M.Sc. M.A.J. MANDL EXPERIMENTAL MEDICINE

AUTOLOGOUS IMMUNE-COMPLEX GLOMERULONEPHRITIS

ABSTRACT

Lewis rats were made nephrotic by intraperitoneal injections of xenogeneic (rabbit) kidney in complete Freund's adjuvant. Disease onset was accentuated by prior splenectomy. Deposition of isogeneic and xenogeneic antigen (FIA) host gamma globulin and complement were demonstrated along the glomerular basement membrane by immunofluorescence techniques. Ultrastructural examination demonstrated deposits beneath the glomerular epithelial cells. An antigen was demonstrated in the serum and urine of nephrotic animals. This antigen exibited immunologic identity with host tubular antigen. Arthus and delayed skin reactions were utilized to demonstrate the active role of the humoral system, in contrast to the lack of specific delayed (cellular) mechanisms. This latter observation was confirmed by the lack of specific monolayer destruction by isogeneic nephrotic lymphocytes in vitro, and by the positive cell transfer of sensitized lymphocytes into isogeneic kidneys.

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science.

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ACKNOWLEDGEMENTS

I wish to extend my appreciation and thanks to Doctor J. I. Watson for his continued helpful advice, assistance and constructive criticism during the period of the experimental work and the preparation of this thesis.

I wish also to thank Dr. Bram Rose for his continued interest in the experimental work.

I wish to express my sincere appreciation and thanks to Miss Annie Katona and Mrs. Gail Deutsch for their unceasing technical assistance and encouragement. My thanks to Miss Deborah Anslow for typing this thesis.

Finally, to my wife Nerida for her usual patience and understanding demonstrated throughout this work.

This work was supported by a grant from the Medical Research Council of Canada.

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I. INTRODUCTION

A. BASIC CONCEPTS AND DEFINITIONS

While studying the secondary immune response in dogs to an extract of sea anemone, Porter and Richet in 1902 realized there was a detrimental aspect to the immune response. They noted many animals died within minutes after exposure to antigen. This phenomenon was later called anaphylaxis (ana - against; phylaxis - protection).

Von Pirquet in 1911 coined the term allergy (altered action) for any altered response to a substance due to previous exposure to it. It was then believed that increased resistance (immunity) and increased susceptibility (hypersensitivity) were two forms of allergic response. However, over the years the two terms became accepted to mean an altered state, induced by an antigen, in which subsequent pathological reactions, when they occur, are induced by challenge with the same or similar antigens.

The allergic or hypersensitivity response has been divided into three classes. This separation is predicated by the time interval between secondary antigenic challenge and the host response. Those reactions which occur within minutes are called immediate, those within hours are called Arthus reactions, and those which occur within days are called delayed. Immediate and Arthus reactions are mediated by serum antibodies, and this type of hypersensitivity can be transferred to normal recipients with serum. The delayed type reactions are mediated by cellular elements, usually by sensitized lymphocytes, and, in certain circumstances, by macrophages. In this latter type, the reactivity cannot be transferred with serum but can be with sensitized cells or cell wall fragments. The exact nature of the factors responsible for the delayed reactions has not yet been elucidated.

The broad classification of humoral type hypersensitivity reactions encompasses three immunological responses which are germane to the work which follows.

These are anaphylaxis, the Arthus reaction and serum sickness.

1. Anaphylaxis - Anaphylaxis occurs when antigen-antibody complexes combine, causing the release of vasoactive substances which in turn have end organ effects.

a. Generalized - Generalized active anaphylaxis occurs when antigen is given intravenously to a previously sensitized animal. The characteristic features of the anaphylactic reaction depend on which particular animal species is involved. The manifestations of anaphylaxis are due to increased capillary permeability and smooth muscle constriction. In the dog, the liver is the main target organ, in the rabbit it is the smooth muscle of the pulmonary blood vessels and in the guinea pig, the smooth muscle of the respiratory airway. The general feature of anaphylaxis over all species encompasses restlessness, sweating, hypotension, constriction, shock and death.

Generalized passive anaphylaxis occurs when antibodies are injected intravenously into a non-sensitized animal followed by intravenous challenge with the appropriate antigen twenty-four to forty-eight hours later.

b. Cutaneous - As with generalized anaphylaxis, the cutaneous reaction can be either active or passive. Active cutaneous anaphylaxis occurs when an immunized animal is challenged intradermally with the appropriate antigen. Passive cutaneous anaphylaxis occurs when antibody is first injected into the skin, and then the animal is challenged after a latent period intravenously, usually with a dye as a marker. Prompt discoloration occurs at the site of the antibody injection.

The property of fixation to elements of the skin has been attributed to "cytotropic antibodies." Two different types of "cytotropic antibodies" are known in most species, homocytotropic and heterocytotropic antibodies. These are responsible for INTRA and INTER

species transfer respectively. In species other than man, homocytotropic antibodies are localized to the fast and slow gamma globulins. In man it appears that homocytotropic antibody belongs to the gamma-E immunoglobulin class. It has been shown that homocytotropic antibody does not require complement for in vitro or in vivo action. Heterocytotropic antibodies do bind complement in passive cutaneous anaphylaxis, the skin fixation being a characteristic of the Fc portion of the heavy chain of Y globulin. This subject will be enlarged upon later in the manuscript.

The efferent arc of anaphylaxis, namely the release of vasoactive substances, can be inhibited by specific antagonists, antihistamines and antiserotonic agents. Platelet and mast cell depletion will also suppress the reaction. This is not totally unexpected as both these tissues are rich in histamine and serotonin.

2. The Arthus Reaction - In 1903, Maurice Arthus described a skin reaction in rabbits which were being immunized subcutaneously with horse serum. After three or four weekly injections, a local reaction developed two to three hours after injection. The reaction evolved slowly, persisted for hours to days, often became necrotic, occasionally sloughed and healed by fibrosis. This reaction is referred to as the Arthus Reaction and is now known to be antibody mediated. An Arthus reaction may be active or passive. An active Arthus reaction occurs in an immunized animal when challenged with antigen. A passive Arthus reaction is induced by injecting antibody intravenously into a non-sensitized animal, followed by the subcutaneous injection of antigen. A Reversed Passive Arthus reaction is induced when the antibody is injected subcutaneously into the recipient, and the antigen intravenously or into the same site at the same time.

Although the anaphylactic and Arthus reactions are both dependent on antibodies, there are several

important differences between them:

a. Type of Antibody - In anaphylaxis either precipitating or non-precipitating antibody is effective. In the Arthus reaction, precipitating antibody is mandatory.

b. Complement Dependence - In anaphylaxis homocytotropic antibody does not bind and activate complement. By contrast, complement binding and activation is essential to the pathophysiology of the Arthus reaction.

c. Efferent Arc - Anaphylaxis is intimately dependent upon the release of vasoactive amines, whereas, in the Arthus reaction, vasoactive amines are released later in the reaction. Accordingly, antihistamines will inhibit some manifestations of anaphylaxis but will not affect Arthus reaction.

d. Time Course - The onset and evolution of anaphylaxis is usually rapid. The cutaneous mani-

festation of the Arthus is delayed for approximately two hours, and the reaction can persist for 18-24 hours.

3. Serum Sickness - The term serum sickness was first used to describe a syndrome occurring in patients treated with large volumes of either horse or rabbit serum. The illness occurred seven to fourteen days after the initial injection and was usually characterized by fever, arthralgia, nephritis, splenomegaly, lymphadenopathy and urticarial lesions.

The pathophysiology of serum sickness has been extensively investigated and more than one pathogenetic mechanism appears to be operative. The mechanism is apparently dependent on the deposition of soluble antigen-antibody complexes in moderate antigen excess. These complexes are capable of activating complement. In addition they are responsible for release of vasoactive substances from mast cells and platelets. The syndrome thus appears

to have a dual mechanism, i.e., complex deposition and vasoactive amine release. It could be considered, therefore, as a combination of the Arthus reaction and the anaphylactic response.

4. Delayed Hypersensitivity - Delayed hypersensitivity reactions differ from the three previous immunologic mechanisms in that this type of reaction is cell mediated. The time sequence of the cutaneous reaction is longer than those previously described, taking twenty-four to forty-eight hours to reach its peak. To transfer delayed hypersensitivity in animals one requires living cells, predominantly lymphocytes; however macrophages have also been implicated. In the human however, delayed hypersensitivity can be transferred by sensitized non-living cells, cell fragments and small molecular-weight poly-peptides. The recent work of Lawrence and Baram et al have shown that both dialysable and non-dialysable extract of sensitized lymphocytes are capable of transferring delayed hypersensitivity and the

dialysable fraction has a molecular weight of approximately 10,000. The efferent arc of delayed hypersensitivity appears to center around stimulation of a sensitized cell. This cell then "liberates" or makes available a small molecular weight polypeptide which alters or reacts with macrophages. Macrophages may then cause tissue injury by the release of lysosomal enzymes. What sensitizes the cells primarily still remains to be clarified. We know that either contact or infection will sensitize; however, what takes place at the cellular level is still not clear.

The histopathology of delayed hypersensitivity is characterized by an infiltrate of mono-nuclear cells with a few polymorphonuclear leucocytes. This contrasts significantly to the cellular morphology of an Arthus reaction as discussed previously. Moreover, one finds very little complement and gamma globulin in tissue sections taken from delayed hypersensitivity reactions. Tissue damage occurs probably through

release of lysosomal enzymes contained in macrophages; however, the pathogenesis of tissue injury in delayed hypersensitivity has yet not been fully clarified.

II. HISTORICAL REVIEW

A. INTRODUCTION

There are two main categories of experimental glomerulonephritis. The first includes those diseases initiated by specific anti-kidney antibodies, while the second encompasses nephritidies produced by soluble, circulating antigen-antibody complexes. In this latter category, the antigens can be of either non-renal or renal origin. This thesis is concerned with the nephritis produced in rats by allogeneic or xenogeneic renal antigens, that is, a model belonging in the second pathogenetic class. The model is referred to here as autologous immune complex nephritis (AIC NEPHRITIS). The significant sequela in each of the diseases caused by complexes is the damage or disruption of basement membrane. The localization of complexes along the basement membrane is influenced by several factors. After localization, those pathways leading up to the final sequela appear to be

the same in each of the models. Accordingly, these factors and pathways will be reviewed prior to discussing individual disorders.

B. FACTORS INFLUENCING BASEMENT MEMBRANE DAMAGE

1. Complex Size - In classic complex-induced glomerulonephritis, the pathogenetic mechanisms of basement membrane damage are now more clearly delineated. The deposition of antigen-antibody complexes in tissue occurs as the primary event. This deposition is dependent on both the physical characteristics of the complex and the release of various vasoactive amines as a secondary event. McCluskey studied in detail the influence of complex characteristics on the induction of disease (1, 2, 3). In moderate antigen excess these complexes caused the pathologic changes of serum sickness nephritis. Similar changes had been noted by Germuth and later by Dixon in actively immunized animals (4, 5). Only complexes produced in

moderate antigen excess (20 x equivalence or less) would deposit, whereas complexes produced in great antigen excess (100 x) failed to localize. The equivalence zone of an antigen-antibody reaction is the point of maximal precipitation, where neither antigen nor antibody can be detected in the supernatant. The specific factor responsible for the localization of the 20 x type of complex appeared to be a rapidly sedimenting component in this group which was absent in the 100 x group. With respect to size, it seemed that a molecular weight of at least 1×10^6 with a sedimentation coefficient of 19 or more was necessary for localization. Cochrane felt that some of these small complexes (100 x equivalence) are still able to bind complement, but that they do not have any increased ability to localize by the addition of bound complement. The inability of some very small complexes (those prepared in 100 x equivalence) to bind complement has been discussed by Ishizaka (6).

2. Vasoactive Amines - The vasoactive amines that are released by antigen-antibody interaction are also important pathogenetic vectors. These amines can produce anaphylaxis in guinea pigs (7) and mice (8), contraction of isolated guinea pig smooth muscle (9) and inflammatory changes within the skin (7). These actions are all dose dependent. Only recently, it has been shown that pathologic changes occur only after the circulating complexes accumulate in vascular structures (10). Therefore, in any damage occurring to basement membranes, the permeability of the vessel wall must be increased initially to allow the macromolecular complex to gain entry. For this reason, although the smaller complexes do not localize, they nevertheless are able to increase vascular permeability and therefore may play an important role in the deposition of the larger complexes (11). Once the latter are deposited,

often in non-phagocytosable areas in vessel walls, the complexes cause continuing damage in a passive manner.

Knicker and Cochrane (10) have shown that by inhibiting the release of vasoactive substances, one can prevent deposition of complexes and the development of lesions. Thus when the circulating platelet level was depressed, rabbits were protected from developing vascular lesions in a serum sickness model, although they still responded to tissue fixed reactants as seen in the Arthus and Nephrotoxic antibody reactions. Depletion of platelets did not inhibit the deposition of 7S gamma globulins, but inhibited the deposition of macromolecular complexes. The most potent amine stores in various experimental animals have been shown to be the mast cells and platelets (12, 13, 14). In the rabbit, immune complexes cause clumping (14, 15, 16) and lysis of

platelets (16, 17) and vasoactive amines are released in their presence (13, 14, 18). It therefore seems possible that in serum sickness-like reactions, the complexes circulate, cause clumping of platelets and release of vasoactive amines, probably both histamine and serotonin. These in turn cause macromolecular complex trapping within vessel walls with the resultant damage. It has been stated that platelet clumping alone will cause similar lesions to those seen in serum sickness (19, 20). However with complexes, adequate platelets, and no inhibition of platelet clumping, tissue damage was prevented by vasoactive amine antagonists alone (21).

3. Hydrodynamic Forces - In 1957 Germuth et al (22) observed that glomeruli at the cortico-medullary junction appeared to bear the brunt of any antigen-antibody complex injury. It was also noted that in chronic glomerulonephritis, areas of localized internal hydronephrosis occurred with sparing of the

associated glomeruli, possibly resulting from plugging due to proteineaous casts. It was felt that the greater penetration and accumulation of complexes as a consequence of the higher intraglomerular hydrostatic pressure in this area.

Germuth further reported that after decreasing the filtration pressure by either ureteric or arterial obstruction, glomerular sparing did occur (23). Knicker et al (10), however, in a slightly different model, found that complex deposition occurred above and below an aortic coarctation. They felt that turbulence per se was responsible for the release of permeability factors and facilitated subsequent complex deposition. By decreasing platelet numbers of by treatment with vasoactive amine antagonists the incidence of complex deposition distal to the coarctation was decreased. The role that shearing forces play at points of high pressure, in contrast to the above in the induction of initial damage and complex deposition still needs to be evaluated.

4. Complement - In the Arthus reaction, experimental serum sickness, nephrotoxic nephritis, and the various antigen-antibody induced nephritidies the interaction of antigen and antibody initiates a series of steps which appear to have final common pathways. The first is the activation of a cascade of interactions in the complement system.

The term "complement" refers to a series of serum protein components (11 at the moment) which when activated have the capacity of causing tissue damage either directly or indirectly (24). These various components may be activated by the combination of antigen and antibody, providing that the antibody is capable of binding the first component (25-27). The binding of C1 by antibody also causes the activation of this component and this initiates a series of quite well understood molecular reactions which involve the uptake and activation of the remaining complement components in the order C4, 2, 3, 5, 6, 7, 8, 9. It has now been shown that complement activation is man-

datory in the pathogenesis of the Arthus reaction (28), a reaction with specific connotations to cutaneous reactions (29), but it has also become apparent that similar mechanisms apply in other anatomic locations, including the arteritis and nephritis of serum sickness (30). Ward et al (28) have shown that tissue damage occurs only when complement is bound, in particular the $C^{1}567$ complex, the major chemotactic agent (30). The role of complement has been questioned by some authors (31). Keller et al found a poor correlation between levels of serum complement and the Arthus reaction using complement depleting agents. In addition, he found no consistent relationship between complement fraction and the release of chemotactic factors (32).

The consensus of opinion however, is that complement activation is essential. Moreover, the complement activation and polymorphonuclear cell (PMN) migration appear closely inter-dependent. Thus if animals were made complement deficient, but adequate

white counts maintained, Ag-Ab complexes did not result in tissue damage. Conversely animals rendered leucopenic, but complement sufficient, did not manifest tissue damage either. These observations indicate that both complement activation and PMN activity are necessary for damage to occur (29). It now appears that complement activation leads to the production of chemotactic factors (primarily the macromolecular complex of activated components C5, 6 and 7). In the absence of this, polymorphonuclear leucocytes (PMN's), which are essential for the immunopathologic sequelae, will not enter the area.

4. Polymorphonuclear Leucocytes and Enzymes - As just discussed, the activation and release of chemotactic factors will attract polymorphonuclear leucocytes (PMN) to the site of complex deposition (33, 34, 35). Polymprphonuclear leucocytes are

essential for subsequent damage to occur (1), and their actual role in membrane destruction has recently been clarified. It has been shown that in immunologic inflammation the ability of carbon particles to pass through vessel walls is dependent on the presence of PMN's (36-38). Moreover, the disruption of the arterial internal elastic lamina in experimental serum sickness is also dependent on the presence of PMN's (33).

It appears that PMN's function in large part by the release of various enzymes. Evidence from in vitro work indicates that basement membranes are profoundly affected by cathepsins and cationic proteins together with other minor proteases released from PMN's (36, 39). When isolated glomerular basement membrane (GBM) and fractions of PMN are incubated together, the protein structure of the GBM is broken down to release four distinct bands as seen on cellulose acetate electrophoresis. These four bands fuse

and form a single line of identity with the whole GBM when run against anti-GBM antiserum in agar gell (36). Thus, PMN's (or contained enzymes) appear able to cause GBM destruction. Moreover, Movat et al feel that the same cathepsins are responsible for the in vitro catabolism of immune complexes (40), and could therefore play a role in removing complexes from these damaged sites (41). When isolated, the cathepsins D and E were active in digesting basement membranes in vitro at Ph 3.4 and 2.4 but inactive at Ph 7.0, and were also inactivated by heat. This apparently differs from the enzyme that Hayashi et al have isolated from 12-24 hour Arthus reactions. This enzyme was unrelated to any found in PMN's (42). What relevance the in vitro Ph activity has to the in vivo situation remains to be clarified (42).

In addition to the proteases, several basic polypeptides have been identified that bring about increase in vascular permeability. Part of their

activity is mediated through histamine release from mast cells, but their entire mechanism of action is incompletely understood (43).

Two groups of basic peptides have been isolated. One of M.W. 9,600 increases vascular permeability without affecting mast cells or releasing histamine. This peptide has been identified in the urine during polymorphonuclear leucocyte mediated glomerulonephritis. The second group of peptides of lower molecular weight, (approximately 1500-5000) contain both histamine dependent and histamine independent permeability producing factors (43-47).

It appears then that protease and cationic proteins are responsible for the breakdown of various tissues, including the internal elastic lamina in arteries and the vascular basement membranes in vasculitis.

C. EXPERIMENTAL GLOMERULONEPHRITIS

Experimentally induced glomerulonephritis may be divided into two major immunopathologic classes. The first is Nephrotoxic Nephritis and the second class is made up of the various antigen-antibody complex induced nephritidies, of which experimental allergic glomerulonephritis induced by Aubular antigen is an example. It is this latter model which will be considered in some detail subsequently. However, since nephrotoxid nephritis has a distinctly different immunopathogenesis it will be discussed initially in order to clearly differentiate it from the antigen-antibody complex induced diseases.

1. Nephrotoxic Nephritis: (N.T.N.)

a. Definition - Classic nephrotoxic nephritis develops when antiserum produced against heterologous glomerular basement membrane is injected back into the donor species. If the nephrotoxic serum is

sufficiently potent, a progressive glomerulonephritis develops.

It is now recognized that NTN is a biphasic disease, comprised of a heterologous phase and an autologous phase. The heterologous, or initial phase results from the kidney-fixation of the injected nephrotoxic serum, and is responsible for the immediate proteinuria seen in the recipient animal. The autologous, or secondary, phase is due to the production of host antibodies to the injected serum and their interaction with the kidney-fixed fraction (i.e., the immunoglobulin) of this serum. This phase is responsible for the persistance and progression of the disease.

b. Background - The initial experiments of NTN were performed in the laboratory of Metchnikoff by Linderman in 1900. He produced disease by injecting rabbits with the serum from guinea pigs immunized

with rabbit kidney (48). From 1900 till around 1957 much work was done attempting to elucidate the pathogenetic mechanisms of this nephritis. The first major contribution was that of Pearce, who reported that the disease was produced with renal cortex, (i.e., glomerular containing tissue) (49). Wilson and Oliver showed that it was the glomerulus which was specifically damaged in this disease (50). Masugi made the next significant contribution to our knowledge in the early 1930's. He showed that a single injection of specific antiserum, if of adequate nephrotoxic potency, produced both an immediate and a persistant disease when given to rats or rabbits (51, 52). He considered the clinical and histopathological course of this experimental disease to be similar to human glomerulonephritis. Moreover, he hypothesized that the pathogenesis of nephritis in man depended upon an antigen-antibody reaction, and noted that Veil and Bucholz in 1932 had noted a drop

in serum complement in patients with acute glomerulonephritis (53).

Smadel added further information in the late thirties and early forties. He showed that different strains of rats have different responses to the antiserum. He also demonstrated that prior intravenous injection of saline extract of ground rat kidneys protected rats from the nephritogenic action of antirenal sera. Finally, he confirmed Pearce's earlier work that the antigen responsible for stimulating production of nephrotoxic antibody was present in the renal cortex (54-56).

Kay in the early 1940's and Pressman in the 1950's were able to differentiate the biphasic nature of the model. Kay felt that the heterologous antiserum fixed to the kidney but did not cause injury (57). He postulated that the injury was caused by the host response to the heterologous kidney-fixed protein, that is, an autologous response alone, a view shown later to be incorrect. Pressman utilized

radioiodinated nephrotoxic serum and established its primary interaction with and persistence on glomerular tissue (58). Earlier investigators such as Masugi and Kay, later Simonson (59) and Lange (60) had failed to establish the role of the heterologous serum because of a unique feature of the particular antiserum duck they used. It has been subsequently shown that in relation to rabbit antiserum, duck antiserum is approximately one third as effective (61, 62). Moreover, not only does the amount of nephrotoxic antibody determine the occurrence of the heterologous phase and its severity, but also its time of onset. With large doses of antibody the disease begins immediately; with lesser amounts a day or two later, and with still less, the disease may begin up to three days later, but still begins before the autologous phase is initiated (2).

c. Immunology of the Heterologous and Autologous Phases

1) Heterologous Phase

a) Antigen - One of the major difficulties which arose in the elucidation of the immunologic mechanisms of the heterologous phase of NTN was its apparent tissue non-specificity. Nephrotoxic serum could be produced with a variety of tissue antigens. These include medulla (63), lung (63, 64), muscle (64, 65) and placenta (64, 66, 67). This apparent discrepancy has been slowly clarified. Basement membranes throughout the body have been shown to have cross-reactivity, the components having common cell origins (68). This cross-reactivity has been confirmed by several investigators. It was this complex antigenicity of the GBM that led to much of the earlier confusion. By cross absorption studies (64, 69) and immunofluorescent procedures (70-72), it has now been shown that the nephrotoxic antigen exists in the greatest concentration in the glomerular basement membrane and in less

concentration in lung, placenta and muscle. Moreover, nephrotoxic antigens are not species-specific and nephritis has been induced in mice by anti-rat nephrotoxic antibodies (64, 73) and in rabbits by injections of antidog kidney antibodies (59) and anti-human antibodies (74). Moreover, animals in which the antiserum is being produced themselves developed nephritis secondarily to the antigen-antibody complexes they produced.

The nephrotoxic antigen also has certain common antigenic determinants with collagen. Antisera to canine tendon will agglutinate GBM (69). Rabbit anti-rat collagen antiserum has complement fixing antibodies to rat kidney <u>in vitro</u> and fixed <u>in vivo</u> to GBM (75). These antisera, although they fixed to glomeruli <u>in vivo</u>, did not induce nephritis unless the animals had been previously sensitized with complete Freund's adjuvant (76). In this situation the nephritis developed late, apparently representing only

the autologous phase. Immediate nephritis possibly did not occur because of the very small amount of antigen present in the glomerular basement membrane which could combine with the anticollagen antibody. The hypothesis is that with complete Freund's adjuvant sensitization, more sites were made available on GBM with which the antibodies could combine, leading to earlier nephritis.

The GBM antigen is poorly soluble in water and salt solutions, resists heating at 60°C for 30 minutes but is partially denatured at 100°C for 30 minutes (77). Tryptic digestion, ultrasonic disintegration, or allowing the membrane to stand for several days, will solubilize the material. Two groups of renal antigens were noted following tryptic digestion. One was soluble and could neutralize 75% of <u>in vivo</u> fixing antibodies. The second was insoluble, and could react with the remaining antibodies (78, 79).

b) Nephrotoxic Antibody Specificity - The nephrotoxic antibodies, predominantly of 7S gamma-2 variety, are markedly heterogeneous with regard to organ specificity (79, 80). By labelling the antibody with either I^{131} or fluoresceine isothiocyanate, it is possible to determine the in vivo localization of nephrotoxic antibody in non-renal sites by autoradiography or fluorescence microscopy respectively. In addition, by the former method, the quantitative aspects of this binding can be assessed. With these methods it was found that nephrotoxic antibody (NTAb) fixed predominantly in the kidney and to a lesser extent in other tissues (notably the lung and liver). In addition, the anti-GBM antibody appeared to be more firmly bound to kidney. Following an injection of NTAb, the kidney fixed fraction formed a larger percentage of total tissue fixed antibody when the interval between the injection and the assay was increased. Blau studied

the fate of labelled NTAb in normal and bilaterally nephrectomized animals, and found that in nephrectomized animals 50% of injected NTAb remained in the circulation. He concluded that this portion represented specific anti-renal antibodies (81).

Unanue injected rats with NTAb and then transplanted their kidneys into normal isologous recipients. He found that the antibody which dissociated from these transplanted kidneys had a high degree of specfficity on relocalization in tissue. He showed that the fraction of NTAb which fixed to GBM was more specific than that fraction of NTAb which dissociated from non-renal tissue (82).

c) Rate of Fixation of Nephrotoxic Antibody - The fact that heterologous antibodies fixed rapidly was initially suggested by Sarre et al in 1942 (83). In rabbits one kidney was clamped for 30 minutes following injection of NTAb. When the disease became manifest days later (using duck NTS), these authors noted less severe disease in the clamped

side. Pressman et al confirmed that NTAb fixed rapidly, probably within 30 minutes (84). Unanue and Dixon in 1965, using duck NTAb in rats, found that renal NTAb fixed maximally in one hour, whereas it fixed to lung and liver more rapidly. The antibodies remaining in the circulation after one hour appeared to be of low avidity for renal tissue. A state of equilibrium existed between antibodies fixed to the kidney, those fixed to extrarenal antigens, and those in the circulation. Unanue et al, and Seegal have shown that NTAb will detach and refix at another renal site in the presence or absence of complement (82, 85); for damage to continue, however, complement binding and activation is mandatory. This suggests that though a specific amount of antibody may have glomerular basement membrane fixing properties, the antibody molecules may detach and refix at different sites, causing continuing damage for possibly weeks depending on their half life.

This concept is not unique, for it is known that in the complement system molecules of $C^{1}3$ can be continuously fixed and bound, released and rebound to basement membrane, causing continuing damage.

d) Role of Complement - In the study of NTAb it rapidly became apparent that avian NTAb did not fix complement, whereas non-avian antibodies fixed complement well (86, 87). When non-avian NTAb was injected intravenously to rabbits or rats, a rapid fall in serum complement was seen. This occurred even in bilaterally nephrectomized animals, presumably due to the fixation of NTAb in non-renal tissue (87, 88), or alternately bound by altered or aggregated gamma globulin. Injection of duck NTAb did not cause a fall in serum complement (88, 89). With both species of NTAb there was a later fall in serum complement during the autologous phase (89). When rats and rabbits were injected with a non-avian nephrotoxic serum, complement was fixed immediately

and is detected by immunofluorescence in a linear pattern along the GBM (13, 89, 90). In animals injected with duck NTAb no complement could be detected along the glomerular basement membrane by this method. However, minute quantities were fixed in the axial or mesangial area in a spotty fashion (89).

The pathogenetic involvement of complement has been studied by two different means. The first method is to attempt to decomplement animals and then inject them with a standardized amount of NTAb. The second is to alter the NTAb so that its complement-binding ability is lost. In both situations, the heterologous phase of NTN was either abolished or reduced.

By decomplementing rats with either zymosan, antigen-antibody complexes or heat aggregated gamma globulin (35, 89, 90), complement was reduced for a

few hours and normal amounts of NTAb did not produce proteinuria if injected during this period. Zymosan is an insoluble glucose polymer prepared from the cell wall of yeast. The rats rendered hypocomplementemic developed nephritis in the autologous phase but complement levels had returned to normal by this time.

Pepsin or papain treated NTAb retains the antibody combining site but the complement fixing Fc piece is lost (91-93). When injected into rats, papain-treated NTAb did not cause any fall in serum complement, nor did it cause nephritis even when injected in very large doses (91, 94). Pepsin treated antibody did lower complement transiently and if injected in large doses into rats a mild nephritis was produced (91, 92). It has been reported that pepsin treated antibody may fix small amounts of complement (95).

The pathogenesis of basement membrane destruction in this model (in non-avian NTN) is initiated

by antibody attachment, complement activation, release of chemotactic agents, polymorphoneutrophil attraction with enzyme release and resultant destruction of basement membranes (90, 96). Animals made leucopenic prior to the induction of the disease have a markedly decreased response, but a mild nephritis still develops (90). Complement thus may exert a direct toxic effect on basement membrane, analogous to that found in red cell or bacterial cell lysis.

The above experimental models utilized non-avian NTAb. Duck NTAb does not fix complement in rats, and yet can induce immediate proteinuria, as shown in decomplemented animals (8). There must be, therefore, other mechanisms at work here which do not utilize complement with resultant PMN attraction and basement membrane destruction. These mechanisms still remain unsolved.

2) Autologous Phase - The dual nature of the renal disease induced by the injection of

nephrotoxic antibodies was well established by Kay in 1942 (97). The second component of this form of nephritis is the autologous phase. The host makes antibodies to the injected heterologous protein which appear approximately seven days later. These antibodies will react with the NTAb, which is fixed to the GBM, causing a second antigen-antibody interaction in the kidney. It has been shown that the appearance of autologous antibodies in the circulation and their kidney deposition coincided temporarily (94, 98). The host globulin was deposited in the same linear fashion as was the original NTAb (35) and it did not appear if the host did not have an immune response (35, 98). As has been discussed earlier, small doses of duck NTAb will not cause immediate nephritis in rats. If the autologous phase was prevented from occurring, then disease was inhibited. Kay did this in 1940, using irradiation to prevent an immune response (97). Hammer and Dixon made rats tolerant to rabbit gamma globulin. Accord-

ingly, when given rabbit NTAb these rats did not have an immune response nor an autologous phase. However, if they were then given rat anti-rabbit gamma globulin passively, they exhibited severe proteinuria (35). It appears that with an adequate host immune response the autologous phase of nephritis occurs even when only 1% of the glomerular basement membrane surface is coated with NTAb (99). What role complement plays in the autologous response is still uncertain. During this phase serum complement drops, and complement can be found in a linear pattern along GBM (89). However, the autologous phase can be induced in congenitally deficient rabbits lacking the sixth component of complement. The inference is that the active component of complement is not C 6 or a later component, and that only a partial fixation of complement is necessary.

d. Non-Immunologic Factors Influencing NTN -

1) Physiologic Loading of Kidney - Following induction of NTN, if a functional fluid load is placed on the kidney, exacerbation of renal failure and pregression of the pathological picture may result (35). Similarly a high protein and high salt diet will cause worsening of the nephritis (100, 101).

2) Coagulation - What role the coagulation process plays is not clear. It is known that small areas of thrombosis appear both early and late in NTN (52, 56, 102). However, the thrombotic component is only seen when large amounts of NTAb are injected (i.e. giving rise to a more marked antigen-antibody reaction locally)(102). It is usually inconspicuous in the nephritis seen in rats. If intravascular coagulation plays an important role, then anticoagulants should ameliorate the disease. Both Warfarin sodium and heparin (102, 103) improved the

histopathology of the disease (102, 103); however, damage to the basement membrane still occurred as reflected by the proteinuria (102). It thus appears that the coagulation process may cause fibrosis and scarring as a consequence of the basic pathologic lesion, but anticoagulation does not appear to have a major effect on the basic disease.

3) Freund's Adjuvant - Watson in 1966 showed that a non-nephritogenic dose of NTAb could induce disease in rats previously injected with complete Freund's adjuvant. By electron microscopy slight damage was seen in the glomerular basement membrane after complete Freund's adjuvant alone, with occasional loss of the lamina lucida and occasional endothelial separation and fragmentation. With this initial damage, a subsequent subthreshold dose of NTAb resulted in overt proteinuria (104).

e. Pathology of Nephrotoxic Nephritis - The pathologic features of this type of nephritis are

variable depending upon the species of animal. In the rat, an early exudative response is followed by a mixed pattern of proliferative and membranous glomerulonephritis (90, 105, 106). Mice have little proliferative changes, but develop marked thrombotic lesions, with associated mesangial and membranous lesions (107, 108).

The pathology seen in the rat is the best studied. Within twelve hours of the injection of NTAb both complement and heterologous gamma globulin are localized along the basement membrane by fluorescence microscopy. Ultrastructurally, wispy and poorly delineated deposits can be seen in many, but not all, of the thickened glomerular basement membranes. Faint densities are present, usually on the luminal side of the basement membrane. Endothelial cell change is minimal, with occasional distortion of the fenestrations and some cytoplasmic swelling.

Over the next few days there is progressive endothelial cytoplasmic swelling and proliferation of

mesangial cells. There is no further change in the GBM. Epithelial cell changes are minor, only occasional broadening of the foot processes being present. This alteration probably reflects protein leakage. With fluorescence microscopy host complement and heterologous NTAb can be seen in a smooth linear pattern along the basement membrane. Host globulin cannot be detected at this stage.

The autologous phase begins about five to seven days after injection of NTAb. Endothelial cells become grossly swollen and mesangial cells hypertrophy. Ultrastructurally, dense granular subendothelial deposits are seen closely applied to the basement membrane and there is marked distortion of fenestrae. By fluorescence microscopy, host gamma globulin is now found in a linear (membranous) pattern (109). There are still no gross epithelial cell changes. This is significant as will be seen later, when the pathologic changes of antigen-antibody disease are discussed.

After two to three months, the basement membrane may become markedly thickened, presumably by the incorporation of the sub-endothelial deposits and by the formation of new basement membrane (110, 111). Moreover, some swelling and proliferation may occur of epithelial cells, and, together with the mesangial and basement membrane swelling, Bowman's space may be occluded. Synechiae may develop. The end result is often architectural distortion or destruction.

g. Methods of Induction and Enhancement

1) Induction of NTN in Immunized Animals - The classic experiments of Steblay and Smadel, later confirmed by Lerner and Dixon, demonstrated that sheep injected subcutaneously or intradermally with heterologous or homologous glomerular basement membrane in complete Freund's adjuvant produced antibodies against injected antigens and developed a

progressive and usually fulminant proliferative glomerulonephritis (54, 112-114).

2) Antibody and Transfer Studies - These antibodies referred to above can be passively transferred to lambs. Using immunofluorescence, it was possible to show that these transferred antibodies fixed toglomerular basement membrane (114). This fixation was linear and was similar to that seen in the kidneys of the nephrotic sheep themselves. Moreover, the recipient lambs developed nephritis.

Lerner showed that by bilaterally nephrectomizing sheep, the serum antibody titer could be increased (114), and this increased titer enhanced the induction of disease in normal lamb recipients. Absorption of the nephritogenic serum with glomerular basement membrane prior to transfer abolished its nephritogenic property, demonstrating the specificity of the reaction. In the recipients, host complement was present in the same pattern as the NTAb.

The sheep has been the most utilized animal for the induction of nephritis, but monkeys have also been studied because of their closer phylogenetic relationship to man (115). Heterologous basement membrane was used, but variable results were obtained and no definite conclusion drawn. Some monkeys developed a nephritis but it was morphologically dissimilar to that seen in sheep.

g. Extrarenal Sites of Antigen - Seegal found that two very vascular organs, placenta and lung, were sites of a nephritogenic antigen (116-118). Antisera produced against these organs resulted in a nephritis similar to that caused by an antiglomerular basement membrane antiserum. The pathologic features included basement membrane thickening with endothelial cell proliferation. Nephritis following the administration of specific anti-lung antisera has been described in the rat and rabbit (118).

Moreover, the absorption of anti-kidney antiserum by placenta and lung reduces the potency of the antiserum significantly (64). Pressman et al, by means of radioactively labelled antisera, have shown that antibody to rat kidney, placenta or lung have a similar distribution in vivo (119). By immunofluorescence, Seegal has shown that nephrotoxic antisera to rat placenta, lung and kidney all localize on the capillary vessels of the rat glomerulus in a smooth linear pattern (120).

Recently, Steblay and Rudofsky have immunized sheep with human lung basement membrane (LBM) and have induced a nephritis of similar, if not identical character to that observed with glomerular antigens. Moreover, they were able to demonstrate autologous gamma globulin and complement deposited in a linear fashion on the glomerular basement membrane as well as on the basement membrane of tubules. They eluted antibodies from the glomeruli which subsequently

fixed to both sheep and human glomeruli and human lung in a linear fashion. Sheep lung stained weakly. Absorption with human GBM or LBM greatly decreased fluorescence to both sheep and human tissue. Corresponding absorption with sheep GBM or LBM abolished the sheep but not the human staining. This demonstrates that both species - specific and cross reactive antibodies - were present (121).

D. GLOMERULONEPHRITIS CAUSED BY ANTIGEN-ANTIBODY COMPLEXES

1. Definition - This type of glomerulonephritis develops secondary to the deposition of soluble antigen-antibody complexes along the glomerular basement membrane. The antigen is not necessarily immunologically related to the glomerular basement membrane, and can be either renal or non-renal in origin. The nephritis can be acute or chronic, depending upon the method of antigen administration. If antigen is

administered as a single large injection then "one shot" serum sickness may occur with its associated acute nephritis. If the antigen is given as a series of small injections then a chronic form of the disease may occur. The passive intravenous administration of complexes directly also causes a proliferative glomerulonephritis.

2. Methods of Induction

a. Non-Renal Antigens

1) Acute Glomerulonephritis

Active Immunization - Following a single injection of suitable antigen, immunologically reactive animals or man will produce antibodies to it five to seven days later. Whether the animal develops renal disease however, depends on three other facts:

a. The amount of antigen given must be such that the animal produces a good immune response.

b. The antigen must not be rapidly destroyed (i.e., eliminated from the circulation) so that antigen-antibody complexes can be formed.

c. The complexes formed must be of sufficient size to be deposited and cause disease (122-124).

This experimental model has been studied by many investigators (4, 5, 125-132). It was found that injected foreign antigen disappeared from the host's circulation in three phases. The first was the equilibrium phase. Following an intravenous injection the protein equilibrated with both intra- and extra-vascular tissues. This caused an initial rapid decline in intravascular concentration over the first 24 hours. Following equilibration the phase of non immune catobolism occurs. Here the disappearance rate of the antigen is constant, dependent on the catabolic half life of the protein. These two phases together constitute the immunologic induction period of the animal. Five to seven days follow-

ing immunization, the animal begins to produce antibodies which combine with the antigen. Antibody then combines with the antigen, and once equivalence is reached antigen is rapidly removed from the circulation in the immune elimination phase. It is during this phase that soluble Ag-Ab complexes are deposited and cause the damage of acute serum sickness. The third phase begins prior to the detection of free circulating antibody as the initial antibody produced is complexed to the circulating antigen (4, 128-130).

The lesions of acute serum sickness appear concomitant with the immune elimination phase of the antigen. As the complexes become larger, dependant upon more antibody production, they become more readily phagocytosable and are rapidly removed from the circulation. Without further introduction of antigen, the disease rapidly subsides. As in the chronic form, the antibody response of the host determined the severity of the disease (131). The

correlation in the acute model is not as striking, but those animals with a good antibody response usually develop the most pronounced lesions. Recently Cochrane and Hawkins have shown in rabbits that complexes formed in moderate antigen excess (20x) have a heavier component than complexes formed in great antigen excess (100x) (11). They correlated the deposition of heavy complexes (19S) with pathologic changes, and though complexes of both sizes activated complement and both were able to increase vascular permeability, only those animals with 19S complexes developed disease.

It thus appears that a similar, if not identical, mechanism for the localization of immune complexes exists as has been investigated more recently in the chronic disease model (24).

The major importance of the host response was shown in 1950 by Schwabe and later by Dixon et al. Development of nephritis was prevented by abolition

of the immune response by irradiation or corticosteroids. The latter agents probably had a dual effect: the reduction of antibody production and second, a general anti-inflammatory effect (133-140).

Intravenous injection of soluble Ag-Ab complexes in rabbits and rats produces acute glomerulonephritis when given in moderate antigen excess (2, 11, 135). If injected over a period of 24 hours, it produces a disease lasting for several weeks. The acute passive disease is identical to the actively produced one.

Pathology of Acute Glomerulonephritis - Light microscopic changes in the kidney of acute glomerulonephritis involve cellular proliferation with little change in the basement membrane (23, 127, 136). The endothelial cell proliferation can be minimal or marked. In the more marked cases, if excessive, some focal necrosis and thrombosis is seen.

The major change ultrastructurally was endothelial cell hyperplasia. No deposits were seen in contact

with the basement membrane. This may possibly be due to their small size.(137, 138). On fluorescence microscopy, it was possible to demonstrate the presence of antigen, host complement and host gamma globulin. In marked contrast to the fluorescent pattern seen in NTN, the pattern here was granular, in a "lumpy-bumby" fashion. Occasionally the deposition appeared to be in a linear pattern. This was felt to be due either to a heavy deposition of immune reactants or to a thick section, both of which give a confluent appearance to the granular deposits.

2) Chronic Glomerulonephritis - It has been adequately shown that repeated injections of foreign proteins over an extended period of time will cause a chronic form of glomerulonephritis (24, 139, 142, 143).

a) Rabbits - Dixon in 1961 and Germuth in 1967 made detailed studies correlating antibody response to various purified antigen with patho-

logic changes found in the kidneys. Dixon found three types of response to a given dose of antigen.

a. One group produced no antibody and had no renal disease.

b. The second group had a moderate antibody response and these became nephrotic.

c. The third group had a marked antibody response, had minimal chronic disease, but had relatively severe episodes of anaphylaxis.

These "high antibody producers" had an initial acute proliferative glomerulonephritis, but prolonged immunization in surviving animals did not result in chronic changes, presumably because the complexes formed were aggregated, rapidly phagocytosed and not deposited within the glomerulus.

Germuth in 1967, in a similar study on rabbits, was able to divide the antibody response into five types.

a. Those with no response and no disease.

b. Those with a marked response with an initial proliferative reaction but without chronic disease.

c. Those with an initial marked response and early proliferative disease. As immunization was continued these animals had a decreased antibody response and associated chronic disease.

d. Those with a moderate response, with resulting chronic disease.

e. Those with a response similar to the fourth group but with a subsequent complete cessation of antibody production. These animals developed a second transient episode of acute glomerulitis as the stage of equivalence was reached during the diminution of antibody production.

Germuth also found he could correlate the pathologic lesions produced with antigen concentration. With larger doses of antigen progressive obliterative changes were produced, whereas with lower doses, membranous changes were seen more frequently. Dixon,

on the other hand, found a poor correlation between antigen concentration and resultant pathology. Boyns et al immunized rabbits with BSA and prevented the appearance of free antibody by the continuous administration of antigen. They found no chronic changes pathologically over a several month period (144).

b) Rats - Fennel produced chronic nephritis with BSA in rats. The animals were not in antigen excess and he could not demonstrate circulating antigen. Moreover the higher the dose of antigen used the more severely scarred were the glomeruli. By fluorescence microscopy he was able to detect antigen, host gamma globulin, and host complement in the basement membrane. Ultrastructural changes were consistent with those previously described and showed sub-epithelial granules along the basement membrane (145).

c) Pathology - There were slight differences in interpretation, and in the timing of

the appearance of pathologic changes, but all authors except Boyns et al agreed that animals in antigen excess over a period of time will develop chronic glomerular changes. By light microscopy in rabbits one sees both proliferative and membranous changes, possibly dose dependent. In rats there is a tendency for membranous changes to be dominant, with less proliferative changes.

Glomerular lesions cover a broad spectrum of pathologic changes. These are characterized by:

1. Irregular thickening of glomerular basement membranes, or

2. Regular and thickened glomerular basement membranes;

3. Mild proliferative epithelial changes, with fibrous adhesions within the tuft;

4. In the chronic forms of nephritis polymorphonuclear leucocyte infiltration may be seen;

5. Focal areas of necrosis are occasionally seen within the tuft, characterized by loss of structure,

nuclear pyknosis, PMN infiltration and "fibrinoid" deposition. When this occurs peripherally, the fibrinoid may extend into Bowman's space and partially occlude it.

6. Glomeruli which show chronic changes with scarring, with many adhesions between the capsule and tuft and obliteration of Bowman's space.

Until the chronic changes predominate, the capillary lumen remains patent, with minimal distortion of architecture except for GBM thickening. Only later do these obliterative changes take place. Using fluorescence microscopy, host gamma globulin, complement and antigen may be detected in a "lumpy" granular fashion along the basement membrane. The amount of immune reactant deposition correlates with the severity of disease. Ultrastructurally, one sees a gradual transition from slight foot process blunting and associated loss of basement membrane definition, to granular deposition subepithelially, with associated

foot process flattening and marked basement membrane thickening. It appears that these granular deposits are trapped within the basement membrane, migrate through it to accumulate in the subepithelial area. (109, 143, 145).

b. Renal Antigens - A chronic disease, similar pathologically to BSA-induced nephritis, develops when rats are immunized with kidney suspensions in complete Freund's adjuvant. The pathogenic mechanisms of this disease are still being clarified. This model appears quite similar to chronic serum sickness and quite dissimilar to nephrotoxic nephritis. The points to be made initially contrasting this disease to nephrotoxic nephritis are:

1. The onset and character of the disease;
2. The site and character of the antigen;
3. The role of complete Freund's adjuvant;
4. The pathology of the lesion.

1) The Onset and Character of the Disease - The original nephritis induced in rats with homologous or heterologous kidney and streptococcus (146) could not be reproduced (147, 148). Smith et al in 1964 produced disease with living streptococcus; however, this work also could not be reproduced (152). Frick (149) produced the disease with M. Tuberculosum in Freund's adjuvant and kidney suspensions. It was Heymann et al who first developed a reproducible model in 1959. Rats immunized intraperitoneally twice a month with homologous kidney in complete Freund's adjuvant become nephrotic after seven to ten injections. Autologous kidney also seemed to be partially effective as an antigen. In addition, when liver was used as the antigen, a small percentage of rats (3 of 21) became nephrotic, and a similar small number (3 of 10) became slightly proteinuric with large doses of complete Freund's adjuvant alone (150). However, with the doses of complete Freund's used in the accepted experimental model no proteinuria was

seen though slight histologic changes were evident. The role that adjuvants play will be discussed in a later section. Once manifest, the disease was chronic. Blozis (151), replacing the M. Tuberculosum with H. Pertussus, produced a nephrotic syndrome in rats, though only in about 30% of the animals. As the disease could be produced in the accepted model only after seven to twelve injections, several procedures were introduced which both hastened and increased the severity of the disease. Heymann found that increasing the dose of antigen would increase the incidence of disease to 100% from the normal 80% (150). By increasing the frequency of injections to weekly rather than bi-monthly, Watson and Dixon also increased the severity of the disease (152). These authors also found that Arlacel A rather than Arlacel C produced a clinically more severe disease. Host factors are also important in the development of disease. Watson showed that of the four strains of isologous rats he tested, the Lewis strain was most prone to disease,

particularly when Sprague Dawley kidney was used as antigen. This finding of increased incidence in the inbred strain was proven to be most fortuitious. In later work, it proved invaluable in the elucidation of cellular mechanisms in the pathogenesis of the disease.

2) The Site and Character of the Antigen - In the original experiments a crude suspension of kidney was used (150, 152). After homogenization, the kidneys were centrifuged at 1600 x G, and the supernatant used as antigen. Subsequently it was found that the responsible nephritogenic antigen was located in a tubular cytoplasmic fraction (153). Recently, Edgington et al have isolated a nephritogenic antigen from Sprague Dawley kidneys which he named RTEa5 to distinguish it from other non-nephritogenic antigens RTEa3 and a4. This antigen has a sedimentation coefficient of 28.6, is a lipoprotein, migrates in the alpha globulin region, and is very

potent. As little as 3 µg. of this antigen in complete Freund's adjuvant in the foot pad is able to produce disease. The antigen is localized to the brush border of the proximal convuluted tubule of the kidney and small amounts are present in the brush border of the small bowel mucosa (154). He did not detect the antigen in liver or other tissues tested. However, human lung but not liver (155, 156) has caused disease in a small percentage of animals.

This disease model is generally accepted as one of antigen-antibody complex disease (150, 151, 155). It is refuted by some on the grounds that there is no correlation between circulating antibody levels and disease indidence (156-158). Holm found that lymphocytes from nephrotic rats would destroy rat kidney monolayers and stated that this in vitro manifestation of delayed hypersensitivity confirmed the in vivo situation. This latter point will be discussed in more detail later. The recent papers

of Boss et al appear to have some confusion in terminology. The authors seemed unaware that nephrotoxic nephritis and this model are induced by different antigens, easily distinguishable. Moreover, in the induction of disease in their model, a relatively crude antigen is used and since the nephritogenic antigen is but one component of kidney, an evaluation of the antibody response to disease induction has minimal meaning. Moreover, in none of their recent papers were any fluorescent or ultrastructural results shown (159-161).

3) Role of Adjuvants - Complete Freund's adjuvant causes damage to the glomerular basement membrane of rats. Depending on the dosage and the route of injection, proteinuria can become evident. Heymann claimed that large doses of complete Freund's adjuvant produced proteinuria, whereas Watson et al produced light microscopic and ultrastructural damage without causing overt proteinuria.

The latter investigators showed, however, that small doses of complete Freund's adjuvant appeared to enhance the nephrotoxicity of a subthreshold dose of nephrotoxic antibody, and the two agents together caused proteinuria (150, 162).

In the complex disease model, it appears that Mycobacterium Tuberculosum is an essential part of the adjuvant, as Mycobacterium Butyricum is without effect (152).

Blozis has shown that H. Pertussis, when used in adjuvant, will produce disease in about 30% of rats. It should be determined whether there is any common antigenic determinants between the two organisms (H. Pertussus and M. Tuberculosum) used in the adjuvant. This might clarify what role the mandatory adjuvant plays in the disease pathogenesis.

4) Pathology - Typically on light microscopy this disease is a membranous glomerulonephritis with little exudative or proliferative changes (155).

The basement membrane is thickened and stains positively with periodic acid-shiff reagent. On fluorescence microscopy there is a deposition of gamma globulin, complement and antigen in a granular pattern along the basement membrane, in contrast to the linear pattern seen in NTN (152, 154, 155). Ultrastructurally, there is thickening of the basement membrane with numerous electron dense deposits situated within or on the subepithelial side of the basement membrane. They are usually amorphous and irregular in size and outline (109). These changes are similar to those described in the previous section.

E. THE ROLE OF CELLULAR (DELAYED) MECHANISMS

1. Transfer of Disease Between Homologous Rats - Though it is generally accepted that antigen-antibody complexes play the major role in the pathogenesis of AIC nephritis, cellular mechanisms are still believed

by some to be causally related. In support of this view, successful transfer of disease using homologous rats has been reported (157, 158). The nephritis seen in the recipients was not that of AIC nephritis. There were no immunofluorescent granular deposits seen along the basement membrane, nor were there the classical ultramicroscopic changes seen within the glomerular basement membrane nor along its epithelial aspect. The changes that were seen in the kidney of recipients could possibly be explained on the basis of a graft-versus-host reaction. Homologous rats were used and the recipients had been rendered tolerant to donor lymphocytes. These transferred sensitized immunocompetent cells, however, would have been able to cause a graft-versus-host reaction.

2. Transfer of Disease Between Isologous Rats - Later workers, utilizing inbred strains of animals demonstrated minimal disease transfer with sensitized lymphocytes. When disease was transferred, the lymphoid cells were obtained from animals which had been

immunized either in footpads or intracutaneously. The recipient animals developed mild nephritis only after a latent period equal to or longer than that required to induce the original disease. The immunohistopathology was identical to that described in the original model (141, 153, 152).

The interpretation placed on these results was that possibly sufficient antigen was transferred in association with the cell transfer, and in fact, the animals were immunized with free antigen or antigen in association with the cells. This rationale follows the demonstration by Edgington et al that very small doses of autologous antigen are capable of inducing the disease, specifically when the antigen used is injected in the footpad. Possibly small doses of autologous antigen given in association with lymphocytes may also be as effective in the induction of AIC nephritis.

3. Transfer of Disease by Parabiosis - Nephritis has also been transferred to normal recipients by parabiosis, and these observations were interpreted as showing transferrability of the disease by cells (163). These findings, however, are not conclusive. Although peritoneal cavities were not joined in these experiments, complete cross circulation was nevertheless established in a few days. This allowed transfer not only of cells, but also antigen-antibody complexes, antibodies and other humoral factors, to the normal isologous parabiont. Therefore, although disease did occur in the normal parabiont after two to five weeks, the pathogenesis was still unclear. Glassock and Watson ran short-term experiments of eleven days of parabiosis. Though they transferred large numbers of lymphocytes, mild disease occurred. (153). To attempt to cut down on the possibility of antigen-antibody complex transfer or depot antigen transfer, a further model was utilized. Following

induction of nephritis by long-term parabiosis, the first recipient (now nephrotic) was separated and joined to a second normal isologous parabiont. This second recipient did not become diseased. They considered, therefore, that sensitized cells alone were not responsible, but that a considerable depot of antigen or complexes must be transferred to the normal recipient. That cellular mechanisms are not significant may also be inferred from the lack of cellular infiltrate in the glomeruli of diseased rats, quite dissimilar from other diseases involving delayed mechanisms (162, 163). One other manifestation of cellular hypersensitivity is the delayed skin test. It has been shown that rats rendered nephrotic have uniformly absent 24-48 hour skin test when challenged intradermally with the antigen to which they are sensitized (159, 164), although they are capable of mounting a good delayed reaction.

4. In Vitro Cytotoxicity - Until recently there were no good in vitro models for the study of delayed hypersensitivity. The past few years have seen a marked advance in this field (156, 165-171). The utilization of tissue culture as targets for sensitized lymphoid cells has given a readily available in vitro system for assaying the cytotoxicity of these lymphoid cells. The interpretation of the mechanisms operable in these systems has not been agreed upon. In early experiments it was believed that only sensitized cells could destroy target cells in vitro. It soon became evident that normal allogenic cells with the addition of phytohemagglutinin (PHA), also destroyed monolayers (168). Moreover, it was shown that F1 hybrid cells destroyed parental cells when PHA was added. F1 hybrid cells should not destroy a parental monolayer on histocompatibility grounds, as no foreign factors are present in the monolayer cells. In addition, irradiation did not alter this parental destruction by F1 cells.

Moller has shown that prior incubation of the target cells with an isoantiserum directed against the cells protects the cells from lymphoid destruction. This implies that the antibodies combine with the available sites in the target tissue, preventing the attachment of the lymphocytes. On the other hand, Hellstrom and Hellstrom postulated that in certain tumor growth inhibition models, H2 locus histoincompatibility was operative and was not dependent on viable lymphocytes. They felt that possibly the afferent arc of this cytotoxic system was the contactual attachment secondary to the histoincompatibility, and that this attachment was achieved in other systems by PHA (172). That histoincompatibility is not necessary has been underlined recently by the demonstration that normal isologous and autologous lymphocytes in the presence of PHA can be cytotoxic (169). Holm has also shown that homologous lymphocytes from patients with Hodgkins disease or chronic lymphatic leukemia,

though histoincompatable, did not cause cytotoxic effects in culture. This may be due to some defective synthetic process in these abnormal lymphocytes, but it has now been shown that synthetic processes and cytotoxicity represent different mechanisms.

If the attachment to the target cells by normal or sensitized lymphocytes is the operative mechanism, it was postulated that these cells when stimulated might secrete a protein or immunoglobulin which would be the ultimate damaging agent. When cells in culture were exposed to the supernatant of a previous cytotoxic system, no cytotoxicity was evident (165). Swedish workers have shown cytotoxicity and DNA synthesis appear to reflect a final common pathway of a similar process, i.e. stimulation. These two mechanisms however, do not appear to be causally related (169, 171, 173). It was demonstrated that DNA synthesis occurs well after cytotoxicity has begun, and though ultimately DNA synthesis and cytotoxicity appear to reflect a similar process, they

manifest peaks at different times. Moller et al showed chloroquin could decrease DNA synthesis but cytotoxicity was maintained (169).

It thus appears that for cytoxicity in tissue culture, histoincompatibility is not essential, though in some systems, namely tumor inhibition, it may play a role. Some fine recognition step is required, as was shown by Perlman in his isologous experiments (171). This is not related to antibody or protein synthesis. It is possible that on the surface of tissue culture cells are sites which are recognized by stimulated lymphocytes, which then attach and cause destruction. The mechanism of this process still remains to be clarified.

Holm utilized the tissue culture system to measure cytotoxicity of sensitized lymphocytes (from nephrotic rats) utilizing as the target a kidney monolayer. He used a homologous model and often the control cells gave as much or more destruction as did the sensitized

cells. He did show some enhanced cytotoxicity in nephrotic peripheral lymphocytes, when kidney was the immunizing antigen, and stated that this suggested a delayed hypersensitivity mechanism. However, as was stated in the report, there was often significant destruction with animals immunized with liver, a tissue from which the nephritogenic antigen cannot be obtained. The fact that an isologous system was not used in this cytotoxic model means that any conclusions made pertaining to cellular mechanisms as vectors in disease etiology must be seriously questioned (156).

5. In Vivo Cytotoxicity - Elkins in 1964 took the mechanics of cellular action one step closer to the in vivo situation. He injected normal or sensitized parental lymphocytes under the kidney capsule of an F1 hybrid. He showed that initially the reaction was "one way" (donor cells were the actively participating component in what was shown to be a graft-

versus-host reaction). The reaction was characterized by an infiltration of mononuclear cells which localized around and destroyed the convoluted tubule cells while sparing glomeruli. The cells were present in cortical tissue in broad sheets with finger-like projections into the deeper cortical tissue. There was congestion, dilation, and plugging of the intertubular capillary circulation by the invading mononuclear cells. Controls (isogeneic, parental, or F_1 lymphocytes into parental kidney) produced either minimal or no reaction. His only interpretation from the experimental data was that lymphocytes, if not destroyed by the host, are capable of initiating a graft-versus-host reaction (174). In later experiments he showed that for continuation of the reaction, an active host component was necessary. This reactivity can be transferred successfully to a second isogeneic F1 recipient (175).

III. MATERIALS AND METHODS

A. ANIMALS

Female Lewis rats of 150 to 200 grams, inbred for approximately 120 generations and completely isogeneic were obtained from Microbiological Associates, U.S.A., and kept in metallic cages, four to six to a cage. For the tissue culture experiments, a female Lewis rat and her litter of less than seven days of age were shipped by Air Express.

Sprague Dawley rats and guinea pigs of 150 to 200 grams were obtained from the Quebec Breeding Farm, Quebec, Canada, and kept in similar cages.

Albino New Zealand rabbits, 3 to 5 kilo, were also obtained from Quebec Breeding Farms, and each rabbit kept in separate animal cages.

All animals were fed with the appropriate Purina animal chow (Ralston Purina); water was changed daily.

B. ANTIGENS

1. Rat Gamma Globulin - Lewis rats were bled by cardiac puncture and the blood allowed to clot over-

night at 4°C. The serum was then aspirated and separated from residual red blood cells by centrifuging at 1,000 x G for 30 minutes. A 5 cc serum aliquot was dialyzed overnight against 250 cc of 0.0175 molar phosphate buffer, pH 6.3. The serum was then applied to a DEAE cellulose column and the gamma globulin fraction eluted with the same buffer (176).

DEAE cellulose with a capacity of 0.91 meq/g was obtained from Bio Rad Chemicals, California. One hundred G of dry material was placed in one liter of normal sodium hydroxide, stirred and then filtered. The cake was then washed with one normal sodium hydroxide till the filtrate was colorless. Normal hydrochloric acid was then added to the suspension to make it strongly acidic. The suspension was filtered and then washed free of acid with distilled water. After restoration to neutrality the cake was suspended in normal sodium hydroxide, filtered, again restored to neutrality. The DEAE was

resuspended in 2 to 3 liters of starting buffer and then the pH of the suspension was adjusted to 6.3 with the acidic component of the starting buffer (NaH_2PO4). Prior to use the DEAE cellulose was again equilibrated with the starting buffer.

Column chromatography was used. A column 30 cm x 2.5 cm was obtained from Pharmacia (Upsala, Sweden). Each column was packed with 5 to 6 grams of DEAE cellulose with a protein absorption capacity of 75 mg per 100 mg of absorbent. Having poured and packed the column evenly, an equilibrated sample of serum was applied. The eluate was monitored with 20% trichloracetic acid which produces white flocculation with protein. The sample was collected till the eluate did not precipitate with TCA.

The eluate of each antiserum was then perevaporated to its original volume, usually 5 cc. Its antibody concentration was then checked by double diffusion in agar, and its purity by immunoelectrophoresis.

2. Rat Complement (C3) - One hundred mg of Zymosan (Fleishman Labs, New York, N.Y.) was boiled in 10 ml of saline for 30 minutes and centrifuged. The sediment was resuspended in 100 mls of saline. 1.35 mg of zymosan was then used for each 1 cc of fresh rat serum. Prior to the addition of the serum, the aliquot of zymosan was centrifuged and the button used. The serum was added and mixed to a smooth suspension and incubated at 37°C for one to two hours with occasional stirring to keep the zymosan in suspension. The mixture was then centrifuged, the supernatant decanted, and the sediment washed x 5 with veronal buffer. The zymosan-C3 complex was then resuspended to the original serum volume. The complement adsorbed by zymosan is predominantly C3 or B_1C.

Each antigen was checked for purity by immunoelectrophoresis against a rabbit anti-rat whole serum.

3. Crude Rabbit Antigen - Albino New Zealand rabbits were killed by 3 cc intravenous Nembutal (Abbot Labs, Canada) and the kidneys removed. The capsules and pelves were removed and the kidneys weighed. The kidneys were then cut into small pieces with scissors and then minced in a Waring blender for 3 to 5 minutes. An equal volume of saline was added and the mixture was thoroughly homogenized for 15 minutes, then centrifuged for 90 minutes at 20G at 4°C. The supernatant was carefully aspirated and stored in 5 cc aliquots at 20°C. The sediment was discarded. The supernatant contained soluble tubular antigens, insoluble tubular mitochondria and microsomes, but did not contain glomerular basement membranes.

4. Rat Fraction IA (FIA) - This tubular antigen was isolated by an adaption of the method of Krakower and Greenspon (177). The cortices of kidneys from

Lewis rats were cut into small pieces with scissors and then with a scalpel blade. These were then pressed through a 150 gauge Monel metal sieve. The pulp was suspended in four volumes of normal saline and centrifuged at 400 x G for 10 minutes to sediment glomeruli and heavy tubular remnants. The supernatant consisting of tubular cytoplasmic remnants was centrifuged at 78,680 x G for 60 minutes and the supernatant was aspirated and lyophilized. This consists of soluble tubular constituents and was called FIB. The sediment (FIA) consisting predominantly of tubular mitochondria, microsomes and tubular membranes, was washed x 2 in distilled water and lyophilized and stored at -20°C.

C. ANTISERA

1. Rabbit Anti-Rat Gamma Globulin - Three male Albino New Zealand rabbits of 3 to 4 kilograms were

immunized with rat gamma globulin in complete Freund's adjuvant containing Mycobacterium Tuberculosm, H 37 Ra (Difco Labs, U.S.A.) at 2 mg/ml of final volume. The solution was thoroughly mixed and a good emulsion produced. The animals received the first injection in the foot pad and two subsequent injections subcutaneously at weekly intervals. The concentration of gamma globulin in the initial volume was 10 mg/ml, and the final emulsion was prepared using a 50:50 mixture of gamma globulin and complete Freund's adjuvant. Each animal received 1 cc per injection. Ten days after the 3rd injection the rabbits received a booster injection, and were bled ten days later.

2. Guinea Pig Anti-Rabbit Kidney - Three guinea pigs were immunized with 1 cc of crude rabbit kidney suspension in complete Freund's adjuvant (50:50 mixture). Immunization consisted of 2 courses of 3 weekly subcutaneous injections, with 1 month separating the two. Ten days after the sixth injection the

animals were bled every three days for 5 cc by intracardial puncture. The gamma globulin fraction of the antiserum was isolated by DEAE chromatography and absorptions were performed to increase specificity. The antibody was first absorbed with normal rabbit serum, 0.5 cc of whole rabbit serum was used for each 100 cc of original antiserum; the mixture was incubated at 37°C for half an hour and then centrifuged at 500 x G for 30 minutes. The precipitate was discarded. This procedure was repeated, and stored overnight at 4°C, then centrifuged at 500 x G/30 min. This absorbed antibody was then absorbed with Lewis rat kidney fractions. Absorptions were performed initially with lyophilized kidney supernatant and after duplicate absorbtions, centrifugation at 4,000 x G for 30 minutes, an aliquot was saved. A second absorption was done with Lewis rat sediment in a similar fashion. The specificity in immunofluorescent staining following these absorptions was

compared. The absorptions were done using 10 mg of kidney tissue powder per ml of original antiserum.

3. Rabbit Anti-Lewis FIA - Three male New Zealand albino rabbits were immunized as in (1); however, with Lewis rat FIA in complete Freund's adjuvant as antigen. The rabbits were bled by intracardiac puncture.

4. Rabbit Anti-Lewis BIC - Two white New Zealand albino rabbits were immunized as in (1), however with the Lewis rat zymosan-B_1C complex as antigen. One half cc of zymosan and one half cc of complete Freund's adjuvant were incorporated into an emulsion and injected into the rabbits in the foot pad and then subcutaneously. The rabbits were bled via intracardiac puncture.

D. DOUBLE DIFFUSION IN AGAR

The technique of double diffusion in agar on a micro-ouchterlony scale was utilized to determine potency of antisera. Inexpensive material was used. Thirty-five mm motion picture safety P 40B leader film was obtained from Du Pont of Canada and cut into 12 cm strips as required. The agar used was "Tonagar" No. 2 (Consolidated Laboratories, Inc.). It was completely dissolved in distilled water by heat and constant stirring. It was made up as a 1% stock solution, with 0.7 glycine as preservative, and was stored at 4°C in 5 cc aliquots. For each procedure an aliquot of the agar gel was liquified and poured evenly over the 35 mm film strips and allowed to harden. Using specific well cutters, six peripheral and one central well were made in the agar, and the wells sucked clean with a pipette attached to vacuum. Each well was 2.5 mm in diameter, the distance between peripheral wells 4.5 mm,

and the distance between the central and peripheral well 8 mm. To titer an antiserum, the pure antigen was placed in the center well at a 1 mg/ml concentration and the antiserum placed in the peripheral wells in 2-folding dilutions. The titer of a given antiserum was the reciprocal of the highest dilution which showed a precipitin line, and the antiserum was used at this dilution in all subsequent fluorescent work (178).

E. IMMUNOELECTROPHORESIS

Immunoelectrophoresis was performed using agar gel as the suppprting medium, poured onto the celluloid film strip as described above. Each strip was 125 mm in length. The trough was 90 mm in length. Two wells, 2 mm in diameter, were cut at the middle of the trough, each being 5 mm from it. However, the agar was made up in a sodium barbital buffer pH 8.8, ionic strength 0.06. A Spinco Model R

paper electrophoresis cell was attached to a Heath-kit power supply. For the electrophoresis, 5 milli-amps of current per strip was used till the serum had migrated 30 mm from the point of application, using bromphenol blue as a marker. The strips were then washed for 24 hours in 0.9% NaCl, and stained with Ammido Black, rinsed and cleared in a methanol-glacial acetic acid clearing solution and then dried.

F. INDUCTION OF NEPHRITIS

1. Experimental Model - Figure 1 - Female Lewis rats of approximately 200 grams were marked by ear punching, and their 24-hour urinary protein checked. Each animal then received weekly intraperitoneal injections. These injections consisted initially of the crude rabbit supernatant, stored frozen at -20°C, and mixed with equal volumes of complete Freund's adjuvant. Early in the experiments, however, a dial-yzed and lyophilized supernatant was used. The final

EXPERIMENTAL MODEL

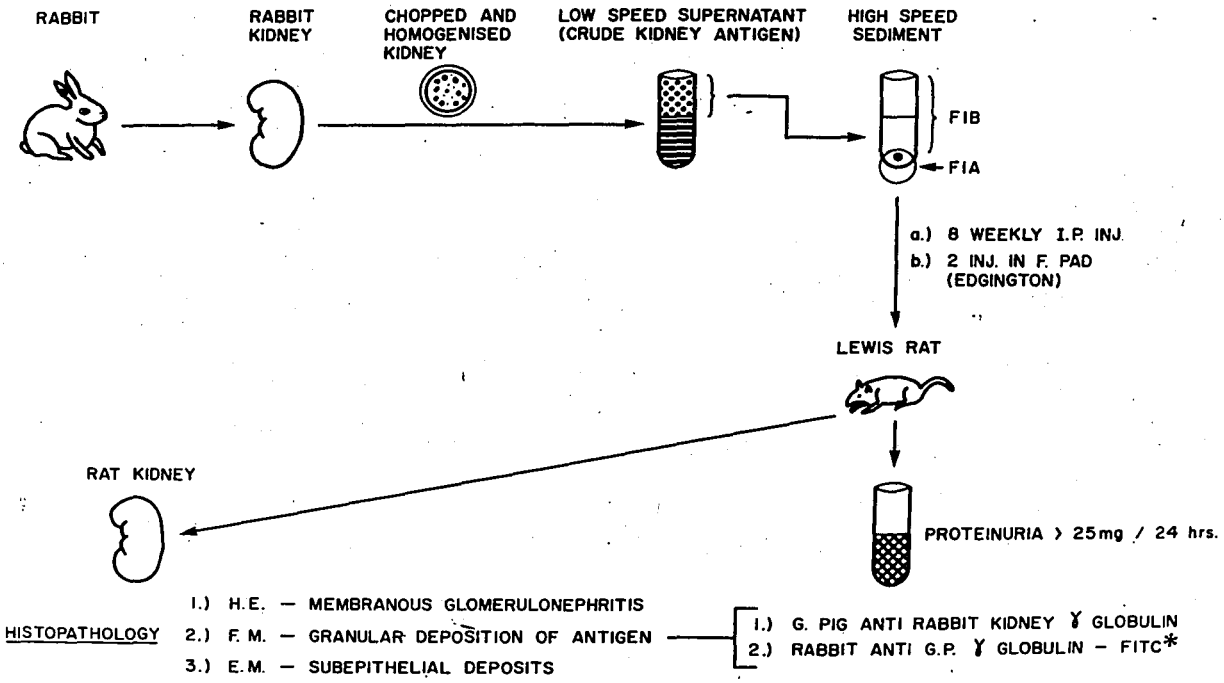

FIGURE I

concentration of antigen being 5 mg/cc in complete Freund's adjuvant. The emulsion contained 2 mg/ml of Mycobacterium Tuberculosum H37 Ra. Each animal received 8 to 12 injections, each of 0.25 cc, to a total of 10 to 15 mg of antigen.

2. Accentuation of Disease - To attempt to increase the severity of the disease, 20 Lewis rats were splenectomized. Under ether anesthesia the rats were shaved and a left lateral incision was made under clean but not sterile conditions. The spleen was located and freed. The arterial and venous supply was tied off with 3-0 catgut and the spleen was excised. The incision was sutured in two layers, muscle to muscle, and skin to skin with 4-0 dermalon thread with a CE - 2-3/8 circle reverse cutting needle. The animals received 500,000 U procaine penicillin intramuscularly after splenectomy and were allowed to recover for three weeks. Nephrosis

was then induced by 8 intraperitoneal injections of rabbit kidney in complete Freund's adjuvant.

3. Progression of Disease - Animals were unilaterally nephrectomized. Part of the kidney was snap frozen, part was fixed in Lillie's buffered formalin and another part was cut into small 1 mm blocks and placed in 2% gluteraldehyde for ultrastructural examination. Prenephrotic, borderline nephrotic and nephrotic animals were chosen for the study. Prenephrotic animals were those which were not proteinuric having completed the series of injections or in the middle of the course. Borderline nephrotic were immunized animals which had proteinuria above their baseline values, but not greater than 20 mg per 24 hours. Nephrotic animals had proteinuria greater than 20 mg per 24 hours.

4. Measurement of Proteinuria - Protein excretion was measured on 24 hour urine specimens by precipitation with 3% sulfosalicyclic acid. Rats normally

have proteinuria of 10 mgs per 24 hours or less. Abnormal proteinuria was considered to be 20 mg or more for a 24 hour period.

G. HISTOLOGY

1. All kidney sections for light microscopy were fixed in Lillie's buffered formalin, paraffin sections were cut and stained with hematoxylin and eosin or with periodic acid Schiff reagents. When sections were examined for fat droplets, Oil Red O stain was used (179).

2. Hyaline Droplet Formation - On examining kidney sections during the prenephrotic phase of the previously discussed groups, many so-called "hyaline droplets" were seen. It has been claimed that hyaline droplets are a reflection of protein reabsorption by the proximal tubules, or "toxic" effects of large doses of complete Freund's adjuvant

(173). As we had observed, these droplets in pre-nephrotic animals, it would appear that excessive protein reabsorption may not play a role in droplet formation. We attempted to determine when "hyaline droplets" appear. Lewis rats were divided into three groups.

Group A - Three rats each received one injection of kidney supernatant in complete Freund's adjuvant in the foot pad.

Group B - Four rats, each received one injection of 0.5 cc of kidney supernatant in complete Freund's adjuvant intraperitoneally.

Group C - Three rats received one injection of 0.5 cc of complete Freund's adjuvant without kidney supernatant.

Groups A and B received 4 mg of antigen in the injection; Group C received no antigen. One animal from each group was nephrectomized every three days and sacrificed if necessary.

3. Arthus skin reactions were formalin fixed for examination by light microscopy. The sections were stained with hematoxylin and eosin or with the May-Grunwald-Giemsa-stain (179).

H. PREPARATION OF FLUORESCENT ANTISERA

The method of Clark and Shepard was used to label pure IgG with fluorescein isothiocyanate (180). The IgG was concentrated to 10 mg/ml in 0.025 M Na_2CO_3 and 0.025 M Na HCO_3 buffer (approximately 4 to 1) having a final pH of 9.0. This was placed in dialysis tubing and dialyzed against 10 volumes of the same buffer containing fluorescein isothiocyanate (Baltimore Biological Laboratories) at a concentration of 0.1 mg/ml. Dialysis was carried out at 4°C for 24 hours with constant stirring. The conjugated protein was then dialyzed against phosphate buffered saline, pH 7.3 until no free fluorescein could be seen in the eluate. Molecular fluorescein to protein

ratios were calculated and this usually was between 3 to one. Occasionally tissue powder absorption of the conjugates was done to decrease non-specific staining.

I. ELUTION PROCEDURES

1. Glomerular Fixed Gamma Globulin - Whole kidneys removed from nephrotic Lewis rats were separated into cortex and medulla. The cortices were minced in phosphate buffered saline (PBS) and washed five times in PBS until the final supernatant was clear. The mince was then resuspended in phosphate buffered saline at 20 ml/gm of kidney and homogenized at medium speed for five minutes in a Virtis homogenizer. The suspension was then centrifuged at 3,500 x G for 15 minutes and the cake washed three times in PBS and recentrifuged. The washed cake was then suspended in 0.02 M citrate buffer pH 3.2 at 20 ml/gm of kidney and incubated for 90 minutes at 37°C with

constant stirring. The suspension was then centrifuged at 3,500 G for 15 minutes and the supernatant aspirated. This supernatant was dialyzed against PBS for 2 days, perevaporated and the protein concentration determined in a Beckman spectrophotometer at a wave length of 280λ. The protein was characterized by immunoelectrophoresis.

2. Glomerular Fixed Antigen - To demonstrate heterologous antigen within the glomerular tuft, the procedure outlined by Edgington et al (181) was attempted. Kidneys were obtained from nephrotic rats and snap frozen in isopentane and dry ice. Four micron sections were cut in the cryostat and fixed to microscope slides by finger heat. After routine fixation (see below), sections were placed in 2.5 M KSCN pH 6.5 for 2 hours at 37°C and 15 minutes at 56°C. The slides were then washed three times in PBS and then stained for antigen deposition by the indirect fluorescent

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method. Tissue morphology, however, was not maintained and a series of elutions was performed to determine the optimal concentration and time (Table I).

TABLE I

K.S.C.N. ELUTION OF 4 U CRYOSTAT KIDNEY SECTIONS

Elution Temperature	Time	Comment
50°C	5 Min.	Glomerular cellular morphology lost but cells present - basement membrane intact.
	10 Min.	Glomerular cellular morphology slightly more disrupted. B.M. intact.
	15 Min.	Complete disruption of cellular elements, B.M. disrupted.
	20 Min.	As for 15 minutes.
37°C	15 Min.	Tissue intact, glomerular all detail good, tubules intact.
	35 Min.	Tissue intact, glomerular cells slightly disrupted, B.M. intact. Tubular cells disrupted, Tubular B.M. intact.
	45 Min.	Glomerular cells disrupted focally; glomerular B.M. also disrupted focally; T.B.M. disrupted.
	55 Min.	As for 45, with larger focal areas.
37°C + 56°C	15 Min. + 10 Min.	Complete cellular destruction.
37°C	30 Min.	Complete cellular destruction.
56°C	10 Min.	Complete cellular destruction.

J. ASSAY OF ELUTED GAMMA GLOBULINS

1. In Vivo - Four normal Lewis rats were placed in metabolic cages and their proteinuria checked. Then each rat received an intravenous injection, via the tail vein, of 1 ml of the eluted gamma globulin. Proteinuria was checked at 4 hours, 24 hours and at 7 days. At 4 hours, each animal was unilaterally nephrectomized, the kidney snap frozen, and sections examined for tissue fixed gamma globulin and complement.

2. In Vitro - The eluted gamma globulin was divided to 4 aliquots. One aliquot was absorbed with Lewis rat FIA, a second was absorbed with crude rabbit supernatant, a third with both and a fourth was left unabsorbed. All absorptions were done in duplicate using 5 mg of tissue powder/ml of eluate. For the indirect fluorescent staining, rabbit anti-rat gamma globulin FITC was the top layer, overlaying the absorbed eluate.

K. IMMUNOFLUORESCENCE MICROSCOPY

1. Demonstration of Injected Antigen in Glomeruli - Guinea pig anti-rabbit kidney gamma globulin was absorbed twice with the supernatant preparation of Lewis Kidney (containing FIA). A second absorption was made with normal rabbit serum. These absorptions were made using 5 mg wet weight of tissue/ml of antiserum or lyophilized rabbit serum. This then served as the middle layer in indirect immunofluorescence. The top layer was column purified rabbit anti-guinea pig gamma globulin, labelled with fluorescein isothiocyanate and having an F/P ratio of between two and three to one. The staining techniques employed were much as originally described by Coons and Kaplan (182). The kidney was snap frozen as described previously, and 4 μ sections were fixed to slides by finger heat. They were then fixed in ether-ethanol (1:1) for 10 minutes followed by 20 mintues in 95% ethanol. Follow-

ing three five-minute washes in PBS, the sections were incubated with the guinea pig anti-rabbit kidney globulin fractions for 30 minutes in a humidified chamber. The slides were then washed for ten minutes x 3 in PBS and the third layer of rabbit anti-guinea pig gamma globulin - FITC applied for 30 minutes in a humidified chamber. Again the antiserum was washed for 10 minutes x 3 with PBS, dried, coverslipped and examined under UV light.

2. Demonstration of Host Immunologic Reactants in Glomeruli - A similar procedure was followed for detection of autologous gamma globulin and C3 except that the antisera were rabbit anti-rat gamma globulin - FITC, and rabbit anti-rat BIC - FITC, respectively. The slides were examined with a Reichert Fluorpan Microscope (Reichert, Austria) using an Osram HBO 50 mercury vapor lamp with a bluepassing 3 mm BG 12 exciter filter and a UV-blue absorption 1.5 mm OG 1 + 1 mm GG 9 blocking filter.

L. ELECTRON MICROSCOPY

1. Materials

a. Sorenson's Phosphate Buffer

I 0.076 M Na H_2 PO_4 $H_2$0 - 10.488 G to 1,000cc with dist. $H_2$0

II 0.324 M Na_2 HPO_4 - 46.001 G to 1,000cc with dist. $H_2$0

b. 2% Glutaraldehyde Fixative pH 7.5

10cc I Na H_2 PO_4
10cc II Na_2 H PO_4 Sorenson's phosphate buffer
10cc of 25% glutaraldehyde, pH 7.5 (Fisher)
94cc of Distilled Water

This results in a buffered solution of pH 7.4 and an osmolarity of 400 milliosmoles. The solution is stored at 4°C.

c. Stock Veronol Acetate Buffer

Na Veronol (Sod. Barbital) 14.714 G

Na Acetate .3 H_2O 9.714 G

Dilute to 500 cc with distilled water.

d. Osmic Acid and Sucrose - Break one gram vial of Osmic acid (Fisher) inside a stoppered brown glass bottle, add 10 cc stock Veronol acetate buffer, then make up to pH 7.5 with approximately 10 cc of OIN hydrochloric acid, then add distilled water to 50 cc. Add 2.25 G of sucrose to the 50 cc solution and store at 4°C.

e. Epon Mixture - The epoxy equivalent of the epon 812 was 157 and from this the volume of the anlysoides to be added was calculated

Solution A - 146 ml DDSA/100 ml of Epon

Solution B - 79 ml NMA/100 ml of Epon

A 50:50 mixture of solution A and Solution B was used. A catalyst DMP-30 to speed the reaction was added to the resin mixture on a 2% volume-to-volume basis.

DDSA - Dodecenyl succense anhydride

NMA - Nadic methyl anhydride (both obtained from Fisher Laboratories, Montreal)

DMP-30 - Rohm & Hoas Co., Washington Square, Philadelphia, Pa.

2. Dehydration and Embedding - Tissues upon which ultrastructural studies were to be made were obtained fresh, cut into small blocks of approximately 1 mm square, then placed in 2% buffered glutaraldehyde (Fisher, Montreal) for 2 hours at room temperature. The pieces were then rinsed in three changes of Sorenson's Phosphate Buffer for approximately 15 minutes. They were then post-fixed for two hours in 2% osmic acid, rinsed again in distilled water x 3 for 15 minutes, then dehydrated in graded ethyl alcohols increasing from 40% to 100%, each, for 15 to 20 minutes x 3. The tissue was then immersed in 2 changes of propylene oxide (British Drug House)

for 15 minutes each, the change of propylene oxide poured off and 1 to 2 cc of fresh propylene oxide and an equal volume of freshly catalyzed Epon resin (Epon 812 - Shell Chemical Company, New York 17, N.Y.) added for 1/2 hour. The tissue was then placed in fresh catalyzed resin mixture for 1 to 2 hours or overnight, then placed in No. 2 gelatin capsules filled with catalyzed resin. The resin was hardened overnight at 37°C, the next day at 45°C and for 2 to 3 days at 60°C. They could be cut upon the next day.

3. Cutting and Staining - The specimens were trimmed and sections cut with glass knives, using a Reichert OM U2 ultramicrotome. To determine the positions of glomeruli within a particular block, a 1 U section was cut, flattened onto a glass slide by heating, stained with 1% tolidine blue in 1% sodium borate solution for 20 minutes, then examined in a light microscope. The block was then trimmed to the desired area and sections approximately 800A thick were cut and collected on 300-mesh copper grids (Fisher). These sections were then stained by immers-

ing the grids in a saturated absolute ethyl alcohol solution of uranyl acetate (Fisher) for 5 minutes followed by rinses in 50% ethyl alcohol and CO_2-free distilled water, then placed in a 0.1% lead citrate solution (K. and K. Laboratories, Inc,, Plainview, N.Y.) in a CO_2-free atmosphere for 5 minutes. The sections were then thoroughly washed in three changes of CO_2-free distilled water and dried on filter paper.

The sections were examined in a JEM T7 electron microscope (Japan Electron Optics Laboratory Co. Ltd., Japan). Pictures were taken using Kodak contrast projector slide plates.

M. ARTHUS REACTION

Two groups of Lewis rats were used, one prenephrotic and the other nephrotic. Prenephrotic animals are those which have had the full series of injections and had mild proteinuria. They did not have proteinuria greater than 15 mg%. An area on the

posterior flank was shaved and the skin cleaned with 70% alcohol. Then 0.1 cc of rabbit kidney in a concentration of 2.5 mg/ml was injected intradermally into the skin of both groups of animals. As controls, saline was injected at one site, and 0.1 cc of ultrasonically disrupted Mycobacterium Tuberculosum 37 Ra, into another at 2.5 mg/ml concentration. The sites were examined at 30 minutes, 2-4 hours, 24 and 48 hours, and induration measured. Skin bippsies were taken at 2 to 4 hours and part was snap frozen for fluorescence microscopy and the other part fixed in buffered formalin for light microscopic examination.

In a second experiment, the response to isogeneic and xenogeneic kidney was compared, nephrotic rats were challenged intradermally at two different sites with xenogenic and isogeneic kidney and the reactions at 30 min., 2 to 4 hours, 24 and 48 hours recorded.

N. CIRCULATING ANTIBODIES

The sera of nephrotic animals were evaluated for antibody production by double diffusion in agar. The animals were bled during the induction phase of the disease and again when they were nephrotic. Sera were checked for antibodies to xenogeneic and isogeneic kidney and to Mycobacterium Tuberculosum.

O. CIRCULATING ANTIGEN

This disease is believed to be due to the deposition of antigen-antibody complexes, then possibly there was release into the circulation of endogenous antigen from the host's kidney.

1. An attempt was made to determine whether autologous antigen (tubular antigen) was released during the disease. This was assessed by double diffusion in agar using rabbit anti-Lewis rat FIA

antiserum. Nephrotic rats were bled by intracardiac puncture several times both before and after they became nephrotic and the serum evaluated for the presence of circulating antigen.

2. A nephrotic animal suspected of having autologous circulating antigen was bilaterally nephrectomized. The rat was then injected intravenously with 1 cc of potent rabbit anti-Lewis rat FIA. The rat was bled at 30 minutes, 1 hour, 4 hours, 16, 24 and 48 hours and the serum checked for antigen concentration utilizing the agar gel method.

A nephrotic serum suspected of containing antigen was absorbed with rabbit anti-rat FIA in equal volumes and the suspension centrifuged at 10,000 x G for 30 minutes. The serum was then

evaluated for the presence of or decrease in antigen concentration in the serum by agar gel diffusion.

P. TISSUE CULTURE

1. Materials and Methods - Figure II - The whole procedure was done with sterile technique. Neonatal Lewis kidneys were decapsulated and cut into small pieces on a petri dish. Medium 199 was added and then poured into a baffled Erlenmeyer flask containing a magnet. The medium was decanted and the sediment was washed with Medium 199 till no more fat or fibrous tissue floated to the surface. The sediment was washed with non-citrated trypsin at 37°C (0.25% of 1.250 Difco Labs) for about 20 to 30 minutes and decanted. Then 10 to 15 ml of non-citrated trypsin per gram of tissue was added, and the mixture was stirred slowly for 1-1/2 hours at room temperature. The heavy particles were allowed to settle and the suspended cells were decanted and then spun down at

1000 x G for 5 minutes. The supernatant was discarded and the cells were resuspended in Medium 199 with 10% fetal calf serum and left at 4°C overnight at a concentration of 50 ml/gram of original kidney. The heavy fragments were stirred overnight with more trypsin, at 4°C, with 10% fetal calf serum. The following morning the suspension was centrifuged at 1000 x G rpm for five minutes and the sediment added to the first suspension. The cells were then counted in a Neubach hemocytometer. A trypan blue exclusion (viability) test was done using 0.4 cc of cells and 0.1 cc trypan blue and read before 15 minutes. The viability was calculated by the following formula:

$$\frac{\text{No. of living - No. dead}}{\text{Total No. Cells}} \times 5/4 \times 100\% = \% \text{ viability}$$

Following this procedure viability approached 90 - 95% on repeated experiments.

The cells were then adjusted to a final concentration of 600,000/cc with medium 199, 10% fetal calf serum, penicillin and streptomycin/(100 U/cc of medium). The cell suspension was then innoculated into Bellco tissue culture tubes, each tube containing 1.5 cc. The caps were screwed tightly and the cells allowed to grow at 37°C for 3 to 5 days till partial monolayer confluency was achieved. At this stage the monolayers were used for the assay.

Lymphocytes from rats and guinea pigs were obtained by intracardiac puncture. The syringes were heparinized with 0.5 cc of 1 to 10,000 aqueous heparin (Riker Labs, Montreal). The blood was collected under clean, but not sterile conditions. The blood was placed in sterile plastic tubes (Falcon) and allowed to sediment at a 45 degree angle at 37°C for one hour. The buffy coat and plasma were aspirated and washed with medium 199 for five washes. After each wash the cells were centrifuged at 75 x G for

six minutes and the supernatant discarded. Following the final wash the lymphocytes were counted and a trypan blue dye exclusion test done. To each tube, 5 to 10 million lymphocytes were added. Where indicated phytohemagglutinin M was added (PHA M - Difco). Each vial contained 10 mg of dessicated PHA M powder and was solubilized by the addition of 5 cc of medium 199. Where indicated, 0.05 cc of this was added to tubes.

Following the addition of the lymphocytes, the cultures were examined at 18, 24 and 48 hours. For cultures examined only at 48 hours, medium was changed after 24 hours. At the specified time the coverslip, upon which was a partial kidney monolayer, was removed from the tube and air dried, and strained by the May Grunwald Giemsa method. The coverslips were then inverted onto regular slides and examined for morphologic changes.

At attempt to quantitate monolayer destruction by labelling with $NaCr^{51}$ (Charles E. Frosst, Montreal)

was unsuccessful. The difficulties initially were due to lack of viability of the monolayer once labelled.

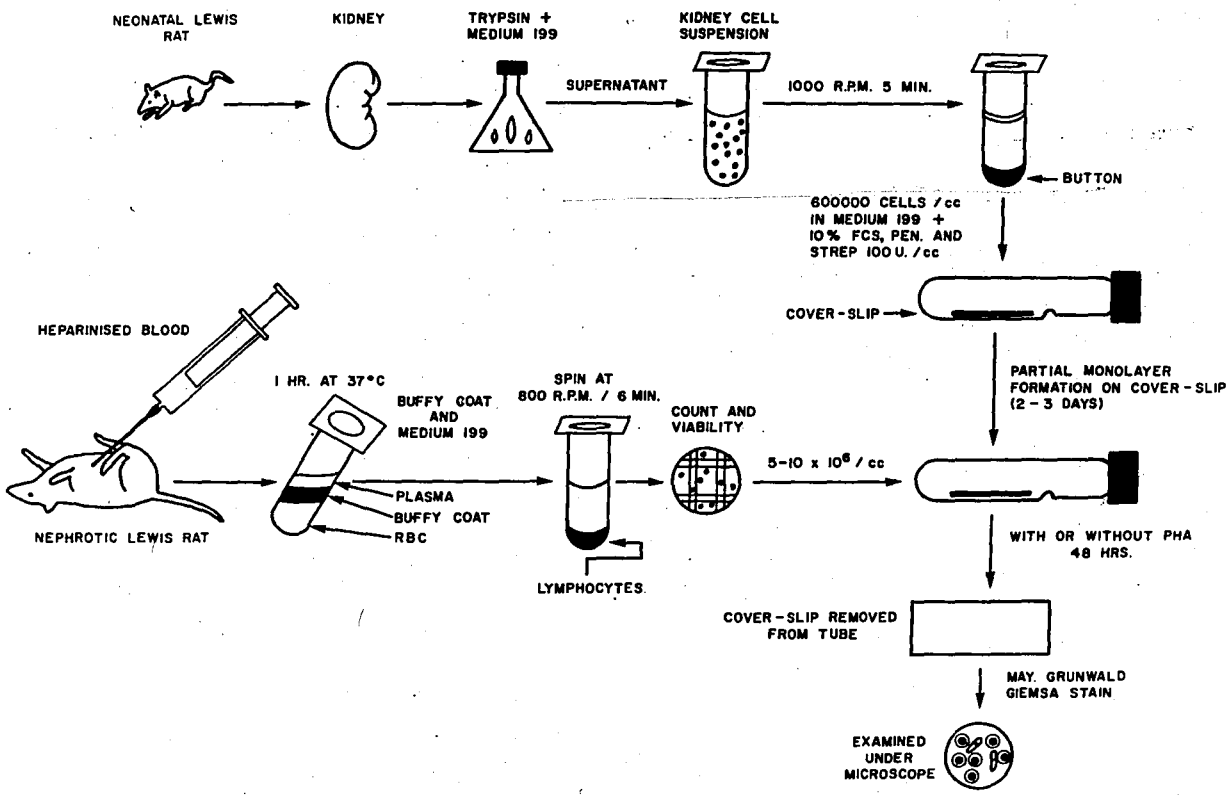
NEONATAL LEWIS RAT
KIDNEY
TRYPSIN + MEDIUM 199
SUPERNATANT
KIDNEY CELL SUSPENSION
1000 R.P.M. 5 MIN.
BUTTON
600000 CELLS / cc IN MEDIUM 199 + 10% FCS, PEN. AND STREP 100 U. / cc
COVER-SLIP
PARTIAL MONOLAYER FORMATION ON COVER-SLIP (2 - 3 DAYS)
HEPARINISED BLOOD
1 HR. AT 37°C
BUFFY COAT AND MEDIUM 199
SPIN AT 800 R.P.M. / 6 MIN.
COUNT AND VIABILITY
5-10 x 10⁶ / cc
NEPHROTIC LEWIS RAT
PLASMA
BUFFY COAT
RBC
LYMPHOCYTES
WITH OR WITHOUT PHA 48 HRS.
COVER-SLIP REMOVED FROM TUBE
MAY. GRUNWALD GIEMSA STAIN
EXAMINED UNDER MICROSCOPE

FIGURE II

2. Assay of Tissue Culture Supernatant - The supernatants from cytotoxic cultures were saved and appropriately pooled. Supernatants from monolayers to which isogeneic lymphocytes from nephrotic animals had been added were pooled separately, as were the supernatants from monolayer cultures which had received allogeneic lymphocytes from normal animals. These pooled supernatants were assayed for antibody and antigen by double diffusion in agar. The supernatants were concentrated ten times by pervaporation.

Q. IN VIVO CYTOTOXICITY OF LYMPHOCYTES

Preliminary experiments were performed using nephrotic Lewis rats. The animals were sacrificed by ether anesthesia and exsanguination by intracardiac puncture. The blood was collected in a heparinized syringe and allowed to stand for one hour at 37°C at a 45-degree angle. The spleen and lymph nodes were excised using a clean technique and placed in a petri dish containing 3 to 5 cc of Medium 199

with 100 U/cc penicillin and streptomycin. The spleen and lymph nodes were cut into small pieces and then passed through a 1mm stainless steel mesh with the use of a metal spatula. The mesh was rinsed with medium 199, and the resultant suspension was spun at 1000 x G rpm for ten minutes. The supernatant was discarded and the cells, predominantly lymphocytes, were washed x 2 with medium 199. Following the final wash, the cells were resuspended and viability and cell counts were made. The final volume was adjusted to approximately 50 x 10^6/0.1 or 0.2 cc.

Normal Lewis rats, with normal urine protein excretion, were anesthetized and shaved. A left lateral incision was made and the left kidney isolated. With a 30 gauge needle, a subcapsular innoculation of cells with medium was made. The kidney was returned to the abdomen and the incision sutured with 3-0 dermalon. Seven days later protein excretion

was measured, the animals were sacrificed and both kidneys and spleen removed and weighed. Part of the recipient kidney was snap frozen for fluorescence microscopic study, and an attempt to demonstrate gamma globulin and complement deposition was made. The remaining tissue was fixed in buffered formulin and stained with hematoxalin and eosin, P.A.S. and methyl green pyronin.

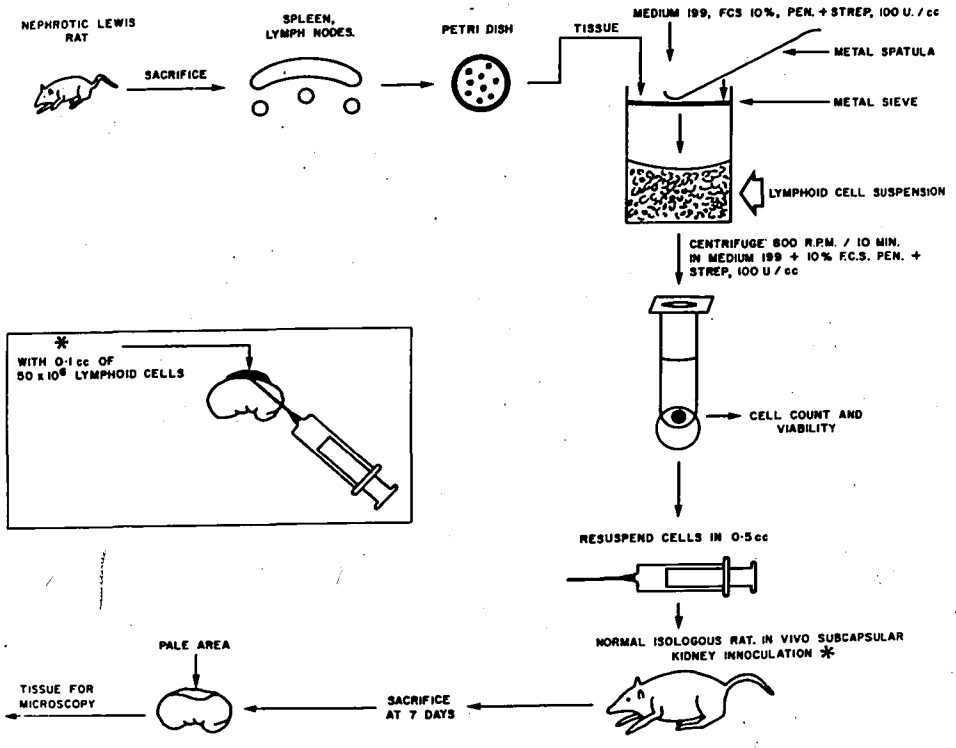

FIGURE III

IV. EXPERIMENTAL RESULTS

A. NEPHRITIS

1. Disease

a. Induction - This experiment was performed in an attempt to induce nephritis with a xenogeneic antigen and to localize the site of antigen deposition. The animals were anesthetized with ether and injections given intraperitoneally as described in Section F of Materials and Methods.

Figure IV demonstrates the relative onset of proteinuria in four groups of non-splenectomized rats and in one group which was splenectomized. Two points should be noted. The onset of proteinuria in the non-splenectomized animals began anywhere from 78 days to 125 days after the first injection, with a mean onset of 104 days for the four groups. The second point was that the degree of proteinuria was never extremely heavy, usually less than 100 mg per 24 hours; with one

exception when it was very heavy, up to 260 mg/24 hours. As was seen groups 3 and 4 required 12 injections compared to 8 in the later experiment. The former two groups received the non-dialyzed, frozen supernatant, whereas the latter groups received a more concentrated and purer kidney fraction.

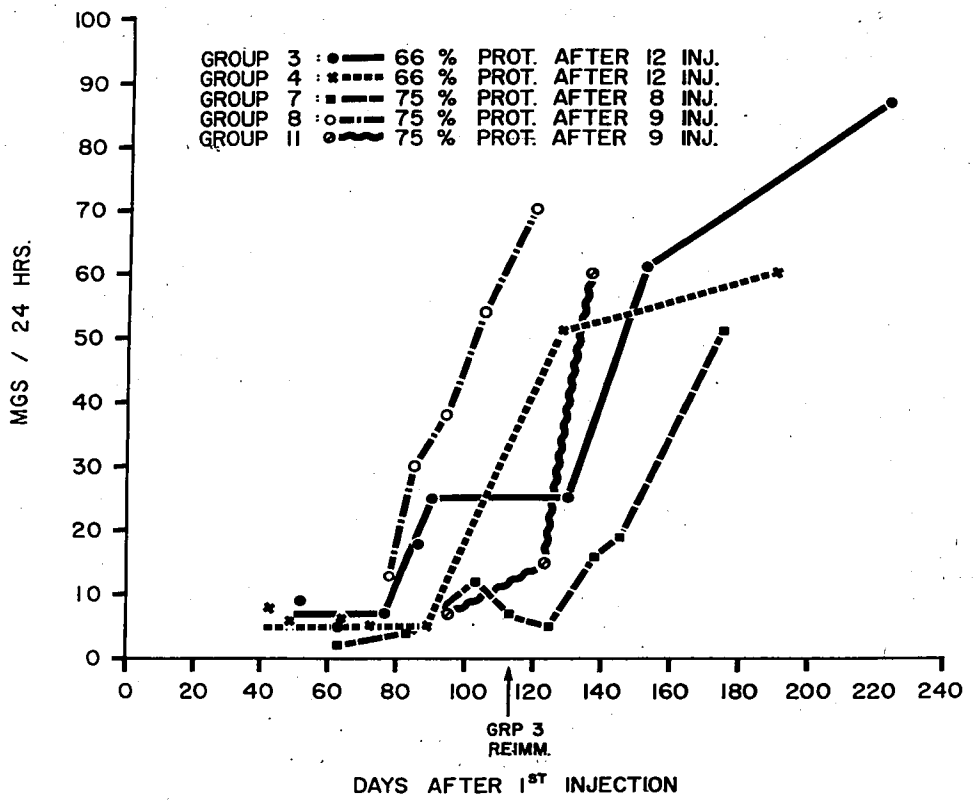

FIGURE IV

Graph relating onset of disease in splenectomised and non-splenectomised Lewis rats. Groups 3, 4, 7, and 11 are non-splenectomised.

b. Accentuation - Having induced the disease with the xenogeneic antigen, we attempted to increase the severity of the disease and advance its onset.

Seventeen rats were anesthetized and splenectomized, as described in Section D (2) of Materials and Methods. The first group of ten animals all died, possibly because of immaturity with increased risk of infection. The subsequent group of 17, a little more mature, all survived.

As can be seen from Figure IV, though the disease incidence was not greater than in the intact group, two significant observations were made. Firstly, once the disease began it was quite fulminant in character. Secondly, comparing groups 8 and 11, it was seen that in the splenectomized group (8), the disease onset was six weeks earlier.

2. Pathology -

a. Light Microscopy - Kidneys from nephrotic animals appeared slightly enlarged on gross examina-

tion. When examined by light microscopy there was no increase in cellularity of the glomerular tuft, no synechae or crescent formation. There was moderate to marked thickening of the glomerular basement membrane seen best with the periodic-acid-Schiff stain (Figure V). There was an occasional P.A.S. positive case in the tubular lumen. The proximal tubular cells had lost the P.A.S. positive brush border (Figure VI).

FIGURE V

Nephrotic Lewis rat demonstrating glomerular basement membrane thickening (HE x 650).

FIGUPE V

Nephrotic Lewis rat demonstrating glomerular basement membrane thickening (HE x 650).

FIGURE VI

Proximal renal tubular cell, demonstrating decreased brush border staining (P.A.S. Stain x 860)

b. Fluorescence Microscopy - More obvious abnormalities were seen when the kidneys were examined by fluorescence microscopy. In the pre-nephrotic animals no deposition of gamma globulin, complement, or antigen could be detected within glomerulae. When the kidneys of nephrotic animals were examined, a heavy granular deposition of host gamma globulin and complement could be seen (Figure VII). Figure VIII illustrates a high power view of a glomerular loop. When kidney sections were examined for xenogeneic antigen, a similar granular pattern was noted (Figure IX). Similar staining for isogeneic antigen was achieved. However, it was very faint and patchy.

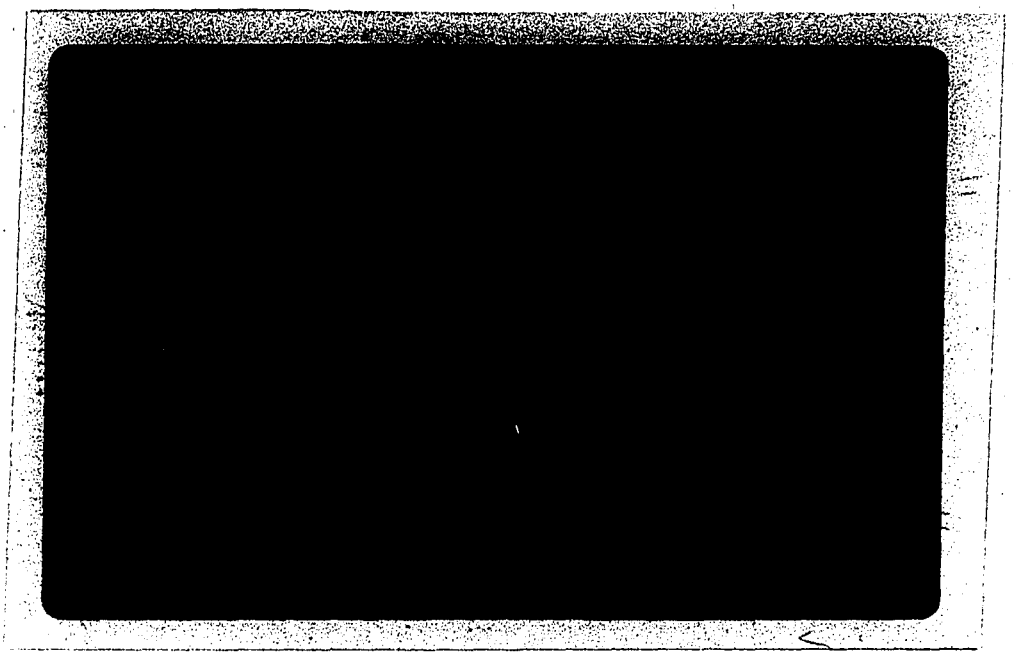

FIGURE VII

Nephrotic Lewis rat kidney stained with fluoresceinated rabbit anti-rat gamma globulin. (Mag x 380)

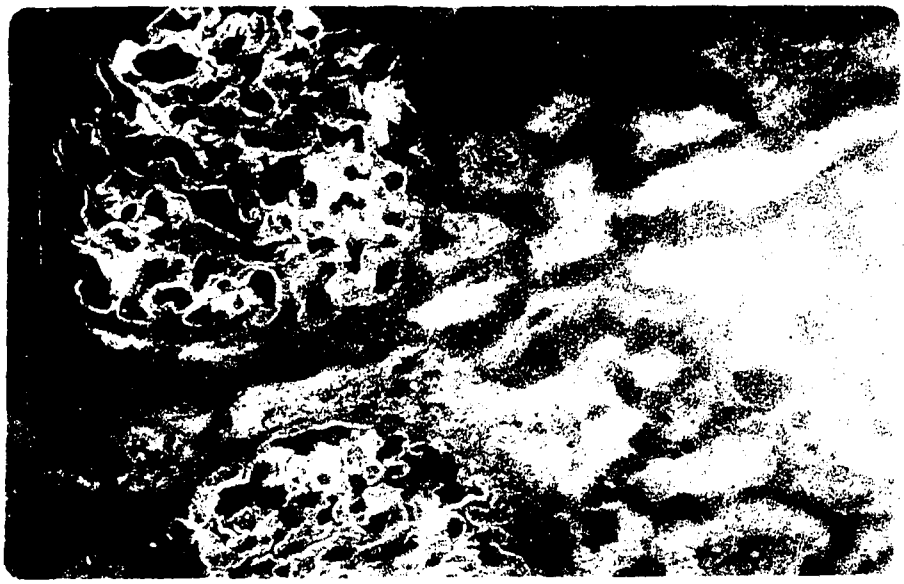

FIGURE VII

Nephrotic Lewis rat kidney stained with fluoresceinated rabbit anti-rat gamma globulin. (Mag x 380)

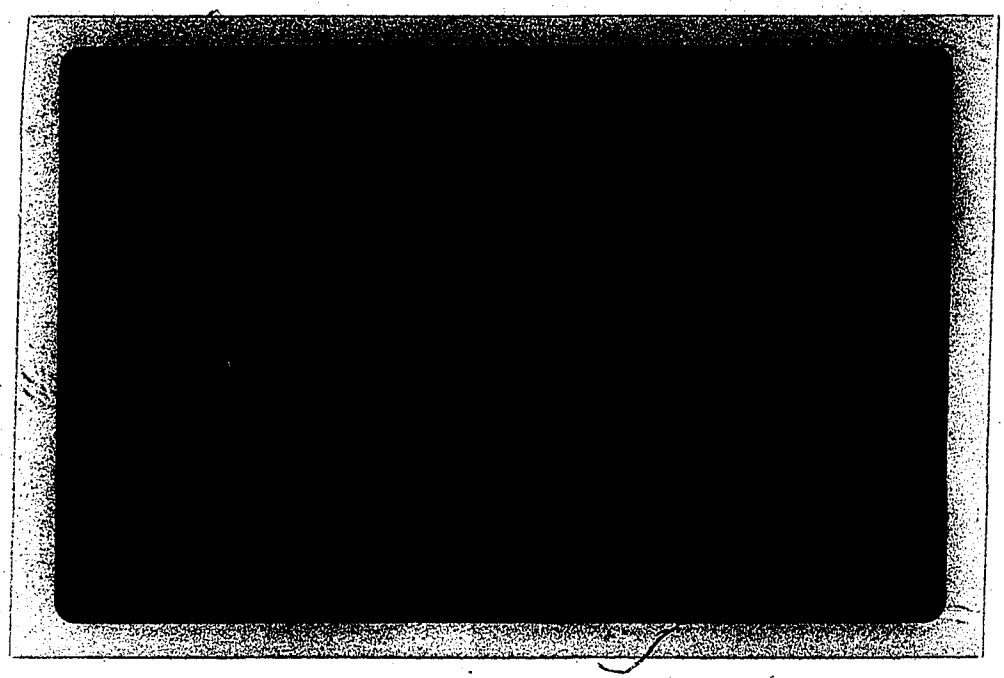

FIGURE VIII

High power view of nephrotic Lewis rat glomerulus stained with fluoresceinated rabbit anti-rat gamma globulin. Note the granular pattern seen along the glomerular basement membrane. (x 860)

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High power view of nephrotic Lewis rat glomerulus stained with fluoresceinated rabbit anti-rat gamma globulin. Note the granular pattern seen along the glomerular basement membrane. (x 860)

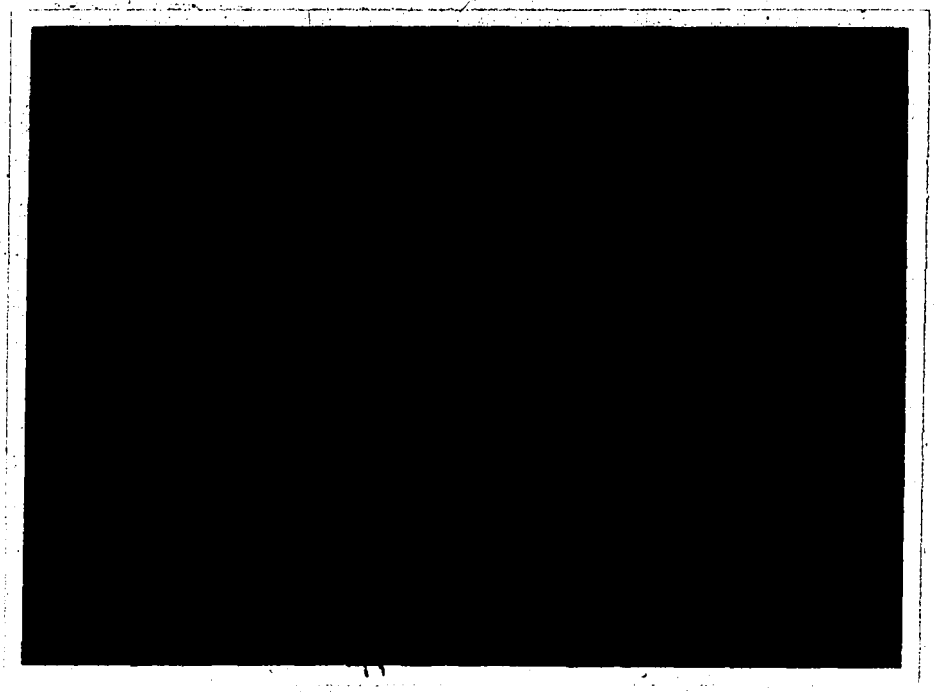

FIGURE IX

Nephrotic Lewis rat kidney glomerulus demonstrating the deposition of xenogeneic antigen by indirect immunofluorescence. (x 560)

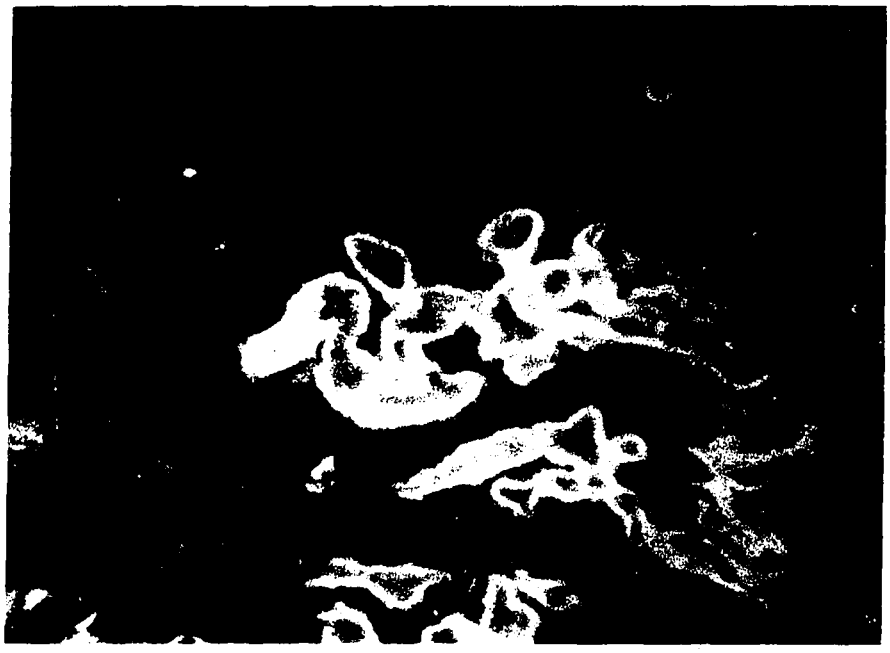

FIGURE IX

Nephrotic Lewis rat kidnev glomerulus demonstrating the deposition of xenogeneic antigen by indirect immunofluorescence. (x 560)

c. Ultrastructural examination of pre-nephrotic glomeruli revealed occasional focal areas of thickened basement membrane. No clear granular deposition was observed, though some areas were very questionable, and it can be seen that the epithelial foot processes were slightly blunted in these areas in contrast to normal. The glomerulus from a nephrotic animal revealed a thickened basement membrane, containing deposits within it and beneath the epithelial cells. There was associated flattening of the epithelial foot processes and loss of definition of the lamina lucida (Figures X, XI, and XII).

FIGURE X

Ultra structural photomicrograph of glomerular basement membrane of prenephrotic Lewis rat. Note slight blunting of epithelial foot processes. There is no evidence of dense deposits within the basement membrane. Note preservation of the lamina densa and lamina lucida. (x 20,000) fp = Epithelial Foot Process; bm = Basement Membrane; bs = Bowman's Space.

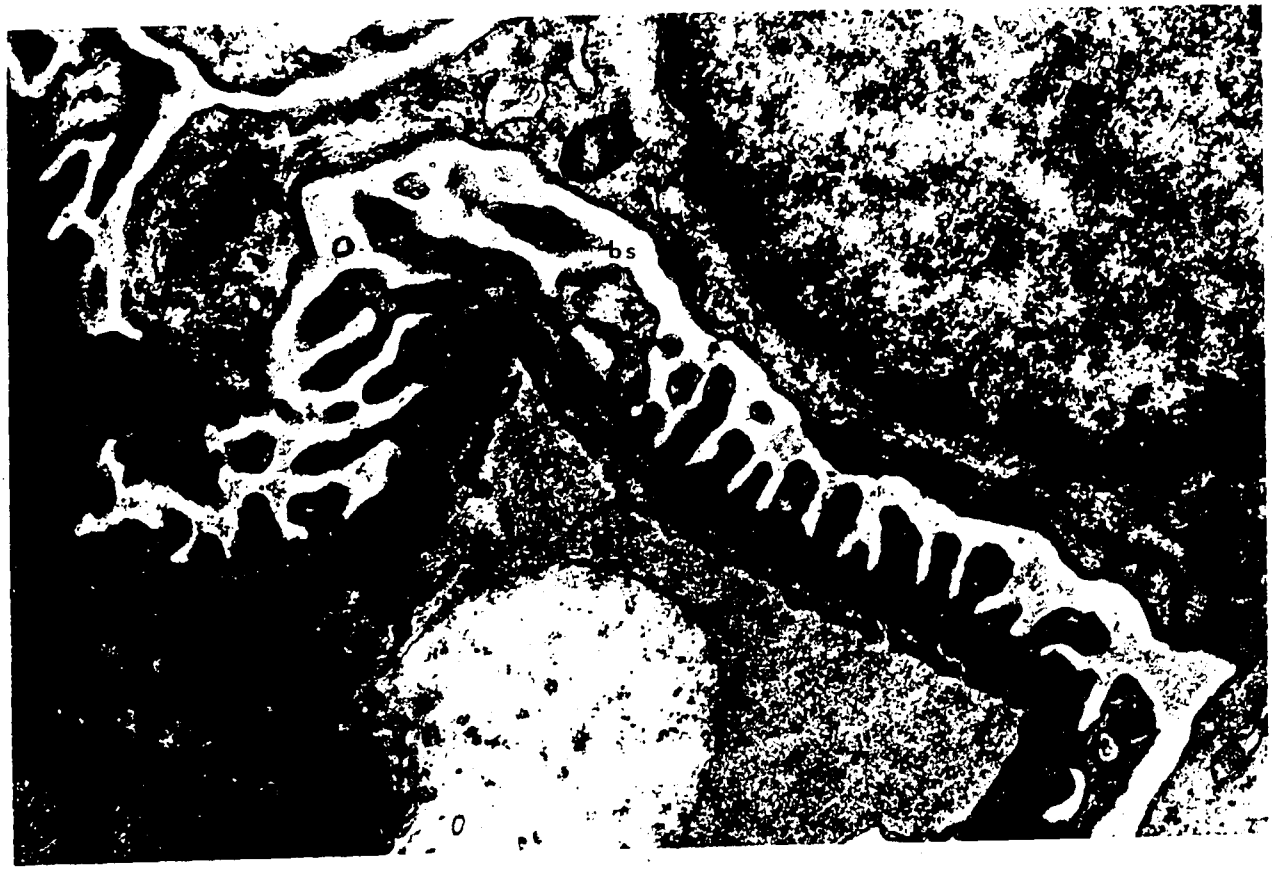

FIGURE X

Ultra structural photomicrograph of glomerular basement membrane of prenephrotic Lewis rat. Note slight blunting of epithelial foot processes. There is no evidence of dense deposits within the basement membrane. Note preservation of the lamina densa and lamina lucida. (x 20,000) fp = Epithelial Foot Process; bm = Basement Membrane; bs = Bowman's Space.

FIGURE XI

Ultrastructural photomicrograph of a nephrotic rat glomerulus. Note the flattening of the epithelial foot processes, the subepithelial dense deposits and thickening of basement membrane. (Mag x 15,000)

EP = Epithelial Cell D = Deposit

ENDO = Endothelial L = Lumen

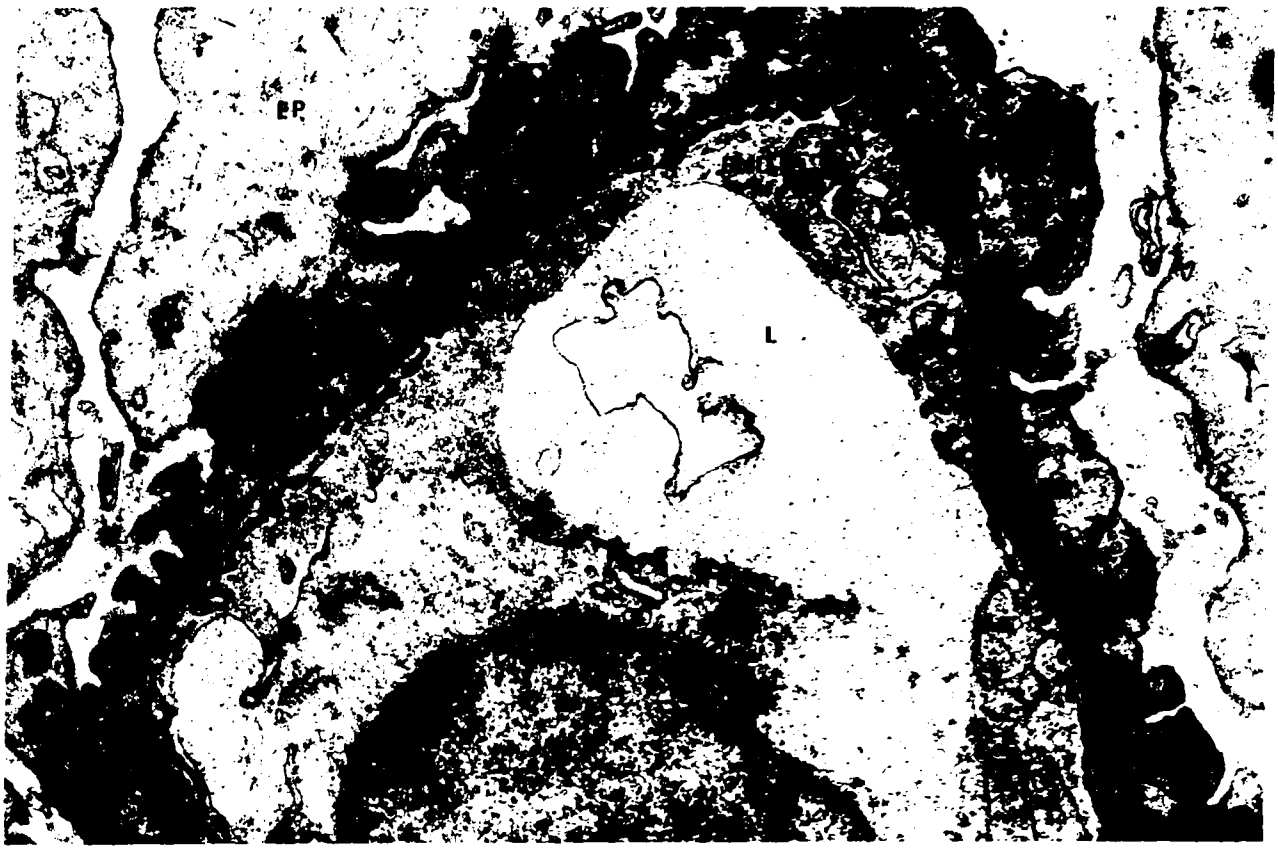

FIGURE XI

Ultrastructural photomicrograph of a nephrotic rat glomerulus. Note the flattening of the epithelial foot processes, the subepithelial dense deposits and thickening of basement membrane. (Mag x 15,000)

EP = Epithelial Cell

ENDO = Endothelial

D = Deposit

L = Lumen

FIGURE XII

High power to show thickening of basement membrane and flattening of the epithelial foot processes.

(Mag x 31,000)

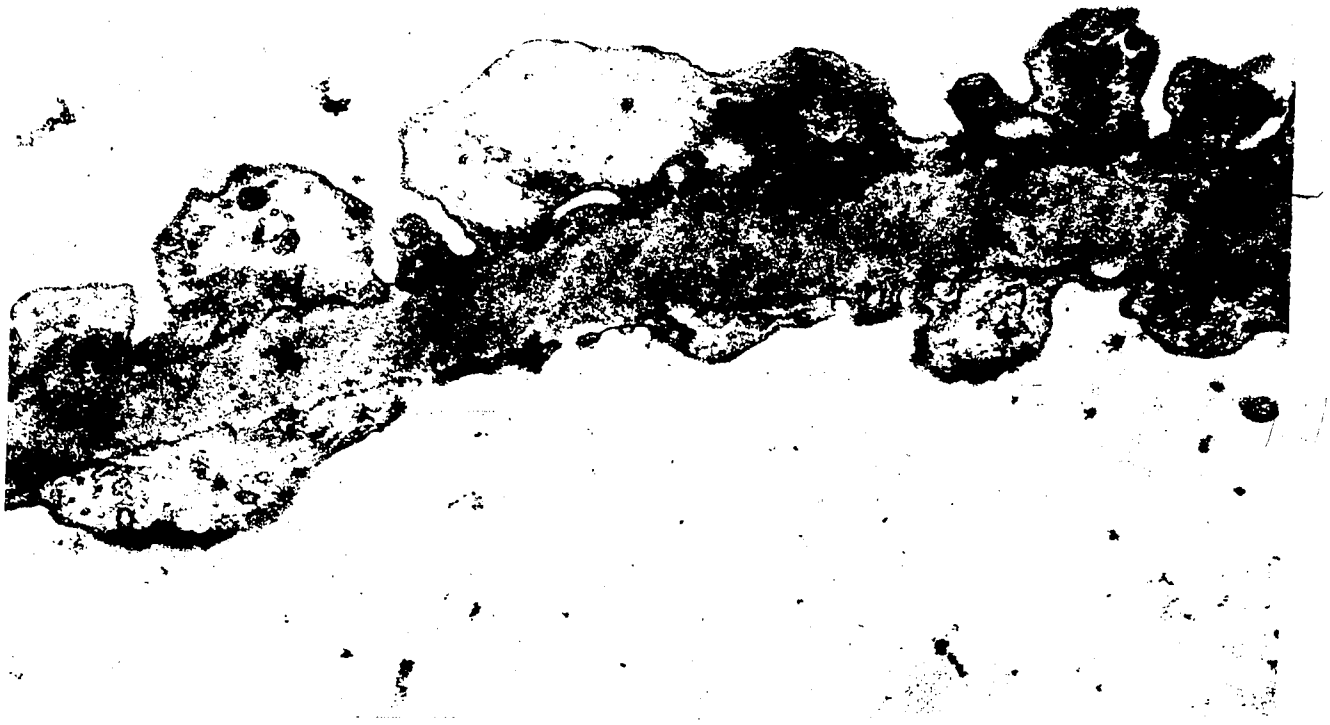

FIGURE XII

High power to show thickening of basement membrane and flattening of the epithelial foot processes. (Mag x 31,000)

3. Hyaline Droplet Formation - Animals from each group had "hyaline granule" deposition by day seven, including Group C. No animal was proteinuric at seven days.

Kidney sections were examined by light microscopy and immunofluorescence. The granules were P.A.S. positive and when stained for fat with oil red-O they were negative. When examined under the fluorescence microscope the granules demonstrated brilliant yellow autofluorescence. When attempting to detect gamma globulin, complement, or antigen within the granules utilizing fluoresceine isothiocyanate conjugated antisera, no change in emission spectrum could be seen. Using a rhodamminated conjugate in order to utilize a different emission spectrum, fluorescence was too weak for any conclusions to be made.

4. Discussion - These immunohistopathological results confirm work from other laboratories that

both xenogeneic and isogeneic tubular antigen, host gamma globulin and complement can be found in a granular pattern along the glomerular basement membrane in AIC nephritis. The ultrastructural changes seen are also compatible with this disease.

The hyaline droplet formation, on the other hand, though confirming what had been seen previously in proteinuric animals, has not been explained. Cup-page correlated glomerular basement membrane changes and proteinuria with resultant secondary proximal tubular brush border changes.

We demonstrated these changes occur prior to the onset of proteinuria. The changes however could be toxic, secondary to complete Freund's adjuvant. If the hyaline droplets in fact contain portions of the brush border (that area which contains the specific tubular antigen responsible in Lewis rats for induction of disease), these may be important in disease pathogenesis.

In this disease model, with the use of exogenous antigen there is release of the autologous antigen from the brush border of the proximal tubule (154). The animal synthesizes antibodies which form soluble complexes; these are then filtered and deposited within the glomerular basement membrane. If the loss of the brush border and the simultaneous appearrance of hyaline droplets within the proximal tubular are related to antigen release, then the hyaline droplet formation seen here may not be a result of, but a cause of, further disease progression in this model.

B. ELUTION EXPERIMENTS

1. Elution to Demonstrate Autologous Tubular Antigen within GBM - In an attempt to demonstrate more clearly the deposition of autologous antigen, potassium thiocyanate elution procedures were undertaken. When utilizing the method of Edgington et al, tissue morphology was not maintained (181).

Accordingly, an experiment was performed to determine optimal time and temperature for the elution step, maintaining adequate tissue morphology and still elute sufficient host gamma globulin, so that antigenic sites were made available. Table I in Materials and Methods illustrates the combinations used. The final time of 30 minutes at 37°C was found to be optimal and the basement membrane remained intact. The post elution tissue is compared to a non-eluted nephrotic glomerulus (Figures XIII and XIV).

On immunofluorescence, autologous antigen was also more easily demonstrable on the eluted kidney sections, though still quite faint (Figure XV).

FIGURE XIII

Nephrotic Lewis rat glomerulus prior to elution procedures. Note thickened basement membrane with intact epithelial and endothelial cells. (P.A.S. x 860)

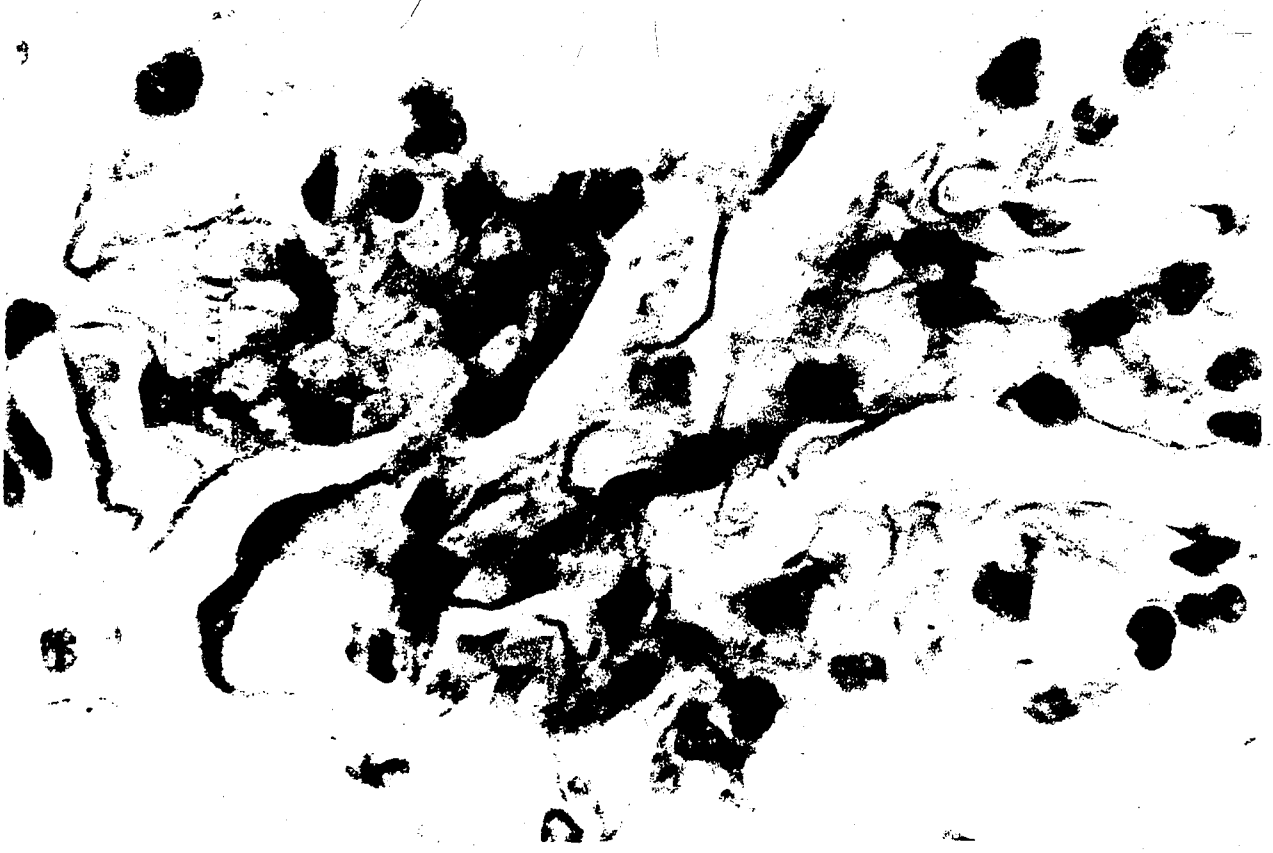

FIGURE XIII

Nephrotic Lewis rat glomerulus prior to elution procedures. Note thickened basement membrane with intact epithelial and endothelial cells. (P.A.S. x 860)

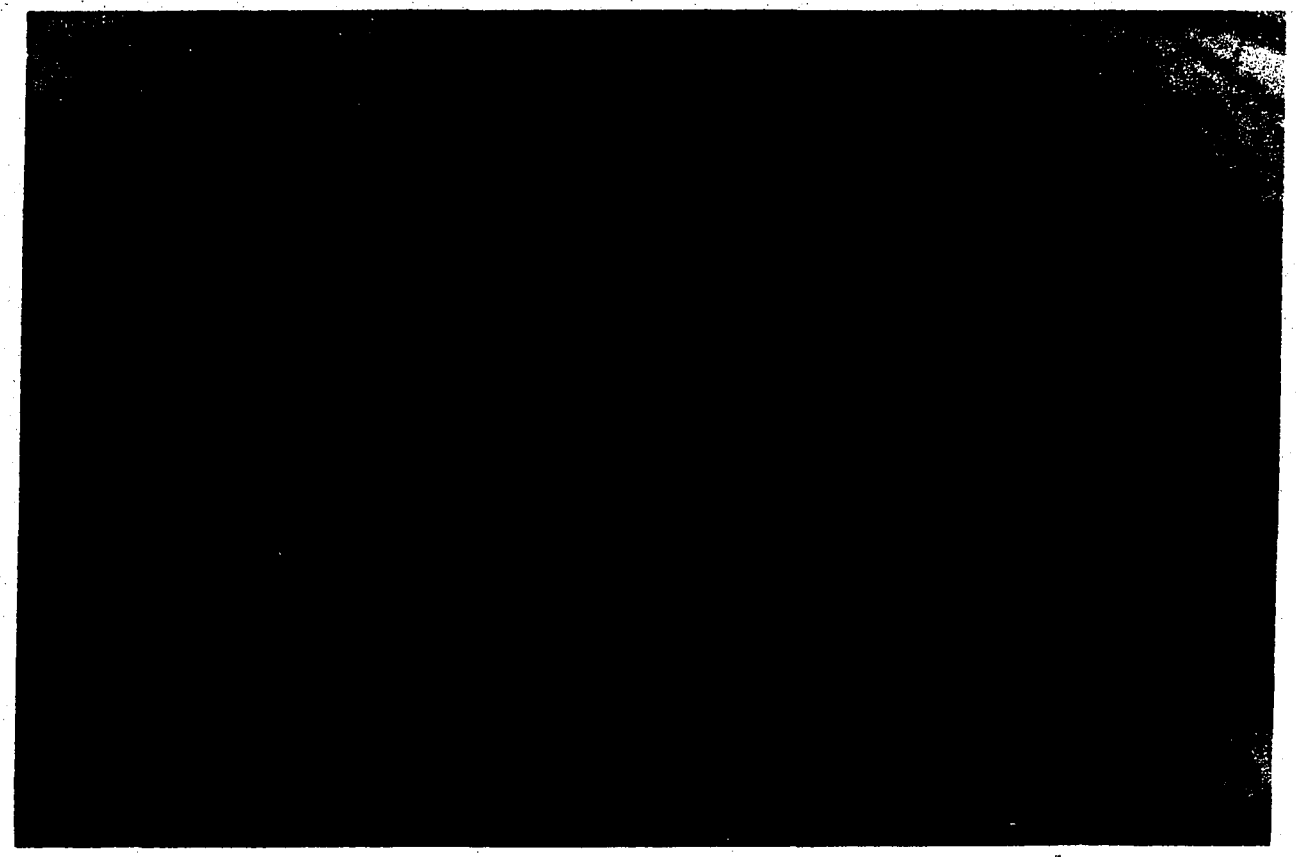

FIGURE XIV

Post elution glomerular basement membrane. Note that the glomerular basement membranes are devoid of epithelial and endothelial cells. (PAS mag x 860)

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Post elution glomerular basement membrane. Note that the glomerular basement membranes are devoid of epithelial and endothelial cells. (PAS mag x 860)

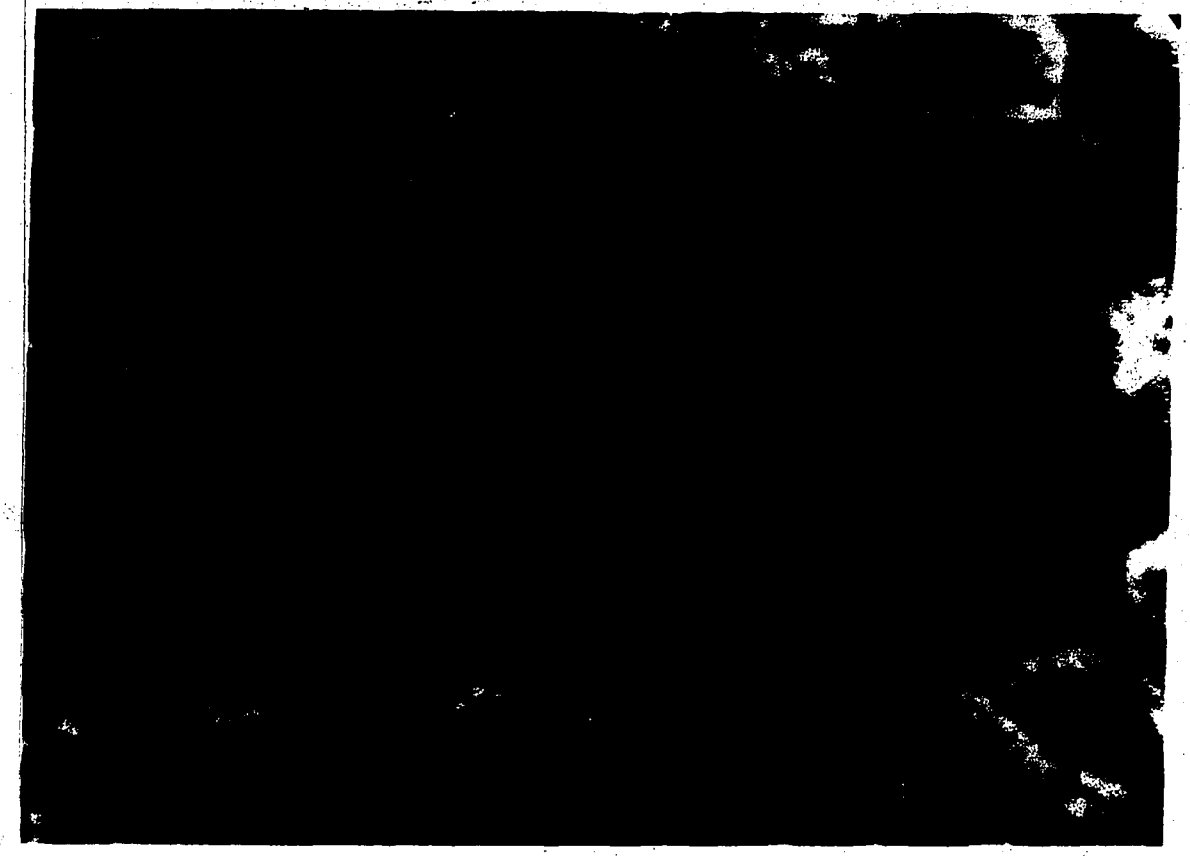

FIGURE XV

Nephrotic Lewis rat kidney demonstrating the granular deposition of autologous tubular antigen within the glomerular basement membrane. (x 650)

2. Elution to Demonstrate Antibody - Sixteen grams of kidney cortex from nephrotic Lewis rats was subjected to the elution procedure as described previously in H (1) of Materials and Methods. The volume of the eluate was 210 cc; 60 cc were per-evaporated to 15 cc, read in a D.U. spectrophotometer at a wave length of 280. The protein concentration was 0.35 mg/ml, resulting in a total yield of 36.4 mg, a yield of 0.23%. Ouchterlony and immunoelectrophoresis analyses were then performed. The eluate was diffused against rabbit anti-rat whole serum. On the Ouchterlony one line was seen and on the immunoelectrophoresis one line was seen migrating in the γ region (Figure XVI).

In an attempt to determine the specificity of the antibody, the eluate was diffused against rat F.I.A. and rabbit supernatant in Ouchterlony. However, no precipitin lines appeared after 48 hours. Negative results were also obtained using two-fold dilutions of the antigens to a dilution of one in 16.

FIGURE XVI

Cellulose acetate electrophoresis demonstrating a single precipitin line migrating in the gamma region from the kidney eluate in alpha. The troughs contain rabbit anti-whole rat antiserum. Normal rat serum was placed in well c.

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Cellulose acetate electrophoresis demonstrating a single precipitin line migrating in the gamma region from the kidney eluate in alpha. The troughs contain rabbit anti-whole rat antiserum. Normal rat serum was placed in well c.

3. In Vivo Assay of Eluate - Four normal Lewis rats were injected intravenously with 1 cc of the eluted gamma globulin (0.35 mg/ml). Proteinuria was checked prior to injection; 4 hours after injection; 24 hours after and 7 days after. Unilateral nephrectomy on the four animals was performed four hours after the intravenous injections, and tissue examined by light and immunofluorescence microscopy.

No animal became oliguric or proteinuric at any time measured. Two of the four kidneys showed moderate interstitial hemorrhage by light microscopy. However, no other tubular nor glomerular changes were seen. Immunofluorescent staining of the tubules for gamma globulin and complement deposition were negative.

4. In Vitro Assay of Eluate - Normal frozen rat kidney sections 4 U thickness were cut on the cryostat in routine fashion. The nephrotic eluate was

divided into 4 aliquots and absorbed as in H (2) of Materials and Methods. Indirect immunofluorescent staining was then performed using goat anti-rat gamma globulin F.I.T.C. as the top layer. Different qualitative staining was seen. Glomeruli were negative. Tubular cells stained brightly with the unabsorbed eluate. There was not, however, any definite accentuation of staining in the area of the brush border. When the eluate was absorbed with the Lewis rat F.I.A., the tubular cytoplasmic staining was considerably less intense and the basement membrane staining persisted. When the rabbit antigen was used to absorb the eluate, there was minimal tubular cytoplasmic staining; however, the tubular cell basement membrane appeared quite bright. When sections were stained with eluate that had been absorbed with both rabbit kidney antigen and rat kidney F.I.A., faint tubular basement staining only was visible (Figures XVII-XX).

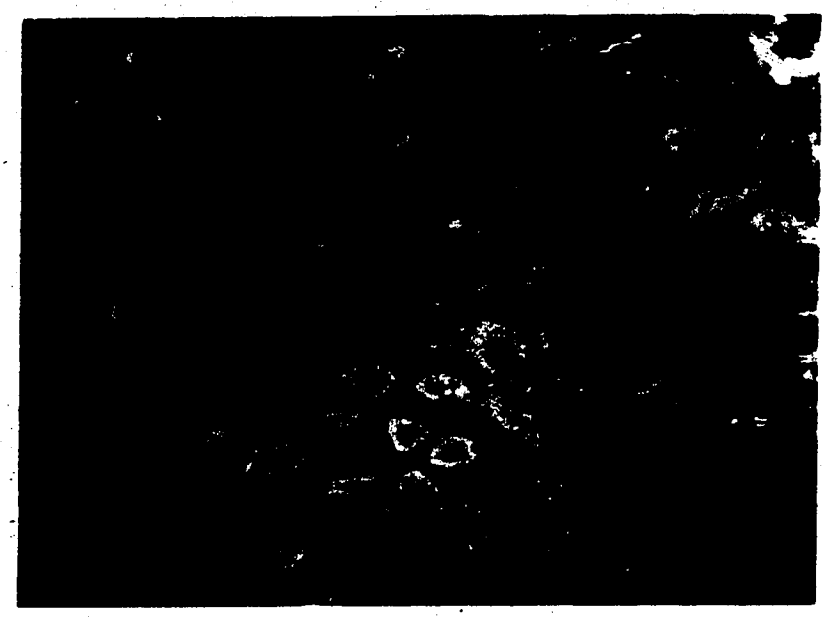

FIGURE XVII

Normal Lewis rat kidney overlayed with nephrotic kidney eluate non-absorbed then overlayed with goat anti-rat gammaglobulin FITC (Figures XVII-XX). Note diffuse tubular staining with absent glomerular basement membrane staining. (x 320)

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Normal Lewis rat kidney overlayed with nephrotic kidney eluate non-absorbed then overlayed with goat anti-rat gammaglobulin FITC (Figures XVII-XX). Note diffuse tubular staining with absent glomerular basement membrane staining. (x 320)

FIGURE XVIII

Eluate absorbed with Lewis rat FIA. Note lack of glomerular staining again. There is less tubular cytoplasmic staining here than in Figure XVII, though some tubular cytoplasmic staining persists. (x 320)

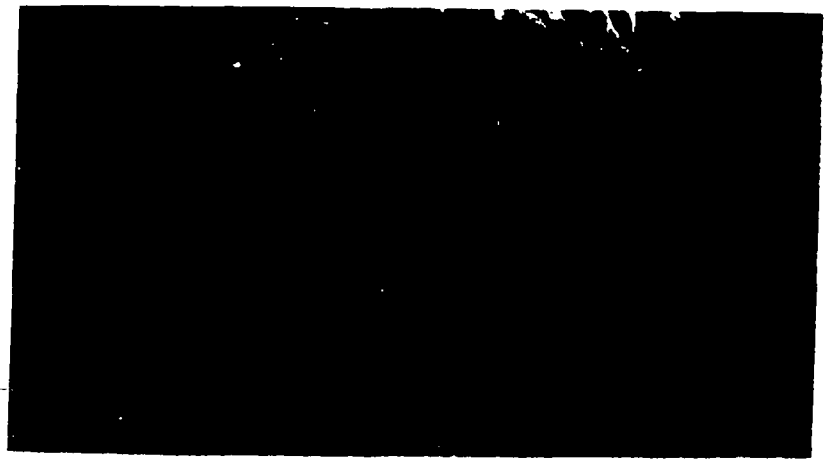

FIGURE XVIII

Eluate absorbed with Lewis rat FIA. Note lack of glomerular staining again. There is less tubular cytoplasmic staining here than in Figure XVII, though some tubular cytoplasmic staining persists. (x 320)

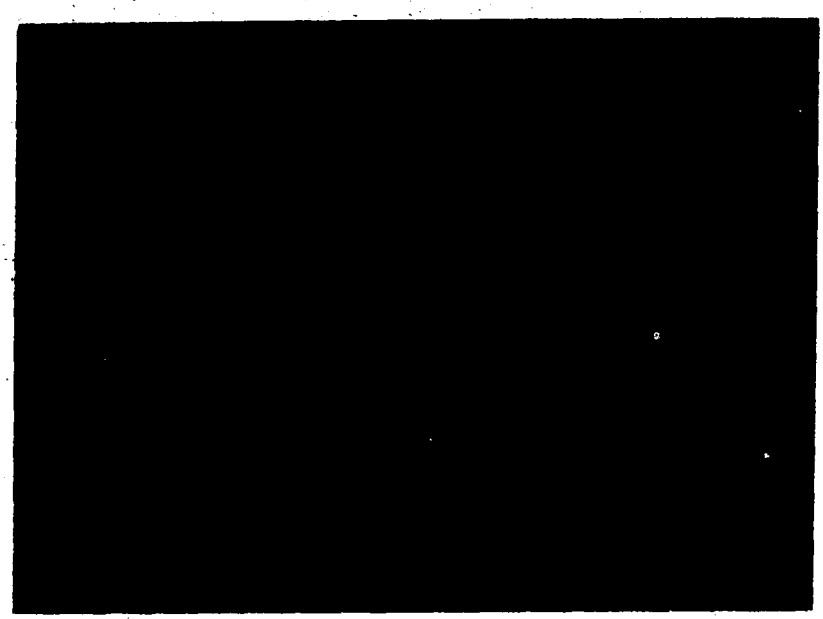

FIGURE XIX

Nephrotic kidney eluate absorbed with crude rabbit antigen. Again tubular staining is less intense, but still evident. (x 320)

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Nephrotic kidney eluate absorbed with crude rabbit antigen. Again tubular staining is less intense, but still evident. (x 320)

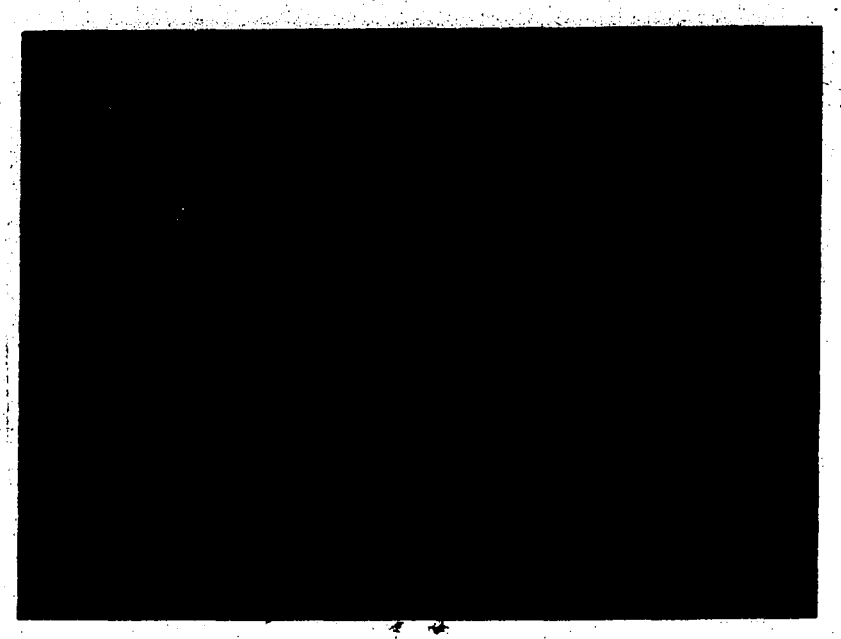

FIGURE XX

Normal Lewis rat kidney overlayed with nephrotic kidney eluate absorbed with both the rat and rabbit kidney antigens. Here we see minimal tubular cytoplasmic staining, the glomerular again remaining unstained. (x 320)

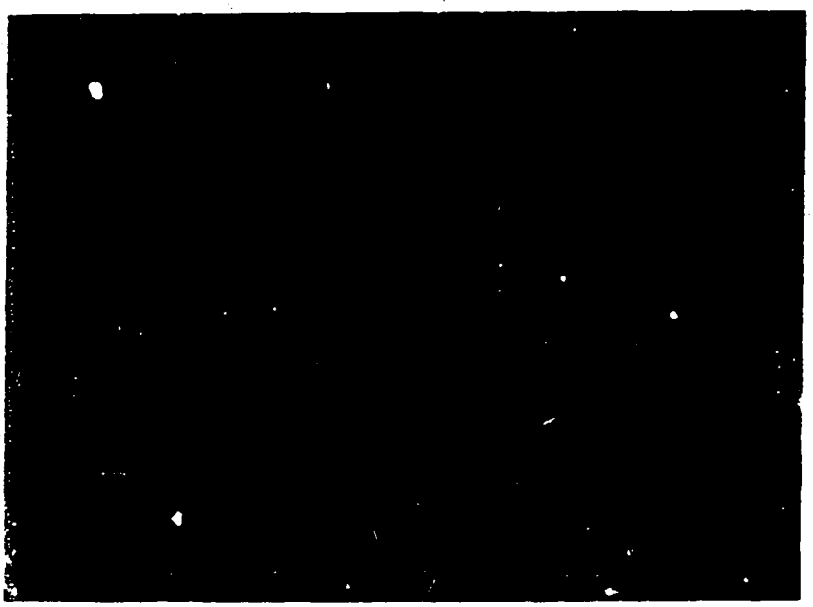

FIGURE XX

Normal Lewis rat kidney overlayed with nephrotic kidney eluate absorbed with both the rat and rabbit kidney antigens. Here we see minimal tubular cytoplasmic staining, the glomerular again remaining unstained. (x 320)

5. Discussion - From previous experiments, it appears in this model xenogeneic antigen is more heavily deposited than is autologous antigen, although both are present along the GBM. From the above experiments, several other points may be made. By differential absorption of the eluted antibody, more antibody directed toward the tubular antigen is removed by absorption with xenogeneic kidney than is with isogeneic kidney. This is in keeping with the quantitative tubular antigen deposition seen earlier in the nephrotic rat glomeruli. Absorption of the eluate with both antigens would be expected to remove all staining; however, minimal staining remained, for which we have no explanation. It should be pointed out that in the in vitro system only questionable brush border accentuation was seen (the site of the nephritogenic antigen). Only in one section was there suggestion of brush border accentuation when stained with unabsorbed eluate. Brush border

accentuation was expected as the immunizing fraction contained the nephritogenic antigen. However, as the rabbit fraction was a crude suspension of soluble and insoluble antigens, the specific antibodies produced were probably masked by the antibodies produced to the other tubular antigens. Since both antigen preparations contained the brush border constituents, brush border accentuation would be abolished following absorptions of the eluate with either antigen.

C. CUTANEOUS MANIFESTATIONS IN AIC

Intradermal skin tests were performed with xenogeneic kidney antigen as in Materials and Methods, Section K. When the kidney sites were examined after 15-30 minutes, there was no reaction. After two to three hours the initial small bleb had been replaced by a markedly indurated and elevated area, often with an area of central hemorrhage (Figure XXI).

This reaction often persisted for 24 hours and by this time it had begun to disappear. No further reaction was noted at 48 hours.

The control site injected with Mycobacterium Tuberculosum had no immediate reaction; however, at 24 to 48 hours an indurated button appeared without central hemorrhage, a delayed cutaneous reaction.

To compare the skin response of the rats to xenogenic and isogeneic kidney, the rabbit antigen and Lewis rat F.I.A. were injected intradermally as previously. Good 2 to 4 hour reactions were again seen to the xenogeneic kidney; no reaction was seen to the isologous kidney (Figure XXII).

Table II summarizes the results of the skin reactions.

Having noted the skin response, the 2 to 4 hour reactions were biopsied. On light microscopy intense oedema was seen throughout the dermis, associated with

a marked polymorphonuclear infiltrate often within the vessel wall.

A high percentage of eosinophils were also seen. It can be noted that even at this early time, namely 2 to 4 hours after the injection, the integrity of the basement membrane had been compromised (Figures XXIII and XXIV).

When examined for the deposition of gamma globulin and C3 by fluorescence microscopy, there was a heavy deposition of both within the vessel wall. A biopsy taken from a site which had been challenged with isogeneic kidney was negative for immune reactants and contrasts to the positive biopsy (Figures XXV and XXVI).

Discussion - In our model of AIC nephritis, delayed hypersensitivity could not be elicited as demonstrated by skin reactivity, even though the rats were sensitized to xenogeneic antigen. The animals did have a delayed skin reaction to Mycobac-

terium Tuberculosum, illustrating that animals were capable of this type of a response.

The demonstration of a typical Arthus reaction with xenogeneic antigen is in keeping with the immediate hypersensitivity pathogenetic mechanisms operative in AIC nephritis, namely one of antigen-antibody complexes rather than that of delayed hypersensitivity. No Arthus reaction could be elicited to the isogeneic kidney. This was expected as we were unable to detect circulating precipitins to this antigen.

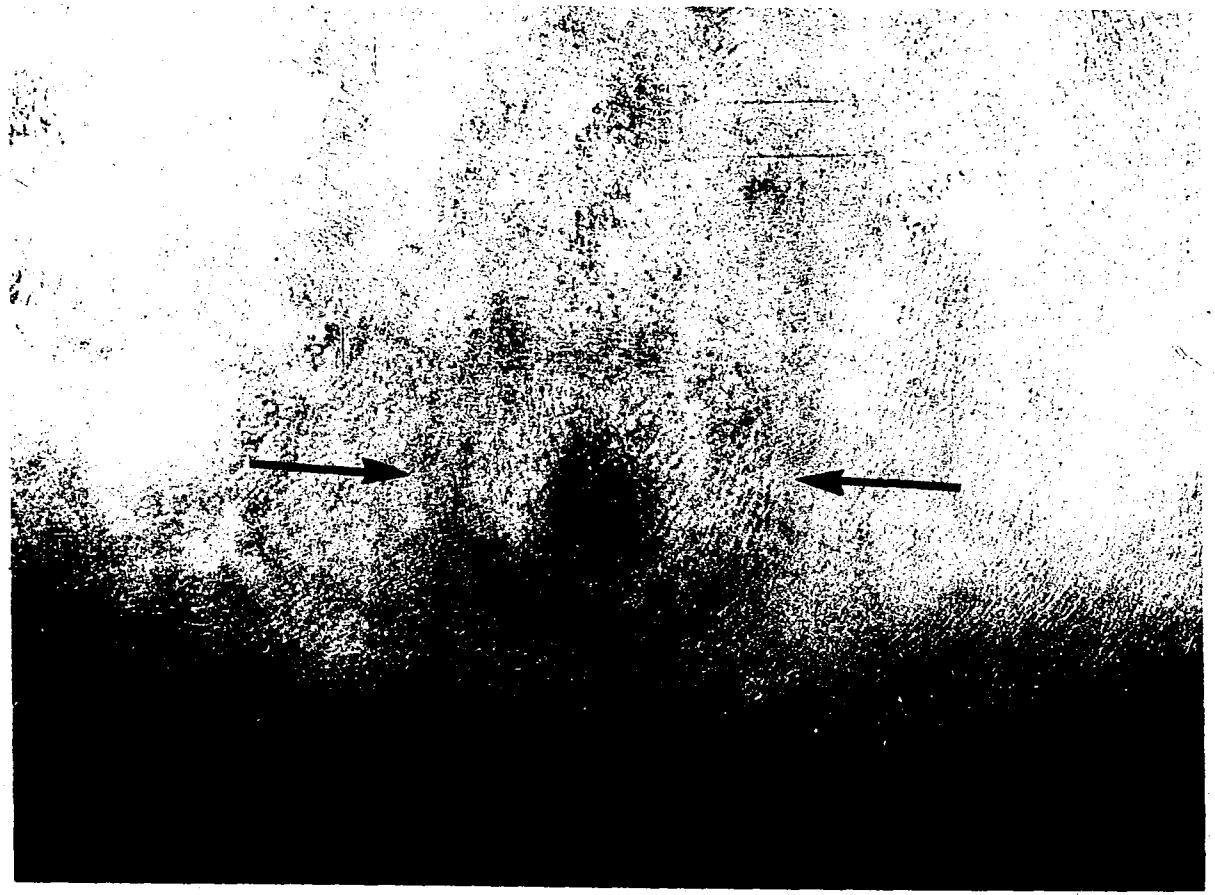

FIGURE XXI

Rat skin site examined at 3 hours following intradermal injection of xenogeneic antigen. Note indurate area with central area of hemorrhage.

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Rat skin site examined at 3 hours following intradermal injection of xenogeneic antigen. Note indurate area with central area of hemorrhage.

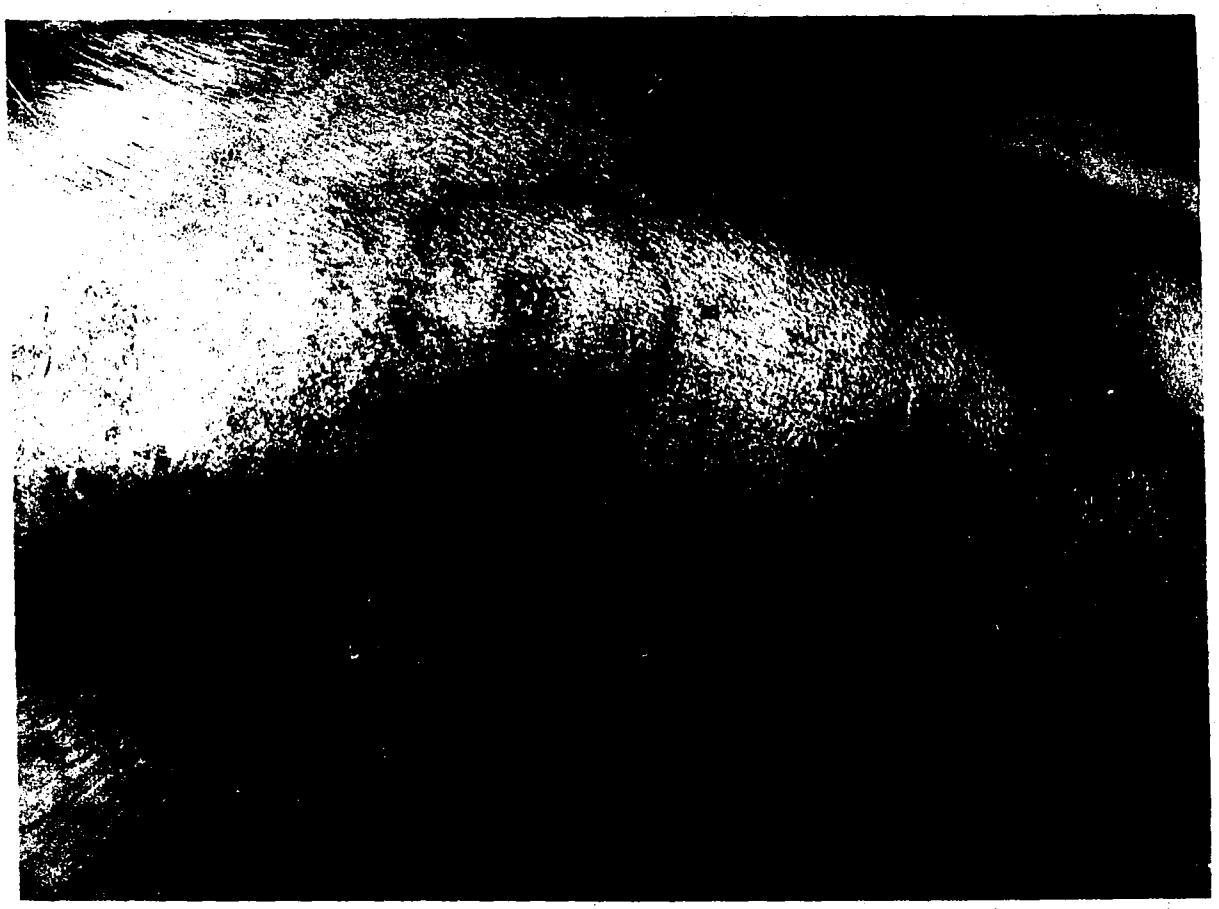


FIGURE XXII

Lewis rat skin test sites at 3 hours. Isogeneic antigen skin test at a. Xenogeneic antigen skin test at b. Note lack of reaction at a and the Arthus reaction at b.

FIGURE XXII

Lewis rat skin test sites at 3 hours. Isogeneic antigen skin test at a. Xenogeneic antigen skin test at b. Note lack of reaction at a and the Arthus reaction at b.

TABLE II

CUTANEOUS MANIFESTATIONS IN AUTOLOGOUS IMMUNE COMPLEX NEPHRITIS

Rat No.	Antigen	Cutaneous Response		
		Immediate (15-30 Min)	Arthus (2-3 Hr)	Delayed (24-48 Hr)
4	Xenogeneic Kid.	Negative	1.9 x 2.2 cm	Negative
	Isogeneic (FIA)	Negative	Negative	Negative
	TBC (Sonicated)	Negative	2 x 2 cm	0.8 x .6 cm
10	Xenogeneic	Negative	1 x .5 cm	Negative
	Isogeneic	Negative	Negative	Negative
	TBC (Sonicated)	Negative	1.5 x 1.3 cm	.3 x .6 cm
19	Xenogeneic	Negative	2.1 x 2.4 cm	Negative
	Isogeneic	Negative	Negative	Negative
	TBC (Sonicated)	Negative	2.0 x 2.0 cm	.8 x 1.0 cm
28	Xenogeneic	Negative	Not Done	Negative
	Isogeneic	Negative	Negative	Negative
	TBC (Sonicated)	Negative	1.5 x 0.5 cm	?
36	Xenogeneic	Negative	2.2 x 2.4 cm	Negative
	Isogeneic	Negative	Negative	Negative
	TBC (Sonicated)	Negative	2.0 x 2.0 cm	?
21	Xenogeneic	Negative	1.5 x .8 cm	Negative
	Isogeneic	Negative	Negative	Negative
	TBC (Sonicated)	Negative	1.5 x 1.3 cm	1.3 x 1.0 cm
6 Normal Rats	Xenogeneic	All Negative	All Negative	All Negative
	Isogeneic			
	TBC (Sonicated)			

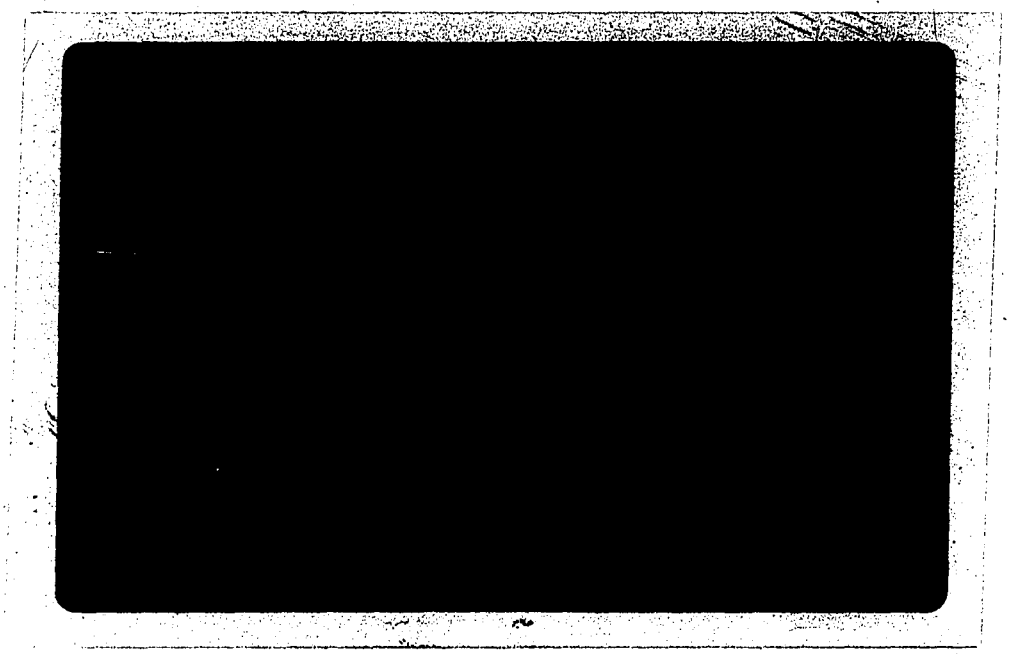
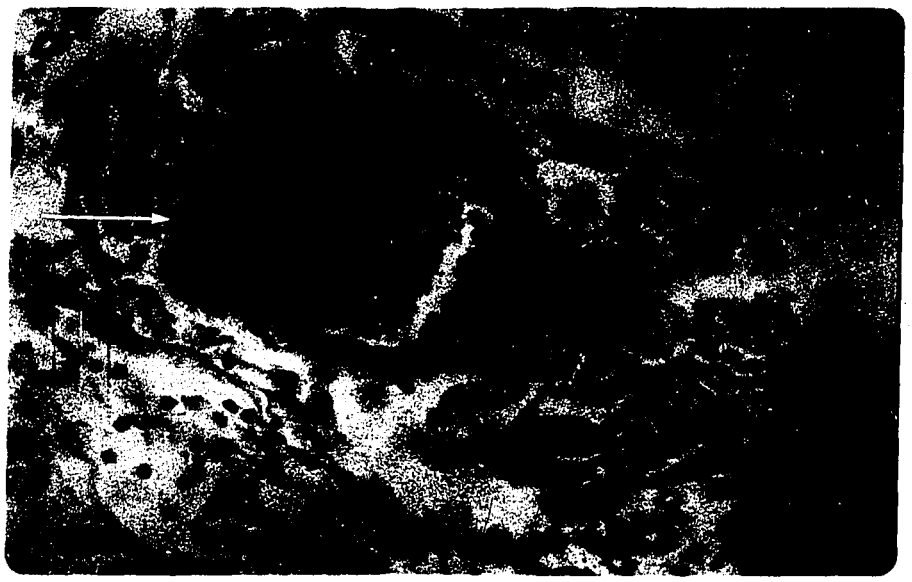


FIGURE XXIII

Photomicrograph of section of Lewis rat skin biopsied from a 4-hour reaction. Note the intense edema of the dermis with the clumps of PMN's to one side of vessel wall. (x 360)



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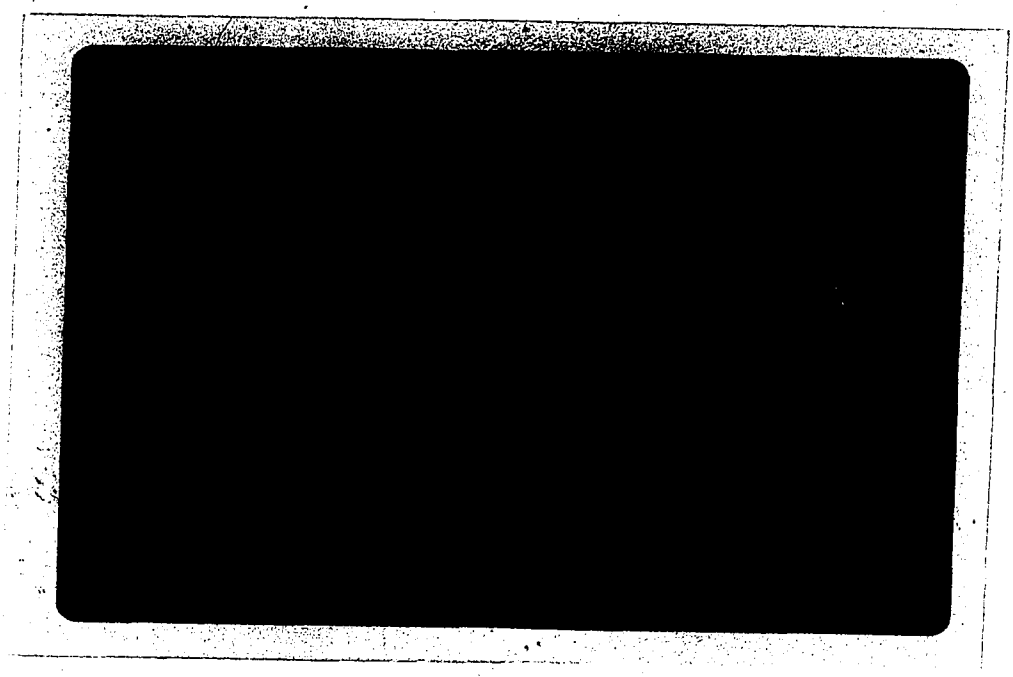


FIGURE XXIV

High power of Figure XXIII. Note rupture of the lining of vessel, the predominantly PMN infiltrate with significant percentage of eosinophils present (arrow), breaking through the vessel wall into the interstitial tissue. (x 650)

FIGURE XXIV

High power of Figure XXIII. Note rupture of the lining of vessel, the predominantly PMN infiltrate with significant percentage of eosinophils present (arrow), breaking through the vessel wall into the interstitial tissue. (x 650)

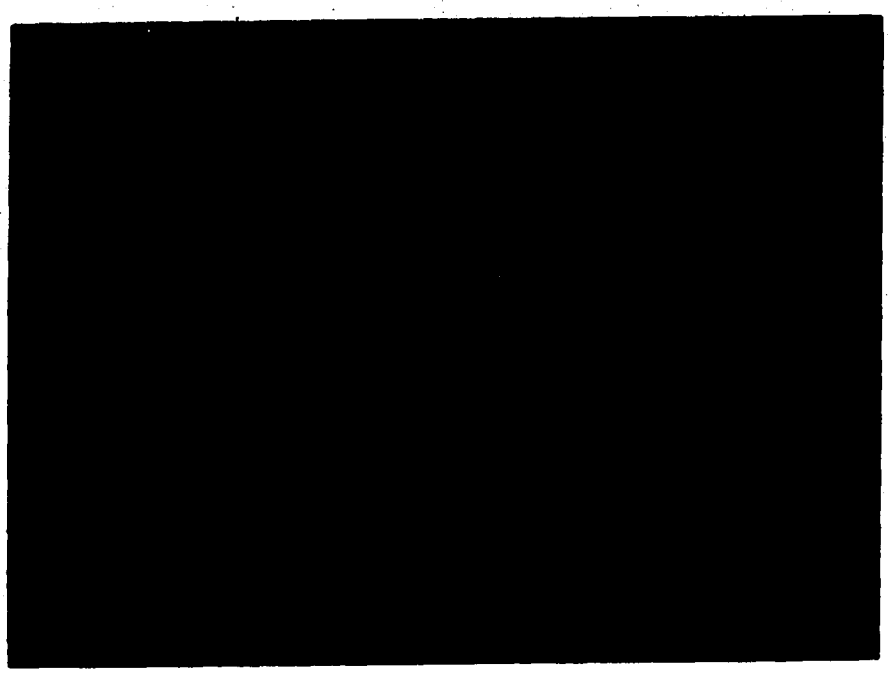
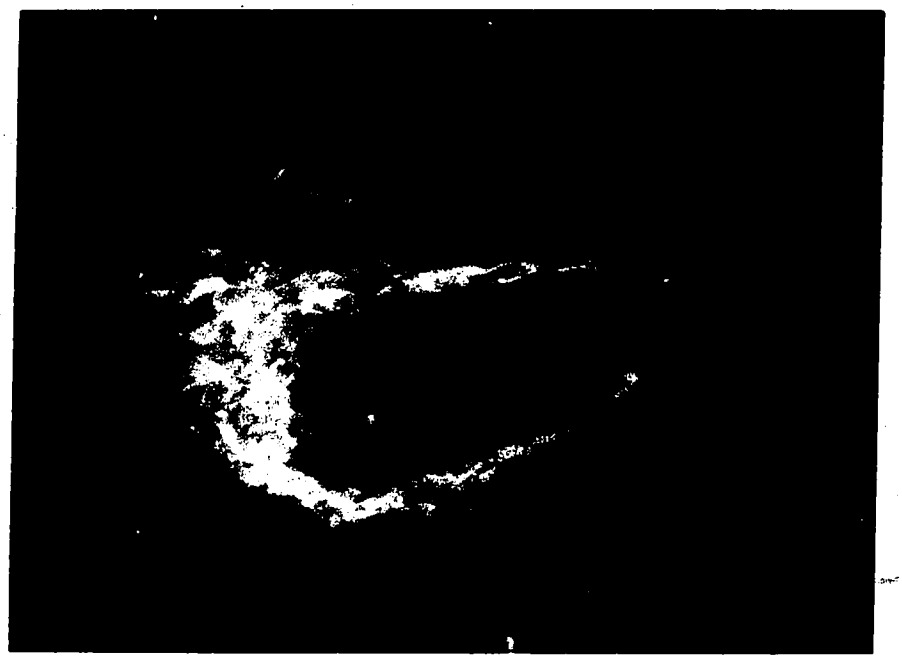


FIGURE XXV

Skin biopsy of Lewis rat Arthus reaction elicited by intradermal injection of xenogeneic kidney. Tissue stained for deposition of B_1C. Note the heavy deposition of complement within the vessel wall. There also appears to be a break in the vessel wall, analagous to the area of PMN infiltration seen in Figure XXIV. (x 650)



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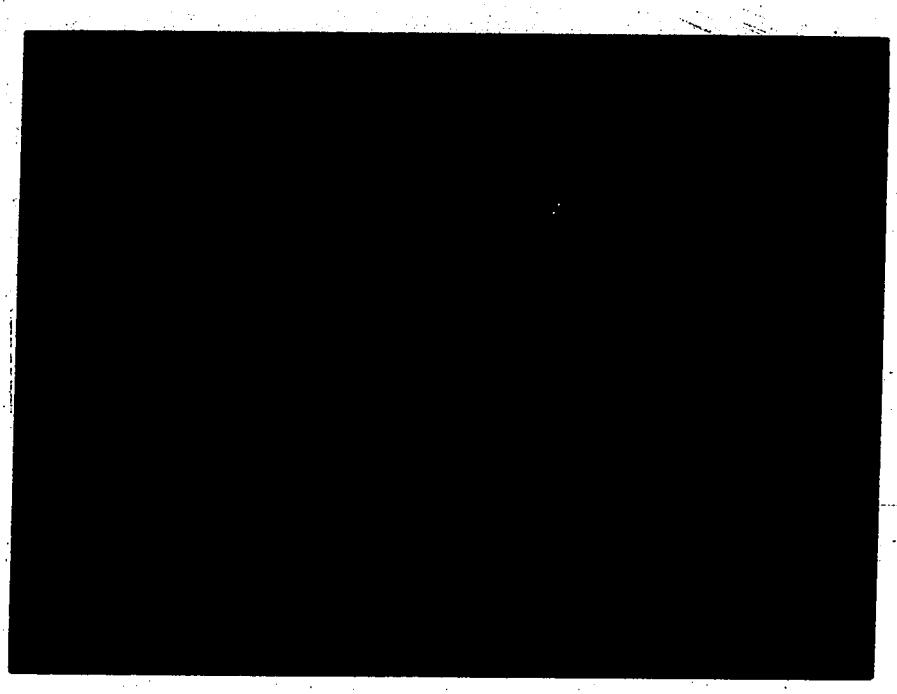

FIGURE XXVI

Rat skin demonstrating a negative biopsy following injection of isogeneic kidney. (x 650)

D. DETECTION OF CIRCULATING ANTIGENS -

During induction but prior to onset of disease, the rats of groups 8 and 11 were bled every two weeks. The serum was diffused against rabbit anti-rat F.I.A. antiserum by double diffusion in agar. A precipitin line appeared 3 to 4 weeks prior to the onset of disease in a high percentage of animals. Again utilizing agar gel diffusion, antibody to isogeneic antigen could not be detected with Lewis rat F.I.A. as antigen.

If the precipitin line demonstrated represented circulating antigen, then it should be removable with specific antiserum. Two procedures were utilized to demonstrate this.

A nephrotic rat was bled for 1 cc by the tail vein. It was then injected intravenously with 1 cc of rabbit anti-rat F.I.A. The rat was then bled for 0.5 cc every 30 minutes for 2 hours. The serum was then separated and diffused in agar gel against

rabbit anti-rat F.I.A. As can be seen in the 30 minute bleed the precipitin line is faint and with increasing time it becomes more pronounced (Figure XXVII). In vitro absorptions with anti-rat F.I.A. also abolished the precipitin line when agar gel diffusion was used.

It has been claimed that the nephritogenic antigen has a half life of less than 24 hours. If what we have demonstrated is the nephritogenic antigen, it should be catabolized in a similar fashion once the major source of the antigen is removed. A nephrotic rat was bilaterally nephrectomized and bled at 0, 16, 19, 22 and 48 hours. The sera were placed in peripheral wells of an Ouchterlony plate, except in well 5 where the nephrotic rat's urine prior to nephrectomy (concentrated x 10) was placed. The antigen was present at 48 hours, though slightly decreased in concentration. In addition we demonstrated an antigen present in the urine which cross-reacts with that in the serum (Figure XXVIII).

Discussion - The substance demonstrated in the circulation of nephrotic rats may be a renal antigen. The in vivo absorption results are interpreted as positive, since when assaying the absorbed serum equal volumes were used in the peripheral wells. Within 2 hours the antigen concentration had returned to its original level.

We were unable to demonstrate any significant change in concentration of the antigen over a 48 hour period. This makes the antigen probably dissimilar to the nephritogenic antigen of Edgington, the half life of which was less than 24 hours.

We were also not able to demonstrate circulating rat anti F.I.A., possibly because all the antibody was bound to antigen utilizing the available antibody binding sites but still leaving antigen combining sites available. If this hypothesis is true, then the animal would indeed be in antigen excess with a reference to the endogenous antigen F.I.A., the optimal state for disease induction.

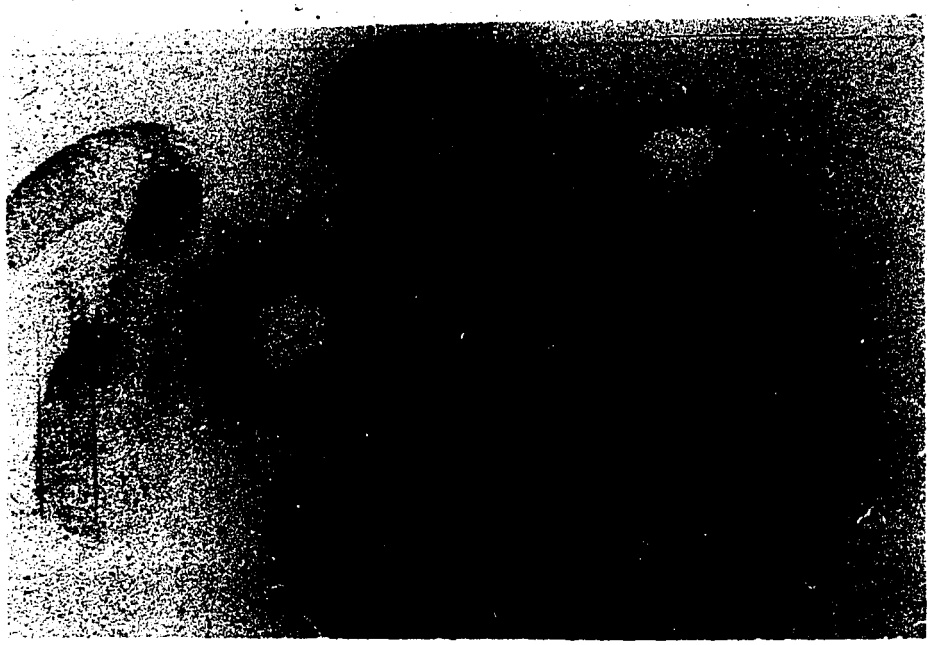

FIGURE XXVII

Double diffusion in agar gel. Rabbit anti-Lewis rat FIA in center well, diffused against sera taken every 30 minutes following an I.V. injection. The animal was injected with rabbit anti-rat FIA. Note the lack of clear definition of precipitin line in 1, with subsequent re-establishment of distinct precipitin line.

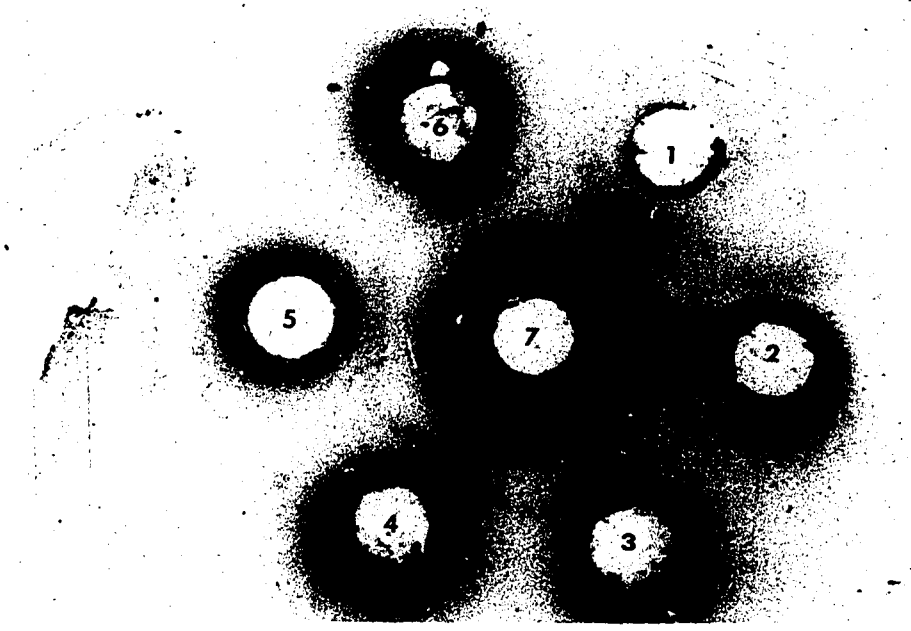

FIGURE XXVII

Double diffusion in agar gel. Rabbit anti-Lewis rat FIA in center well, diffused against sera taken every 30 minutes following an I.V. injection. The animal was injected with rabbit anti-rat FIA. Note the lack of clear definition of precipitin line in 1, with subsequent re-establishment of distinct precipitin line.

FIGURE XXVIII

Ouchterlony plate demonstrating the persistance of antigen in serial serum samples over a 48-hour period. The rat urine (prior to nephrectomy) is placed in well 5; two precipitin lines are seen, one of which demonstrates identity with that in serum.

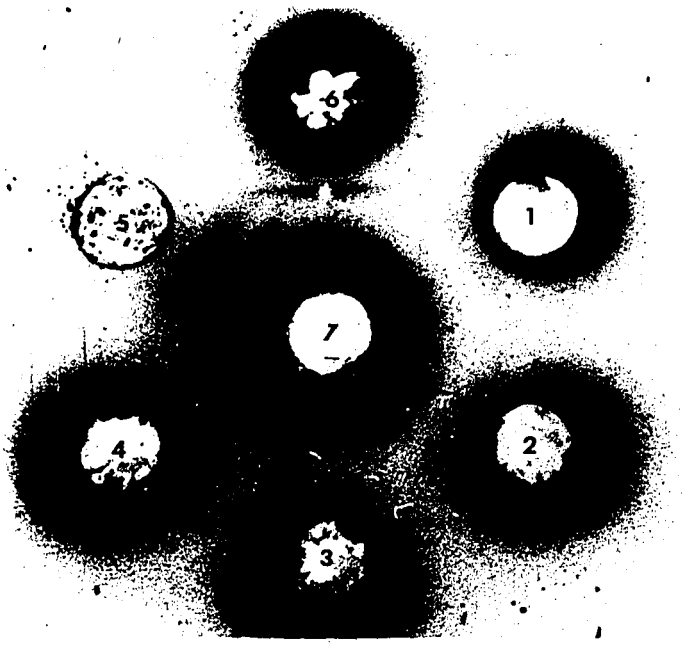

FIGURE XXVIII

Ouchterlony plate demonstrating the persistance of antigen in serial serum samples over a 48-hour period. The rat urine (prior to nephrectomy) is placed in well 5; two precipitin lines are seen, one of which demonstrates identity with that in serum.

E. DETECTION OF ANTIBODY

Nephrotic Lewis rat serum was evaluated for the presence of antibodies as outlined in Section L of Materials and Methods. Precipitating antibody was present after the third or fourth intraperitoneal injection to the xenogeneic antigen during induction of disease. An attempt to demonstrate precipitating antibodies to autologous kidney was negative. Using Lewis F.I.A. as antigen in concentrations of 10, 5 and 1 mg/ml in the center well of serial Ouchterlony immunodiffusion plates the sera of nephrotic rats were allowed to diffuse from the peripheral wells. At no time in the course of induction disease was circulatory precipitating antibody demonstrable to autologous tubular antigen.

F. TISSUE CULTURE

The cytotoxicity of nephrotic rats' lymphoid cells from spleen and peripheral blood were evaluated by the destruction of isogeneic neonatal Lewis kidney

monolayers. Initially a protocol of boosting the animals 8 to 12 days prior to cell harvesting was followed. Results are listed below.

5×10^6 lymphoid cells were added to each tube. All cultures were stained by May-Grunwald-Giemsa cytotoxicity method.

Experiment I	18 Hours
Lewis Rat Monolayer & Normal Lewis Lymphoid Cells	± Destruction
Lewis Rat Monolayer & Nephrotic Lewis Lymphocytes	+ Destruction
Lewis Rat Monolayer & Rabbit Lymphoid Cells	4+ Destruction

Experiment II - Each tube in duplicate	24 Hours
Lewis Rat Monolayer & Nephrotic Spleen Lymphoid Cells + PHA	2 + 3
Lewis Rat Monolayer & Nephrotic Peripheral Lymphoid Cells + PHA	2 + 3+
Lewis Rat Monolayer & Nephrotic Serum* + PHA	0

* 0.3 cc of serum was added to culture tubes.

Experiment III

	24 Hours
Lewis Rat Monolayer & Normal Lewis Lymphoid Cells + PHA	±
Lewis Rat Monolayer & Normal Lewis	±
Lewis Rat Monolayer & Nephrotic Lewis + PHA	3+
Lewis Rat Monolayer & Nephrotic Lewis	2+, 3+
Lewis Rat Monolayer & Normal Sp. Dawley + PHA	2+, 3+
Lewis Rat Monolayer - Normal Sp. Dawley	1+, 2+
PHA Alone	0

Experiment IV

For 48 hour assays it was found that improved monolayer morphology was achieved by changing medium at 24 hours. The experiment outlined below is a representative one.

	-48 Hours
a. Normal Lewis Lymphoid Cell	0+, 1+
Normal Lewis Lymphoid + PHA	1+

b.	Normal Sp. Dawley Lymphs	1-, 2+
	Normal Sp. Dawley Lymphs + PHA	2+
c.	Nephrotic Lewis Lymphs + PHA	3-, 4+
	Nephrotic Lewis Lymphs	2+, 3+
d.	PHA Alone	0

Summary of the above experiments is seen in Table III. (See Figures XXIX - XXXII)

Discussion - Being aware of the shortcomings of morphologic studies, several observations however appeared valid. Firstly, normal isogeneic lymphoid cells in the presence of phytohemagglutinin were slightly cytotoxic for the kidney monolayer. This confirms recent reports of isogeneic lymphoid cytotoxicity in tissue culture, negating previous claims that histoincompatibility is a prerequisite for cytotoxicity. Since cytotoxicity was increased in most instances by the addition of PHA, the inference is that the "recognition" step is facilitated or that "contactual agglutination" enhanced. Secondly,

we have shown that these nephrotic animals did not manifest delayed cutaneous hypersensitivity to the xenogeneic kidney, the immunizing antigen. Thirdly, as normal allogeneic lymphoid cells gave similar cytotoxic reactions *in vitro* as did the isologeneic sensitized lymphoid cells, it appeared that the *in vitro* cytotoxic assay systems do not necessarily reflect *in vivo* pathogenetic mechanisms. To test this theory a further set of experiments was performed, an *in vivo* cytotoxic assay.

TABLE III

SUMMARY ON OBSERVATIONS IN IN VITRO CYTOTOXICITY

	PHA ±	24 Hrs	48 Hrs
Normal Isogeneic Cells	+	±	+
	-	±	±
Normal Allogeneic Cells	+	++	++
	-	+	+±
Normal Xenogeneic Cells	+	++++	——
	-	+++	——
Nephrotic Isogeneic Cells	+	+++	++++
	-	++	+++
PHA Alone	+	0	0
	-	0	0
Nephrotic Serum	+	0	0
	-	0	0

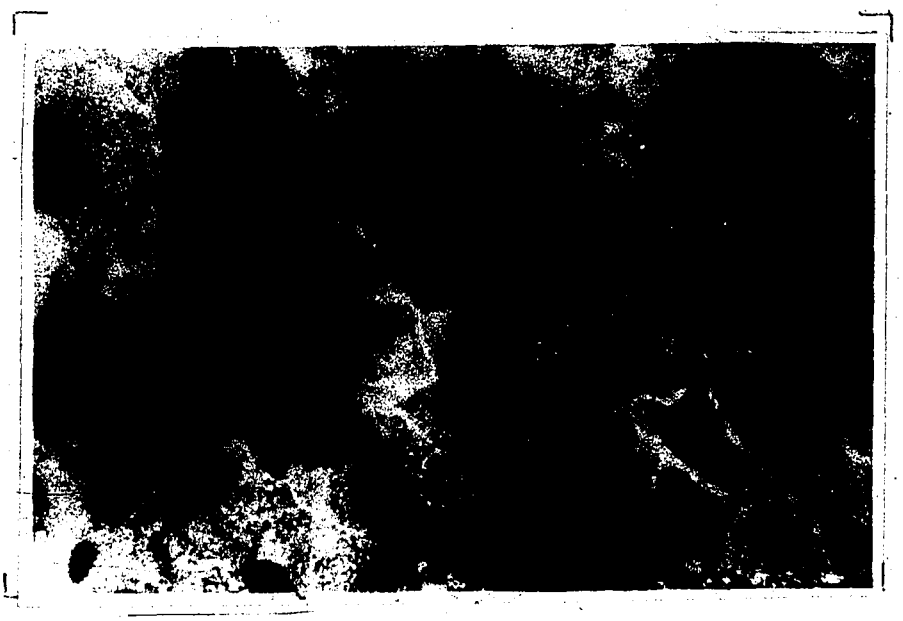

FIGURE XXIX

Neonatal Lewis kidney monolayer at 24 hours with normal isologous lymphocytes. Note minimal but definite vacuolisation of cytoplasm of monolayer (arrow) (May Grunwald Giemsa). (x 630)

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Neonatal Lewis kidney monolayer at 24 hours with normal isologous lymphocytes. Note minimal but definite vacuolisation of cytoplasm of monolayer (arrow) (May Grunwald Giemsa). (x 630)

FIGURE XXX

Lewis kidney monolayer and allogeneic nephrotic rat lymphocytes and PHA at 24 hours. Note increase in monolayer cell vacuolisation with early spindling of cells. (May Grunwald Giemsa x 960)

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Lewis kidney monolayer and allogeneic nephrotic rat lymphocytes and PHA at 24 hours. Note increase in monolayer cell vacuolisation with early spindling of cells. (May Grunwald Giemsa x 960)

FIGURE XXXI

Lewis rat monolayer and isogeneic nephrotic lymphocytes and PHA at 48 hours. Note virtual complete destruction of the monolayer. (May Grunwald Giemsa x 320)

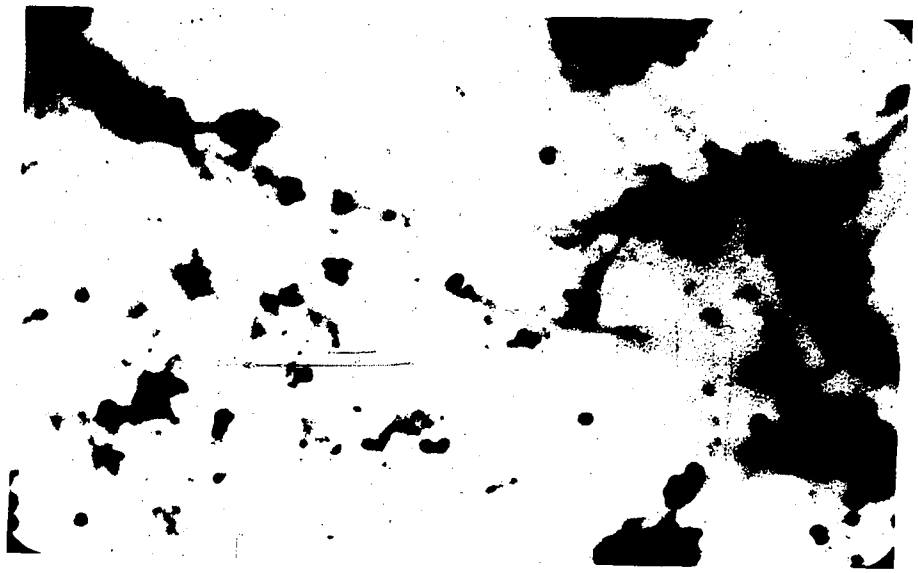


FIGURE XXXI

Lewis rat monolayer and isogeneic nephrotic lymphocytes and PHA at 48 hours. Note virtual complete destruction of the monolayer. (May Grunwald Giemsa x 320)

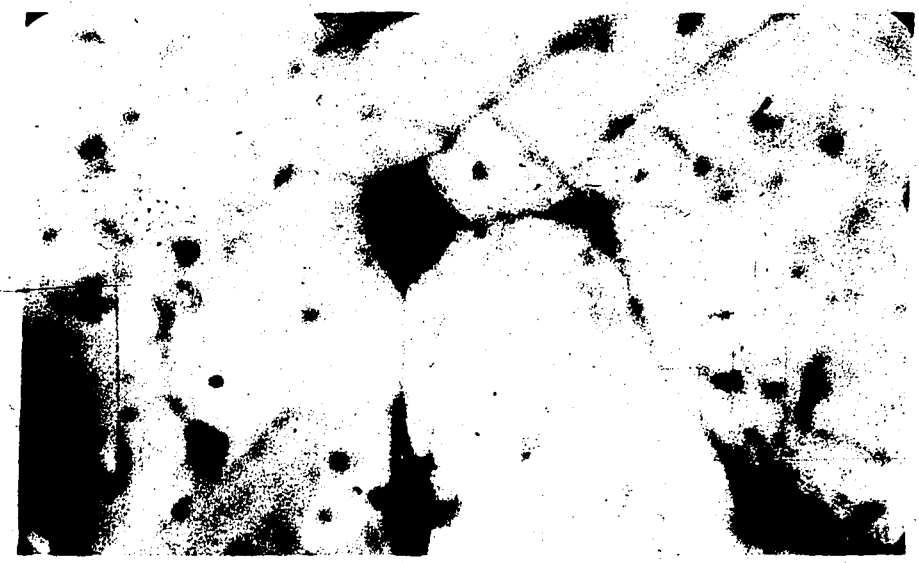


FIGURE XXXII

Residual kidney monolayer cells demonstrating nearly complete cell destruction. Note extreme spindling of cytoplasm, remaining nucleus, marked cell debris surrounding cell. (May Grunwald Giemsa x 960)

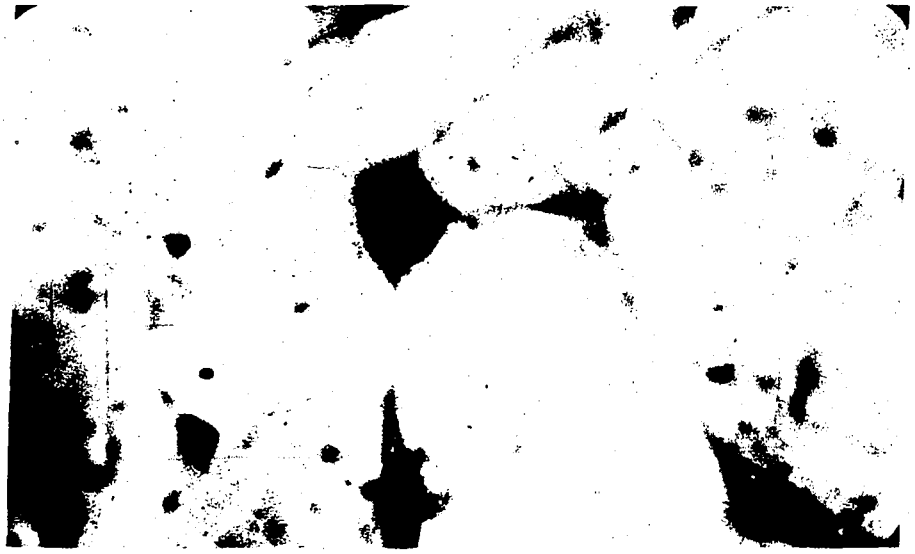


FIGURE XXXII

Residual kidney monolayer cells demonstrating nearly complete cell destruction. Note extreme spindling of cytoplasm, remaining nucleus, marked cell debris surrounding cell. (May Grunwald Giemsa x 960)

G. IN VIVO CYTOTOXICITY

Eight isologous Lewis rats were recipients of nephrotic Lewis lymphoid cells as described previously.

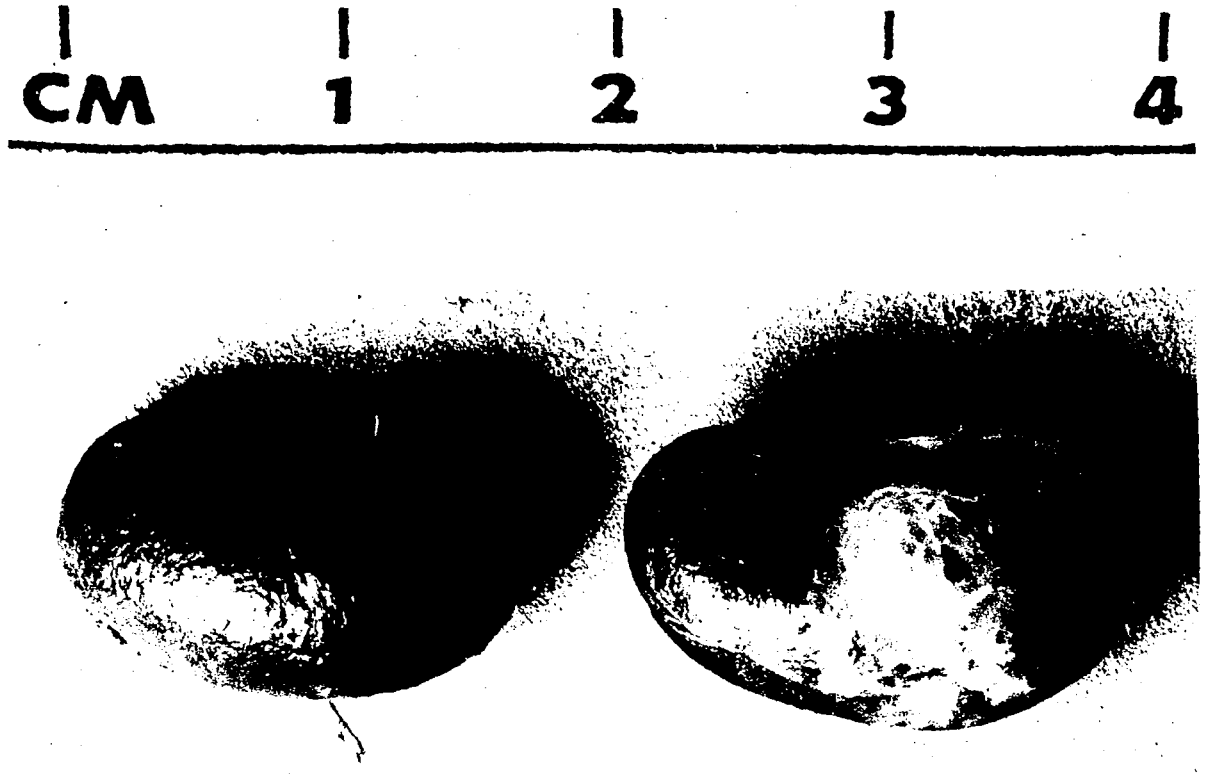
When the injected kidney was examined, a large pale scar was noted. On gross cut section the scar tissue was slightly retracted and appeared to be of cortical thickness (Figures XXXIII and XXXIV). On light microscopy, low power examination revealed a heavy cellular infiltrate with "fingers" of cells invading the deeper cortical tissue (Figures XXXV and XXXVI). At high power these cells were predominantly lymphoid in nature with many cells in active mitosis. Those cells which surrounded the tubules appeared to be destroying the tubular epithelial cells, glomeruli appeared intact (Figure XXXVII). These changes were not seen in normal Lewis to Lewis transfer experiments.

Discussion - This model is patterned after the ELKIN model which demonstrated a graft vs. host reaction in a parental to F_1 hybrid system on a histoincompatability basis. Histoincompatability could not be operative here as this was a completely isogeneic system. As the cellular infiltrate is lymphoid in character and as it occurred only at 6 to 7 days, we must consider the reaction as one of "delayed hypersensitivity." Thus there appeared to be a cellular mechanism operative when sensitized cells were placed in the kidney beneath the capsule. Yet in the experimental nephrotic model, no cellular infiltrate was seen in the kidneys. It must be concluded that in this artificial system where sensitized lymphocytes were placed in apposition to the tubular antigen (that to which they were sensitized), a cell mediated hypersensitivity reaction did appear. These animals had absent skin delayed hypersensitivity to xenogeneic kidney, the immunizing antigen, but were

not anergic for they demonstrated positive delayed skin reactivity to ultrasonically disrupted TBC. Moreover, the disease is not passively transferred with lymphoid cells alone. For these reasons it appeared that though these lymphocytes were capable of manifesting a cellular reaction locally, this feature was unique and contrasted to the disease model. For these reasons it does not appear that sensitized lymphoid cells play a direct or important role in the pathogenesis of this disease.

FIGURE XXXIII

The excised injected and contralateral kidney. Note pale, edematous area at the site of injection (arrow).



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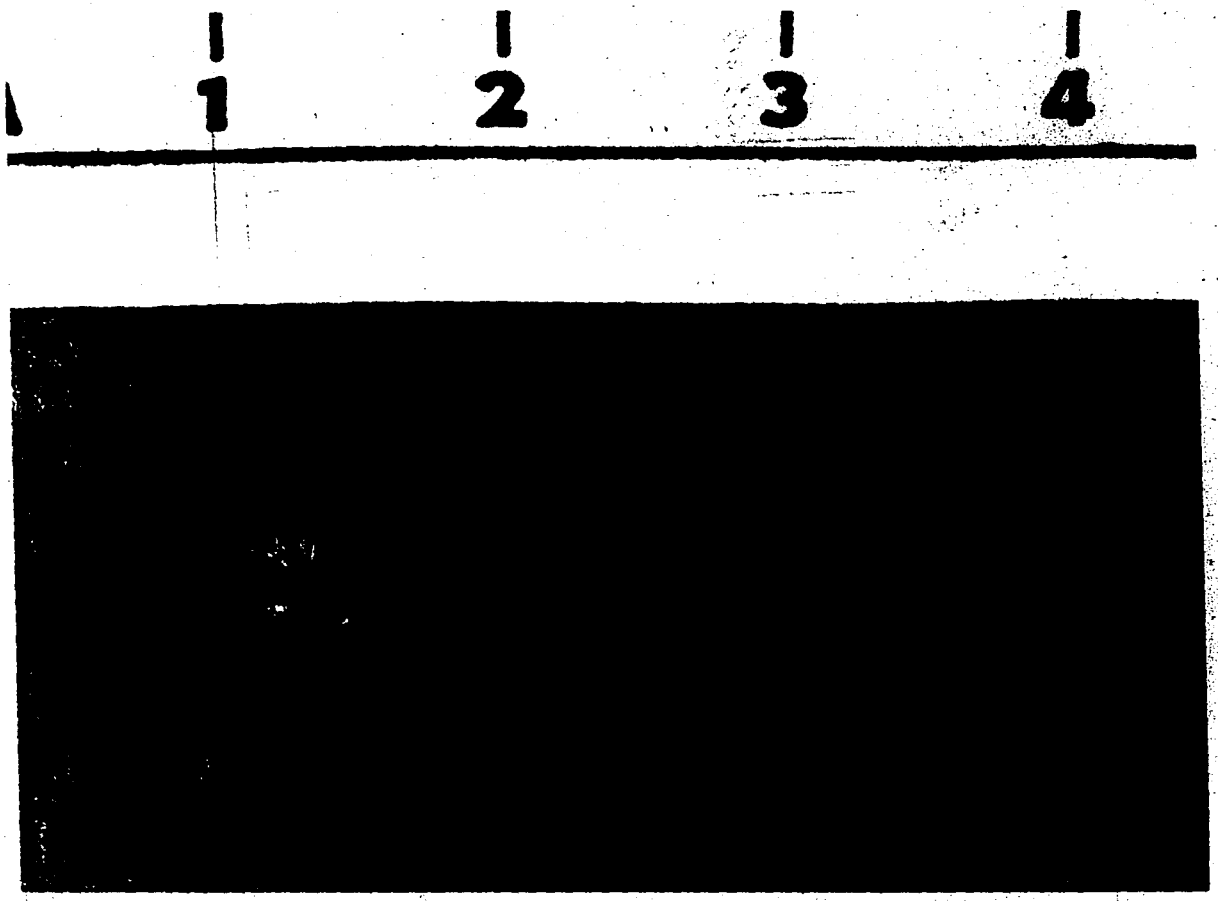
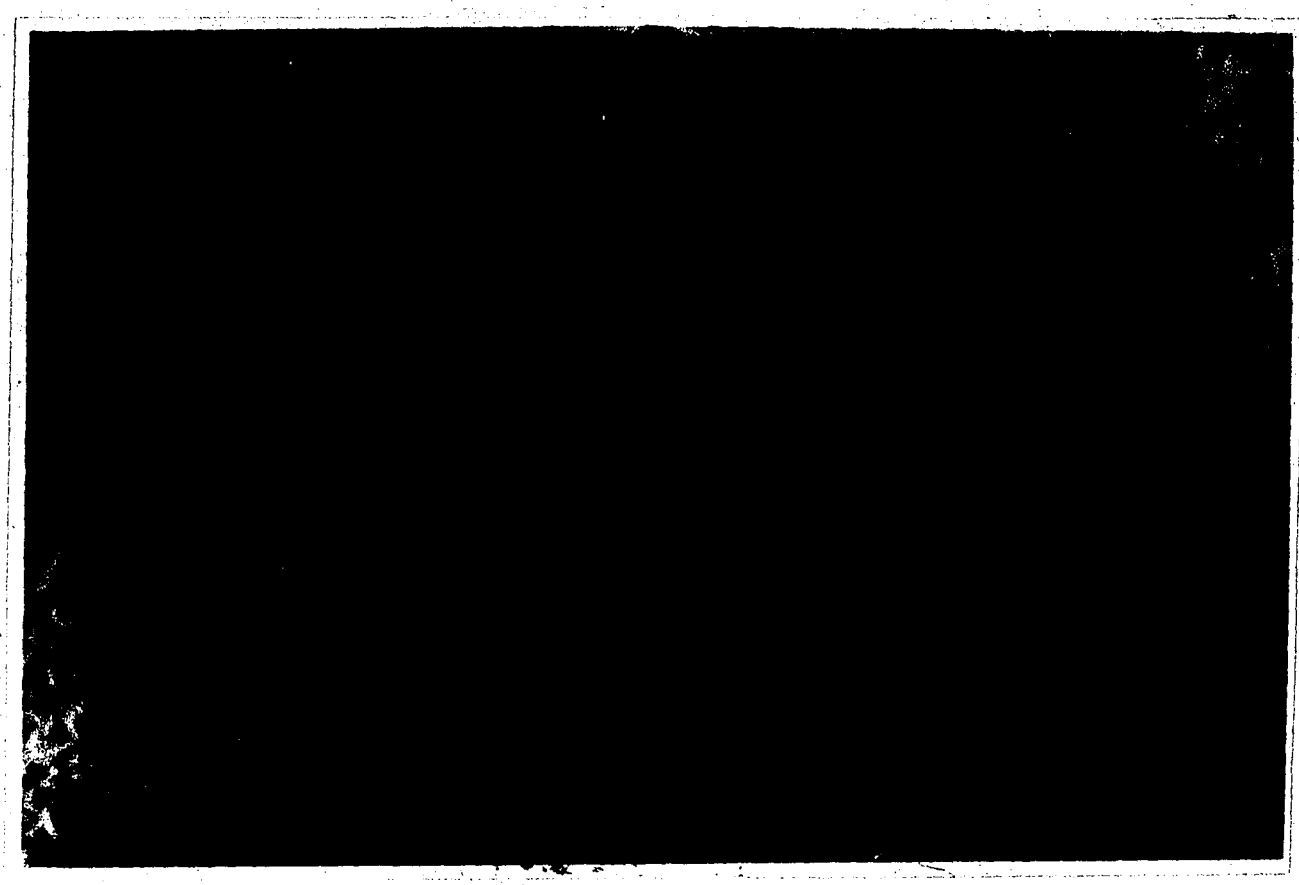


FIGURE XXXIV

Section of kidney to demonstrate complete pallor of cortex.

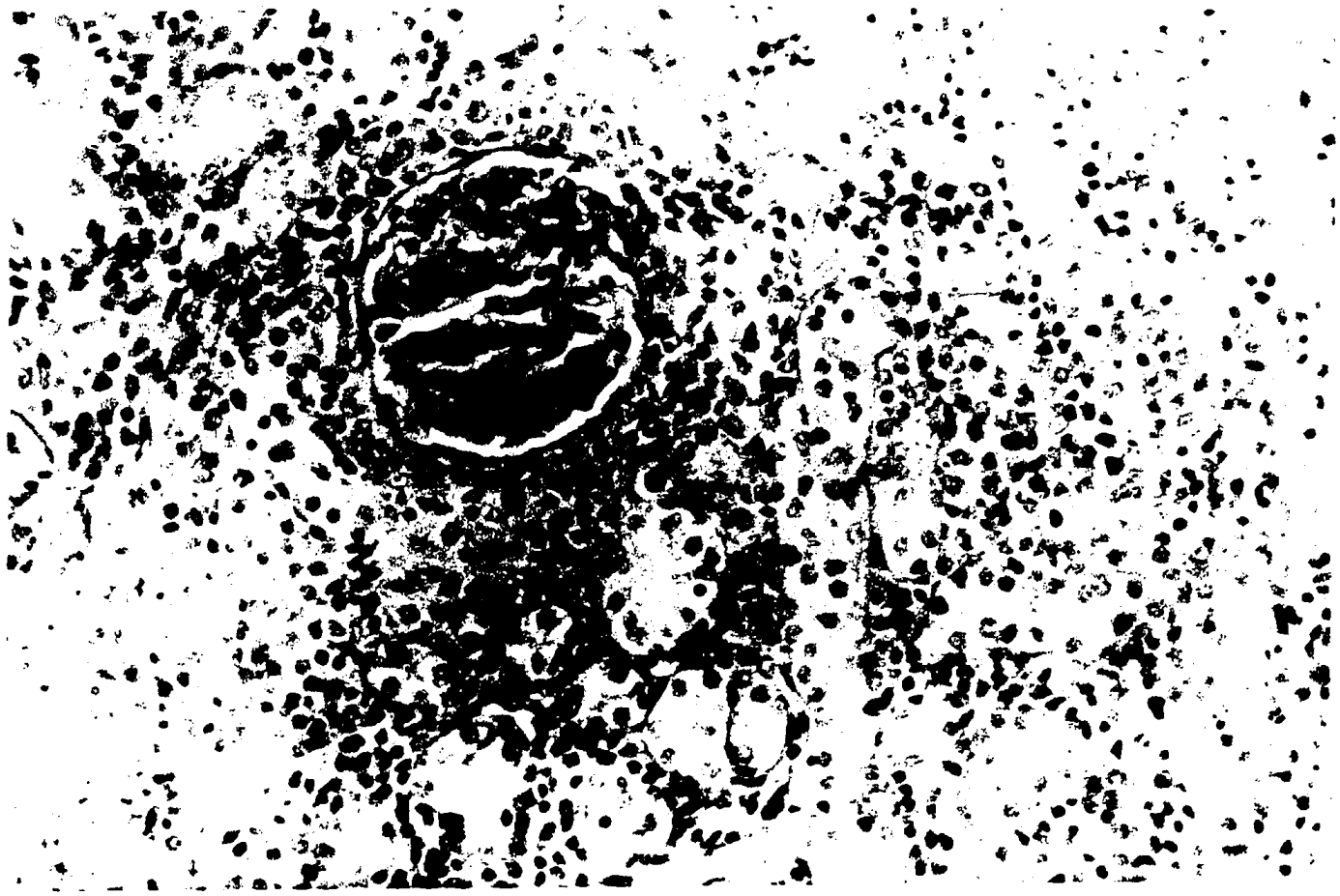
FIGURE XXXIV

Section of kidney to demonstrate complete pallor of cortex.



FIGURES XXXV, VI

Photomicrograph of Lewis kidney in which isologous nephrotic lymphocytes have been injected subcapsularly. Note the fine cord-like fingers surrounding the tubules, seen better in Figure XXXVI. (Mag. x 360 - H & E)



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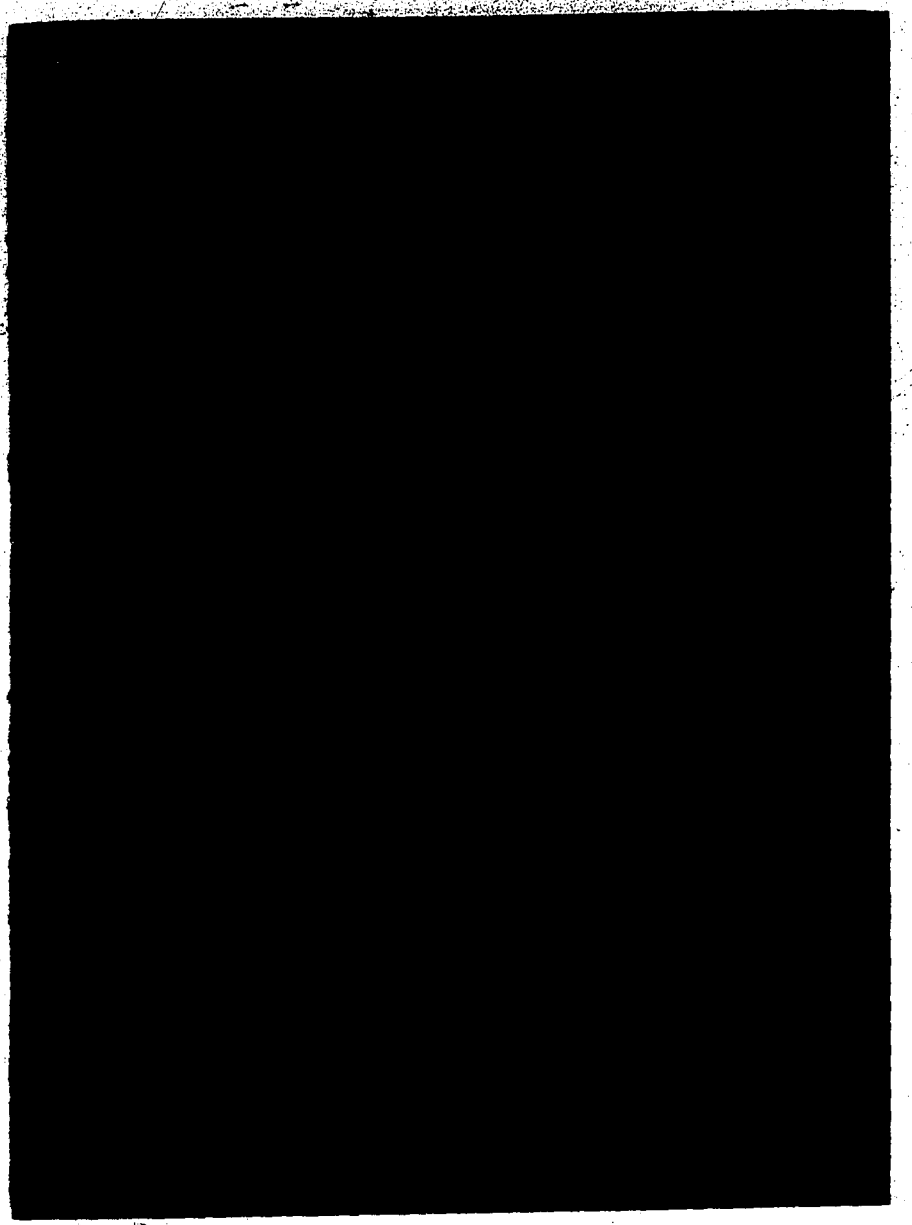

FIGURE XXXVI

(x 360 H & E)

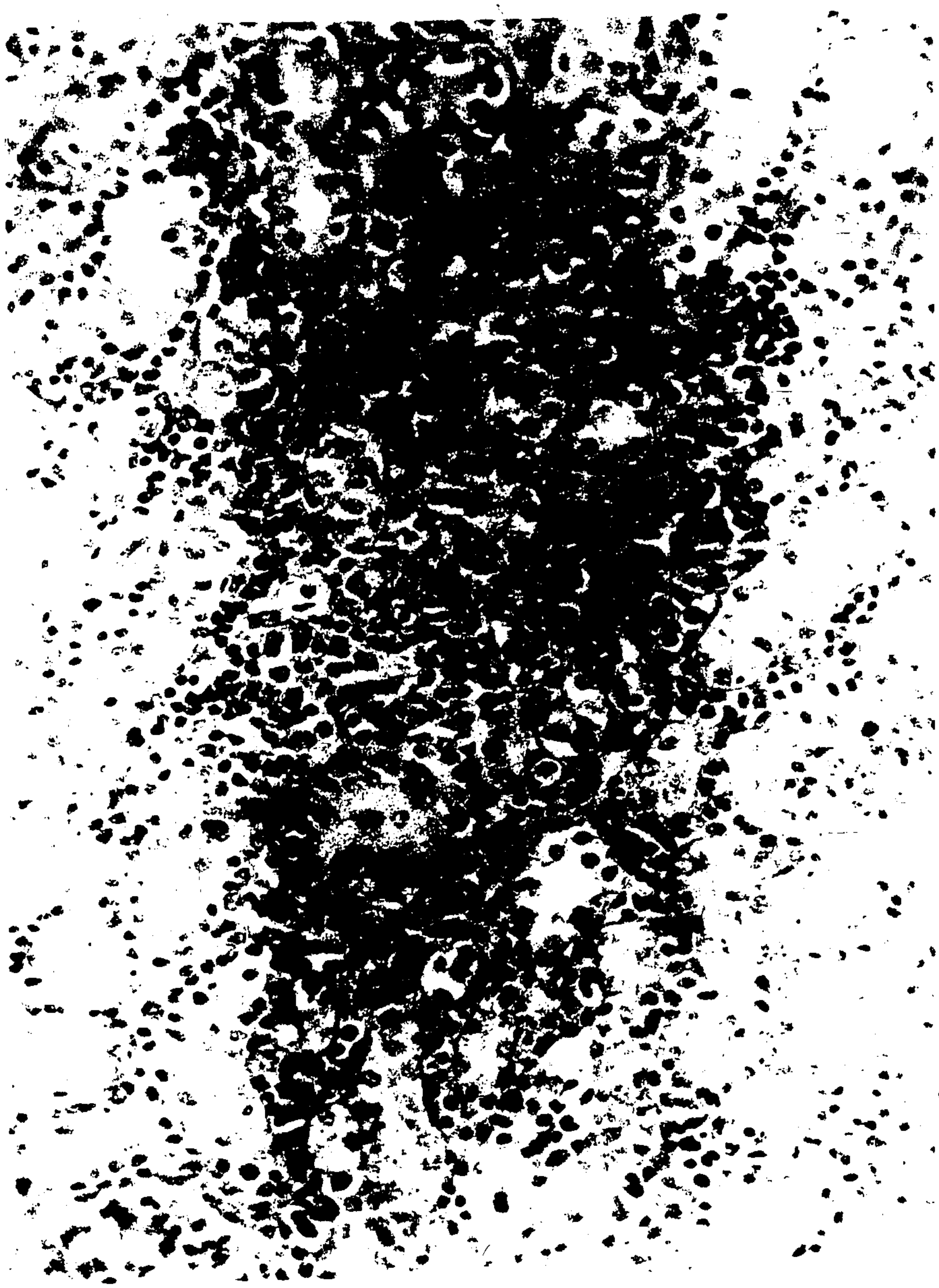

FIGURE XXXVI

(X [illegible]50 H & E)

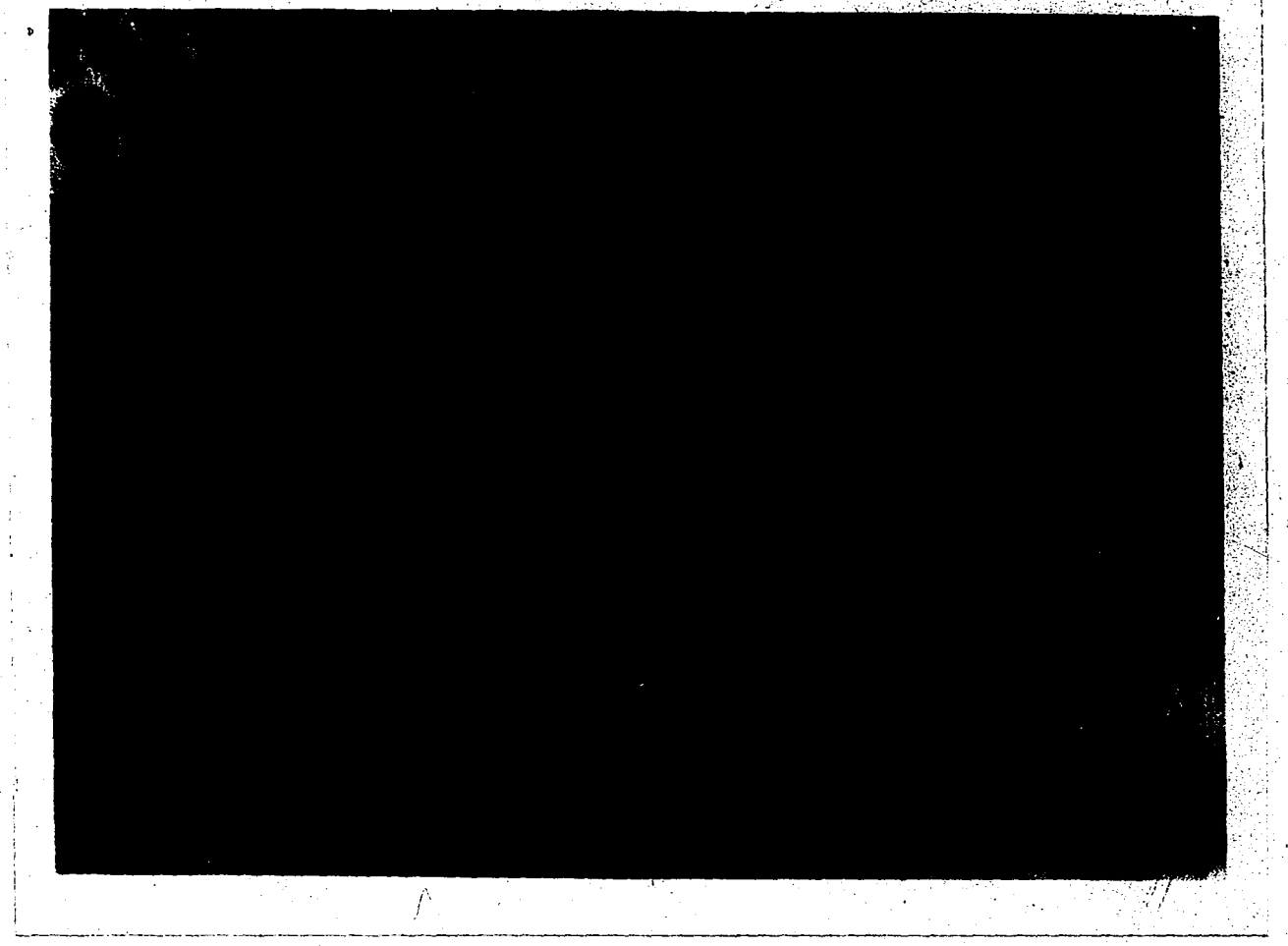

FIGURE XXXVII

High power view of peritubular lymphocytic infiltration. Note the tubular cell swelling and vacuolisation. (x 960 H & E)

V. FINAL DISCUSSION

These experiments have been performed to confirm the concept that AIC nephritis is an antigen-antibody complex disease. This concept is not new and in fact has been well discussed by many previous authors. Though a few others have disputed the pathogenetic role played by complexes, it has become more and more apparent that their view is no longer tenable.

In our experimental model, Lewis rats were immunized with a crude rabbit antigen. The animals so immunized became nephrotic over a period which was comparable with similar models. We also demonstrated that it was possible to potentiate the disease by splenectomy. In the splenectomized group of animals, the disease was much more rapid in onset and animals were markedly wasted at the time of death. The characteristics of the pathology were similar to those seen in the intact group of animals. The rationale for splenectomy was that, with the removal of a large reticuloendothelial organ, large quantities of complexes would

remain in the circulation. Those complexes able to be filtered would then be deposited in greater concentration in the kidney.

In both groups of animals antigen, gamma globulin and complement were demonstrated in a granular fashion along the basement membrane. The deposition of xenogeneic antigen was heavy and easily seen, contrasting to the faint deposition of isogeneic antigen. Ultramicroscopic examination of the kidneys demonstrated that prior to the onset of gross proteinuria, a progressive increase in basement membrane thickening was seen. Though unable to demonstrate antigen-antibody complex deposition by immunofluorescence, we were able to show a patchy granular deposition within the basement membrane by electromicroscopy.

This progressed to the characteristic pattern of deposits beneath the epithelial cells and within

the basement membrane. These complexes appear to increase in density within the basement membrane and at the same time appear to progressively migrate toward the outside surface where these deposits accumulate beneath the epithelial cells. The interpretation is that these complexes initially do not cause disease, but after having reached a specific concentration proteinuria results. The migration of these deposits to the sub-epithelial area prevents subsequent phagocytosis by reticuloendothelial cells. In the splenectomized group, moderately well-demonstrated deposits within the glomerular basement membrane prior to the onset of proteinuria would support this hypothesis. In addition, we demonstrated the deposition of isogeneic antigen. However, the deposition was faint and patchy contrasting to the characteristics of xenogeneic antigen deposition. Edgington claimed that in his model the isogeneic antigen-antibody deposition is the main complex which

causes disease. In our model it would appear that though the isogeneic antigen is deposited with its antibody, this antigen would not play the important role in our model as it did in that of Edgington. He showed that microgram amounts of specific nephritogenic allogeneic antigen in one injection in the footpad were able to cause disease with subsequent moderately heavy isogeneic antigen deposition. It appeared in our model that with xenogeneic antigen, antibodies which were formed cross-react with the antigen present in the proximal tubules of the Lewis rats. This cross-reaction caused subsequent damage to the proximal tubule which resulted in the release of the isogeneic antigen, the breaking of tolerance and antibody formation. The isogeneic antigen then acts as an auto-immunogen causing antigen-antibody complex formation. However, only qualitatively small amounts were deposited, which contrasts to Edgington's earlier work and possibly his conclusions.

We had observed during the induction of nephritis, classical hyaline droplet formation within the proximal tubules of Lewis rats. As had been discussed in the literature these hyaline droplets were thought to be manifestations of protein reabsorption. Cuppage, however, accepting this view, raises the point whether or not these droplets in fact may represent toxic changes. We demonstrated that these hyaline droplets develop prior to the onset of proteinuria, and we attempted to determine what role these droplets played in disease pathogenesis and at what time they initially appeared. We have shown their formation within the first week and in fact appeared with minimal immunization. In three different groups of animals the hyaline droplets appeared even after one intraperitoneal injection of complete Freund's adjuvant alone. We were not able to demonstrate that these droplets contained the specific nephritogenic antigen. When we attempted to do this by immunofluorescence, the droplets themselves had sufficiently

strong autofluorescence to quench the specific emission. An attempt was made to fluoresce these granules with a different conjugate (rhodamine). The red emission from this conjugate was also not sufficiently strong to overcome the intense yellow autofluorescence of the granules. When stained by routine methods the granules appeared as P.A.S. positive; however, they were negative when stained for fat.

What significance these hyaline droplets have in this experimental model remains to be determined. If we had been able to demonstrate that these granules contained the specific nephritogenic antigen then one would be able to hypothesize with confidence that the early rounding up of the brush border to form what appeared to be hyaline "re-absorption" granules was related to the freeing of autologous antigen. As Edgington has shown autologous antigen is released and later deposited within the glomerular basement

membrane as complexes. Finally, the brush border appeared to be lost very early in the immunization process even without the utilization of specific antigens. With complete Freund's adjuvant alone, basement membrane changes are seen similar to those which occur in animals immunized with antigen plus complete Freund's adjuvant.

The release of autologous antigen, the breaking of tolerance and antibody formation is probably potentiated by antibodies formed to the xenogeneic antigen. These antibodies then cross-react with the autologous antigen releasing more antigen. This breaking of tolerance is an essential step for the formation of autologous antigen-antibody complexes.

In our own attempt to demonstrate the deposition of autologous antigen, the elution procedure of Edgington et al was found to be unsatisfactory. Good morphology was maintained with a thirty minute elution, and autologous antigen was shown to be deposited within the glomerular basement membrane.

We confirmed that though xenogeneic antigen is deposited much more heavily, isogeneic antigen is deposited in a similar qualitative fashion. In conclusion we did not show that the hyaline "reabsorption" droplets contained nephritogenic antigen though the droplets appeared parri-passu with the rounding up and droplet formation within the brush border. The deduction is very tempting that in fact the nephritogenic antigen is contained within these hyaline droplets.

Our elution experiment to demonstrate free antibody was successful. We demonstrated that we had a single protein and it migrated on immunoelectrophoresis in the gamma region. We were not able to demonstrate that the antibody obtained was directed against the autologous antigen (rat FIA) nor crude rabbit antigen.

The eluate was then evaluated by an in vivo assay system. This experiment was performed to determine if there was any direct nephrotoxic

activity of the eluate. We had not expected glomerular damage as the antibody initially was complexed within the glomerular basement membrane. One possibility existed that, since the antibody had activity towards the antigen present in the brush border of the proximal tubules, a form of acute tubular necrosis may have developed upon challenge with this antibody. This however was not the case. No animal became nephrotic or proteinuric following the injection of 1 cc (.35 mgm) of the eluted gamma globulin. Microscopic examination of the kidneys of these recipient animals was completely within normal limits except for two of the kidneys which showed mild interstitial hemorrhage. The eluate was then examined by an <u>in vitro</u> system in which the eluate was differentially absorbed and then overlayed onto normal Lewis rat kidneys. The eluate stained the tubules in a differential fashion while the glomerulae never stained.

When stained with the unabsorbed eluate, the brush border appeared to stain slightly more than the whole tubular cell. Upon absorptions of the eluate with rat and then rabbit antigen one saw that the brush border staining was completely abolished as was most of the tubular cytoplasmic staining. Some slight basement membrane staining of the tubular cells remained. Comparison of the staining between eluate absorbed with isogeneic and xenogeneic antigens alone showed that the xenogeneic antigen appeared to absorb out more antibody activity than did the isogeneic antigen. This may have been expected as the immunizing antigen originally was a crude xenogeneic fraction and that more antibody activity would be directed towards the soluble and insoluble antigens present in this immunizing fraction. Consequently, it was expected that specific absorption with a pure isogeneic antigen would absorb less antibody activity, possibly the brush border staining. This was achieved.

Edgington has shown that the brush border contains many antigens, only one of which is nephritogenic. The glomeruli did not stain as the antibodies obtained from our elution procedures were not directed towards glomerular basement membranes but directed towards components of the tubular cells.

CUTANEOUS MANIFESTATIONS IN AIC NEPHRITIS

Continuing our demonstration that AIC nephritis is mediated by antigen-antibody complexes, we then demonstrated that animals which were nephrotic were unable to exhibit a delayed cutaneous reaction to either the immunizing xenogeneic antigen or the isogeneic antigen. The animals were not anergic for they were able to exhibit a delayed cutaneous reaction when challenged with Mycobacterium Tuberculosum. We were able to utilize skin reactivity to demonstrate that precipitating antibodies were circulating *in vivo*. This was done by challenging

the animals with xenogeneic antigen and noting the response at 2 to 3 hours. A classical Arthus reaction was found on biopsy of these 2 to 3 hour reactions; both gamma globulin and complement were found within the vessel walls. We were not able to demonstrate an Arthus reaction, however, when the animals were challenged with an isogeneic tubular antigen fraction. This had been expected as no circulating precipitating antibody could be demonstrated to the isogeneic antigen. The supposition was therefore that if the antibody was present, it was in such a low concentration that it was unable to elicit an Arthus reaction. We had previously demonstrated that isogeneic antigen was deposited within the glomerular basement membrane, presumably together with its antibody so that even though antigen-antibody complexes were circulating, no free antibody was available to combine with that antigen injected subcutaneously.

During induction of disease an antigen was demonstrated in the circulation of some animals about to become nephrotic. This antigen was absorbed out by both in vivo and in vitro techniques. When we attempted to demonstrate that the antigen was similar to that described by Edgington et al, it was apparent that our antigen had a longer half-life than that which he described. Possibly then this antigen was not the nephritogenic antigen but one of the other antigens present in FIA. In the gel diffusion technique that was used to demonstrate the antigen, it was noted that a concentrated nephrotic urine contained an antigen which cross-reacted with that present in the serum of nephrotic rats. This demonstration is not unique, for nephrotic animals may excrete various antigens in the urine - either fragments of glomerular basement membrane or, in our model, tubular antigens which are released into the urine by immunologically destructive processes.

As this disease is humorally mediated, we attempted to disprove recent experimental work which implied strongly that a cell mediated mechanism was of importance in this disease model. Utilizing neonatal kidney rat monolayers, we assayed the cytotoxicity of nephrotic rat lymphocytes. In this system, we were unable to demonstrate any specific cytotoxicity which was unique to these lymphocytes, as normal allogeneic and xenogeneic cells gave similar cytotoxicity. If there had been a specific relationship between disease induction and cytotoxicity, then one would have expected more devastating or more rapid destruction by these sensitized lymphocytes. This, however, was not the case. Since there was similar destruction utilizing sensitized isogeneic lymphocytes and normal allogeneic lymphocytes, the relationship between <u>in vitro</u> destruction and the <u>in vivo</u> situation appears minimal. Cytotoxicity in all cases was enhanced by the addition of PHA, a non-specific contactual agglutinator.

When nephrotic animal kidneys were examined, no cellular infiltrate was seen. This lack of cell infiltrate underlines the absence of cell mediated mechanisms here.

The in vivo cytotoxicity studies were patterned after the model described by Elkins. An isogeneic system rather than an F_1 hybrid into a parental strain was utilized, and positive transfers were demonstrated. This is in contrast to the isogeneic transfers of Elkins in which no positive transfers were demonstrated. Elkins' model was utilized to demonstrate a graft versus host reaction, a cell mediated situation. He demonstrated that the reaction requires the participation of the donor lymphocytes. For extension of the reaction, the host must also participate and contribute to the continuation of the infiltrate. In our isogeneic system, there was no histoincompatability and as a consequence, other mechanisms must be invoked to explain the intense cellular proliferation that was seen. The

antigen responsible for disease induction is located in the brush border of the tubular epithelial cells. As discussed previously, when nephrotic lymphocytes are passively transferred beneath the kidney capsule then the sensitized lymphocytes are placed in intimate contact with host tubular antigen. This tubular antigen is isogeneic to that which had been released in the nephrotic animals and which had sensitized lymphocytes. The conclusions which have been made from this work are that in an artificial situation where lymphocytes are placed in direct opposition to an antigen to which they have been sensitized, then a cellular reaction may become evident. This may not be a dissimilar situation to that seen in the previous cytotoxicity experiments. In the particular model patterned after Elkins, the lymphoid infiltration within the parenchyma of the kidney contrasts greatly to that seen in the intact nephrotic situation where one sees very few cells in the kidney.

In summary, it has been shown that in AIC nephritis antigen-antibody complexes are deposited within the glomerular basement membrane. The antigen involved is both xenogeneic and isogeneic; the latter may be released from the brush border of the proximal tubular cells. The animals manifest an Arthus type reaction which is characterized on light microscopy by intense polymprphonuclear and eosinophilic infiltrate, on fluorescence microscopy by deposition of gamma globulin and complement within the vessel wall. Precipitating antibodies to the xenogeneic antigen were demonstrated in nephrotic rats; however, antibodies to the isogeneic antigen could not be demonstrated. An antigen was found circulating in the serum of both pre-nephrotic and nephrotic animals. In addition, an antigen was found in the urine of these animals which showed a line of identity to that demonstrated in the serum. The role of the sensitized lymphocytes was investigated and it was demonstrated that these sensitized

lymphocytes would destroy an isogeneic kidney monolayer and would also exhibit a reaction not dissimilar to that of a graft versus host reaction in an in vivo isogeneic situation. These results were discussed and conclusions made.

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