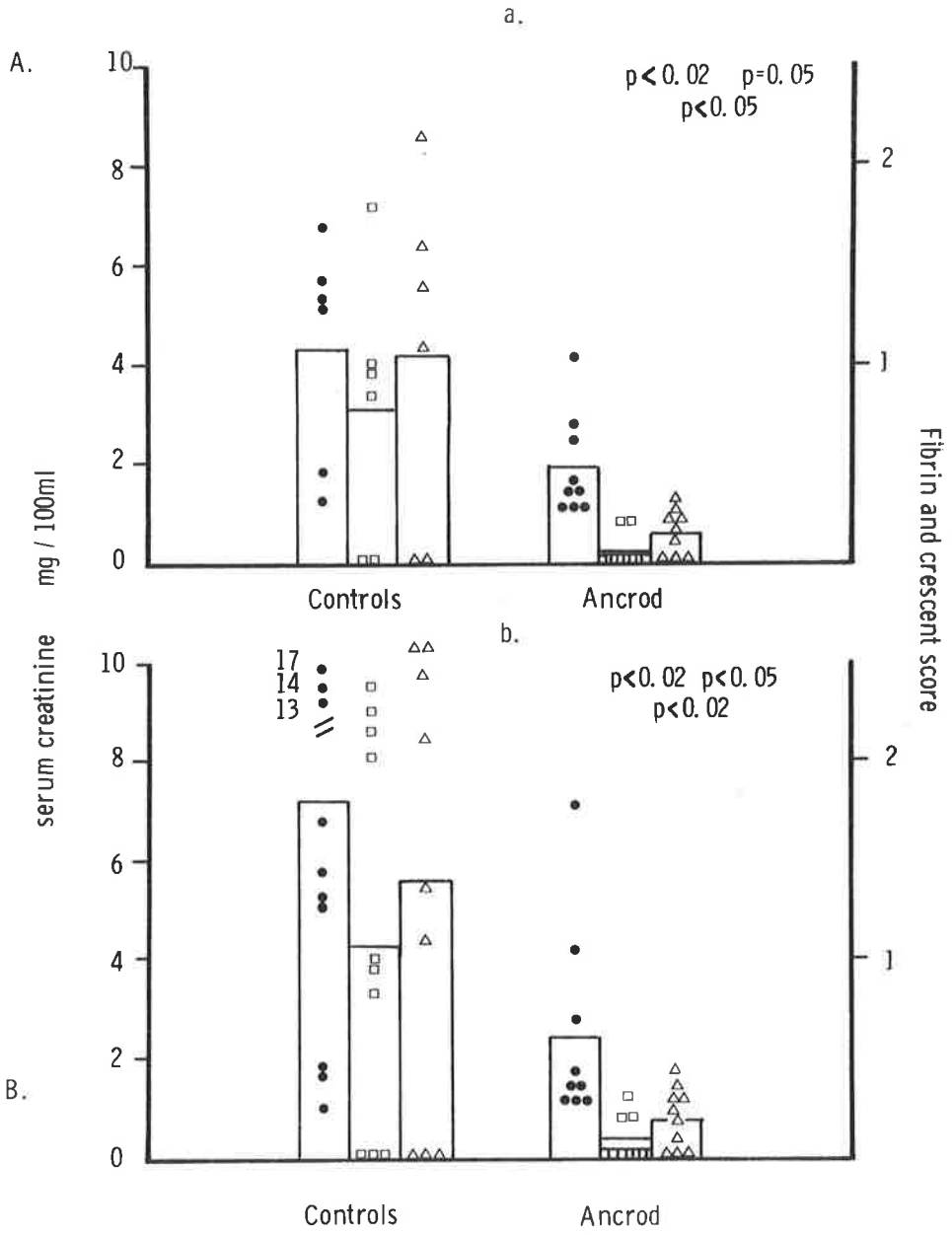

Fig. 6.6 Serum creatinine concentrations (●), glomerular fibrin scores (□) and crescent scores (△) on Day 54 or at death in control and ancrod-treated animals with BSA-induced chronic immune-complex glomerulonephritis :

(a) Animals surviving until Day 54

(b) All twenty animals (ie including those animals dying between Day 35 and Day 54)

ANIMAL GROUP	n	URINE PROTEIN CONCENTRATION mg/ml : Mean (Range) DAY 35	DAY 44	DAY 50	DAY 54
Ancrod-treated	10	10.2 (1.8-21)	21.2 (3.0-49)	20.9 (1.8-49)	12.4 (1.4-38.5)
Controls	10	6.4 (1.5-14.5) P>0.1	18.5 (1.8-56) P>0.1	15.2 (4.6-50) P>0.1	9.9 (1.2 - 50) P>0.1

Table 6.8 (a). Urine Protein Concentrations in Control and Ancrod-treated Rabbits with BSA-Induced Chronic Immune-Complex Glomerulonephritis.

ANIMAL GROUP	n	SERUM C3 CONCENTRATIONS % NORMAL POOL : Mean (Range) DAY 0	DAY 8	DAY 15	DAY 21	DAY 28	DAY 35	DAY 44	DAY 54
Controls	10	100 (82-115)	104 (82-130)	103 (56-125)	82 (54-105)	69 (18-90)	44 (24-49)	51 (19-100)	49 (48-73)
Ancrod-treated	10	108 (88-130) P>0.1	107 (52-130) P>0.1	102 (88-120) P>0.1	79 (54-130) P>0.1	67 (32-105) P>0.1	40 (26-63) P>0.1	50 (36-100) P>0.1	57 (28-100) P>0.1

Table 6.9 Serum C3 Concentrations in Control and Ancrod-Treated Rabbits with BSA-Induced Chronic Immune-Complex Glomerulonephritis.

greater mesangial deposition.

(ii) Fibrin (Fig. 6.6 ; Table 6.10)

Moderately heavy glomerular fibrin deposition, particularly in Bowman's space was observed in seven of the ten controls. Virtually no fibrin was detected in treated animals, only three showing any deposition at all. Quantitative analysis showed that fibrin scores in the treated group were significantly lower than in the control group ($P < 0.02$).

(f) Histology

All animals showed diffused or focal endothelial and mesangial cell proliferation in which polymorphonuclear leucocyte infiltration was prominent, and most animals showed varying degrees of basement membrane thickening. These changes were unaffected by defibrination. Extracapillary cell proliferation with crescent formation was found in seven treated and seven control animals. However, quantitative assessmer showed that crescent scores in the treated animals was significantly lower than in controls ($P < 0.05$)(Fig. 6.6 ; Table 6.10).

(g) Correlation Of Severity Of Nephritis With Dosage Of BSA

Values of serum creatinine greater than 2.0 mg/100 ml were not observed unless an overall mean daily dose of 60 mg of BSA was exceeded. Since none of the six animals (three controls and three treated) receiving a mean daily dose of less than 60 mg showed glomerular fibrin deposition or extracapillary cell proliferation, it seemed approp-

MEAN DAILY DOSE OF BSA (mg)	ANIMAL GROUP	NUMBER OF ANIMALS	MEAN FIBRIN SCORE (Range)	MEAN CRESCENT SCORE (Range)	MEAN SERUM CREATININE VALUE (mg/100 ml on Day 54, or at Death) (Range)
All Doses	Controls	10	1.06 (0 - 2.15)	1.38 (0 - 2.6)	7.2 (1.2 - 17.4)
	Ancrod-treated	10	0.06 ** (0 - 0.2)	0.17 * (0 - 0.4)	2.4 ** (1.1 - 7.1)
Greater than 60 mg	Controls	7	1.52 (1.0 - 2.15)	1.97 (1.08-2.54)	9.7 (5.1 - 17.4)
	Ancrod-treated	7	0.1 *** (0 - 0.3)	0.24 *** (0.08 - 0.4)	2.9 *** (1.4 - 7.1)

Table 6.10 Serum Creatinine Concentrations, Fibrin Scores and Crescent Scores on Day 54 or at Death, in Control and Ancrod-Treated Rabbits with BSA-Induced Chronic Immune-Complex Glomerulonephritis

*** $P < 0.01$ ** $P < 0.02$ * $P < 0.05$

riate in analysing the effect of defibrination to compare the subgroups of animals receiving more than a mean of 60 mg of BSA per day. The fibrin and crescent scores and serum creatinine on Day 54 were then even more highly significantly lower in the treated group than in controls ($P<0.01$) (Table 6.10).

3 : DEFIBRINATION IN NTN AFTER GLOMERULAR FIBRIN DEPOSITION

EXPERIMENT A

(a) Renal Function

Figure 6.7 shows that if the serum creatinine on Day 10 was greater than 8 mg/100 ml the outlook was bad irrespective of whether the animal was defibrinated or not. Thus,three of the five animals (two treated and one control) with serum creatinine of greater than 8 mg/100 ml on Day 10 died from renal failure. It was therefore decided to analyse the results of defibrination in animals in whom the serum creatinine was less than 8 mg/100 ml on Day 10, the day on which defibrination was started. When this was done, a highly significant reduction in serum creatinine (evident from Day 16) was found in the defibrinated group (six animals) when compared to controls (seven animals) (Fig. 6.8 ; Table 6.11).

(b) Proteinuria

At no stage were urine protein concentrations in treated animals significantly different from controls (Table 6.12).

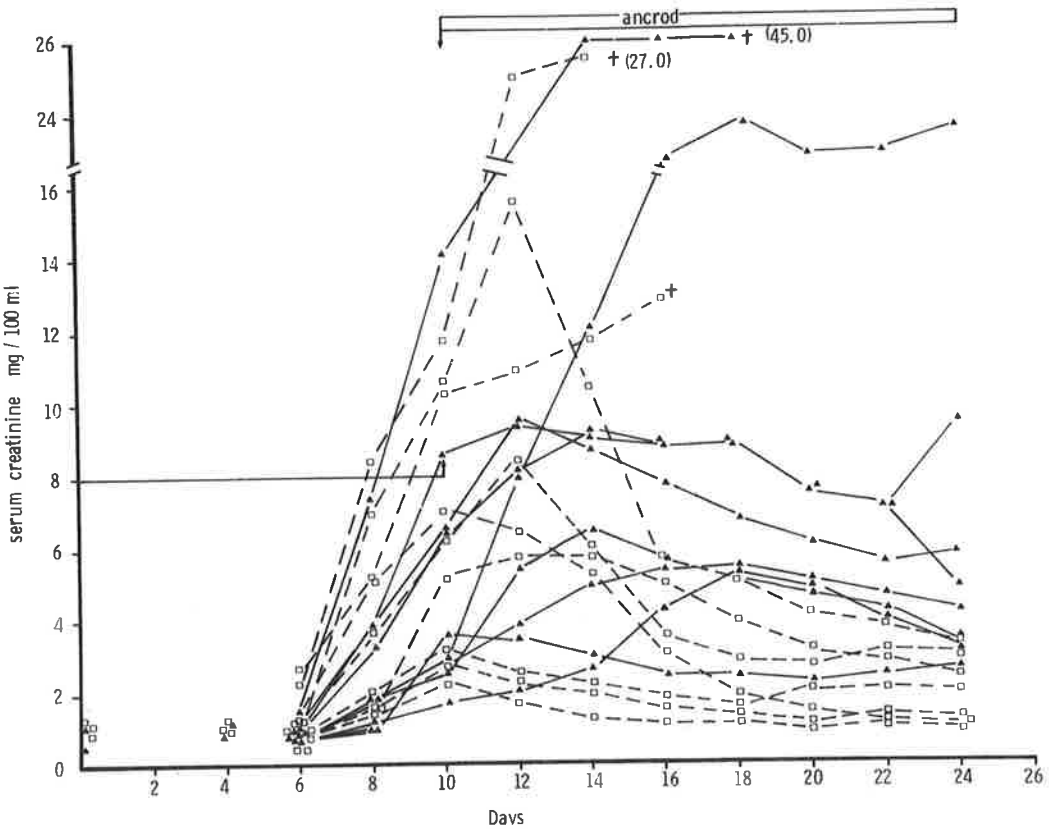

Fig. 6.7 Serum creatinine concentrations in rabbits with NTN : control animals (▲) and animals treated with ancrod from Day 10 of the disease (□). († Died)

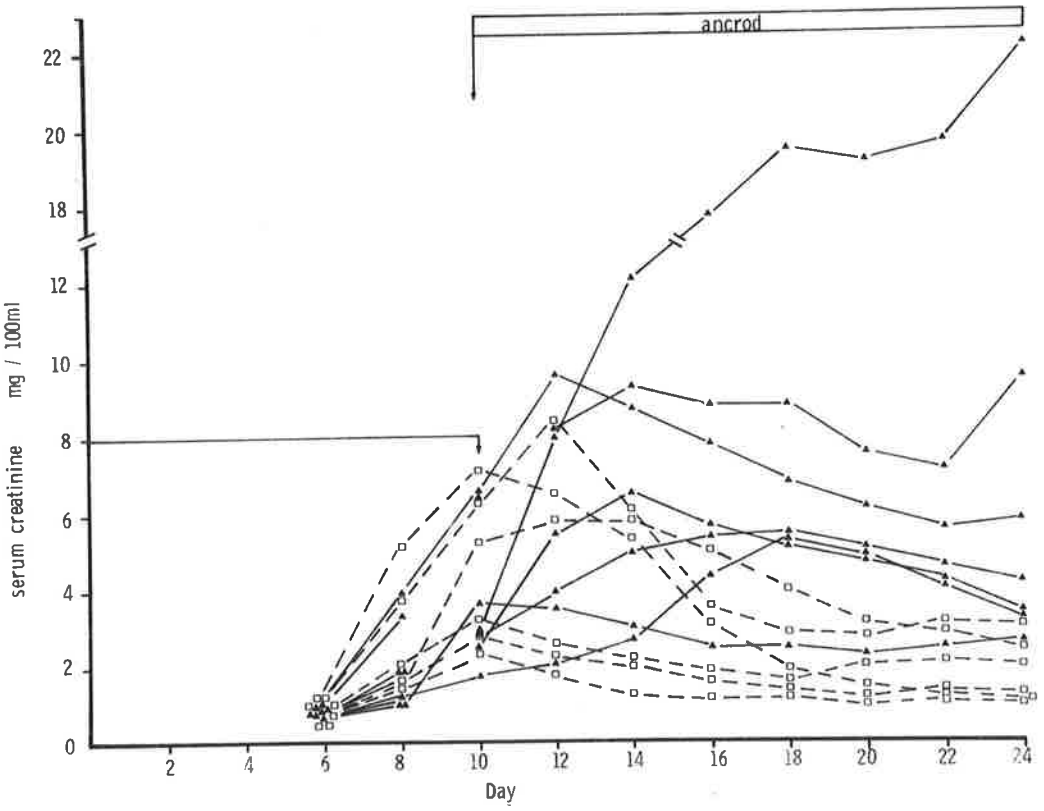

Fig. 6.8 Serum creatinine concentrations in rabbits with NTN, in which the serum creatinine had not risen above 8 mg/100 ml on Day 10 : control animals (▲) and animals treated with ancrod from Day 10 (□).

ANIMAL GROUP	n	SERUM CREATININE CONCENTRATION mg/100 ml : Mean (Range)					
		DAY 10	DAY 12	DAY 14	DAY 16	DAY 20	DAY 24
Control	6	3.79 (1.8-6.5)	5.83 (2.1-9.6)	6.76 (3.0-12.1)	7.4 (2.5-17.4)	7.1 (2.3-19.1)	7.21 (2.2-22.2)
Ancrod-treated	7	4.53 (2.5-7.1)	4.57 (1.8-8.4)	4.35 (1.3-6.0)	2.75** (1.1-5.1)	2.08*** (1.0-3.1)	1.82*** (1.0-3.1)

Table 6.11 Defibrination from Day 10 in NTN. Serum Creatinine Concentrations on Days 10-24 in Control and Ancrod-Treated Animals in which Serum Creatinine Concentrations were less than 8 mg/100 ml on Day 10.

**P< 0.02

***P< 0.01

ANIMAL GROUP	n	URINE PROTEIN CONCENTRATION mg/ml : Mean (Range)					
		DAY 0	DAY 10	DAY 12	DAY 16	DAY 20	DAY 24
Controls	6	< 0.3	12.6 (2.1-18)	7.2 (1.8-18)	5.9 (1.2-10.5)	4.4 (2.1-8.5)	3.8 (1.9-6.8)
Ancrod-treated	7	< 0.3	9.8 (1.9-17)	8.1 (1.6-12)	6.4 (1.8-14.2)	3.2 (1.6-5.9)	2.9 (1.2-4.5)

Table 6.12 Defibrination from Day 10 in NTN.
Urine Protein Concentrations in Control and Ancrod-Treated Animals in which Serum Creatinine Concentrations were less than 8 mg/100 ml on Day 10.

(c) Immunofluorescence

(i) Immunoglobulin And C3

Linear deposition of sheep IgG, rabbit IgG and rabbit C3, along the GBM was seen in all animals. No difference in intensity of staining was evident between the groups of animals.

(ii) Fibrin

Only flecks of glomerular fibrin were detected in treated or control animals on Day 24.

(d) Histology

When assessed on Day 24, crescent formation in the treated animals was significantly less than in controls ($p=0.02$). The mean crescent scores were 1.55 (range 0.55-2.45) and 2.4 (range 1.9-2.8) for the treated and control groups respectively (Fig. 6.9).

EXPERIMENT B

In control animals there appeared to be a steady reduction in the glomerular fibrin deposits with time but fibrin was still prominent in the one animal killed on Day 21. By Day 24 only flecks of fibrin were seen on immunofluorescence or MSB staining. There was a striking difference in animals defibrinated from Day 10, the reduction in glomerular fibrin being more rapid, such that fibrin was not detected in the glomeruli of animals killed after Day 14 (Fig. 6.10).

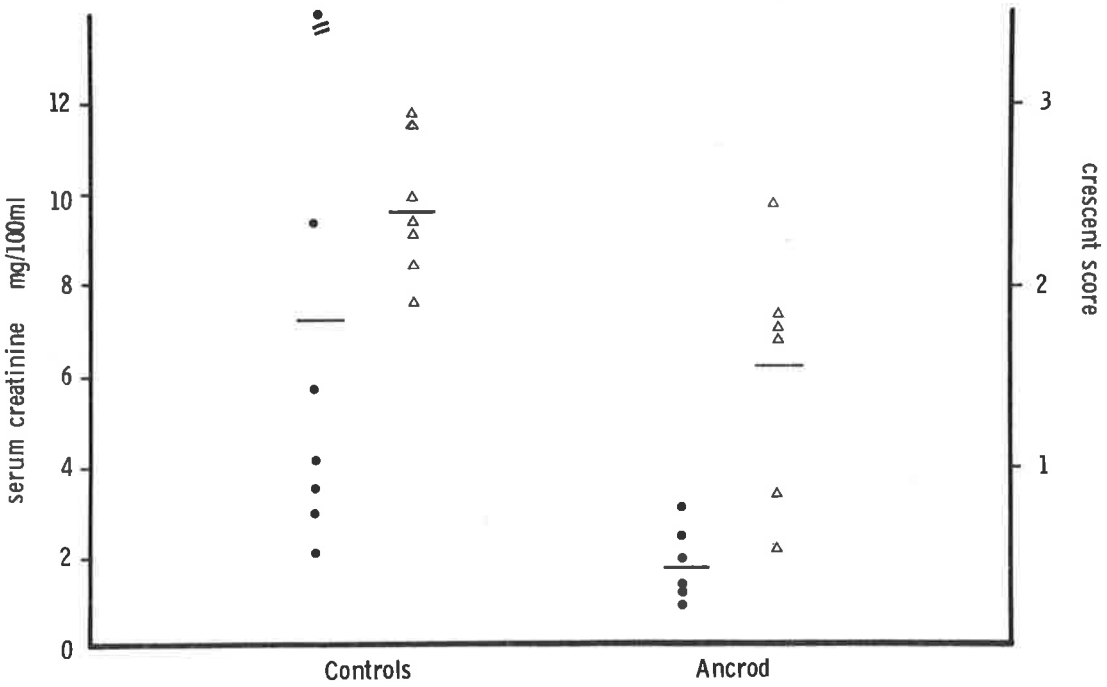

Fig. 6.9 Serum creatinine concentrations (●) and crescent scores (Δ) on Day 24 in rabbits with NTN : control animals and animals treated with ancrod from Day 10 of the disease.

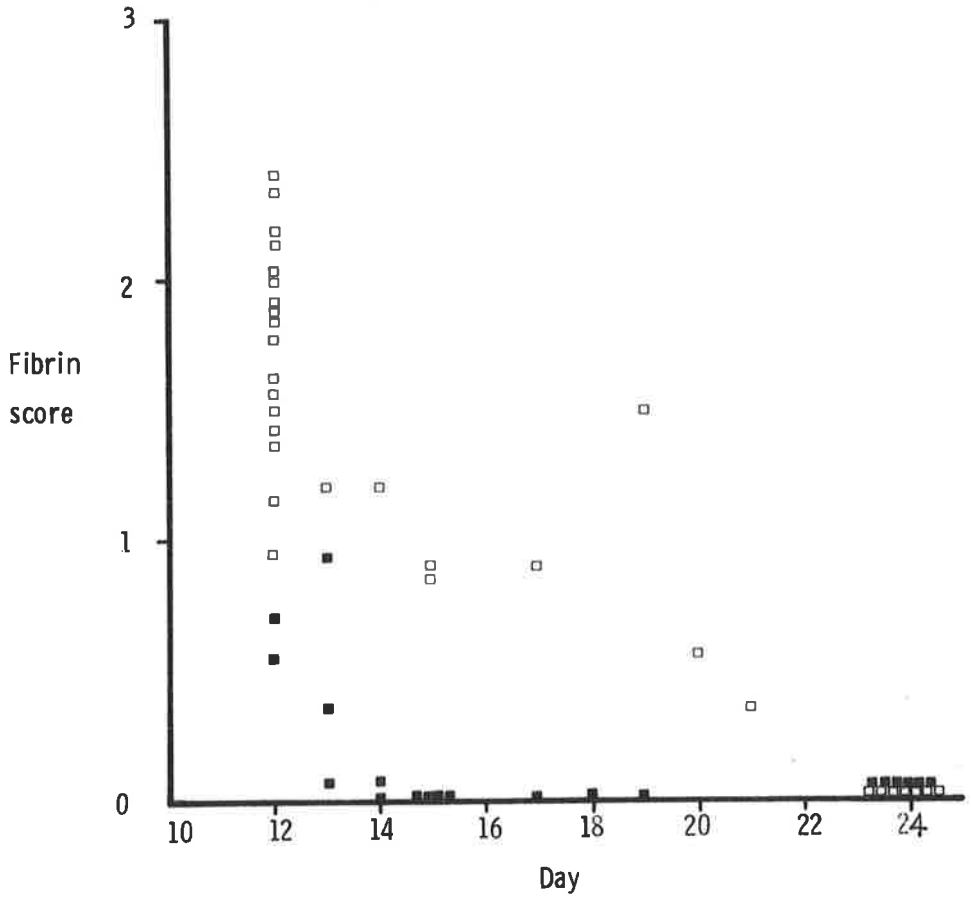

Fig. 6.10 Glomerular fibrin deposition in control (□) and ancrod-treated (■) rabbits with NTN, when killed on successive days. Ancrod was commenced on Day 10 (scores on Day 12 for control animals are those of the control animals in protocol 1).

SUMMARY OF RESULTS

These experiments have shown :

(1) Even when heparin was commenced prior to the glomerular deposition of fibrin in rabbits with NTN, only very large doses of 2000 u/Kg/day which prolonged whole blood clotting times to 15-20x normal, resulted in any reduction in glomerular fibrin deposition, extracapillary cell proliferation and renal failure.

(2) Defibrination with ancrod prior to the glomerular deposition of fibrin in rabbits with NTN provided excellent protection from glomerular fibrin deposition, crescent formation and renal failure, protection significantly greater than that obtained with very large doses of heparin.

(3) A highly significant relationship was observed between the degree of glomerular fibrin deposition and the degree of extracapillary cell proliferation and renal failure.

(4) Defibrination with ancrod in NTN after the glomerular deposition of fibrin had occurred and extracapillary cell proliferation was developing, resulted in a much more rapid disappearance of fibrin from the glomerulus and consequently significantly less extracapillary cell proliferation and renal failure. However, this protection was not as great as when defibrination was commenced before glomerular fibrin deposition.

(5) As in crescentic glomerulonephritis due to anti-GBM antibody (NTN), crescentic glomerulonephritis due to the chronic glomerular deposition of BSA - anti-BSA immune complexes was largely prevented by defibrination with ancrod.

(6) Although anticoagulation with heparin or defibrination with ancrod reduced glomerular fibrin deposition, crescent formation and renal failure of NTN or chronic BSA-induced immune-complex glomerulonephritis, urine protein excretion was unaffected.

(7) Neither defibrination with ancrod nor anticoagulation with heparin significantly altered serum C3 concentrations in either NTN or BSA-induced chronic immune-complex glomerulonephritis.

The mechanism by which glomerular fibrin deposition occurs in crescentic glomerulonephritis is the subject of the next chapter.

Chapter 7

POLYMORPHONUCLEAR LEUCOCYTES IN THE AUTOLOGOUS PHASE OF NTN

Introduction

The importance of the polymorphonuclear leucocyte (PMN) in the mediation of allergic tissue damage has been discussed in Chapter 1. In summary, PMN may be seen to accumulate at the site of antibody-antigen reaction in various types of allergic tissue injury. They induce injury by liberation of catheptic enzymes and other proteins. It has been shown that PMN are the principal injurious mediator in the Arthus reaction, the necrotising arteritis of acute serum sickness and the heterologous phase of NTN. Less obvious glomerular polymorph accumulation also occurs in the autologous phase of NTN (Ehrich, Forman and Seifter, 1952) but the role of the PMN in the autologous phase has not so far been investigated.

Initial evidence that PMN may be important in the autologous phase of NTN came from experiments performed by Dr. P. Naish in our laboratory. He found the depletion of circulating PMN, induced by giving nitrogen mustard, provided some degree of protection from injury during the autologous phase. However, the possibility that other actions of nitrogen mustard were responsible for the protection could not be excluded. These other actions include general cytoxic and anti-inflammatory action and alteration in the immune response (Hitchings, Elion and Singer, 1954).

In this chapter I have described experiments in which the autologous phase of injury has been examined in rabbits in which circulating PMN have been depleted with a specific anti-PMN serum. In the first series of experiments PMN were depleted during the autologous phase of the standard model of NTN. In the second, depletion was achieved throughout the 5-day period of the "telescoped" model of NTN

EXPERIMENTAL PROTOCOLS

1 : PMN DEPLETION DURING THE AUTOLOGOUS PHASE OF THE STANDARD MODEL OF NTN

Twenty-one rabbits were injected IV with 1 ml of NTG/Kg BW on Day 0 :

- five of these received 5 ml of goat anti-rabbit PMN globulin (APG) IV daily from Day 4. On Days 10 and 11, two doses of 5 ml were given.
- five control animals received the same volume of normal goat globulin IV daily.
- eleven other control animals received 5 ml of saline IV daily.

All animals were given prophylactic IV antibiotics (penicillin 20,000 u and streptomycin 50 mg) daily from Day 4.

Serum creatinine and C3 concentrations and titres of anti-sheep

immunoglobulin antibody were estimated on alternate days. Proteinuria was quantitated on urine samples obtained daily by urethral catheterisation. Peripheral blood white cell counts, differential cell counts and platelet counts were estimated daily, extra estimations being carried out in the APG-treated animals at various times after the dose of APG.

Animals were killed on Day 12 and the kidneys removed for histology and immunofluorescence examination for sheep and rabbit immunoglobulin, rabbit C3 and rabbit fibrin.

2 : PMN DEPLETION IN THE "TELESCOPED" MODEL OF NTN

Twelve rabbits were immunised IM on Day -7 with 10 mg of normal sheep globulin in FCA. Seven days later (Day 0) each animal was injected IV with 1 ml/Kg BW of ^{125}I-labelled NTG (containing ^{131}I-labelled normal sheep globulin - see Methods) :

- six of the rabbits were injected IV with 5 ml of APG daily from Day -1
- six control rabbits received the same volume of saline daily from Day -1

As in protocol 1, all animals were given prophylactic antibiotics. Serum concentrations of creatinine and C3 and titres of anti-sheep immunoglobulin antibody were measured daily. Proteinuria was measured daily on urine samples obtained by urethral catheterisation.

Peripheral blood white cell counts, differential cell counts, and platelet counts were estimated daily.

The animals were killed on Day 5 and the kidneys were removed for histology and immunofluorescence as in protocol 1, and also for measurement of fixed nephrotoxic antibody (as described in Methods).

RESULTS

1 : PMN IN THE AUTOLOGOUS PHASE OF THE STANDARD MODEL OF NTN

(a) Morbidity

No deaths occurred. The administration of the first and second doses of APS was associated with some peripheral vasoconstriction lasting 6-12 hours but this did not affect urine output. This was presumed to be the consequence of intravascular release of polymorph constituents before persistent PMN depletion was achieved. On Days 10 and 11 animals receiving APG and the control group receiving normal goat globulin developed minor anaphylactic reactions.

(b) PMN Depletion (Fig. 7.1 a)

Immediate and sustained PMN depletion was obtained after the injection of APG. Mean counts were less than 400/mm^3 at all times except in two animals in which counts of 450 and 500/mm^3 were recorded on Days 10 and 11. In contrast, control animals showed a mild polymorphonuclear leucocytosis. Lymphocyte counts were never reduced by more than 15% after APG administration.

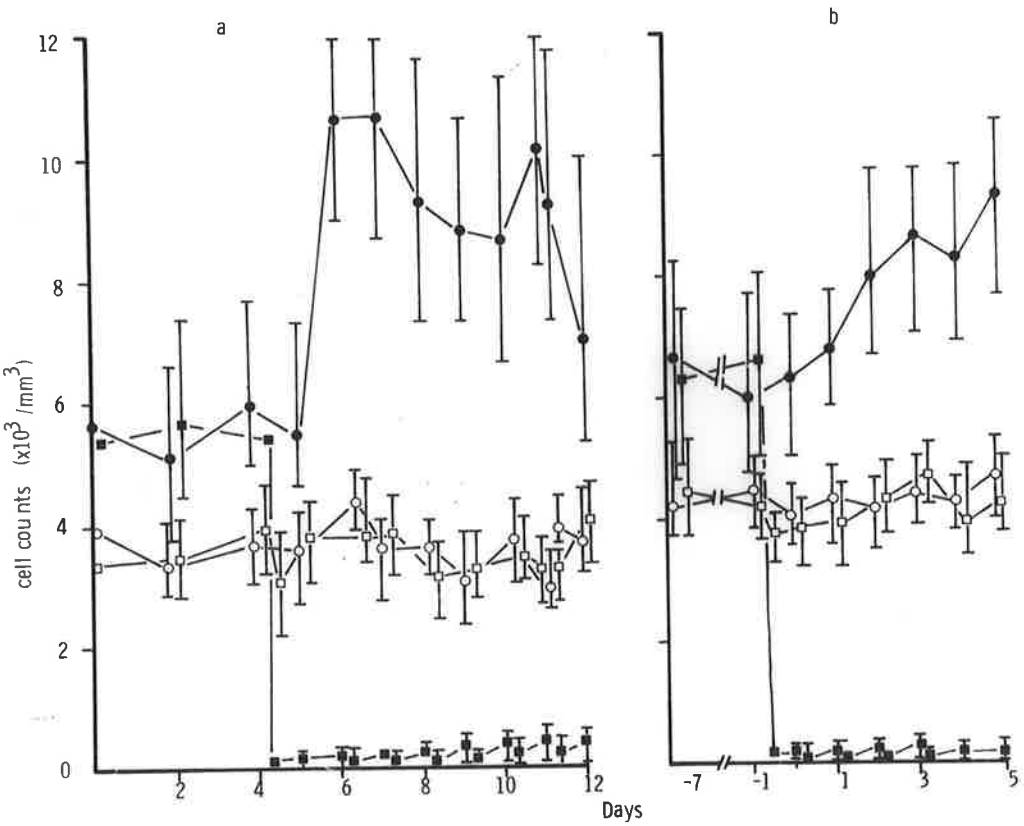

Fig. 7.1 Mean peripheral-blood PMN (solid symbols) and lymphocyte (open symbols) counts for control (● and ○) and APG-treated (■ and □) groups of rabbits with NTN (range I).

(a) Standard model of NTN :- controls given normal goat serum

(b) "Telescoped" model of NTN :- controls given saline

(c) Platelet Counts (Fig. 7.2 a)

APG caused a slight reduction in platelet counts but this was never greater than 10%.

(d) Rabbit Anti-Sheep Immunoglobulin Antibody Titres (Fig. 7.3 a)

No significant difference in the anti-sheep immunoglobulin antibody titres was found between animals given APG and the controls given normal goat globulin ($P > 0.1$).

(e) Renal Function (Fig. 7.4 a ; Table 7.1)

Serum creatinine concentrations rose rapidly and progressively from Day 5 onwards in the control groups given saline and normal goat globulin. There was no significant difference on any day in the serum creatinine concentrations between the two control groups. Animals given APG showed only a small rise in the serum creatinine to a maximum mean value of only 2.0 mg/ml (range 1.3-2.7 mg/ml) on Day 10. The mean serum creatinine concentrations of the treated animals were highly significantly lower ($P < 0.01$) than either control group from Day 8.

(f) Proteinuria (Table 7.2)

Heavy proteinuria was seen in both groups of control animals from Day 8 until death. This contrasted to only very mild autologous-phase proteinuria in APG-treated animals.

(g) Serum C3 Concentrations (Fig. 7.5 a)

There was no significant difference in the serum C3 concentrations

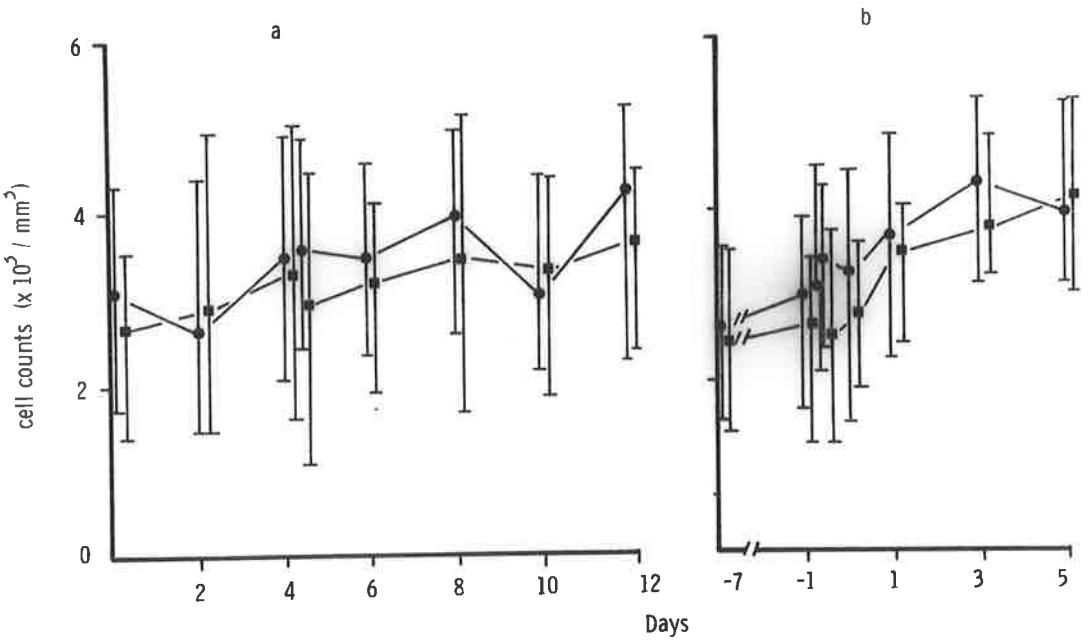

Fig. 7.2 Mean peripheral-blood platelet counts for control (●) and APG-treated (■) groups of rabbits with NTN (range I).

(a) Standard model of NTN:- controls given normal goat serum

(b) "Telescoped" model of NTN:- controls given saline

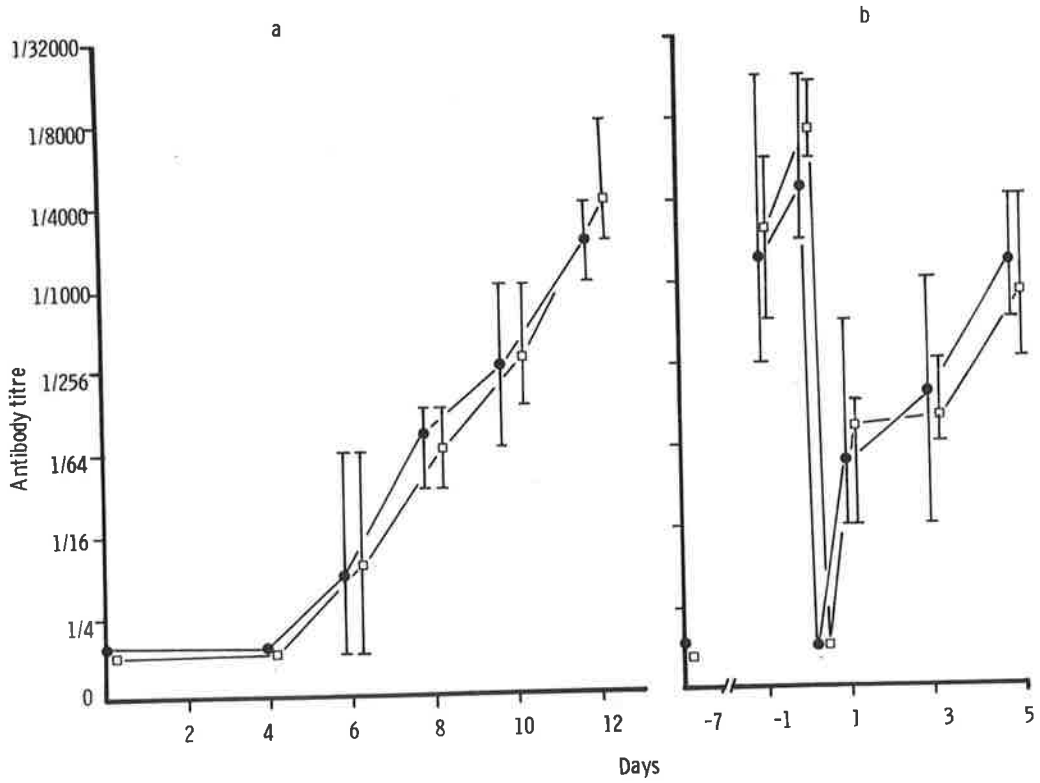

Fig. 7.3 Mean anti-sheep immunoglobulin antibody titres in control (●) and APG-treated (□) rabbits with NTN (range I).

(a) Standard model of NTN:- controls given normal goat serum

(b) "Telescoped" model of NTN:- controls given saline

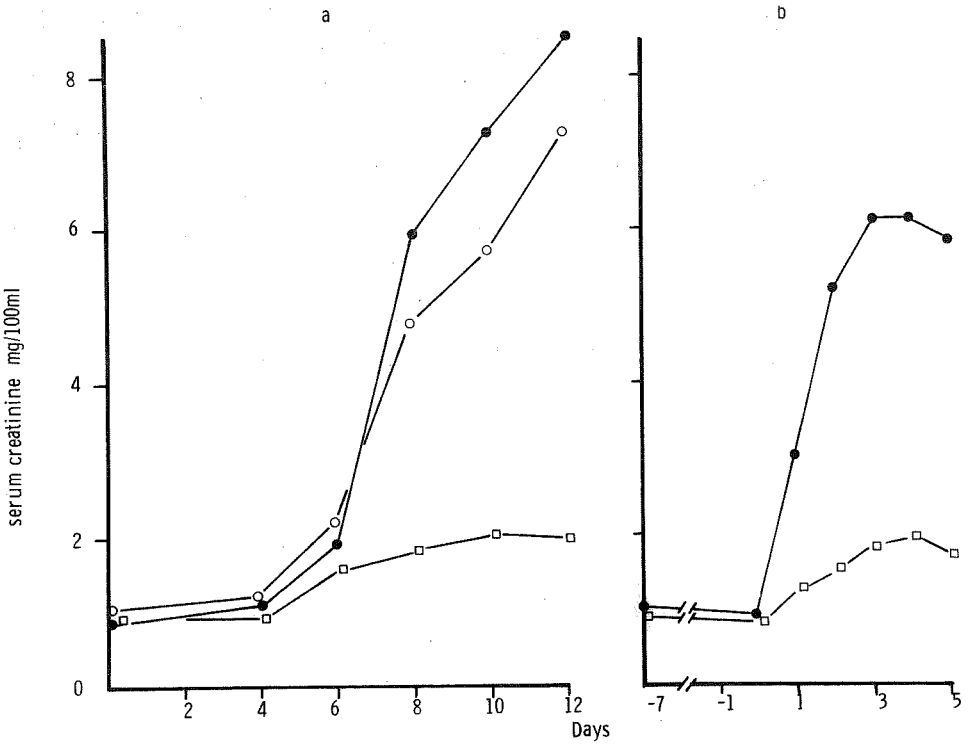

Fig. 7.4 Mean serum creatinine concentrations in control and APG-treated rabbits with NTN.

(a) Standard model of NTN:- controls given normal goat serum (●), controls given saline (○), APG-treated animals (□).

(b) "Telescoped" model of NTN:- controls (●), APG-treated animals (□).

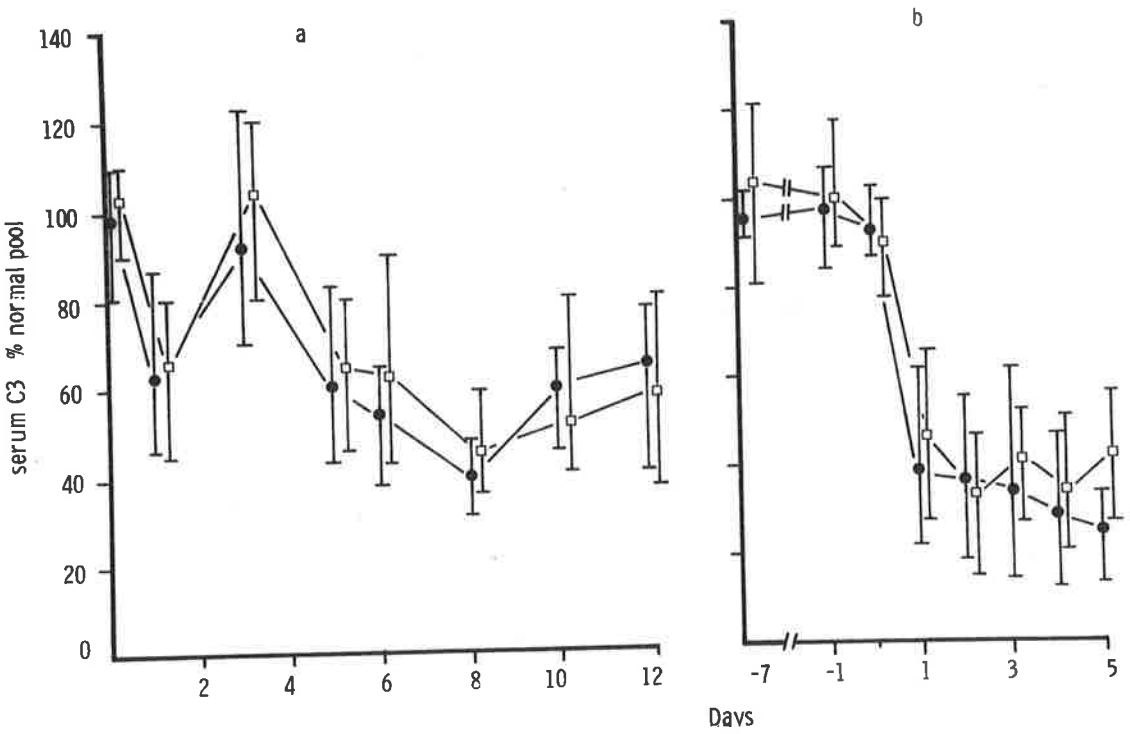

Fig. 7.5 Mean serum C3 concentrations in control (●) and APG-treated (□) rabbits with NTN (range I).

(a) Standard model of NTN:- controls given normal goat serum

(b) "Telescoped" model of NTN:- controls given saline

ANIMAL GROUP		n	SERUM CREATININE CONCENTRATION mg/100 ml : Mean (Range)					
			DAY 0	DAY 4	DAY 6	DAY 8	DAY 10	DAY 12
Controls	Goat Serum	5	0.88 (0.8-1.0)	1.1 (0.9-1.3)	1.86 (0.9-4.5)	5.94 (3.9-7.2)	7.3 (5.8-8.0)	8.56 (7.6-9.5)
	Saline	11	1.0 (0.8-1.3)	1.1 (0.8-1.7)	2.2 (0.9-4.4)	4.5 (3.5-8.5)	5.7 (3.5-11.0)	7.3 (3.9-12.1)
APS-treated		5	0.90 (0.7-1.2)	0.90 (0.7-1.0)	1.56 (0.9-4.4)	1.82*** (1.4-2.7)	2.0*** (1.3-2.7)	1.95*** (1.0-3.2)

Table 7.1 Serum Creatinine Concentrations in APS-Treated and Control Groups of Rabbits with NTN.

***P<0.01

ANIMAL GROUP		n	URINE PROTEIN CONCENTRATION mg/ml : Mean (Range) DAY 0	DAY 5	DAY 7	DAY 9	DAY 11	DAY 12
Controls	Goat Serum	5	<0.3	13.5 (4 - 23)	38.2 (17 - 60)	26.2 (14 - 35)	13.0 (9 - 26)	10.3 (4.2 -15.0)
	Saline	11	<0.3	16.8 (8 - 25)	23.4 (8 - 49)	32.6 (15 - 65)	18.6 (12 - 43)	8.6 (3.5-13.9)
APS - treated		5	<0.3	0.42*** (<0.3-0.68)	0.66*** (<0.3-1.48)	1.48*** (0.35-2.96)	0.83*** (<0.3 -1.68)	1.19*** (0.5 - 2.9)

Table 7.2 Urine Protein Concentration in APS-Treated and Control Groups of Rabbits with NTN.

***P< 0.01

between control groups and the group receiving APG (P 0.1 for all estimations) (Fig. 7.5 a).

(h) Immunofluorescence

(i) Immunoglobulin And C3

Linear deposition of sheep and rabbit immunoglobulin and rabbit C3 along the GBM was seen in all animals. No difference in deposition could be detected between the groups of animals.

(ii) Fibrin (Table 7.3)

No significant difference was observed in glomerular fibrin deposition between the two control groups ($P > 0.1$). However, virtually no fibrin could be detected in the glomeruli of animals given APG, only one animal showing any deposition at all.

(i) Histology (Table 7.4)

No significant difference was observed in the degree of crescent formation between controls given normal goat globulin and those given saline ($P > 0.1$). In contrast, virtually no extracapillary cell proliferation was seen in APG-treated animals. APG-treated animals nevertheless did show focal and diffuse endothelial and mesangial cell proliferation. In APG-treated animals striking and highly significant reductions in glomerular PMN counts were observed ($P < 0.01$). In the control group it was clear that PMN infiltration was focal and showed striking variation from glomerulus to glomerulus. Thus, 16% of glomeruli contained

ANIMAL GROUP		n	PER CENT INCIDENCE OF EACH GRADE OF FIBRIN DEPOSITION : Mean (Range) GRADE 0	GRADE 1	GRADE 2	GRADE 3	MEAN FIBRIN SCORE (Range)
Controls	Goat Serum	5	1.0 (0 - 5)	26.3 (15 - 55)	37.6 (30 - 60)	35.1 (15 - 55)	2.09 (1.6 - 2.4)
	Saline	11	3.1 (0 - 15)	36.2 (15 - 70)	36.6 (25 - 60)	24.1 (0 - 45)	1.81 (1.3 - 2.20)
APS-treated		5	97.1*** (85 - 100)	2.9*** (0 - 15)	0***	0***	0.03*** (0 - 0.15)

Table 7.3 Intraglomerular Fibrin Deposition on Day 12 in APS-Treated and Control Groups of Rabbits with NTN.

***P<0.01

ANIMAL GROUP		n	PER CENT INCIDENCE OF EACH GRADE OF EXTRA-CAPILLARY CELL PROLIFERATION : Mean (Range) GRADE 0	GRADE 1	GRADE 2	GRADE 3	MEAN CRESCENT SCORE (Range)
Controls	Goat Serum	5	3.4 (0 - 12)	24.6 (8 - 40)	28.0 (16 - 52)	44.0 (20 - 72)	2.11 (1.6 - 2.48)
	Saline	11	3.0 (0 - 6)	15.5 (6 - 36)	43.5 (38 - 52)	39.0 (14 - 56)	2.06 (1.5 - 2.4)
APS-treated			91.2*** (72 - 100)	6.4 (0 - 16)	2.4*** (0 - 12)	0***	0.11*** (0 - 0.4)

Table 7.4 Extracapillary Cell Proliferation on Day 12 in APS-Treated and Control Groups of Rabbits with NTN.

***P< 0.01

0-1 PMN, 60% 2-5, and 22% 5-26, whereas in the APG-treated group 98% of glomeruli contained 0-1 PMN. The mean glomerular PMN counts were 4.5 (range 3.0-6.4) and 0.21 (range 0-0.58) in control and APG-treated groups respectively.

2 : PMN IN THE "TELESCOPED" MODEL OF NTN

(a) _ Morbidity

Mild initial vasoconstriction was seen in animals given APG as in the standard model.

(b) _ PMN Depletion (Fig. 7.1 b)

Immediate and sustained PMN depletion was again attained and in all animals PMN counts were less than 400/mm^3 at all times. Lymphocyte and platelet counts were not significantly reduced at any time (Fig. 7.2 b).

(c) _ Rabbit Anti-Sheep Immunoglobulin Antibody Titres (Fig. 7.3 b)

High titres of antibody were present in all animals prior to the administration of NTG. Antibody titres fell to undetectable levels when the NTG was given but rose progressively subsequently. No difference in antibody titres was found at any stage between control and APG-treated groups ($P > 0.1$ on all days).

(d) _ Renal Fixation of Nephrotoxic Antibody

There was no significant difference ($P > 0.1$) in the quantities of antibody fixed in the kidneys between control and APG-treated groups:

mean values per kidney were 135 µg (range 115-156 µg) and 155 µg (range 142-169 µg) in control and APG-treated groups respectively.

(e) Renal Function (Fig. 7.4 b ; Table 7.5)

Immediate and rapidly progressive impairment in renal function was seen in control animals after the injection of NTG. As in the standard model of NTN, APG-treated animals developed only very mild impairment in renal function. The serum creatinine concentrations of APG-treated animals were significantly lower than in controls on all days ($P < 0.01$ for each day).

(f) Proteinuria (Table 7.6)

Again only minimal proteinuria was seen in APG-treated animals, contrasting to the heavy proteinuria seen in controls.

(g) Serum C3 Concentrations (Fig. 7.5 b)

A sustained fall in C3 was seen in both control and APG-treated rabbits. There was no significant difference in serum C3 concentrations between the two groups on any day ($P > 0.1$ for each day).

(h) Immunofluorescence

(i) Immunoglobulins And C3

Linear deposition along the GBM of sheep and rabbit immunoglobulin and rabbit C3 was seen in all animals.

(ii) Fibrin (Table 7.7)

Glomerular fibrin deposition was virtually absent in APG-treated animals and extensive in controls.

ANIMAL GROUP	n	SERUM CREATININE CONCENTRATION mg/100 ml : Mean (Range)					
		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
Control	6	0.9 (0.7-1.0)	3.0 (2.0-4.0)	5.2 (3.7-7.5)	6.1 (3.3-9.1)	6.1 (2.3-9.8)	5.8 (2.4-10.6)
APS-treated	6	0.82 (0.7-1.0)	1.3*** (0.8-1.5)	1.5*** (1.1-2.1)	1.8*** (1.2-2.4)	1.9*** (1.1-2.8)	1.7*** (1.0-2.5)

Table 7.5 Serum Creatinine Concentrations in APS-Treated and Control Groups of Rabbits in the "Telescoped" Model of NTN.

***P< 0.01

ANIMAL GROUP	n	URINE PROTEIN CONCENTRATION mg/ml : Mean (Range) DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
Control	6	< 0.3	28.6 (11.3- 40)	9.6 (5.9-18.3)	11.7 (4.1 - 20.1)	9.0 (3.4 - 14.6)	10.1 (5.1 - 16.2)
APS-treated	6	< 0.3	0.95*** (0.5 - 1.3)	1.0*** (0.5 -2.6)	0.8*** (0.6-2.6)	1.2*** (0.4- 2.8)	0.9*** (0.1 -1.8)

Table 7.6 Urine Protein Concentrations in Control and APS-Treated Rabbits in the "Telescoped" Model of NTN. (*** P< 0.01)

ANIMAL GROUP	n	PER CENT INCIDENCE OF EACH GRADE OF FIBRIN DEPOSITION : Mean (Range)				MEAN FIBRIN SCORE (Range)
		GRADE 0	GRADE 1	GRADE 2	GRADE 3	
Control	6	10.0 (0 - 20)	20.0 (5 - 50)	35.0 (20 - 50)	35.0 (10 - 45)	1.80 (1.2 - 2.3)
APS-treated	6	95.0*** (84 - 100)	3.1*** (0 - 8)	1.9*** (0 - 8)	0***	0.07*** (0 - 0.24)

Table 7.7 Intraglomerular Fibrin Deposition on Day 5 in APS-Treated and Control Groups of Rabbits in the "Telescoped" Model of NTN. ***P<0.01

ANIMAL GROUP	n	PER CENT INCIDENCE OF EACH GRADE OF EXTRA-CAPILLARY CELL PROLIFERATION : Mean (Range)				MEAN CRESCENT SCORE (Range)
		GRADE 0	GRADE 1	GRADE 2	GRADE 3	
Control	6	29.3 (7 - 73)	23.2 (3 - 32)	23.4 (10 - 36)	24.0 (0 - 46)	1.02 (0.43 - 2.06)
APS-treated	6	91.6*** (80 - 100)	8.4** (0 - 20)	0***	0***	0.08*** (0 - 02)

Table 7.8 Extracapillary Cell Proliferation on Day 5 in APS-Treated and Control Groups of Rabbits in the "Telescoped" Model of NTN. (**P=0.02 ; ***P< 0.01)

(i) Histology

(I) Crescent Formation (Table 7.8)

Again in APG-treated animals, a high degree of protection from crescent formation was evident.

(II) Glomerular PMN Infiltration

A highly significant reduction in glomerular PMN counts was seen in APG-treated animals when compared to controls ($P < 0.01$). The mean glomerular PMN counts were 3.9 (range 0.9-10.5) and 0.05 (range 0-0.15) in control and APG-treated groups respectively.

SUMMARY OF RESULTS

It may be seen from these experiments that :

(1) The depletion of circulating PMN by a specific APG, before the onset of the autologous phase of NTN in rabbits, led to marked reduction in proteinuria, intraglomerular fibrin deposition, extra-capillary cell proliferation and renal failure.

(2) This protection was a specific consequence of PMN depletion and not due to an alteration in the immune response, because :
(a) rabbit anti-sheep immunoglobulin antibody titres (b) the fall in C3 (c) renal fixation of nephrotoxic antibody (d) peripheral blood lymphocyte and platelet counts, and (e) deposition of rabbit immunoglobulin and C3 along the GBM were all similar in both control and APG-treated groups of animals.

(3) The same protection was observed when PMN were depleted in the "telescoped" model of NTN. This protection contrasted with the effect of complement depletion in the same model (see Chapter 8).

The mechanism by which PMN are attracted to the glomeruli in crescentic glomerulonephritis is the subject of the investigations outlined in the next chapter.

Chapter 8

COMPLEMENT IN THE AUTOLOGOUS PHASE OF NTN

Introduction

As discussed in Chapter 2, in the heterologous phase of NTN in the rat and rabbit, PMN accumulation is complement-dependent, probably largely due to C3b-induced immune adherence. This conclusion has been based on experiments in which complement (C) depletion with cobra venom factor (CVF) or depletion of PMN provided similar protection (Cochrane, Unanue and Dixon, 1965; Hammer and Dixon, 1963; Unanue and Dixon, 1964; Henson, 1971 c; Cochrane, 1969).

In the autologous phase of NTN there is circumstantial evidence to support a similar C-dependent PMN-mediated system of injury : serum C is reduced, C3 is found fixed on glomerular basement membrane and, as discussed in Chapter 9, injury is mediated by PMN. However, direct experiments on the role of C in this phase have not been reported, presumably because the invariable generation of antibodies to CVF limits its decomplementing action in vivo to a period of 5-6 days.

Rother et al (1967) have shown that rabbits congenitally deficient in the sixth component of complement are not protected from the autologous phase of NTN. In the first series of experiments described in this chapter I have confirmed these findings.

In preliminary experiments performed in our laboratory by Dr. P. Naish, in which rabbits were decomplemented with CVF prior to the autologous phase but after the heterologous phase, protection from

the crescentic glomerulonephritis was not shown. However, C3 was found in the glomeruli of decomplemented rabbits and although this probably represented C3 deposited during the heterologous phase it was still possible that sufficient C3 had been circulating after the CVF-treatment to be fixed to the GBM in the autologous phase. Moreover, because of the short action of CVF it had been necessary to induce C depletion (and therefore a state of massive intravascular complement activation) after some degree of glomerular injury had occurred in the heterologous phase. In the second group of experiments described in this chapter I have examined the role of C in the autologous phase of the "telescoped" model of NTN in the rabbit. In the "telescoped" model, crescentic nephritis is well advanced by Day 5 so it was possible to decomplement animals with CVF prior to the injection of NTG and thus examine the effect of C depletion on both the heterologous and autologous phases in each animal.

Because protection in the autologous phase of NTN was not demonstrated when C was depleted with CVF, the possibility that CVF itself may have been responsible for initiating or augmenting renal damage remained. In experiments in pursuit of this possibility I have assessed the effect of CVF given to rabbits immediately after NTG. The object was to determine whether the initial massive CVF-induced C activation would augment complement deposition and thus proteinuria in the heterologous phase.

EXPERIMENTAL PROTOCOLS

1 : AUTOLOGOUS PHASE OF NTN IN RABBITS CONGENITALLY DEFICIENT IN C6

Twelve rabbits congenitally deficient in C6 and eleven normal NZW rabbits were injected IV with 1 ml of NTG/Kg BW (Day 0). Serum C3 creatinine concentrations and urine protein concentrations were determined on alternate days. All animals were killed on Day 12 and the kidneys were removed for histology and immunofluorescence examination for sheep and rabbit immunoglobulin, rabbit C3 and fibrin.

2 : COMPLEMENT DEPLETION WITH CVF IN THE HETEROLOGOUS AND AUTOLOGOUS PHASES OF THE "TELESCOPED" MODEL OF NTN

Twelve rabbits were immunised IM on Day -7 with 10 mg of normal sheep globulin in FCA. 1 ml/Kg BW of ^{125}I-labelled NTG was given IV 7 days later (Day 0) :

- six of these animals were given 150 u of CVF on Day -1
- six control animals were given saline

Serum concentrations of creatinine and C3 and titres of anti-sheep immunoglobulin antibody were measured daily. Proteinuria was measured daily on urine samples obtained by urethral catheterisation. Animals were killed on Day 5 and the kidneys removed for histology, immunofluorescence - as in protocol 2 - and for measurement of fixed nephrotoxic antibody.

3 : EFFECT OF CVF GIVEN IMMEDIATELY AFTER NTG

Twelve rabbits were injected IV with 1 ml/Kg BW of ^{125}I-labelled NTG, a dose known to result in the glomerular deposition of about 180 µg of nephrotoxic antibody per kidney and induce only mild proteinuria in the heterologous phase.

Five minutes after the injection of NTG, 150 u of CVF were given IV to six rabbits, the remainder reiving saline.

Twenty-four hour urine protein excretion was measured before and after the injection of NTG. The animals were killed at 24 hours and the kidneys were removed for histology, immunofluorescence and estimation of fixed nephrotoxic antibody.

RESULTS

1 : NTN IN C6-DEFICIENT RABBITS

(a) Renal Function (Table 8.1)

As from Day 5, progressive deterioration in renal function was seen in both C6-deficient and normal rabbits. At no stage was there a significant difference in serum creatinine concentration between these two groups of animals.

(b) Serum C3 Concentrations (Fig. 8.1)

The falls in C3 concentrations in the heterologous and autologous phases in C6-deficient animals were not significantly different from the falls in controls ($P > 0.1$ on all days).

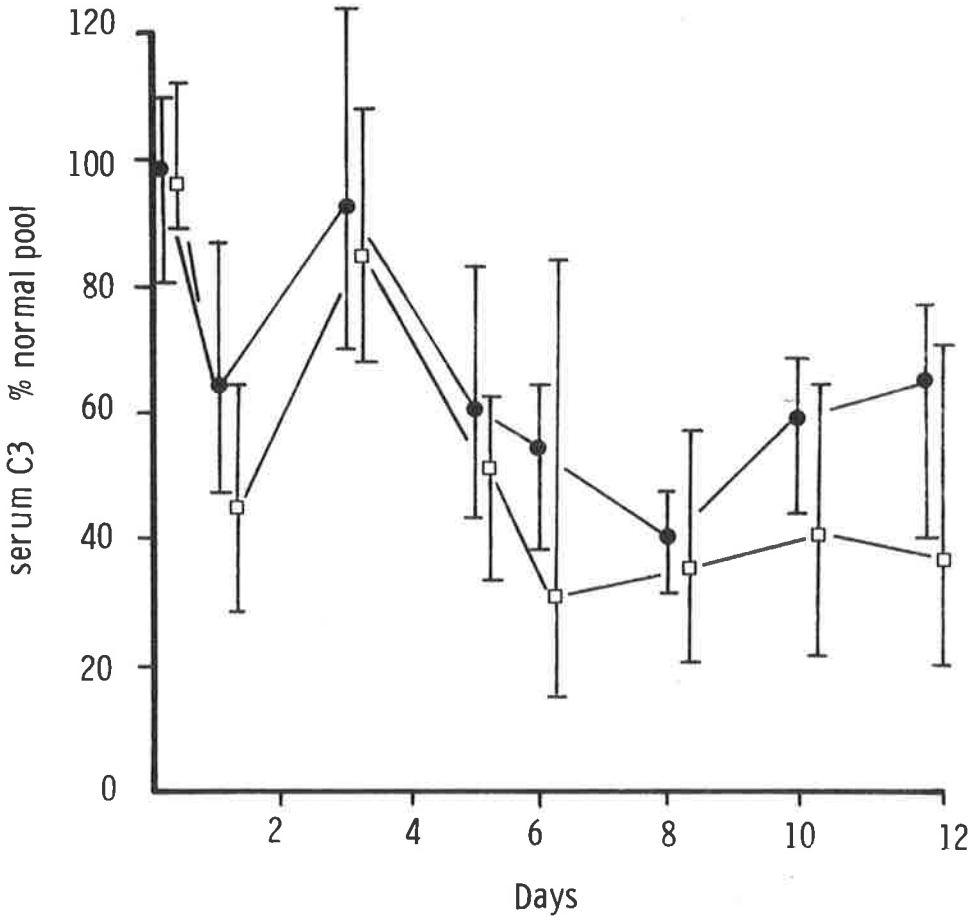

Fig. 8.1 Mean serum C3 concentrations in normal (●) and C6-deficient (□) rabbits with NTN (range I).

ANIMAL GROUP	n	SERUM CREATININE CONCENTRATION mg/100 ml : Mean (Range)				
		DAY 0	DAY 6	DAY 8	DAY 10	DAY 12
Normal	11	1.0 (0.8-1.3)	2.2 (0.9-4.4)	4.5 (3.5-8.5)	5.7 (3.5-11.0)	7.3 (3.9-12.1)
C6-deficient	12	0.9 (0.7-1.1) P>0.1	1.47 (0.8-7.0) P>0.1	3.9 (1.4-7.5) P>0.1	7.0 (2.9-11.4) P>0.1	9.42 (3.0-17.1) P>0.1

Table 8.1 Serum Creatinine Concentrations in Normal and C6-Deficient Rabbits with NTN.

(c) Proteinuria (Table 8.2)

Proteinuria was similar in both groups of animals on all days.

(d) Immunofluorescence

(i) Immunoglobulin And C3

Linear deposition of sheep and rabbit immunoglobulin and rabbit C3 along the GBM was seen in all normal and C6-deficient rabbits.

(ii) Fibrin (Table 8.3)

No significant difference in the degree of glomerular fibrin deposition was detected between normal and C6-deficient animals.

(e) Histology (Table 8.4)

Extracapillary cell proliferation and glomerular PMN infiltration was as extensive in C6-deficient rabbits as in normal rabbits.

2 : C3 DEPLETION IN THE "TELESCOPED" MODEL OF NTN

(a) Titres Of Antibody To Sheep Immunoglobulin (Fig. 8.2)

There was no significant difference in antibody titres between control and CVF-treated animals on any day ($P > 0.1$ on all days).

(b) Renal Function (Fig. 8.3 ; Table 8.5)

Control animals developed immediate and rapidly progressive impairment of renal function after the injection of NTG. CVF-treated animals also developed progressive renal failure but the onset was

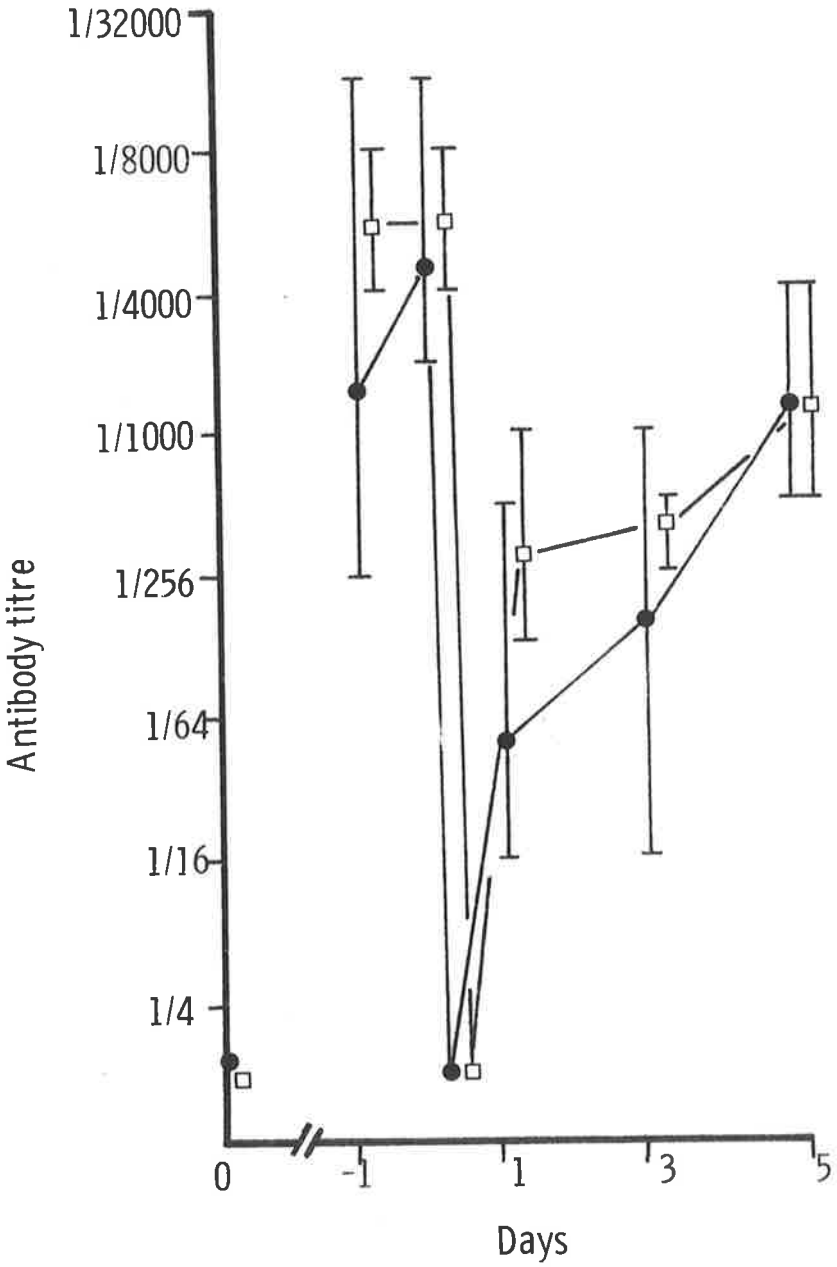

Fig. 8.2 Mean anti-sheep immunoglobulin antibody titres for control (●) and CVF-treated (□) rabbits in the "telescoped" model of NTN (range I).

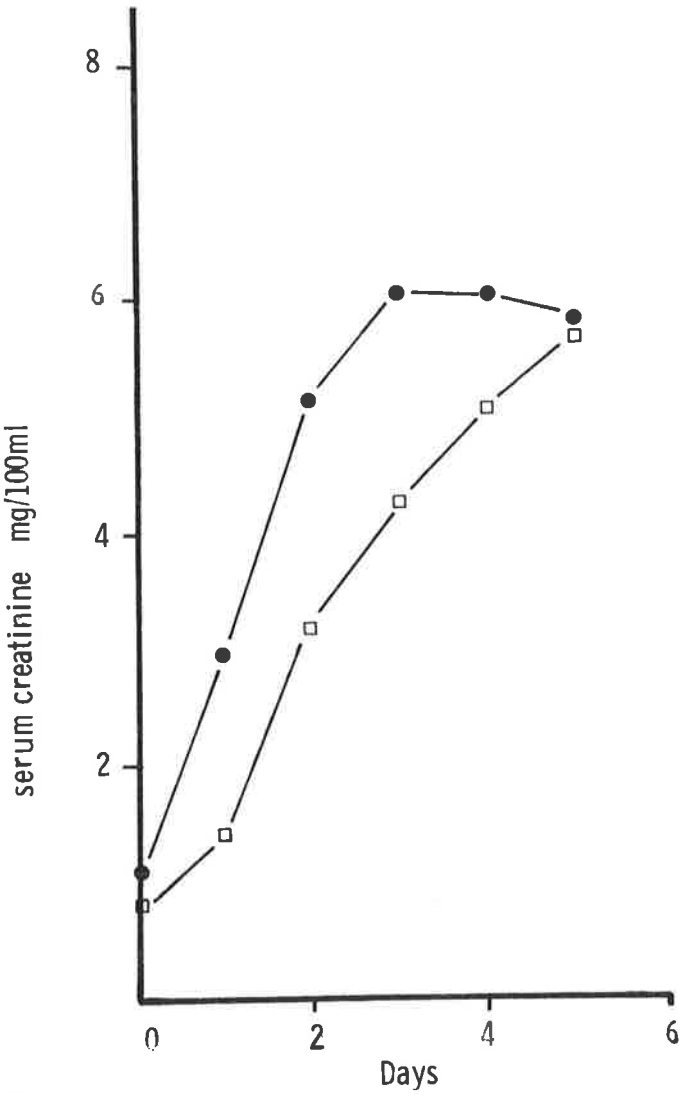

Fig. 8.3 Mean serum creatinine concentrations for control (●) and CVF-treated (□) rabbits in the "telescoped" model of NTN.

ANIMAL GROUP	n	URINE PROTEIN CONCENTRATION mg/ml : Mean (Range)					
		DAY 0	DAY 5	DAY 7	DAY 9	DAY 11	DAY 12
Normal	11	< 0.3	16.8 (8 - 25)	23.4 (8 - 49)	32.6 (15 - 65)	18.6 (12 - 43)	8.6 (3.5 - 13.9)
C6-deficient	12	< 0.3	11.6 (6 - 18)	35.9 (13 - 59)	23.2 (6 - 36)	20.5 (8 - 38)	13.6 (5 - 21)

Table 8.2. Urine Protein Concentrations in Normal and C6-Deficient Rabbits with NTN.

ANIMAL GROUP	n	PER CENT INCIDENCE OF EACH GRADE OF FIBRIN DEPOSITION : Mean (Range)				MEAN FIBRIN SCORE (Range)
		GRADE 0	GRADE 1	GRADE 2	GRADE 3	
Normal	11	3.1 (0 - 15)	36.2 (15 - 70)	36.6 (25 - 60)	24.1 (0 - 45)	1.81 (1.3 - 2.2)
C6-deficient	12	11.9 (0 - 30)	23.5 (5 - 50)	29.6 (20 - 35)	35.0 (0 - 75)	1.86 (1.2 - 2.7)

Table 8.3 Intraglomerular Fibrin Deposition on Day 12 in Normal and C6-Deficient Rabbits with NTN.

ANIMAL GROUP	n	MEAN GLOMERULAR PMN COUNTS (Range)	PER CENT INCIDENCE OF EACH GRADE OF EXTRACAPILLARY CELL PROLIFERATION: Mean (Range)				MEAN CRESCENT SCORE
			GRADE 0	GRADE 1	GRADE 2	GRADE 3	
Normal	11	5.8 (1.8 - 11.5)	3.0 (0 - 6)	15.5 (6 - 36)	43.5 (38 - 52)	39.0 (14 - 56)	2.06 (1.5 - 2.4)
C6-deficient	12	3.6 (2.1 - 5.6)	12.0 (0 - 33)	22.7 (12 - 40)	29.3 (12 - 40)	36.0 (12 - 60)	1.89 (1.16 - 2.3)

Table 8.4 Extracapillary Cell Proliferation and Glomerular PMN Infiltration on Day 12 in Normal and C6-Deficient Rabbits with NTN.

ANIMAL GROUP	n	SERUM CREATININE CONCENTRATION mg/100 ml : Mean (Range)					
		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
Control	6	0.9 (0.7-1.0)	3.0 (2.0-4.0)	5.2 (3.7-7.5)	6.1 (3.3-9.1)	6.1 (2.3-9.8)	5.8 (2.4-10.6)
CVF-treated	6	0.8 (0.5-1.0)	1.4*** (0.5-1.0)	3.2 (1.8-4.3)	4.3 (3.8-5.4)	5.1 (3.1-7.5)	5.7 (3.9-7.8)

Table 8.5 Serum Creatinine Concentrations in CVF-Treated and Control Groups of Rabbits in the "Telescoped" Model of NTN.

***$P < 0.01$

delayed by approximately 24 hours ; thus, the mean serum creatinine in the CVF-treated animals was significantly lower on Day 1 than controls (P 0.01) but not subsequently.

(c) Serum C3 Concentrations (Fig. 8.4)

A sustained fall in C3 was seen in all control animals after the injection of NTS. Decomplementation was almost complete in all CVF-treated animals before the injection of the NTG and for all subsequent days until Day 5, when the C3 concentration had risen to a mean of 11.6% (range 8-24%).

(d) Fixation Of Nephrotoxic Antibody In The Kidney

No significant difference ($P > 0.1$) was found in the quantities of antibody fixed per kidney, between control and CVF-treated groups ; mean values were 135 µg (range 115-156 µg) and 146 µg (range 130-168 µg) in control and CVF-treated groups respectively.

(e) Proteinuria (Table 8.6)

In CVF-treated animals, proteinuria was significantly less than in controls on Day 1 ($P < 0.01$) but not subsequently.

(f) Immunofluorescence

(i) Immunoglobulin And C3

Linear deposition of sheep and rabbit immunoglobulin along the GBM was found in all animals. Of the six animals given CVF, C3 could not be detected in the glomeruli in four. Only minor linear GBM deposition of

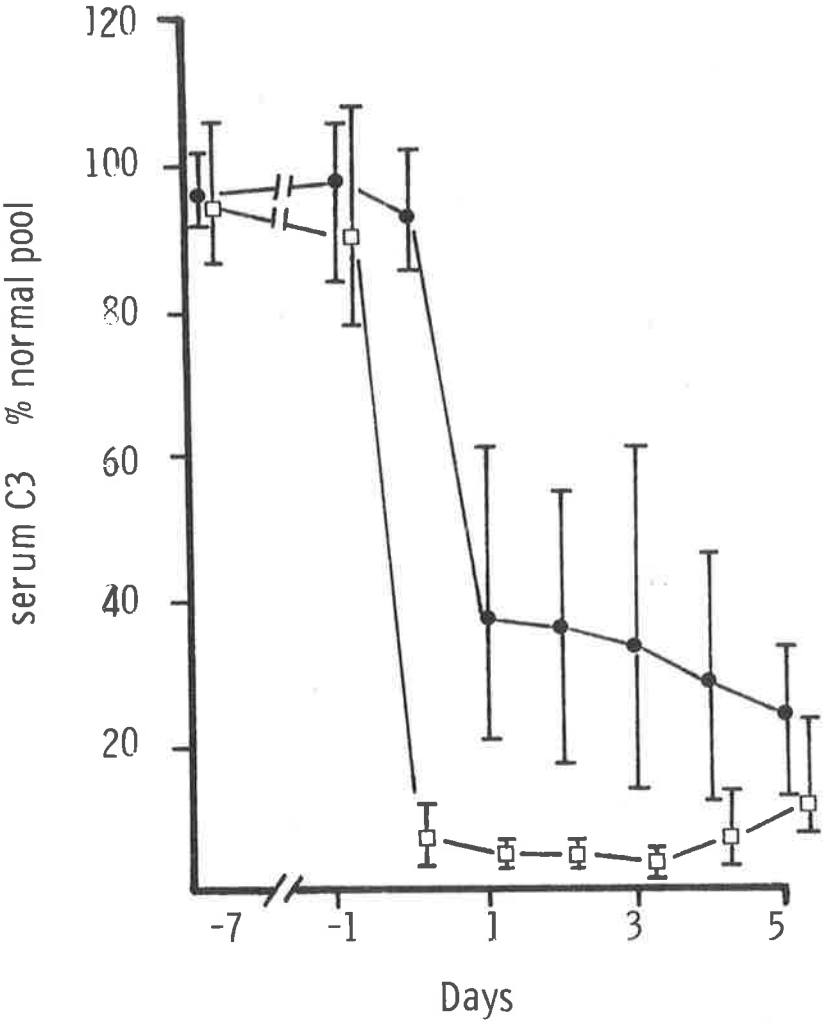

Fig. 8.4 Mean serum C3 concentrations for control (●) and CVF-treated (□) rabbits in the "telescoped" model of NTN (range I).

ANIMAL GROUP	n	URINE PROTEIN CONCENTRATION mg/ml : Mean (Range) DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
Control	6	< 0.3	28.6 (11.3- 40)	9.6 (5.9-18.3)	11.7 (4.1-20.1)	9.0 (3.4-14.6)	10.1 (5.1-16.2)
CVF-treated	6	< 0.3	3.35 *** (1.0 - 5.8)	27.4 (5.6 - 70)	13.8 (7.0 -23.8)	7.5 (6.3 - 9.0)	6.2 (3.9 - 8.5)

Table 8.6 Urine Protein Concentrations in Control and CVF-Treated Rabbits in the "Telescoped" Model of NTN. (*** $P < 0.01$)

of C3 was seen in the remaining two rabbits and in these animals the serum C3 had risen to 20% on Day 5 (Fig. 8.4). Heavy linear fixation of C3 on the GBM was seen in all control animals.

(ii) Fibrin (Table 8.7)

No significant difference in the incidence of each grade of glomerular fibrin deposition or fibrin scores was found between control and CVF-treated groups.

(g) Histology

(i) Crescent Formation (Table 8.8)

There was no significant difference in the incidence of each grade of extracapillary cell proliferation or crescent scores between control and CVF-treated groups.

(ii) Glomerular PMN Infiltration

Glomerular PMN infiltrations were not significantly different between the two groups of rabbits ($P > 0.1$) ; the mean glomerular PMN counts were 3.9 (range 0.9-11-5) and 2.4 (range 1.4-3.2) in control and CVF-treated groups respectively.

3 : CVF GIVEN IMMEDIATELY AFTER NTG

(a) Fixation Of Nephrotoxic Antibody In The Kidneys

The quantities of antibody fixed in the kidneys of the control group were not significantly different from those in the CVF-treated

ANIMAL GROUP	n	PER CENT INCIDENCE OF EACH GRADE OF FIBRIN DEPOSITION : Mean (Range)				MEAN FIBRIN SCORE (Range)
		GRADE 0	GRADE 1	GRADE 2	GRADE 3	
Control	6	10.0 (0 - 20)	20.0 (5 - 50)	35.0 (20 - 50)	35.0 (10 - 45)	1.80 (1.2 - 2.3)
CVF-treated	6	6.7 (0 - 15) P>0.1	30.8 (15 - 45) P>0.1	36.7 (15 - 50) P>0.1	26.0 (15 - 35) P>0.1	1.80 (1.65 - 2.1) P>0.1

Table 8.7 Intraglomerular Fibrin Deposition on Day 5 in CVF-Treated and Control Groups of Rabbits in the "Telescoped" Model of NTN.

ANIMAL GROUP	n	PER CENT INCIDENCE OF EACH GRADE OF EXTRA-CAPILLARY CELL PROLIFERATION : Mean (Range)				MEAN CRESCENT SCORE (Range)
		GRADE 0	GRADE 1	GRADE 2	GRADE 3	
Control	6	29.3 (7 - 73)	23.2 (3 - 32)	23.4 (10 - 36)	24.0 (0 - 46)	1.02 (0.43-2.06)
CVF-treated	6	26.6 (0 - 53) P>0.1	29.4 (20 - 36) P>0.1	24.3 (13 - 33) P>0.1	19.7 (13 - 28) P>0.1	1.17 (0.87-1.63) P>0.1

Table 8.8 Extracapillary Cell Proliferation on Day 12 in CVF-Treated and Control Groups of Rabbits in the "Telescoped" Model of NTN.

group ($P > 0.1$) ; the mean quantities were 191 µg (range 162-250 µg) and 204 µg (range 173-243 µg) in the CVF-treated and control groups respectively.

(b) Proteinuria

The mean 24-hour urine protein excretion prior to the injection of NTG was less than 20 mg in both groups of animals. The mean 24-hour urine protein excretion after the injection of NTG was 36 mg (range 14-73 mg) in the CVF-treated group and 75 mg (range 42-140mg) in the control group. This difference was significant ($P < 0.05$).

(c) Immunofluorescence

Linear deposition of C3 along the GBM was seen in all control animals but not in CVF-treated animals.

SUMMARY OF RESULTS

These experiments concerning the role of complement in the autologous phase of NTN have shown :

(1) In rabbits congenitally deficient in C6, protection was not afforded in the autologous phase of NTN, a finding consistent with previous observations.

(2) When rabbits were depleted of circulating C3 by CVF during the heterologous and autologous phases of the "telescoped" model of NTN, protection from renal damage was seen only in the heterologous phase.

In the autologous phase, proteinuria, glomerular fibrin deposition, crescent formation and renal failure were as severe in complement depleted animals as in non complement depleted animals. That these animals were depleted of complement was shown by the virtual absence of C3 in the circulation and the absence of glomerular C3 fixation in four of the six CVF-treated animals. These results contrast to the protection provided by the removal of circulating PMN described in Chapter 7.

(3) Experiments in which massive intravascular activation of C was induced with CVF at the same time as NTG was fixing to the GBM failed to provide evidence to support the suggestion that this C activation induced by CVF may itself induce renal injury.

Chapter 9

DISCUSSION OF THE EXPERIMENTS ON MEDIATION OF ALLERGIC GLOMERULAR INJURY IN EXPERIMENTAL CRESCENTIC GLOMERULONEPHRITIS

These studies concerning the mediation of glomerular injury in experimental crescentic glomerulonephritis in the rabbit have defined a system of injury in the autologous phase of NTN which is independent of complement but mediated by polymorphonuclear leucocytes and fibrin. They have also shown that fibrin is an important mediator of injury in experimental chronic immune-complex crescentic glomerulonephritis.

I will firstly discuss separately each of these three mediator systems (fibrin, PMN and complement) as regards their involvement in the glomerular injury of experimental crescentic glomerulonephritis and then discuss the interrelationship between these mediator systems.

FIBRIN IN EXPERIMENTAL CRESCENTIC GLOMERULONEPHRITIS

Experiments described in Chapter 6 have shown that defibrination with ancrod in experimental crescentic glomerulonephritis due to either anti-GBM antibody or BSA - anti-BSA immune complexes prevented glomerular fibrin deposition and subsequent extracapillary cell proliferation. However, in both situations proteinuria was unaffected by defibrination. Moreover, other features of the glomerulonephritis, such as endothelial and mesangial cell proliferation and PMN infiltration, were not altered by defibrination. These and other experiments (Naish et al, 1972 : Unpublished data) suggest that fibrin deposition plays no part in the primary allergic events causing capillary damage and

proteinuria but that it is a consequence of capillary damage.

There is strong evidence that the protection by ancrod in these experiments is exclusively due to defibrination : (a) in both NTN and BSA-induced chronic immune-complex glomerulonephritis no difference in in-vivo complement activation - as indicated by a reduction in serum complement - were found between treated and control groups ; (b) in both models of crescentic nephritis, differences in antibody and C3 deposition in the kidney between treated and control animals - as assessed by immunofluorescence - were not apparent ; (c) anti-BSA antibody production - as reflected by the mean daily dose of BSA - was similar in both treated and control animals with BSA-induced chronic glomerulonephritis. Moreover, Naish, Evans and Peters (1975) showed that in NTN, ancrod had no effect on intrarenal fixation of nephrotoxic antibody, complement fixation, or host antibody response to the nephrotoxic antibody. They also found no differences in immune elimination of BSA, circulating immune complexes or intrarenal localisation of immune complexes between defibrinated and control animals with acute serum sickness.

Although defibrination almost completely prevented glomerular fibrin deposition in NTN, only massive doses of heparin provided any reduction in fibrin deposition. Despite prolongation of whole blood clotting time to 2-3 times normal and 4-6 times normal at all times in animals receiving 300 u and 1000 u of heparin/Kg/day respectively, glomerular fibrin deposition was unaltered. Significant reduction in in glomerular fibrin deposition and subsequent crescent formation was

found in rabbits given 2000 u of heparin/Kg/day but this reduction was not as great as that obtained with defibrination. Moreover, this dose of heparin produced virtually unclottable blood (clotting times 15-20 times normal). Why local glomerular fibrin deposition can still occur in the presence of such marked anticoagulation of the blood is not well understood. One possible explanation is that fibrin is being deposited by a mechanism unrelated to coagulation. Supporting this concept are the findings of Hoyer et al (1974) who were unable to demonstrate coagulation factor VIII(antihaemophilic factor, AHG) in the crescents and glomeruli of patients with severe proliferative glomerulonephritis, despite the presence of extensive fibrin deposition. AHG is an essential component of the intrinsic pathway of coagulation and is readily found within the endothelial cells of normal glomerular capillaries, and is also found in conjunction with the glomerular and arterial fibrin deposits that occur in renal diseases associated with intravascular coagulation (eg malignant hypertension, haemolytic-uraemic syndrome, scleroderma and acute homograft rejection). However, other studies, including the demonstrations that collagen and vascular basement membrane activate Hageman factor (FXII) (Wilner, Nossel and LeRoy, 1968; Cochrane et al, 1972) suggest the coagulation sequence may be involved in glomerular fibrin deposition. Another explanation why anticoagulation with heparin is poorly protective is that because heparin is a highly negatively charged molecule it may not penetrate into the mesangium and Bowman's space, the sites at which fibrin deposition is occurring. In normal subjects given heparin, free heparin activity

can only be detected in the urine in those subjects given very large doses of heparin (Douglas, 1962).

It is interesting to note that rabbits congenitally deficient in the sixth component of complement (at least in the Freiburg stain) also have a coagulation abnormality which is manifested by marked prolongation of clotting times to 3-7 times normal (Zimmerman, Arroyave and Müller-Eberhard, 1971). Despite this coagulation defect fibrin deposition is as extensive in C6-deficient rabbits with NTN as in normal rabbit with NTN.

Whatever the mechanism of glomerular fibrin deposition, defibrination with ancrod prevents fibrin deposition because the necessary substrate for fibrin production is absent or markedly reduced.

In other experiments (Naish, Evans and Peters, 1972 : Unpublished observations) sequential renal biopsies in animals given similar doses of NTG have shown that fibrin deposition is a feature of the autologous phase and cannot be detected until Day 5. It is therefore not surprising that no difference was observed between heparinisation starting on Day 1 and heparinisation starting on Day 4, nor is it surprising that Naish et al (1972) found that ancrod provided similar protection when started on Day -1 and Day +5. However, by Day 10, glomerular fibrin deposition is extensive and crescents are developing. I have shown that defibrination from Day 10 still provided some protection from crescent formation and subsequent renal failure, provided the serum creatinine concentration was not greater than 8 mg/100 ml when defibrination was commenced. As might be expected, the protection was not as great as that following defibrination before glomerular fibrin deposition. These results

contrast with those of Kleinerman (1954) and Halpern et al (1965) who were unable to demonstrate protection even when massive doses of heparin were administered in established nephrotoxic nephritis in rabbits, although the same doses of heparin were protective when administered before the induction of disease. This difference may be related to the superiority of ancrod over heparin in preventing glomerular fibrin deposition as discussed in the preceding paragraphs.

The prevention of crescent formation by defibrination is strong evidence that glomerular fibrin deposition, particularly within Bowman's space is the stimulation for extracapillary cell proliferation. This is further supported by the linear relationship found between the degree of glomerular fibrin deposition and the extent of crescent formation (Fig. 6.2).

In the experiments (protocol 3B) designed to examine the disappearance of glomerular deposits of fibrin in NTN it was found that in untreated animals there was a steady reduction in glomerular fibrin deposits so that by Day 24 fibrin became virtually undetectable in all animals. However, in animals defibrinated from Day 10, fibrin was no longer detected in the glomeruli after Day 14, indicating that when further fibrin deposition was prevented the glomerulus was capable of clearing its fibrin deposits rapidly. These observations suggest that the glomerular fibrin-clearing mechanisms still function in severe crescentic nephritis but that their capacity to remove fibrin is saturated.

It is unclear to what extent removal of fibrin from the glomeruli is dependent on local fibrinolysis or phagocytosis. Although renal

vascular endothelium has been demonstrated to have considerable fibrinolytic potential (Holemans, Johnston and Reddick, 1965), for anatomical reasons this endothelial activity may be without effect on the fibrin deposited in Bowman's space. In this connection recent evidence (Kondo, Shigematsu and Kobayashi, 1972; Atkins, Glasgow and Matthews, 1975) suggests that the principal cells constituting crescents are not of epithelial origin but are infiltrating monocytic cells - contrary to earlier views (Heptinstall, 1974). In NTN in rabbits, Kondo et al have identified monocytic clear cells (epithelioid cells) in the glomerular tuft, migrating through ruptures in the GBM and in Bowman's space where they constituted over 50% of the cells of the crescent.

Similar migration of phagocytic cells from the glomerular tuft to Bowman's space has been observed by Atkins et al in cultures of isolated glomeruli from patients with rapidly progressive glomerulonephritis. These cells have been shown to take up colloidal carbon. It has been postulated that such cells accumulate as a phagocytic response to fibrin deposition and produce crescents in the process. Removal of fibrin may not necessarily be just by phagocytosis as there is some evidence that activated but not normal macrophages produce a plasminogen activator (Unkeless, Gordon and Reich, 1974). I am at present investigating the role of macrophages in experimental crescentic glomerulonephritis by using an anti-macrophage serum.

The degree of protection from experimental crescentic glomerulonephritis afforded by defibrination compared to that obtained with

heparin suggests that defibrination with ancrod might be more valuable than anticoagulation with heparin in the treatment of rapidly progressive glomerulonephritis in man. Anticoagulation with either heparin or warfarin, with or without immunosuppressive therapy and antiplatelet drugs, has been widely used in the treatment of the types of human proliferative glomerulonephritis in which glomerular fibrin deposition is found, (Shires et al, 1966; Kincaid-Smith, Saker and Fairley, 1968; Freedman et al, 1970; Kincaid-Smith, Laver and Fairley, 1970; Herdman et al, 1970; Cade et al, 1971; Arieff and Pinggera, 1972; Kincaid-Smith, 1972; Brown et al, 1974). Considerable disagreement still exists as to the value of anticoagulants in proliferative glomerulonephritis. This situation is likely to remain until control trials of anticoagulation have been undertaken. The finding that conventional doses of heparin failed to prevent glomerular fibrin deposition in the severe crescentic nephritis of NTN suggests anticoagulation with heparin may be of little value in human rapidly progressive glomerulonephritis. However, in less aggressive disease (such as progressive focal glomerulonephritis) and in nephritis in which fibrin deposition is mainly within the glomerular tuft (such as membranoproliferative glomerulonephritis) it is possible that anticoagulants in conventional doses may be of benefit. However, experimental models of less aggressive disease in which fibrin deposition occurs have not been well developed. The possibility that anticoagulants in conjunction with immunosuppressive drugs may be more beneficial than anticoagulants alone has not been elucidated in experimental models.

POLYMORPHONUCLEAR LEUCOCYTES IN EXPERIMENTAL CRESCENTIC GLOMERULONEPHRITIS

Experiments described in Chapter 7 have shown that whereas defibrination prevented glomerular fibrin deposition and subsequent crescent formation in the autologous phase of NTN, removal of circulating PMN with a specific APS also substantially prevented proteinuria as well as fibrin deposition and crescent formation. This protection was evident in both the conventional and "telescoped" models of the disease. All evidence suggests that PMN depletion rather than any other mechanism was responsible for the protection : The autologous antibody response, fall in C3 concentration, lymphocyte and platelet counts, renal fixation of nephrotoxic antibody and deposition of autologous antibody and C3 along the GBM were all similar in control and APS-treated groups of animals.

On casual examination the glomerular PMN infiltration in the autologous phase of NTN was not striking and certainly did not compare with the intense accumulation characteristic of the heterologous phase. However, PMN counts were definitely increased in controls compared with the PMN-depleted groups, although the median value was only 2-4 PMN per glomerular section. Furthermore, section counts give a poor impression of the extent of PMN infiltration in a 3-dimensional structure and the section count at death takes no account of destroyed PMN involved at an earlier stage or the possibility that PMN may have contributed to damage without persisting in the glomerulus.

As discussed in Chapter 2, the evidence that PMN mediate glomerular damage in the heterologous phase of NTN is very strong ;

PMN lysosomal enzymes and GBM fragments appear in the urine, and in PMN-depleted animals GBM fragments are not found in the urine and only mild, highly selective proteinuria develops (Hawkins and Cochrane, 1968). It is suggested that in the autologous phase, the process is similar but continues over a much greater period of time. Distortions and breaks in the GBM, often with PMN in close apposition or even protruding through, have been demonstrated in crescentic glomerulonephritis due to anti-GBM antibody, both in the experimental animal and in human Goodpasture's syndrome (Germuth et al, 1972).

The mechanism linking glomerular PMN accumulation and fibrin deposition has not been clarified :

(a) one suggestion , as has been discussed, is that PMN-induced basement membrane damage leads to surface activation of Hageman factor;

(b) another is that PMN are directly responsible for fibrin deposition by releasing procoagulant materials (see Chapter 2) (Rapoport and Hjort, 1967; Kociba, Loeb and Wall, 1972; Niemetz and Fani, 1973). Following exposure to endotoxin, enhanced procoagulant release from PMN occurs if the animal has previously received a "priming" dose of endotoxin (Niemetz, 1972). This may have some bearing on the paucity of fibrin deposition in the heterologous phase of NTN when high glomerular PMN counts are found, whereas fibrin deposition is prominent in the autologous phase in association with less obvious glomerular PMN aggregation. However, the duration of the allergic reaction is probably the major factor responsible for this difference ;

(c) platelet counts were not depressed in association with PMN depletion, although this observation cannot discount a role, such as a PMN-dependent platelet-release reaction, for the platelet in fibrin deposition.

It would have been desirable to add further evidence for the role of PMN by undertaking reconstitution experiments, such as have been carried out in the heterologous phase of NTN (Henson, 1972). However, this is impossible when PMN depletion is induced by APS.

Thus it would seem that the principal injurious agent in the crescentic glomerulonephritis of NTN is the PMN and that glomerular fibrin deposition, which also induces injury by stimulating crescent formation, is a consequence of the PMN mediated damage to the GBM or the PMN release of procoagulants. Unfortunately it was impossible to investigate the role of the PMN in the crescentic glomerulonephritis of BSA-induced chronic immune-complex disease because of the considerably greater time required for the induction of this disease. Depletion of PMN with APS for longer than a week would have resulted in deaths from anaphylaxis due to production of antibody to the APS and from infection. However, as PMN infiltration of the glomeruli is even more conspicuous in chronic BSA glomerulonephritis than in NTN, it seems likely that the PMN is also the major mediator of injury in this disease.

The frequent occurrence of glomerular PMN infiltration in various types of human crescentic glomerulonephritis and the reported findings of breaks in the GBM in these diseases suggests that the PMN may also

be an important mediator of the glomerular injury of human crescentic glomerulonephritis. Although it is obviously impracticable to deplete circulating PMN in man for any length of time, interference with release of PMN lysosomes or inhibition of lysosomal enzyme action by pharmacological agents may well be of therapeutic value in human glomerulonephritis.

COMPLEMENT IN EXPERIMENTAL CRESCENTIC GLOMERULONEPHRITIS

In the first series of experiments described in Chapter 8, glomerular fibrin deposition and crescent formation in rabbits congenitally deficient in C6 with NTN were as severe as in normal rabbits with NTN. These observations in the Cambridge strain of C6-deficient rabbits confirm those in the Freiburg strain reported by Rother et al (1967). These results are not surprising when one considers that the important biologically active fragments of complement (particularly chemotactic factors, anaphylatoxin and fragments mediating immune adherence) are produced before C6 is activated. C3 activation in NTN as assessed by the fall in serum C3 concentration and C3 fixation to the GBM was similar in C6-deficient and normal animals.

In experiments in which rabbits with the "telescoped" model of NTN were depleted of circulating C3 by CVF, protection was seen only in the heterologous phase. Glomerular fibrin deposition, crescent formation and renal failure were as severe in complement depleted animals as in control, non-complement depleted animals. The injury occurred in the absence of C3 fixation in the kidney in four of the six

CVF-treated rabbits. That this injury was nevertheless largely PMN-dependent has been established in the same "telescoped" model (Chapter 7 ; protocol 2).

The possibility that CVF may itself cause glomerular damage was suggested by preliminary experiments in the conventional model of NTN in which animals depleted of complement by CVF suffered significantly worse renal injury than non-decomplemented animals. However, as has been shown in protocol 3 of Chapter 8, CVF when given immediately after NTS did not augment heterologous phase proteinuria in rabbits given a dose of NTS just sufficient to induce proteinuria, despite the massive complement activation due to CVF that was occurring at the time of NTAb deposition in the kidney. Moreover, CVF administration did not augment NTAb fixation in the kidney (as might for example have occurred as a result of exposure of antigenic determinants by vasoactive effects of complement activation). There is little or no evidence that CVF augments renal damage by promoting intravascular coagulation : CVF has been shown to not affect platelet counts or fibrinogen turnover in rabbits with NTN, nor does CVF induce glomerular fibrin deposition when administered to normal rabbits (Thomson et al, 1976). Bergstein and Michael (1973) have shown that glomerular fibrinolytic activity is not reduced in normal rabbits given CVF. In rabbits and rats, CVF is known to cause transient leucopoenia followed by a sustained polymorphonuclear leucocytosis (McCall et al, 1974). Since it has been claimed that leucocytosis enhances injury caused by antibodies to GBM (Goubeaud et al, 1974), a CVF-induced leucocytosis may have augmented

injury by this mechanism. However, there is no experimental data bearing directly on this suggestion.

The mechanism by which antibody fixation induces PMN-mediated injury remains uncertain. Since PMN have receptors for the Fc piece of the immunoglobulin molecule (Henson, 1969 b; Messner and Jelinek, 1970), it is conceivable that no other chemotactic or immune-adherence factors are required, although a role for C4 has not been excluded by my experiments. At first sight it does not appear likely that a sufficient gradient for chemotactic influences to have an important effect could ever be established in a structure as hyperaemic as the glomerulus, and it may be that adherence reactions with passing PMN are along sufficient to account for PMN infiltration.

The failure to detect complement in glomeruli in 25% of cases of Goodpasture's syndrome in man (Wilson and Dixon, 1973; Sissons et al, 1974) suggests that the mechanism of glomerular injury in Goodpasture's syndrome may also be complement-independent but PMN-dependent.

SUMMARY OF THE MECHANISM OF GLOMERULAR INJURY IN EXPERIMENTAL CRESCENTIC GLOMERULONEPHRITIS (Fig. 9.1)

The findings of the experiments described in Chapters 6, 7 and 8 and those of other workers strongly suggest that the following sequence of events leads to the crescentic nephritis of NTN.

1 : Following the reaction of autologous antibody with heterologous anti-GBM antibody fixed to the GBM, PMN infiltration of the glomerulus occurs. The attraction of PMN to the immune reactants in the glomeruli

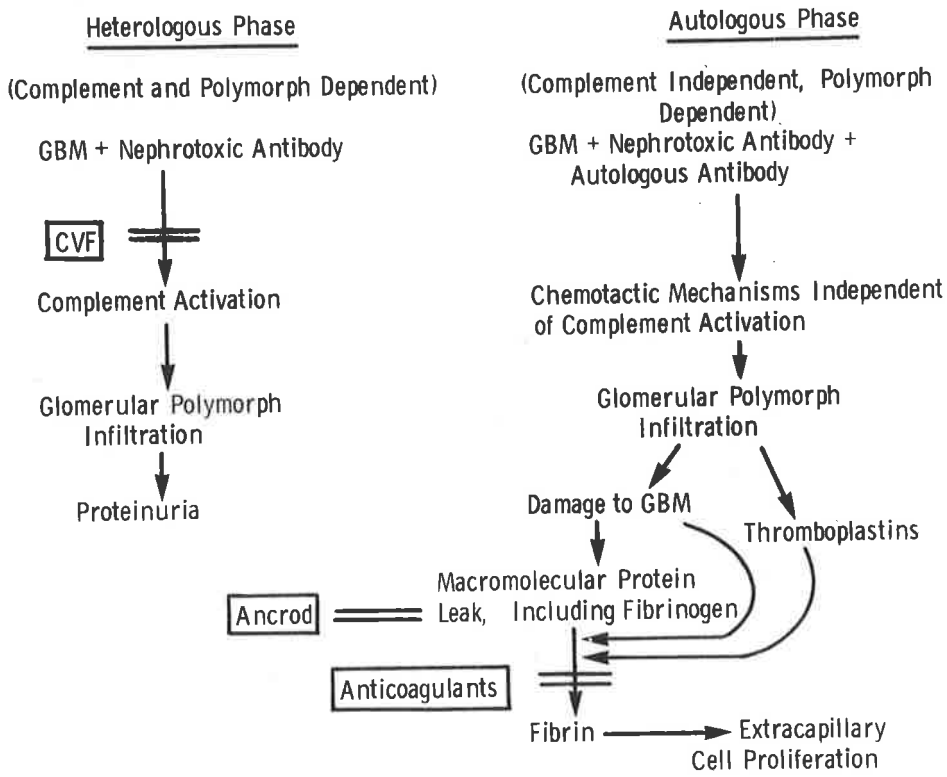

Fig. 9.1 The mechanisms of mediation of glomerular injury in the heterologous and autologous phases of NTN in the rabbit.

appears not to depend on complement activation and may be simply an adherence reaction between the Fc receptor on PMN and the fixed antibody.

2 : PMN, once attracted to the immune reactants along the capillary walls, are activated by the immune reactants and release various injurious agents including lysosomal enzymes. These enzymes damage the GBM such that distortions and even breaks in the GBM develop, heavy proteinuria resulting. PMN also induce glomerular fibrin deposition, particularly within Bowman's space. Whether PMN lead to fibrin deposition because of contact-activation of coagulation by exposure of damaged basement membrane or by release of procoagulant substances is not known. If circulating PMN are depleted by anti-polymorph serum, proteinuria and glomerular fibrin deposition do not develop. Defibrination of the circulating blood or anticoagulation with enormous doses of heparin reduces fibrin deposition but proteinuria is unaffected.

3 : The deposits of fibrin in the glomeruli (particularly those in Bowman's space) induce crescent formation. Whether crescents are simply glomerular epithelial cells proliferating in response to fibrin deposition or are largely composed of infiltrating phagocytic cells whose role is to remove the fibrin is not known.

4 : Crescent formation,and possibly also fibrin deposition in the glomerulus, impedes glomerular filtration and renal failure results.

In experimental crescentic glomerulonephritis due to BSA-induced

chronic immune-complex disease the importance of glomerular fibrin deposition in the induction of crescent formation has also been established. However, in this model it has not yet been proven that PMN are the major mediator of glomerular injury.

It is hoped that this definition of the role of complement, PMN and fibrin in experimental crescentic glomerulonephritis will lead to more specific methods of treating human glomerulonephritis.

ABBREVIATIONS USED

APG	-	anti-polymorphonuclear leucocyte globulin
APS	-	anti-polymorphonuclear leucocyte antiserum
BSA	-	bovine serum albumen
C, C3, C6	-	complement, third component of C, sixth component of C
CFA	-	Freund's complete adjuvant
CVF	-	cobra venom factor
EDTA	-	ethylenediaminetetraacetate disodium
FITC	-	fluoroisothiocyanate
GBM	-	glomerular basement membrane
IEP	-	immunoelectrophoresis
IFA	-	incomplete Freund's adjuvant
NTAb	-	nephrotoxic antibody
NTG	-	nephrotoxic globulin
NTN	-	nephrotoxic nephritis
NTS	-	nephrotoxic serum
PBS	-	phosphate buffered saline
PMN	-	polymorphonuclear leucocyte
RSS	-	rabbit serum saline
TBM	-	tubular basement membrane
TCA	-	trichloroacetic acid

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