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## Effect of Experimental Glomerulonephritis on the Cells in Canine Renal Lymph

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EFFECT OF EXPERIMENTAL GLOMERULONEPHRITIS  
ON THE CELLS IN CANINE RENAL LYMPH

by

Sandra L. Garber

A Dissertation Submitted to the Faculty of the Graduate School  
of Loyola University of Chicago in Partial Fulfillment  
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## VITA

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## INTRODUCTION

Glomerulonephritis is an inflammatory disease of the kidney that is manifested by the presence of protein and blood in the urine. The primary lesion affects the glomerulus, that component of the kidney where blood is filtered and urine formation begins. As the disease progresses entire nephrons may be destroyed and chronic renal failure may develop. This condition is present in approximately fifty percent of all patients with severe renal disease and accounts for about two-thirds of the renal transplant operations performed in the United States (Epstein, 1974).

It is generally accepted that there is an immunologic component to glomerulonephritis that may be manifested in at least two forms. One is characterized by the formation of circulating antigen-antibody complexes of different sizes. The smaller complexes remain in the blood and do not appear to cause glomerular damage; the larger complexes are removed by phagocytic cells. However, those of intermediate size are deposited in the walls of vessels, particularly the glomerular capillaries, where they initiate an inflammatory response characterized by an infiltration of neutrophils. The subsequent release of lysosomal enzymes by these cells damages the capillary basement membrane and leads to an increase in its permeability, which in turn leads to the escape of plasma proteins, and cells from the blood into the urinary space. In a second form of the disease, antibody to glomerular

basement membrane (GBM) formed in response to immunogenic substances of autologous or heterologous origin, or acquired passively by the introduction of heterologous anti-GBM serum, attaches to the membrane and also results in a nephritis. If this antibody is heterologous, a second phase occurs in which the foreign Ig attached to the GBM forms an immunogenic site, and through sensitized lymphocytes stimulates the production of antibody by the host. The product of this response (autoantibody) reacts with the foreign Ig resulting in further damage.

Recently (Rocklin et al., 1970 and Bahn et al., 1978) it has been suggested that cell-mediated immunity may produce a third type of response in which lymphocytes, sensitized against the GBM or one of its constituents, are a direct cause of damage to the renal glomerulus. It is known that cell-mediated immunity and humoral immunity commonly accompany each other during the course of a disease. It is not clear, however, if in an experimental model a level of circulating nephrotoxic antibody too low to cause immediate, direct injury to the glomerulus is capable of stimulating the production of sensitized lymphocytes or if these cells could then be capable of initiating a humoral response.

It has been postulated (Humphrey and White, 1971) that for antibody production to take place, the antigen in some form must first reach lymphoid tissue. During a primary immune response there is a marked increase in the number of cells leaving the stimulated tissue by way of the afferent lymphatics. The response is the same whether the antigen used is a protein capable of initiating a humoral (antibody)

response (Hall and Morris, 1963; Kelly, 1970) or a tissue graft, which stimulates cell-mediated immunity (Pedersen and Morris, 1970; Evans et al., 1978). There also appears to be a qualitative and temporal difference in the immediate response to these two types of antigens.

Following the local administration of an antigen, there is an initial increase in the number of cells in the regional peripheral lymph (Hall and Morris, 1963). Neutrophils comprise fifty percent of this cell population and this neutrophilia persists for approximately twenty-four hours. Gradually, lymphocytes become the predominant cell and the total white cell count reaches a peak on the fourth day following antigenic stimulation. The cell count then rapidly drops towards normal levels by the fifth to the seventh day (Hall and Morris, 1963). In the cell-mediated or tissue allograft model, the initial response is of greater magnitude and ninety percent of the responding cells are lymphocytes. This difference in the cell type seen in the regional lymph could prove to be of great value in the investigation of the early events in immunologically mediated glomerulonephritis.

For a variety of reasons the canine kidney is a suitable model for study of immunologic disease processes. For instance, the dog is frequently used as a model for glomerulonephritis, and the progress of the experimental disease is well established (Stebly et al., 1961; Wright et al., 1973; Wagnild, 1977). Additionally, the canine kidney has a well described system of anastomosing hilar and capsular lymphatics (Holmes et al., 1977; Albertine and O'Morchoe, 1979).

The vessels of the hilar system begin in the renal cortex and are frequently seen in association with an interlobular artery. They then follow along the arcuate and interlobar vessels and leave the kidney at the hilus in company with the neurovascular bundle. Thus the renal hilar lymphatics provide a direct route for cell traffic between the kidney and the regional lymph node. Although several investigators (Haynes and Field, 1931; Cockett et al., 1973) have described the cellular content of canine renal hilar lymph (RHL) in general terms, no comprehensive study on the normal cellular composition has yet been carried out. Renal hilar lymph continues to flow during anesthesia, making any further disturbance of the normal physiology of the kidney unnecessary.

The purpose of this investigation was to define the normal cellular composition of renal hilar lymph, and to study the changes that take place in the numbers and types of cells leaving the kidney during the initial phase of an experimentally induced immunologic glomerulonephritis.

## REVIEW OF LITERATURE

### Normal cellular composition of the lymph

The lymphatic system consists of vessels, and lymphatic tissue. The vessels provide a pathway for the movement of fluid containing dissolved substances, macromolecules and cells from the interstitial spaces of the tissues to the regional lymph nodes. There are three main types of lymphatic vessels: peripheral lymphatics, which carry lymph that has not yet passed through a lymph node; intermediate or efferent lymphatics, which contain post-nodal lymph that may pass through at least one more node before entering the central vessels; and central lymphatics, which empty into the blood stream.

Hewson, in 1774, was the first to note the presence of formed elements in lymph. A century later Ranvier (1875) described lymphocytes in the thoracic duct and referred to them as "une glande mobile unicellulaire". Winternitz in 1895 cannulated lymphatics in the groins of several dogs and induced lymph flow by massage of the limb. In this way he measured lymph flow and white cells in the lymph. The mean white cell count in the lymph was 717 cells/mm<sup>3</sup>.

In an attempt to identify the types of cells found in lymph, Rous in 1908 prepared smears of thoracic duct lymph from 23 dogs. In this case massage was not necessary since thoracic duct lymph continues to flow in the anesthetized animal. He found that 87.6% of the cells in the lymph were lymphocytes. Neutrophils were present only in association with numerous erythrocytes and mast cells

were always absent. Eosinophils accounted for 2.6% of the cells, a figure that was apparently not influenced by the presence or absence of parasites in the dog. Rous (1908) also described a "transitional" leukocyte with a nucleus that was bilobed or deeply cleft into irregular, blunt branches and with a cytoplasm that was blue. Although this cell was rare in central lymph (0.39%) it was frequently seen in the blood (17.8%). The author reported that the smears contained many "distorted" cells that could not be classified and this he attributed to the difficulty in obtaining adequate preparations from lymph because of its lack of "body".

A later study (Haynes and Field, 1931) compared the number of cells in the cervical duct, a central lymphatic, with the peripheral lymphatics of the dog's leg. Whereas central lymph contained 2,800-68,600 cells/mm<sup>3</sup>, most of which were small lymphocytes, the cellular content of peripheral lymph was sparse (0-2,500 cells/mm<sup>3</sup>). Furthermore, it was determined that while massage of the limb had little effect upon the number of cells in peripheral lymph, manipulation of the regional lymph node increased the cellular content of central lymph 4 to 6-fold. This suggested that the lymph node was the source of the cells in central lymph. To confirm this finding, Yoffey and Drinker (1939) sampled peripheral lymph, thoracic duct lymph and blood in the same animal. It was assumed that the cells of peripheral lymph entered the lymphatic from the tissue spaces, having left the blood and migrated through the



interstitium. Cell counts were performed on supravitaly stained preparations, but this procedure did not allow differentiation between lymphocytes and monocytes. Unlike central lymph, which contained only mononuclear cells that were presumed to be lymphocytes, peripheral lymph contained 24% neutrophils and 71% mononuclear cells. Yoffey and Drinker (1939) were unable to find any consistent numerical relationship between the number of mononuclear cells in the peripheral lymph and in the blood. They concluded, therefore, that over 95% of the lymphocytes in the thoracic duct were newly formed cells derived from lymphoid tissue. Later studies by other investigators (Gowans & Knight, 1964), however, showed this assumption to be incorrect. For example, small lymphocytes taken from the thoracic duct and labeled with radioactive tracer could, when re-injected intravenously, be recovered in the thoracic duct. It was shown that these cells left the blood within the lymph node by way of a specialized set of blood vessels, the post-capillary venules. From the lymph node most of the cells entered the efferent lymph and then passed to the central lymph, thus demonstrating the existence of a pool of small lymphocytes, comprising about 70% of the cells in the thoracic duct lymph, that recirculate from blood to lymph. The number of labeled cells found in non-lymphoid tissue was considered to be "trivial". This relegated to afferent lymph the role of returning stray cells from the interstitium, much as it returns protein to the blood. However, because lymph flows through lymph

nodes, other investigators (Hall and Morris, 1963) have suggested that it may play a role in the immune response. Recent studies have confirmed the presence of immunocompetent cells in the afferent lymph of several species including man (Pattengale et al., 1972; Hall et al., 1976; Kotani et al., 1977, Sokolowski et al., 1978).

#### The cellular composition of lymph following antigenic stimulation

Winternitz (1895) injected turpentine oil into dog foot pads and collected lymph from the groin. Samples of lymph and blood were collected for the ensuing 6-8 hours. He concluded that local irritation was followed, after a delay of several hours, by an increase in the number of cells in the lymph but that this increase was not reflected in the blood. It was not until 1962 that a specific function of the lymph node and the efferent lymphatics in the immune response was shown by Hall and Morris (1962). They injected human serum globulins into sheep in an area draining to the popliteal node and reported that within one hour of antigenic stimulation the white cell count of the efferent lymph had fallen to 10% of the pre-injection level. In the unstimulated state neutrophils and eosinophils were rarely seen, but after injection each contributed one percent to the total white cell population. After two to three hours the cell count returned to normal and then rose again to a maximum two days later. At this time many large immature cells as well as "transitional" cells, plasma cells and their precursors were seen. By the sixth day the cell count had returned to normal but plasma cells were still present.

In the unstimulated state most of the cells in the efferent lymph were considered to be part of the recirculating pool, but after antigenic stimulation cells having 'blast-like' characteristics and plasma cells, not seen in blood, were found in the lymph. Therefore, it was assumed that under these conditions, new cells could be formed in the lymph node and added to the efferent lymph. This process was not well understood until 1967 when Hall et al. injected labeled antigen into afferent lymph and failed to recover the tracer in lymph draining from the node. However, when the cells were removed from efferent lymph the occurrence of a systemic immune response was prevented. The authors concluded that the cells in efferent lymph, rather than the antigen itself, were responsible for disseminating the immune response throughout the body.

Earlier studies by the same group (Hall and Morris, 1963) demonstrated that the cell population of peripheral lymph also changed in direct response to the antigenic challenge in the appropriate tissue bed. For example, within a few hours of the local injection of human serum globulins into sheep, the number of neutrophils in the regional lymph rose and accounted for 50% of the white cell population. The count returned to normal twenty to thirty hours later. Eosinophils also increased, and remained at an elevated level in both peripheral and efferent lymph throughout the response. Large lymphocytes and transitional cells with basophilic cytoplasm gradually began to appear in the peripheral lymph, as did

cells undergoing mitosis. "Plasma blasts" and mature plasma cells were present and comprised one percent of the total cell population. These changes were fully developed within three days of injection and returned to normal levels by the fifth day. Simultaneous studies on the blood of these animals were not reported; therefore it is not known whether plasma cells were also present in the peripheral blood. In 1969, Osogoe reported that obstruction of the afferent lymphatics brought about architectural changes in the lymph node, particularly in the germinal centers, a site of lymphocyte proliferation. This suggested a relationship between these structures and the afferent lymph.

Kelly (1970) demonstrated that following peripheral antigenic stimulation in the rabbit, there was an increase in the cellular content of the regional lymph, thereby confirming the work of Hall and Morris (1963). In addition, he reported that the cells became localized within the regional lymph node. Bovine erythrocytes induce a humoral immune response in rabbits and diphtheria toxoid elicits a mixed humoral and cellular response. Using these two immunogens, Kelly (1970) noted a difference in the blast cell population of peripheral lymph. Diphtheria toxoid induced an intense response of short duration and rapid enlargement of the germinal centers. Bovine erythrocytes induced a blast cell response of lesser magnitude that was sustained for a longer period of time. The regional lymph node after this latter type of stimulation showed

few active germinal centers. In a later study Kelly et al. (1972) used antigens that gave rise to either a humoral, mixed or cell-mediated response. The antigen that induced a cell-mediated immunity caused a transient increase of neutrophils in the peripheral lymph, but thereafter the cell content remained at or slightly below normal levels. The response to the other antigens confirmed the earlier study (Kelly, 1970). Following the subcutaneous injection of bacillus Calmette Guérin (BCG), also a stimulator of cellular immunity, Vadas et al. (1979) reported a large increase in the number of mononuclear cells in lymph draining the site of the lesion. They also identified a vasoactive cellular product that was distinct from prostaglandin E<sub>1</sub> and other known hyperemic agents. This factor was present in afferent but not efferent lymph; and since one difference in the cellular composition of these two arms of the lymphatic system is the presence of macrophages in the afferent lymph (Reinhardt and Yoffey, 1957) this latter cell has been implicated as the source of the vasoactive product.

In 1968, Morris et al. used phase contrast microscopy and microcinematography to examine the cells of ovine peripheral lymph. They reported a cell with voluminous cytoplasm that frequently extended out into cytoplasmic "veils" which they presumed to be a macrophage. This cell accounted for 14% of the cells of the lymph. This "veiled" cell was occasionally seen to be in intimate contact with a lymphocyte or to form the center of a lymphocyte rosette. When

examined by electron microscopy it exhibited a well developed Golgi apparatus, numerous lamellae of ergastoplasm, microtubules and phagocytic vacuoles. However, there was no structural evidence that suggested that material passed between the cell and adjacent lymphocytes. Kelly (1970) reported the presence of a "frilly" cell in the lymph afferent to the popliteal node of the rabbit that accounted for 52% of the cells. He felt that it was similar to the "veiled" cell described by Morris et al. (1968), but differed in that the "frilly" cell had villiform cytoplasmic processes covering its surface and adhered to glass when incubated. It was not considered to be phagocytic since it did not ingest carbon particles or labeled immune complexes. The number of "frilly" cells in the afferent lymph increased following antigenic stimulation but no contact relationship with lymphocytes was reported.

Further investigations by the same author (Kelly et al., 1978) showed that the phagocytic activity of the "veiled" or "frilly" cell was dependent on the incubation medium used. For example, when incubated in lymph, 8-10% of the cells were phagocytic. Also, if the "veiled" cells were labeled and returned to afferent lymph, they were found to be localized in the regional lymph node, mainly in the thymus-dependent paracortical areas. They were not recovered in the central lymph or blood. Kelly et al. (1978) also observed by electron microscopy that approximately 3% of the "veiled" cells contained Langerhans (Birbeck) granules, which are highly characteristic of the Langerhans cell. This latter cell is usually associated with the

upper layers of the stratum malpighii of the epidermis and possesses limited, if any, phagocytic capabilities. A similar type cell of unknown function, also containing Birbeck granules has been described in normal lymph nodes (Vernon et al., 1973; Kobayashi and Hoshino, 1976) and the thymus (van Haelst, 1969), where they have been called interdigitating cells.

It has been suggested that the Langerhans cell plays a role in the uptake of contact allergens (Shelly and Juhlin, 1977). In an attempt to elucidate the role of the "veiled" cell and its possible relationship to the Langerhans cell, Drexhage et al. (1979) applied the contact sensitizing agent 1-fluoro-2,4-dinitrobenzine (DNFB) to the skin of pigs. The regional pre-nodal lymph showed not only an increase in the total number of "veiled" cells but also an increase in the proportion of these cells that formed aggregates with other lymph-borne cells. The number of these aggregates increased with the duration of the response. Seeger and Oppenheim (1970) had previously reported this tendency of macrophages to form aggregates during delayed-type hypersensitivity reactions in vitro. The regulatory role of the macrophage in the response to antigen has been well established (Unanue, 1972). Drexhage, therefore suggested that the "veiled" cell may serve to process the antigen, at least in contact hypersensitivity. A recent study of "veiled" cells in human afferent lymph (Spry et al., 1980) also indicated that the cell may play a role in the transport of antigens and the stimulation of T-lymphocytes in the lymph node.

## Renal Lymph

The majority of experiments concerning the cells of peripheral lymph has been performed on lymphatics draining to the popliteal lymph node. It is known, however that there is a comparable system of lymphatics draining other areas of the body, including the kidney. As early as 1931, Haynes and Field cannulated one renal lymphatic and reported a white cell count of 1,200 cells/mm<sup>3</sup>. However, a differential count was not made on this sample. More recently, Smith et al. (1970) sampled renal lymph in unanesthetized sheep and found a white cell count ranging from 100-700 cells/mm<sup>3</sup>, 80% of which were lymphocytes. Although generally comparable to lymph from other regions, renal lymph was reported to contain a greater number of macrophages and more erythrocytes. A brief report by O'Morchoe (1972) confirmed that in the dog, as in the sheep (Smith et al., 1970), the majority of cells in renal lymph are lymphocytes. Most information on renal lymph has been obtained from animals subjected to renal transplantation. In the most comprehensive study Pederson and Morris (1970) transplanted kidneys, with the hilar lymphatics intact, into allogeneic sheep and collected renal lymph for the life of the graft, which was approximately 7-9 days. Within the first 48-hours the lymph cell population was characterized by a transient influx of neutrophils. By 24-hours the cellular component mostly in the form of lymphocytes, began to increase in number, but at this time no blast cells were noted. However, if the small lymphocytes leaving the kidney were cultured in vitro blast trans-



formation did occur. Similarly, if these cells were injected into a distant, non-stimulated lymph node, they evoked a cellular and antibody response in that node, indicating that these cells had become sensitized to the graft. After a period of 2-3 days, medium to large lymphocytes with intensely basophilic cytoplasm appeared in the renal lymph. At the height of the rejection response these cells accounted for 60% of the cells present. Granulocytes were rare and macrophages, many of which possessed cytoplasmic veils, accounted for 3-10% of the cells. Lymphocyte-agglutinating antibody production, as estimated by the capillary tube migration test, did not begin until 2-3 days after the graft was in place, and antibody was not detectable in the blood until close to the time of graft rejection.

Cannulation of renal hilar lymphatics in dogs with transplanted kidneys has yielded similar results (Cockett et al., 1973). The greatest number of lymphoblasts was seen on the fourth day after transplantation, when these large cells ( $> 13 \mu\text{m}$ ) accounted for 25% of the cell population. However, by the eighth day these cells had decreased in number to only 5% of the cells present. Evans et al. (1978) used the sheep model and reported that although there is a marked rise in the white cell count in renal lymph during rejection, this increase is not reflected in the number of lymphocytes in thoracic duct lymph. Indeed, if any change occurred, it was a slight decrease in the number of circulating lymphocytes. Immunosuppressive therapy failed to decrease further the number of cells in thoracic

duct lymph but it did appear to reduce the traffic of lymphocytes through the kidney. An earlier study by Anderson et al. (1977) in the rat had shown that thoracic duct drainage could be used to increase the survival time of renal allografts. Their report amplified the findings of Hall et al. (1967) to include central lymph and substantiated the suggestion that if "committed" lymphocytes are not allowed to remain in the body, a systemic immune response can be prevented.

#### Experimental glomerulonephritis

In the early nineteen hundreds the etiology of diffuse glomerulonephritis was a source of controversy. One theory proposed that the disease resulted from anemia of the glomeruli effected by constriction of the afferent arterioles. Another theory stated that the disease resulted from glomerular inflammation. A tool with which to investigate the pathogenesis of the disease was provided by Matazo Masugi when in 1931, he succeeded in developing a reproducible experimental model of glomerulonephritis in several animal species (Masugi, 1933, 1934). The injection of heterologous anti-kidney serum into rabbits or rats resulted in a lesion of the kidney. On microscopic examination it was characterized by a thickening of the capillary loops, followed 9-15 days later by endothelial proliferation, proteinuria and a decrease in renal function. Today it is known that this thickening of the capillary loops is actually due to a thickening of the basement membrane, a

structure not recognized in Masugi's time. Masugi thought that the mechanism for the antibody action on the glomerulus was a "reverse anaphylaxis" in which the injected antibody acted as an antigen that led to the production of autologous antibody and that the latent period of one to two weeks represented the time needed for the antibody to fix to the kidney. That this time frame was inaccurate was demonstrated by Pressman et al. (1950), who showed that in the mouse the serum is cleared of circulating antibody within 18 minutes of intravenous injection.

Although the effects of nephrotoxic serum had been examined in depth, the specific nature of the kidney antigen responsible for the antibody production was unknown. Heymann et al. (1950) localized the antigen to the renal cortex, and Pressman and Eisen (1950) found it to be restricted to the glomerulus. Using various crude fractions of isolated glomeruli, Krakower and Greenspon (1951) demonstrated that the basement membrane was twenty times more antigenically active in the production of nephrotoxic serum than the cellular elements of the glomerulus. With the advent of the fluorescent antibody technique (Coons & Kaplan, 1950), it became possible to localize specifically the nephrotoxic antibody, to the glomerular basement membrane (Mellors et al., 1955a, 1955b).

Although thirty years had elapsed since the discovery of an experimental model for glomerulonephritis, the pathogenesis of the disease was still in doubt in 1954, but it was then clear that the

process was an inflammatory one. The applicability of the Masugi model to the human condition presupposes that (1) other proteins have antigenic determinants in common with the glomerular basement membrane and that these antigens can stimulate an antibody response or (2) that the body is capable of producing antibodies against its own glomerular basement membrane. This latter tenet is in conflict with Ehrlich's doctrine of "horror autotoxicus", which states that immunity is directed against foreign materials, but not against the constituents of one's own body (Ehrlich, 1900). Attempts to find anti-kidney antibodies in human patients with glomerulonephritis did not yield convincing results. Indeed, a new controversy arose because of a second experimental model developed by Germuth in 1953. In this model, known as serum sickness nephritis, glomerulonephritis was produced by the repeated injection of foreign proteins that lacked any anti-kidney or anti-basement membrane activity. In order to define the mechanisms involved in serum sickness nephritis, Dixon et al. (1961) gave daily injections of various serum proteins to rabbits and monitored the resultant glomerulonephritis relative to the levels of antigen and antibody in the animal's circulation. They demonstrated that the type of disease developed was related to the amount of antibody formed. In antibody excess there was an acute, self-limiting glomerulonephritis. In antigen excess, no renal damage was noted. When there were approximately equal quantities of antigen and antibody, a chronic glomerulonephritis developed. Dixon et al. (1961) postulated that antigen-antibody complexes that

lodged in the glomerulus could be the etiologic agent of the renal injury.

In 1967, Lerner et al. were able to demonstrate anti-glomerular basement membrane antibodies in either the kidneys or serum of some patients with glomerulonephritis. They found that an IgG eluate to the antibody could induce nephritis in experimental animals. The antibody was present in all patients with Goodpasture's syndrome, believed to be an immune-complex disease characterized by pulmonary hemorrhage and renal failure, and in slightly less than 50% of patients with subacute or chronic glomerulonephritis. Thus, it appeared that there were at least two distinct immunopathogenic mechanisms operating in glomerulonephritis and that both involved the presence of humoral antibody (Dixon et al., 1971).

The question then arose as to what role, if any, cellular hypersensitivity played in glomerulonephritis. Rocklin et al. (1970) took lymphocytes from patients suffering from glomerulonephritis and incubated them with glomerular basement membrane. They found that in the presence of this antigen the cells of some patients were capable of producing migration inhibitory factor (MIF) indicating that the cells had been sensitized in vivo to glomerular basement membrane. It was not known whether these sensitized lymphocytes were the result of glomerulonephritic damage or whether they represented yet a third immunopathologic mechanism. It was argued that since there was not a "noteworthy" infiltrate of lymphoid cells into the glomerulus, the

possibility of a cell-mediated disease was unlikely (Dixon, 1970).

Jones (1951, 1953) thought that the hypercellularity seen in the glomerulus was due to an accumulation of migrant macrophages rather than the in situ proliferation of a fixed glomerular element. More recently it has been suggested that sensitized lymphocytes releasing MIF are responsible for this macrophage accumulation (Kondo and Shigematsu, 1980). While a mononuclear cell infiltrate may not be a significant finding in human glomerulonephritis, it has been seen in some experimental forms of the disease (Kondo et al., 1972; Shigematsu, 1970). In one such model (Schreiner et al., 1978) rats were pre-immunized with rabbit IgG and then given subnephrotoxic doses of anti-kidney serum. After a transient neutrophilia, the glomeruli became infiltrated with mononuclear cells. Experiments with [<sup>3</sup>H] thymidine indicated that these cells originated from precursors outside the kidney. A similar study was reported by Bahn et al. (1978). In this instance the rats were not pre-immunized but were given (1) a single subnephrotoxic dose of rabbit anti-rat kidney serum, or (2) lymph node cells from syngeneic donors previously sensitized to rabbit gamma globulin, or (3) both antibody and cells. Those animals receiving only cells or antibody did not develop demonstratable kidney lesions, although in the latter case rabbit gamma globulin was seen by immunofluorescence in the rat kidney. Only those animals that received both antibody and cells developed glomerular lesions that were characterized by segmental hypercellularity and necrosis. These findings indicated that

"interaction of specifically sensitized lymphocytes with glomerular-bound antigen can induce a cell-mediated (delayed-type) reaction in glomeruli" (Bahn et al., 1978).

Although not as extensively studied as the rabbit and rat, the dog is also a suitable model for the study of glomerulonephritis. Wright et al. (1973) examined the sequential pathology in a canine model of Masugi nephritis using light, immunofluorescent and electron microscopy. The animals were monitored from 4 hours up to 60 days after the administration of antiserum. The glomerular changes were similar to those seen in other experimental animals. An earlier report (Movat et al., 1961) noted that, using an anti-renal cortex preparation, no changes were seen in the kidney by light microscopy until 7 days after antiserum administration; however, changes at the ultrastructural level were apparent as early as 12 hours. Using a serum prepared against glomerular basement membrane, Wright et al. (1973) reported that aggregates of neutrophils could be seen in the glomerular capillaries as soon as 4 hours after injection. At this time immunofluorescent microscopy revealed a linear deposit of rabbit gamma globulin along the basement membrane. McPhaul et al. (1974) investigated the incidence and severity of the disease in the dog by a quantitative approach. They concluded that the extent of immediate injury depended directly on the amount of nephrotoxic antibody administered. In another study the morphologic changes were shown to be accompanied by changes in renal function (Wagnild,

1977). A comparative study of naturally occurring glomerulonephritis in several species confirmed that the canine experimental model corresponded morphologically to the natural disease (Slauson and Lewis, 1979).

### Chronobiologic Variation

A circadian rhythm in total white blood cell count has been reported for man (Shaw, 1927; Sharp, 1960), mouse (Brown, 1962), and rat (Pauly and Scheving, 1965). Unshelm and Hagemester (1971), however, failed to find a similar rhythm in bovine counts assessed at two-hour intervals between 0800 and 1800. A circannual rhythm in murine white blood cell count has also been reported (Berger, 1980). While there is no apparent rhythm in the relative percentages of lymphocytes, neutrophils, or eosinophils in the animals studied, absolute counts of these cells do show a circadian fluctuation (Elmadjian and Pincus, 1946; Halberg and Visscher, 1950; Brown and Dougherty, 1956; Dahl, 1977). A rhythm in blood monocytes has also been reported in mice (Soliman and Walker, 1977).

Both humoral and cell-mediated immune functions have been shown to vary in a circadian fashion. For example, studies of antibody production in mice immunized with sheep red blood cells indicated the existence of a circadian rhythm in the plaque-forming cell response (Fernandes et al., 1974; Fernandes et al., 1976). Furthermore it has been shown that the survival time of skin (Halberg et al., 1974) or kidney (Ratte et al., 1973) allografts in rodents is strongly influenced



by the circadian-phase dependent timing of transplantation. A chronosusceptibility rhythm has also been reported for killer cell function (Fernandes, et al., 1979), PHA-induced lymphocyte transformation (Tavadia et al., 1975), delayed-hypersensitivity (Knapp and Pownall, 1978) and phagocytosis (Szašo et al., 1978).

The kidney also shows circadian changes in such variables as urine (Mills and Stanbury, 1952), renin production (Gomez-Sanchez et al., 1976; Hilfenhaus, 1976), electrolyte excretion (Mills, 1963), and glomerular filtration both in man (Wesson and Lauler, 1961) and the rat (Cambar et al., 1979).

## PURPOSE OF STUDY

- 1.) To determine the normal cellular content of renal lymph in the dog. Specifically, to determine the total and differential counts of the white blood cells present. Particular attention will be paid to the lymphoid cell regarding size and nuclear-cytoplasmic ratio in order to establish the presence or absence of blast cells.
- 2.) To establish whether or not a relationship exists among the numbers and types of cells in renal lymph, thoracic duct lymph and blood. This information is important in order to establish whether there is a selective traffic of cells into renal lymph or if this is a random process dependent on the number and type of cells present in the blood.
- 3.) To determine if there is a circadian rhythm in the white blood cell count of the renal lymph, thoracic duct lymph and/or blood of the dog during anesthesia.
- 4.) To determine the effect of cytocentrifugation on cell size and morphology.
- 5.) To investigate the effect of a low dose of rabbit anti-canine glomerular serum on the number and type of cells found in renal lymph, thoracic duct lymph and blood over a two week time span.

Specific attention will be focused on the lymphoid cells and on the presence or absence of macrophages and "veiled" cells. If any cellular changes result from the treatment, an attempt will be made to correlate these changes with either a delayed-type hypersensitivity or a humoral response.

## MATERIAL AND METHODS

### Animals

Studies were carried out on adult mongrel dogs, ranging in weight from 20-35 kg. All animals were maintained on a standard 12 hr.:12 hr. light-dark schedule (lights on 0700-1900), fed laboratory dog chow and allowed water ad libitum. When necessary, urine collection was facilitated by keeping the animals in metabolism cages.

### Surgical procedure

Anesthesia was induced by the intravenous injection of sodium pentobarbital, 32.5mg/kg body weight. An endotracheal tube was inserted, the saphenous vein cannulated, and an infusion of 0.9% saline was given during the experimental procedure at the rate of 2ml/min. The left renal hilar lymphatic was cannulated with nylon tubing (ID 0.02 in.). Renal lymph was collected into capped tubes (12 x 75 mm) containing 0.2ml of a 2.7% solution of EDTA over ice, for consecutive 4 hour periods. In the initial experiments, three to four samples of 1-2ml each were collected from each animal and processed separately, but because little fluctuation in the white cell counts was found over the four hour periods, this procedure was discontinued, except as noted below.

The thoracic duct was isolated in the neck and cannulated with PE 90 polyethylene tubing. Samples, each of 7ml of lymph, were collected over ice in tubes (100 x 13 mm) containing 0.07ml of 15% EDTA. The residual thoracic duct lymph was returned to the circulation by way of the saphenous or cephalic vein. During each lymph collection period, blood samples from the saphenous vein were collected into similar EDTA containing tubes as for thoracic duct lymph. All samples were processed immediately to minimize any ex vivo morphological alterations. At the end of the experiment the animal was sacrificed and tissue taken from the left kidney.

Renal tissue for light microscopy was fixed in formalin, embedded in paraffin and sections were stained with (1) hematoxylin and eosin, (2) periodic acid Schiff reagent and (3) Mallory's trichrome. Tissue for fluorescent microscopy was frozen and stored at  $-80^{\circ}\text{C}$ . Sections cut on a Cryostat were stained with fluorescein-conjugated anti-rabbit IgG produced in goat according to the technique of Coons and Kaplan (1950). Tissue for electron microscopy was fixed in 4% glutaraldehyde, post-fixed in 2% osmic acid and embedded in Epon 812. Thin sections were mounted on copper grids and stained with uranyl acetate and lead citrate.

In order to establish a base-line for the 24-hour experimental studies, 5 control experiments were designed to examine the effect of prolonged anesthesia and circadian influences on the white cell count

of blood and lymph. Animals were anesthetized and cannulated as previously described. Samples of blood, thoracic duct and renal lymph were collected every four hours for 24 hours and where possible the initial time point was repeated.

#### Processing of cell samples

Prior to cell separation and cytocentrifugation, a small volume of fluid was taken from each sample and total white cell counts were performed using a standard hemocytometer. All blood samples plus thoracic duct lymph samples contaminated with blood were centrifuged over a Ficoll-Hypaque gradient (density 1.077gm/ml). The paucity of cells in renal lymph precluded similar centrifugation, but rarely was erythrocyte contamination a problem in hilar lymph.

The cell suspensions were washed in phosphate-buffered saline (PBS) supplemented with 6% bovine serum albumin (BSA), resuspended in this medium and incubated for 30 minutes at room temperature with latex particles (0.804  $\mu\text{m}$  in diameter). This procedure aided in the differentiation of lymphocytes from actively phagocytic mononuclear cells. Next, the uningested latex particles were removed during three successive washes and centrifugations at 160 x g for 10 minutes.

The cell suspensions were then diluted with the BSA supplemented PBS in order to obtain a solution containing  $1.0 \times 10^6$  cells per ml. Monolayered cell preparations were made from these suspensions using

a Shandon-Elliott cytocentrifuge. There is an inherent difficulty in obtaining adequate smears for differential counts from lymph because of its low protein content (Rous, 1908). This problem is compounded by the fact that the rate of spread and air drying of the conventional smear results in a distortion of cell morphology (Dacie and Lewis, 1975). Vossen et al. (1976) have shown that the cytocentrifuge yields highly reproducible results with a minimal amount of non-selective cell loss. In our hands, the cytocentrifuge method consistently provided superior monolayered preparations for analysis. To determine the effects of cytocentrifugation on cell size, comparative measurements were made of lymphocytes in whole blood smears and washed cytocentrifuged cell suspensions prepared from the blood of the same animal. In addition, similar comparative erythrocyte measurements were made.

Preparations from whole blood smears and cytocentrifuged monolayers of blood and lymph were stained with Mac Neal's tetrachrome and differential cell counts were performed. Cells were classified on the basis of standard hematologic criteria with the exception of the monocyte/macrophage type where the presence of latex within the cytoplasm of a mononuclear cell was used as the criterion for assigning a cell to this category. On each slide a minimum of 200 cells was counted. Cell and nuclear diameters of 31 randomly chosen lymphoid cells were determined using a micrometer eyepiece. Two measurements at right angles to each other were made, and the

average of the measurements taken. These data were also used to calculate nuclear-cytoplasmic ratios.

### Antiserum<sup>1</sup>

Purified glomeruli were obtained from the kidneys of normal dogs by a modification of the method of Krakower and Greenspon (1951). Antiserum was produced in New Zealand white rabbits by injecting them with 2.5-3.0 mg of purified glomeruli in complete adjuvant. Animals received two injections of antigen 14 days apart. If in 21 days the antibody titer was 1:16 or greater the animal was exsanguinated, otherwise a third injection of antigen was given at this time and these animals were exsanguinated on the 30th day. The serum was heated at 56°C for 30 minutes to remove complement activity. Species specific activity directed against non-glomerular canine antigens was absorbed using washed canine red blood cells. Adsorptions were continued until a negative titer was macroscopically observed. The antiglomerular activity and specificity were assessed by titration of the antiserum against normal dog kidney, lymph node, spleen, skeletal muscle and lung using the indirect immunofluorescent technique of Coons et al. (1950). Those antisera showing titers of

<sup>1</sup>The antiserum used in the initial part of this investigation was kindly provided by Dr. C.F. Lange of the Department of Microbiology of Loyola University.



1:16 or greater against renal glomeruli were pooled. Total gamma globulin level was determined spectrophotometrically at 280 nm after precipitation by ammonium sulfate.

### Experimental procedures

For at least two days before the injection of antiserum, animals to be injected were housed in metabolism cages, and 24-hour urine samples were collected. A routine urinalysis was performed, and 24-hour protein levels were determined using the sulfosalicylic acid method (Davidsohn and Henry, 1974). Animals in which the urine was essentially protein-free and the sediment within normal limits, were considered to be free from clinically evident renal disease. A control blood sample was drawn, after which the animals were injected with the equivalent of one mg rabbit gamma globulin/ kg body weight. Inasmuch as a circadian variation in immunosusceptibility has been demonstrated in several laboratory animal species (Fernandes et al., 1974; Stinson et al., 1980; Willey and Ushyima, 1980), the above procedure was carried out on four dogs during four phases of their circadian physiological system. These four animals were sacrificed four days after injection. All other animals were injected at 0900 hours unless otherwise noted. Twenty-four hour urine collection and analysis continued following the injection of antiserum. Groups of dogs (n=3-5) were examined at 1, 2, 4, 7 and 14 days after injection.

The above procedure was modified for those time points of less than 24-hours. Nine animals were prepared for surgery and the lymphatics were cannulated as described above. In a group of five of these dogs, one control sample each of blood, thoracic duct lymph and renal hilar lymph was obtained. Immediately following this collection, the animals were injected with 1mg/kg body weight of anti-glomerular serum. Samples of blood and lymph were collected one hour later and every two hours thereafter until 16 hours after the injection. The four remaining dogs were used to determine the effect, if any, of anesthesia and/or cannulation on the control and experimental values. In these cases, control samples of lymph and blood were collected every two hours for six hours. The animals were then injected with 1mg/kg body weight antiglomerular serum and collections were continued at two-hour intervals over the next six hours.

## RESULTS

### Controls

#### Total white blood cell counts

The total white blood cell counts from the three compartments are shown in Table 1. The mean white cell count in the blood was 11,947 cells/mm<sup>3</sup> (S.E.  $\pm$  1,003), which is within the normal range (6-18,000 cells/mm<sup>3</sup>) for canine peripheral blood (Hime, 1976). The mean count in thoracic duct lymph was 7,709 cells/mm<sup>3</sup> (S.E.  $\pm$  1,226), which is in general agreement with published values (Yoffey and Drinker, 1939; Leeds et al., 1971), and in peripheral renal lymph it was 133 cells/mm<sup>3</sup> (S.E.  $\pm$  25). As may be seen from the standard deviations there was greater variability in the cell density of the lymph than of the blood. No correlation in total white cell counts among the three compartments was found. For example, in animal number 3, although there was a marked peripheral leukocytosis, the thoracic duct cell count was within normal limits, whereas the hilar lymph cell count was low. In contrast, in animal number 8, the renal lymph cell count was elevated, whereas the peripheral blood count was normal and the cellular content of the thoracic duct lymph was depressed.

TABLE 1

Total white cell counts of the control animals. The white cell counts of the blood, thoracic duct lymph, and renal hilar lymph are compared within the same animal.

TABLE 1  
TOTAL WHITE CELL COUNTS<sup>1</sup>

Animal Number	Blood	Thoracic Duct Lymph	Renal Hilar Lymph
1	11,650	7,150	30
2	15,400	21,500	448
3	25,750	8,600	58
4	10,200	5,200	285
5	17,950	10,850	265
6	7,500	2,925	105
7	10,450	2,500	20
8	15,400	3,900	375
9	9,850	14,800	29
10	10,000	3,900	28
11	19,750	3,700	35
12	6,500	5,600	79
13	13,425	4,975	88
14	13,750	6,025	105
15	7,175	7,475	86
16	12,325	15,800	130
17	8,400	5,525	171
18	6,550	1,325	47
19	11,925	4,025	120
20	7,975	1,825	48
21	10,325	12,150	218
22	10,575	19,850	158
Mean	11,947	7,709	133
Standard Deviation	4,704	5,749	118
Standard Error	1,003	1,226	25

<sup>1</sup>All values are expressed in cells/mm<sup>3</sup>.

### Cellular profile

The mean differential cell counts for blood and lymph are shown in Table 2. Blood counts were consistent with those performed routinely by the Animal Research Facility of this institution on all dogs admitted to the animal colony. The majority of the cells in the peripheral blood were neutrophils (Fig. 1), which were often so filled with ingested latex particles that the nucleus lay adjacent to the cell membrane. Twelve percent of the cells were lymphocytes, and in the conventional film preparations they exhibited the clumped chromatin pattern and sparse homogenous cytoplasm typical of the small lymphocyte. However, one advantage of the cytocentrifuge is that it accentuates cell characteristics without distorting cell size, and by this method lymphocytes frequently exhibited the notched nucleus and prominent nucleolus that is usually not evident in routine blood films (Fig. 1). Monocytes, as identified by latex particle ingestion were infrequent (2%), and when present, never contained the quantity of latex seen in the neutrophil (Fig. 2). Eosinophils comprised 9% of the cell population, and were occasionally noted to contain latex particles, indicating some phagocytic capability (Fig 2).

In the thoracic duct lymph, 97% of the cells were of the lymphoid line, the remainder being mainly eosinophils. Monocytes, as identified by latex particle ingestion, were not seen. Neutrophils were present only in samples that also contained

TABLE 2

Mean differential cell counts, in percentages and absolute numbers for the three compartments sampled. The lymphocyte group includes all lymphoid cells noted, regardless of the state of maturation.

TABLE 2

## MEAN DIFFERENTIAL CELL COUNTS--PERCENTAGES

	Neut.	Lymph.	Mono.*	Eos.
Peripheral Blood	77 $\pm$ 2.7 <sup>1</sup>	12 $\pm$ 1.5	2 $\pm$ 0.3	9 $\pm$ 1.5
Thoracic Duct Lymph	1 $\pm$ 0.4	97 $\pm$ 0.8	--	2 $\pm$ 0.7
Renal Hilar Lymph	27 $\pm$ 5.6	63 $\pm$ 6.4	7 $\pm$ 1.4	3 $\pm$ 0.9

## MEAN DIFFERENTIAL CELL COUNTS--ABSOLUTE

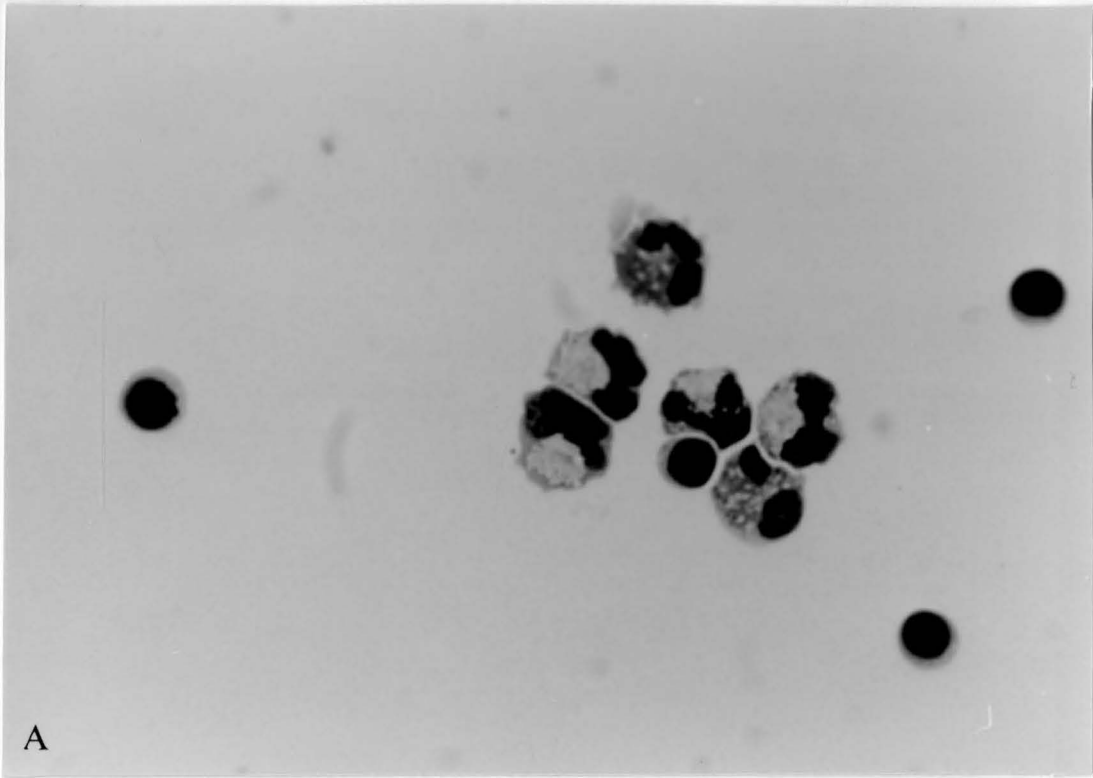
	Neut.	Lymph.	Mono.*	Eos.
Peripheral Blood	9,200 $\pm$ 1,090	1,434 $\pm$ 222	239 $\pm$ 42	1,075 $\pm$ 177
Thoracic Duct Lymph	72 $\pm$ 63	7,455 $\pm$ 1,493	--	200 $\pm$ 120
Renal Hilar Lymph	35 $\pm$ 19	84 $\pm$ 19	10 $\pm$ 2.3	4 $\pm$ 1.9

<sup>1</sup>Standard errors  
\*Monocyte/Macrophage



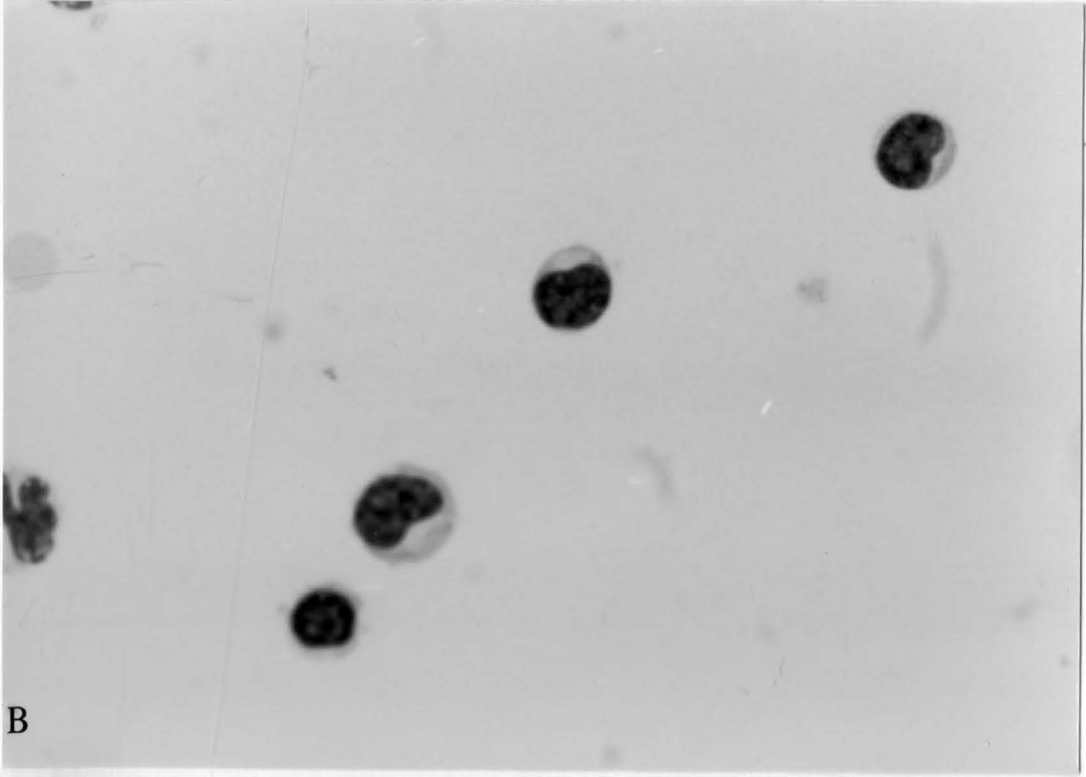
## FIGURE 1

Cytocentrifuge preparations of cells obtained from whole blood. A) The cytoplasm of the neutrophils is filled with latex particles. x600. B) Four lymphocytes, three of which show a notched nucleus. The clear area at the notch is probably indicative of the Golgi complex. x600.



A

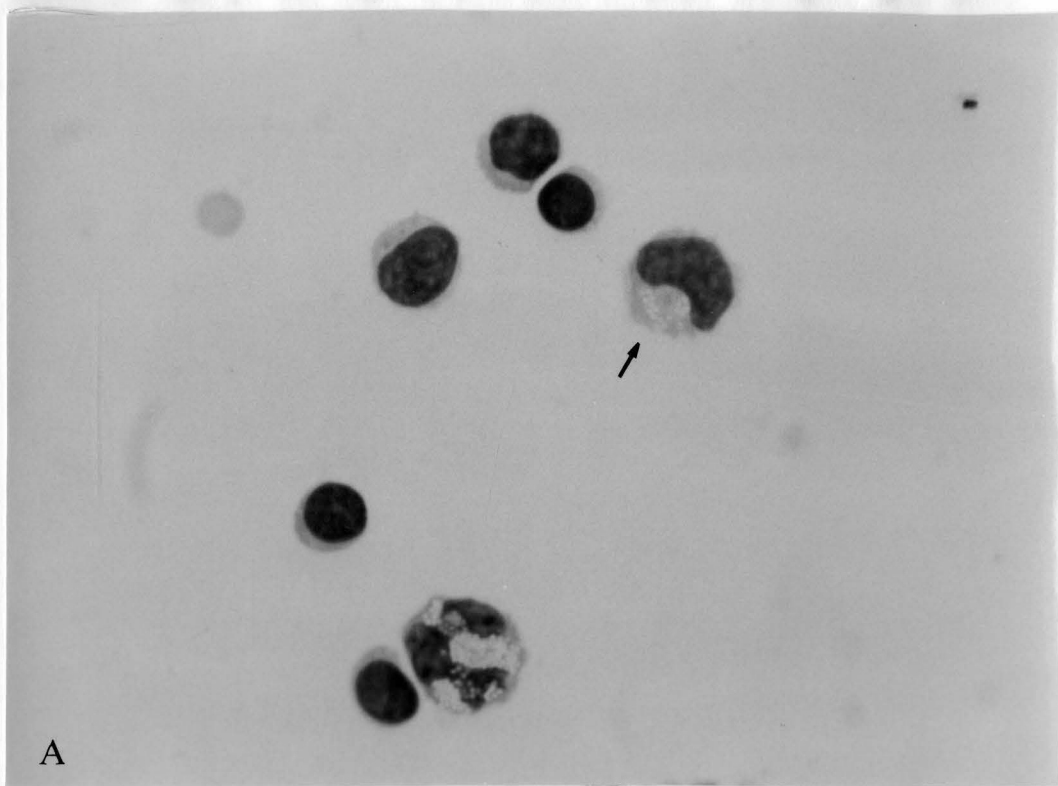
particles (arrow) counterstained with a latex-laden  
basophil. x600. B) Basophil with some ingested



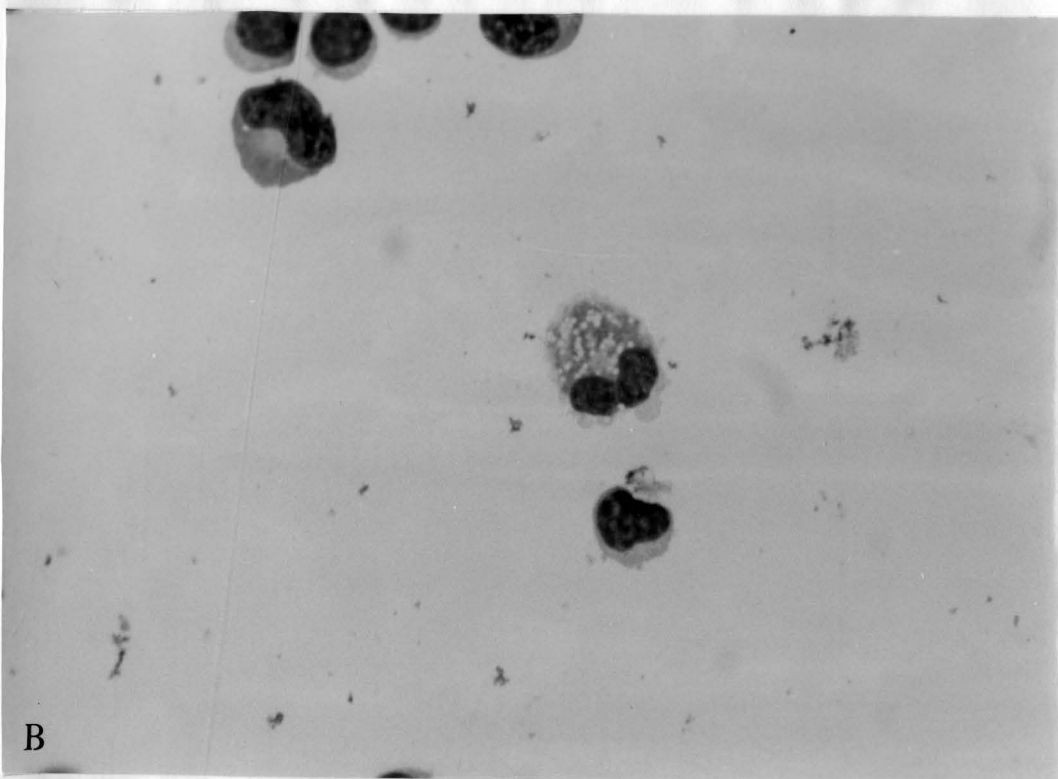
B

## FIGURE 2

Cytocentrifuge preparations of cells obtained from whole blood. A) Monocyte containing a few latex particles (arrow) contrasted with a latex-laden neutrophil. x600. B) Eosinophil with some ingested latex, indicating that these cells have some phagocytic capability. x600.



A



B

numerous erythrocytes and were assumed to reflect contamination from the blood. Cells in various stages of mitosis were also occasionally present (Fig. 3).

In renal hilar lymph the lymphocyte was the predominant cell type (63%), many of which were of the medium-to-large variety. The other cell types commonly found in the blood were also present but in different proportions. Blast-like cells with basophilic cytoplasm and prominent nucleoli were also seen (Fig. 3). Another group of cells was observed that was difficult to classify morphologically. They were greater than 13  $\mu\text{m}$  in diameter with a horseshoe shaped nucleus, the inner margin of which was smooth, but which had a convoluted outer edge. The cytoplasm possessed the "ground-glass" appearance typically seen in the blood monocyte, but the cell was rarely seen to contain more than one or two latex particles (Fig. 4).

The absolute number of lymphocytes in each compartment was calculated for each animal. There was no correlation among the three samples according to the Pearson product-moment correlation, ( $p > 0.05$ ).

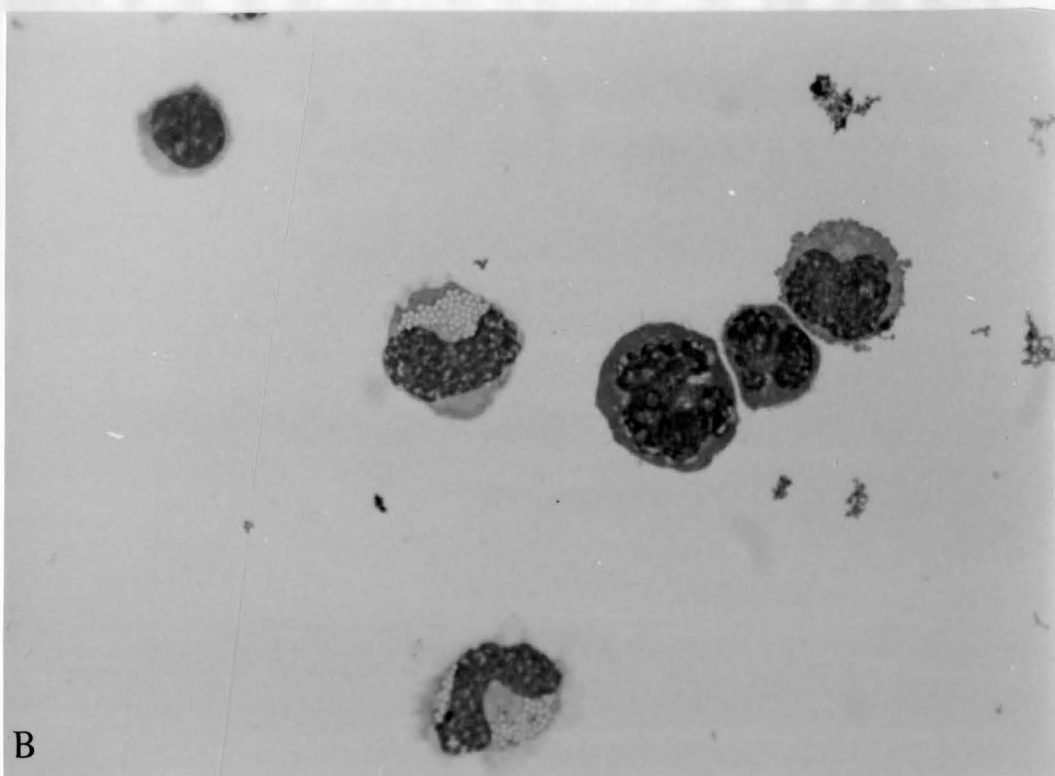
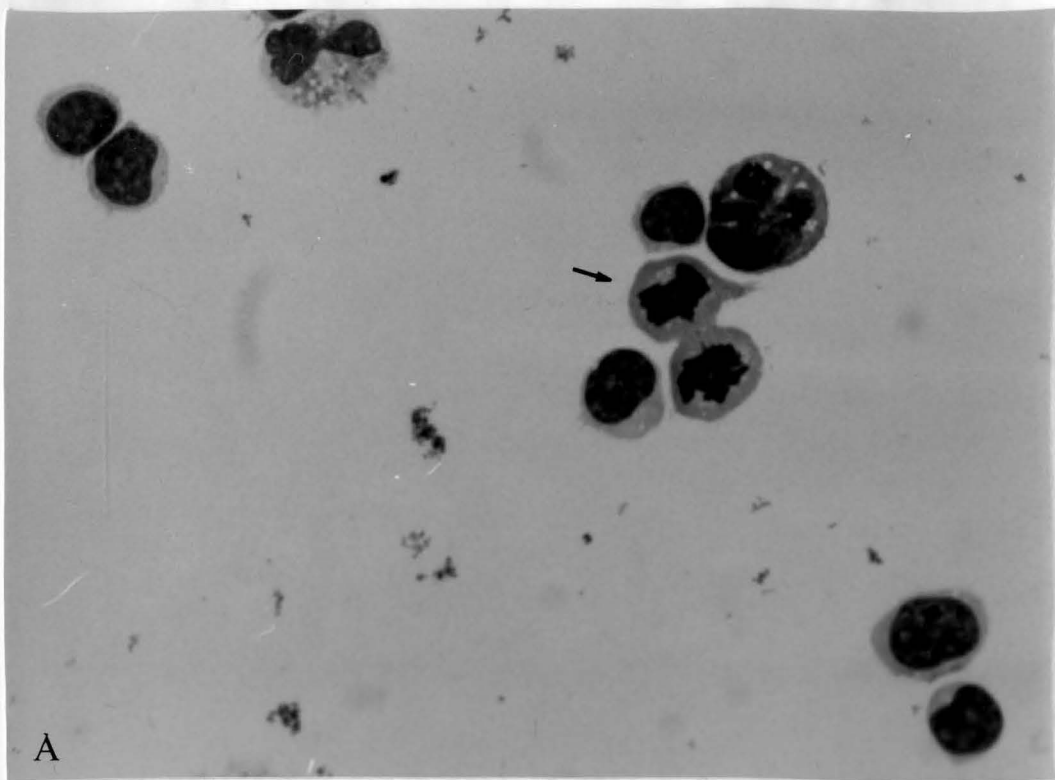
#### Cell Size

Measurements of the lymphocyte cell diameter from whole blood smears and from the cytocentrifuged preparations of the same sample after separation revealed that the cells in smears were larger

## FIGURE 3

Cytocentrifuge preparations of the cells of the lymph.

A) Cells of the thoracic duct. The majority of the cells are lymphocytes. Cells in mitosis (arrow) are occasionally encountered. x600. B) Cells of renal hilar lymph with three large blast cells. x600.



A

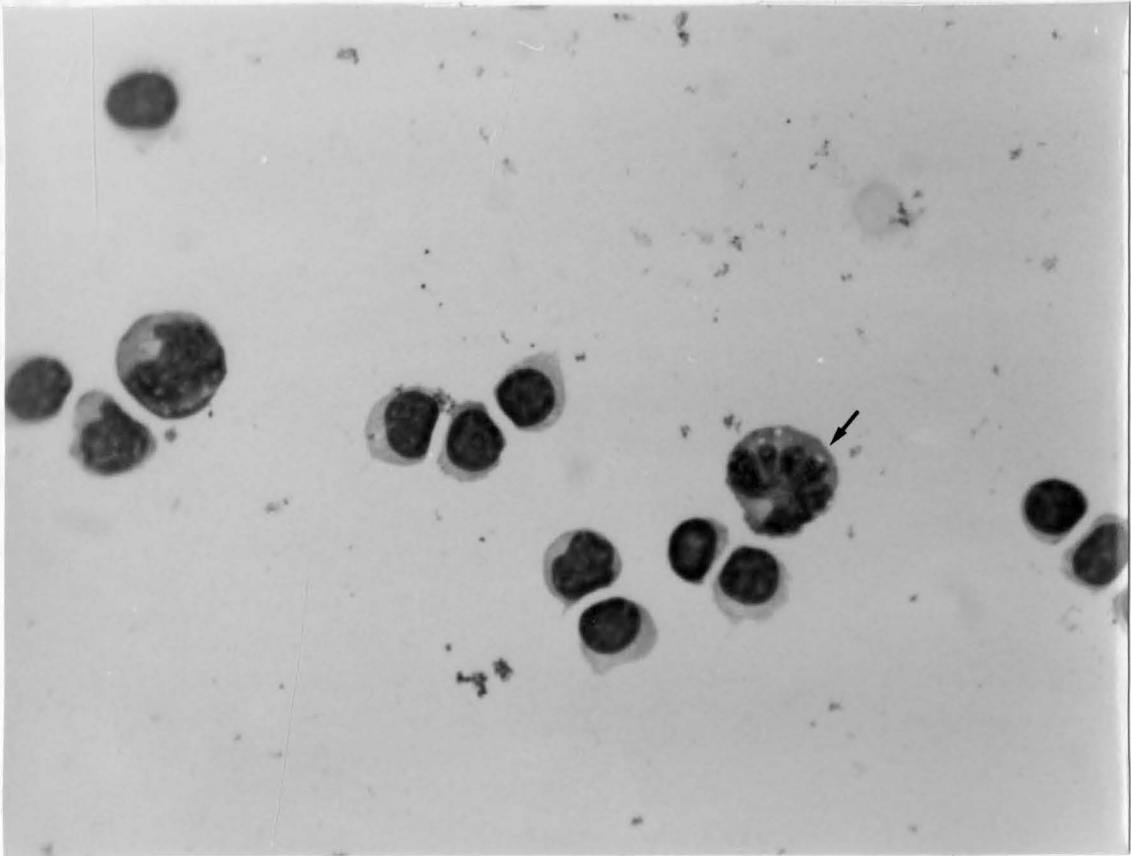
B

## FIGURE 4

Monocytoid cell type seen in renal hilar lymph. Note the convoluted outer edge (arrow) and the smoother inner margin. x600.



(11.60  $\mu$ , S.D. = 0.39) than those on the cytocentrifuged monolayer (10.32  $\mu$  + 0.33). Erythrocyte diameters, on the other hand, were the same in both preparations. Due to the known difficulties with distention and distribution associated with piped films (Dacie and



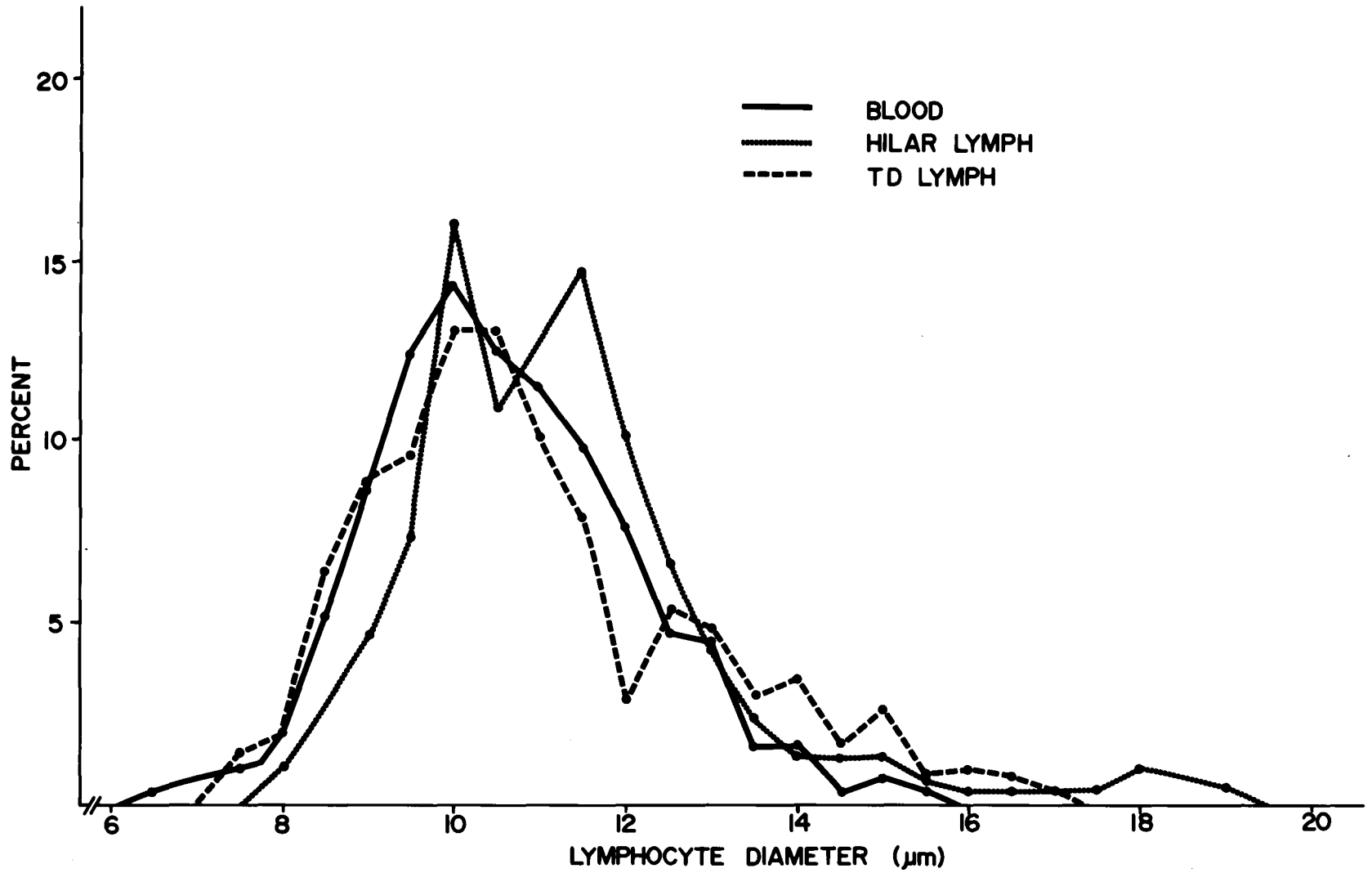
incidence of large cells. The curve for the piped films was different from the other two. It was still wider on the right, was clearly bimodal, and had a tail that represented a group of larger cells that were seen in the blood on slides. The piped films were possibly accounted for the differences between the piped and centrifuged films, although there was no significant difference in the erythrocyte to platelet ratio among the three groups, there was a greater

(11.60  $\mu\text{m}$ , S.E.  $\pm$  0.39) than those on the cytocentrifuged monolayers (10.32  $\mu\text{m}$   $\pm$  0.38). Erythrocyte diameters, on the other hand, were the same in both preparations. Due to the known difficulties with distortion and distribution associated with blood films (Dacie and Lewis, 1975), the cytocentrifuge preparations were taken to represent a more accurate picture of cell size and were therefore used in order to obtain cell measurements in this study.

Measurements of lymphocytes from the three compartments are depicted in Figure 5. The mean lymphocyte diameter in the blood was 10.62  $\mu\text{m}$  (S.E.  $\pm$  0.18), and that in the hilar lymph was 11.17  $\mu\text{m}$  (S.E.  $\pm$  0.14). This difference, while not large, was statistically significant at the .05 level, and was consistently seen in all animals. The thoracic duct lymphocytes had a mean diameter of 10.94  $\mu\text{m}$  (S.E.  $\pm$  0.19), and apparently represented an intermediate between the other two compartments. Figure 5 reveals that the frequency curves for lymphocytes in the blood and thoracic duct lymph were generally similar although the lymph contained a higher incidence of large cells. The curve for the hilar lymph was different from the other two. It was shifted to the right, was clearly bimodal, and had a tail that represented a group of larger cells than were seen in the blood or thoracic duct lymph. This group possibly accounted for the differences in mean cell diameters. Although there was no significant difference in the mean nuclear-cytoplasmic ratios among the three groups, there was a greater

## FIGURE 5

Frequency distribution of the mean cell diameters of lymphocytes from blood, renal hilar, and thoracic duct (TD) lymph.



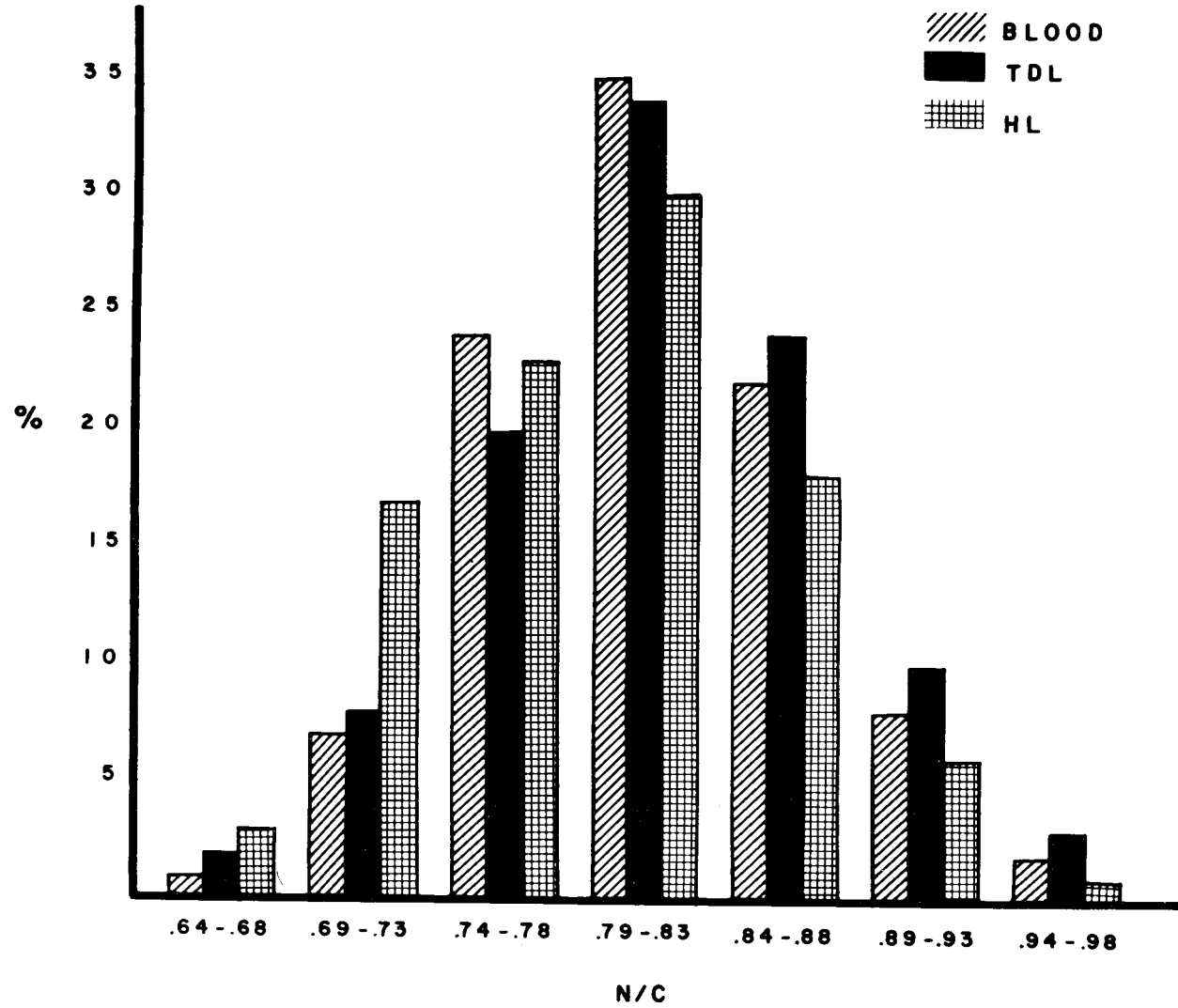
frequency of cells with an increased nuclear-cytoplasmic ratio in the thoracic duct lymph (Fig. 6).

#### 24-hour studies

There was no apparent circadian rhythm in the total white blood cell count of the peripheral blood of the anesthetized dog. There was, however, a marked effect of the anesthetic on this variable. White cell counts are shown in Figure 7 as a function of the hours post-induction of anesthesia. Time zero represents white cell counts performed on unanesthetized dogs at 0900 hours. Following an initial drop, the number of cells in the blood began to rise, reaching peak values of 28,000-29,000 cells/mm<sup>3</sup> 16 hours after the administration of sodium pentobarbital. From the differential and total white cell counts it was calculated that this increase was due to a rise in the absolute number of neutrophils. Cell counts were not available for time points earlier than 4 hours after the induction of anesthesia in the thoracic duct or renal hilar lymph because of the time required to perform the surgical procedures. On the other hand, the white cell count of the thoracic duct lymph appeared to be unaffected by anesthesia. Sequential deviations of the renal lymph cell count with increasing time of anesthesia did not depart significantly ( $p > 0.05$ ) from randomness according to a runs test (Sokal and Rohlf, 1969).

## FIGURE 6

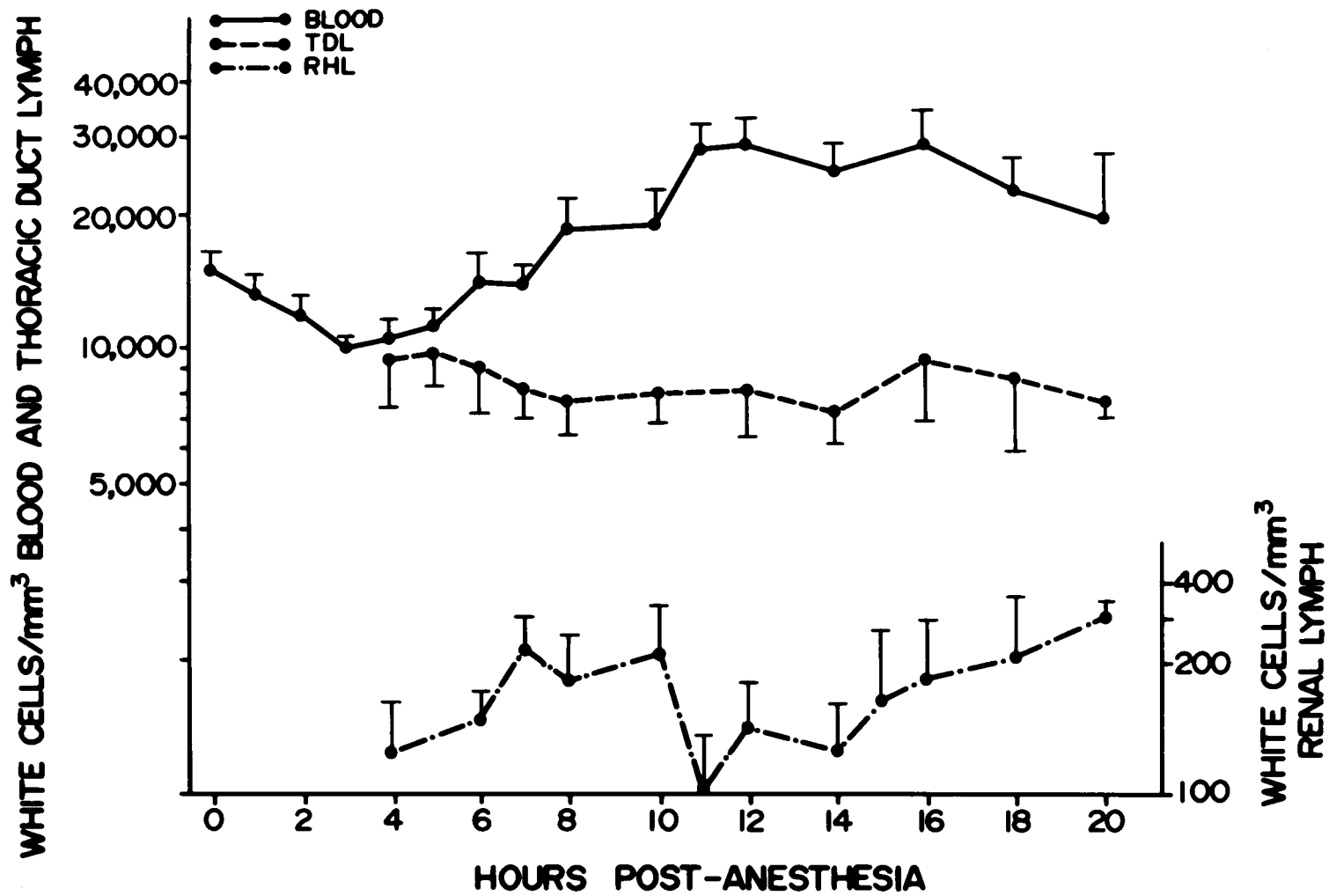
Frequency distribution of the nuclear-cytoplasmic ratios (N/C) of the lymphocytes from the blood, thoracic duct lymph (TDL) and renal hilar lymph (HL).



## FIGURE 7

Means of the total white cell counts of the blood, thoracic duct (TDL), and renal hilar (RHL) lymph as a function of the duration of anesthesia with sodium pentobarbital. The ordinate scale is logarithmic.





### Evaluation of Antiserum

When tested in vitro using the indirect immunofluorescent technique, our antiserum gave a positive reaction with all epithelial basement membranes, sarcolemma and reticular tissue of lymph nodes and spleen. However the in vivo localization of the antiserum was quite specific. Examination of dog kidney, skeletal muscle, spleen and lymph node following the injection of antiserum revealed that the fluorescence was confined to the glomerular basement membrane.

### Experimental Animals

#### Urine

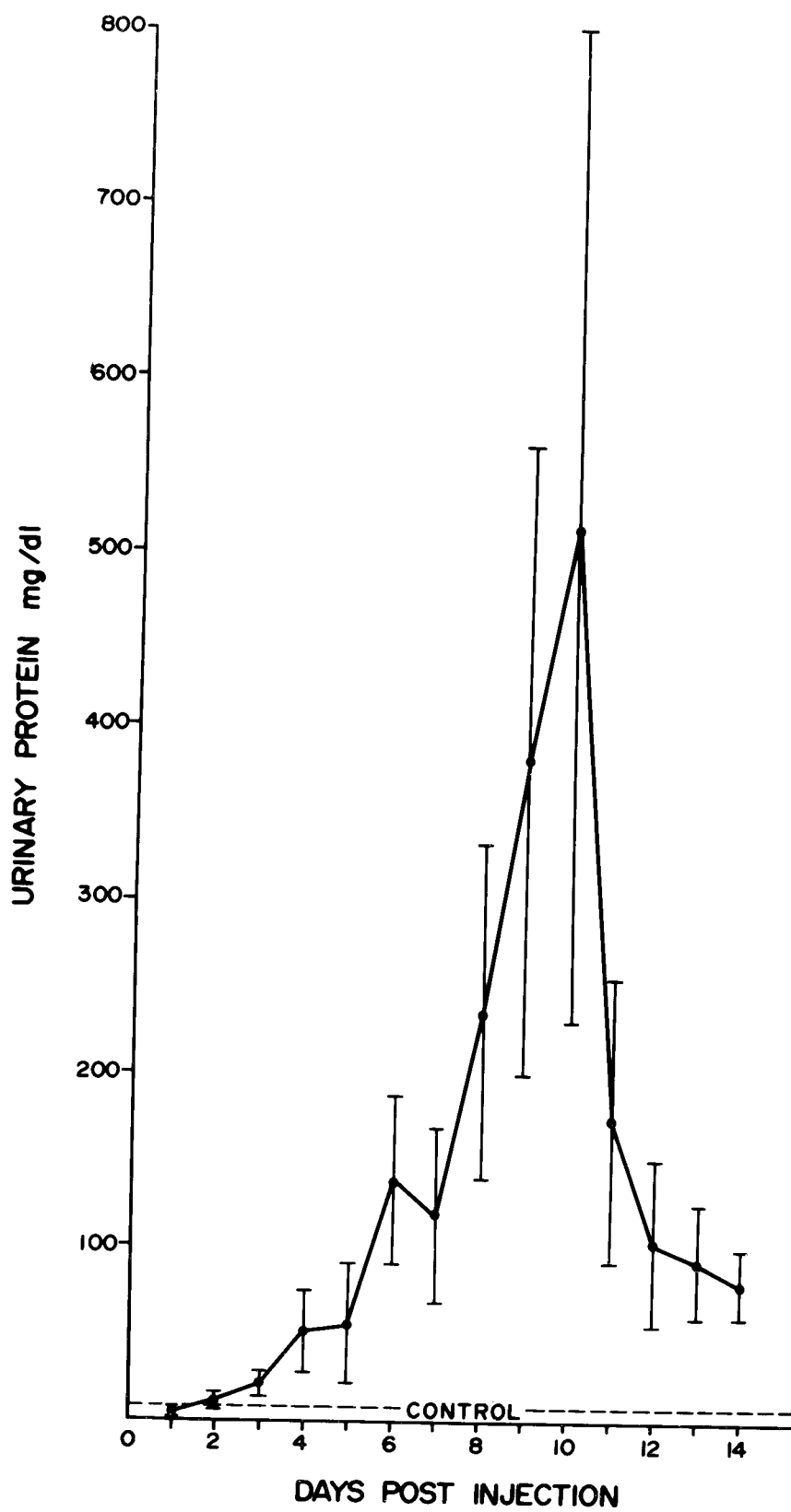
Changes in the protein concentration of the 24-hour urine specimens with time after the injection of antiserum are seen in Figure 8. The protein concentration began to increase in most animals on the third day, reached a peak on the 10th day and then dropped rapidly but remained at elevated levels throughout the experimental period. This proteinuria was accompanied by the presence of numerous white blood cells in the urine, and some animals displayed a transient hematuria unrelated to the dog's estrus cycle. Proteinaceous casts were rarely seen, which was not surprising since canine urine normally has a very alkaline pH and casts dissolve under these conditions.

#### Kidney

On the first day following the injection of antiserum there

## FIGURE 8

Urinary protein of dogs injected with anti-glomerular serum. The protein in mg/dl is plotted against the days after injection. The dashed line represents the mean urinary protein during the control period.



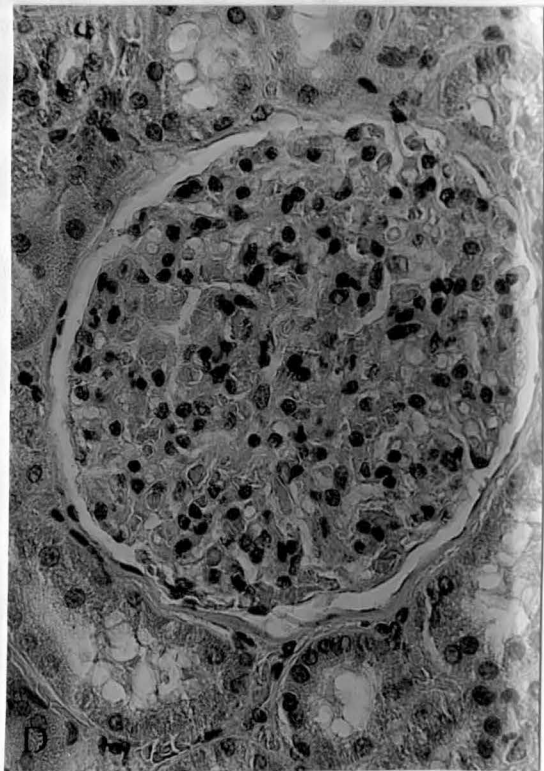
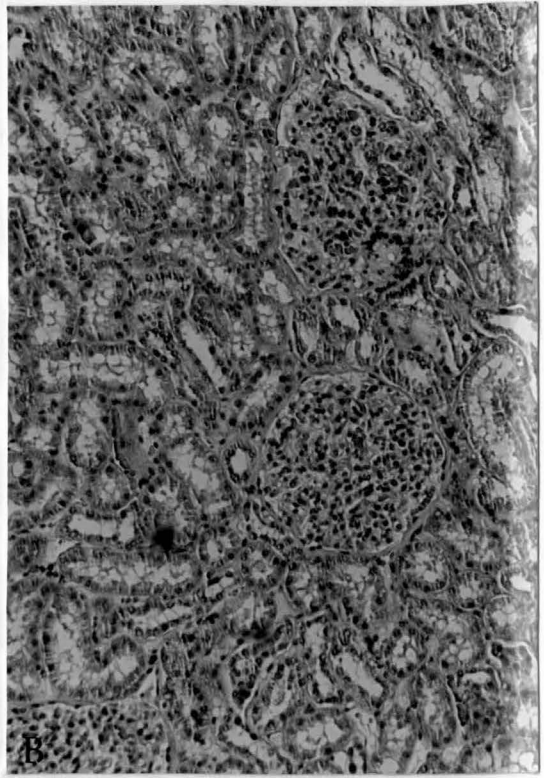
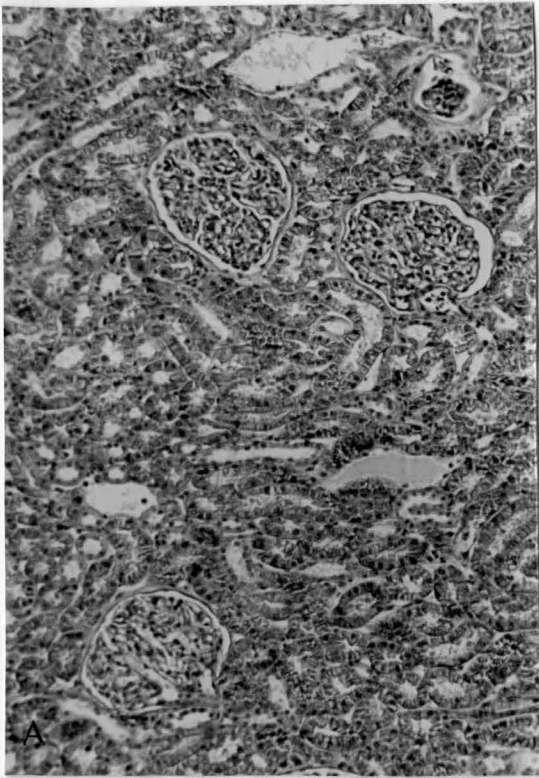
was no noticeable alteration in renal morphology although immunofluorescence showed a linear deposit of rabbit gamma globulin in the renal glomerulus. By the fourth day there was some focal thickening of the glomerular basement membrane accompanied by a fusion of some podocyte foot processes. After 14 days there was a mixed membranoproliferative lesion discernible at the light microscopic level. This was characterized by diffuse enlargement of the glomerular tufts with lobulation (Fig. 9). Epithelial crescents were not noted and the degree of neutrophil infiltration was not remarkable. However, in some instances there was a periglomerular mononuclear cell infiltrate, which often contained plasma cells (Fig. 10). Some of the glomerular tufts appeared to be shrunken and a proteinaceous deposit was seen in Bowman's space (Fig 11). In others the increased mesangial cellularity was accompanied by an increase in PAS-positive deposits (Fig. 12).

### The Blood

In Figure 13 white cell counts in the blood of the experimental animals, during the post-injection period are contrasted with control animals anesthetized for a comparable time. Although the experimental animals demonstrated a rise in the white cell count that was attributed to the effect of anesthesia, the magnitude of this response when compared to control results was depressed. This depression persisted until the second day after injection. By the fourth day the count in the experimental animals had risen to control levels and continued to rise until the experiments were

## FIGURE 9

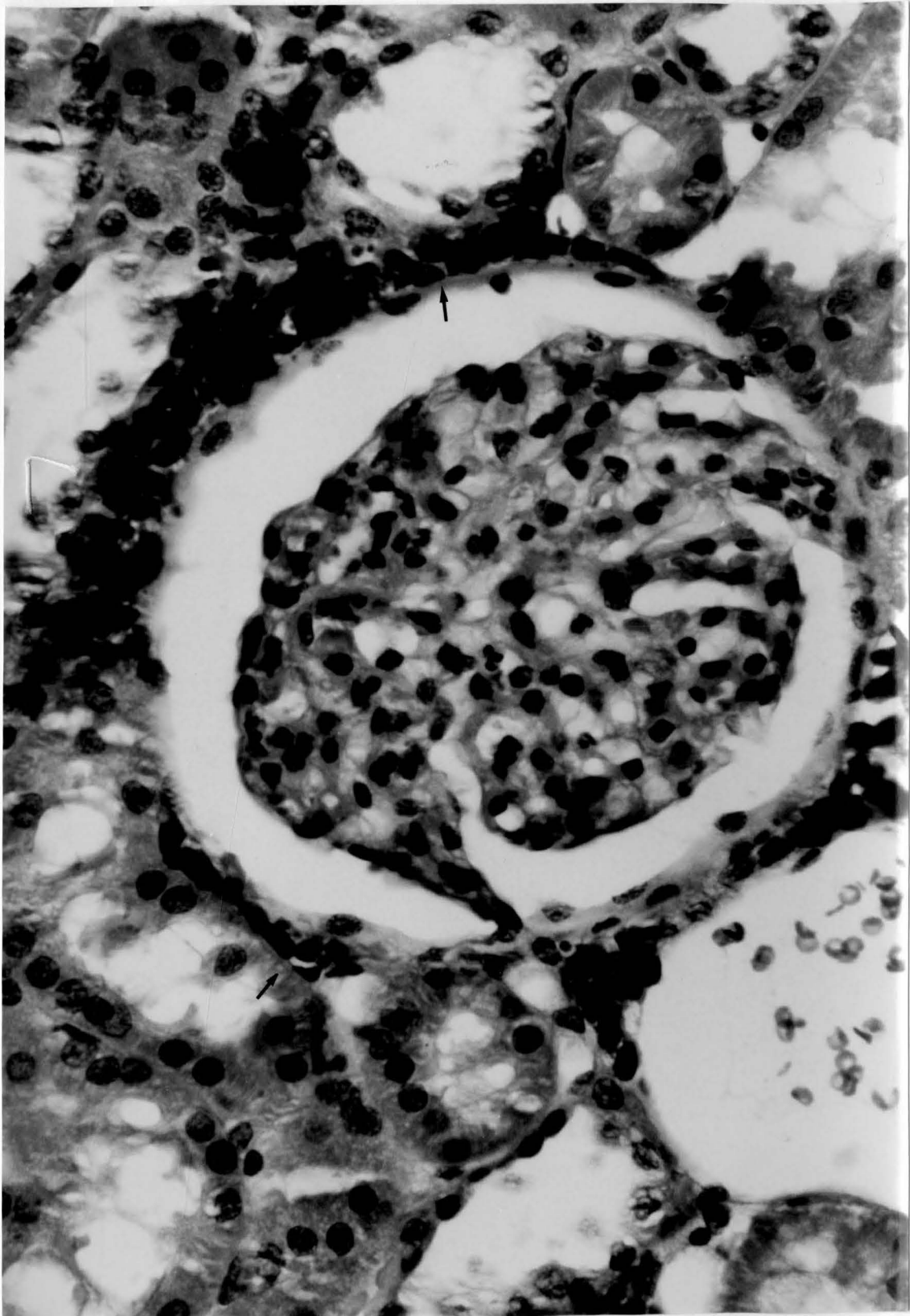
A comparison of the renal cortex from control and experimental animals. A) Overview of normal canine renal cortex. x130. B) Overview of renal cortex 14 days after the injection of antiserum. There is a marked hypercellularity of the glomerular tufts. x130. C) Normal canine glomerulus. x400. D) Hypercellular glomerulus from an experimental animal 14 days after antiserum injection. x350.



## FIGURE 10

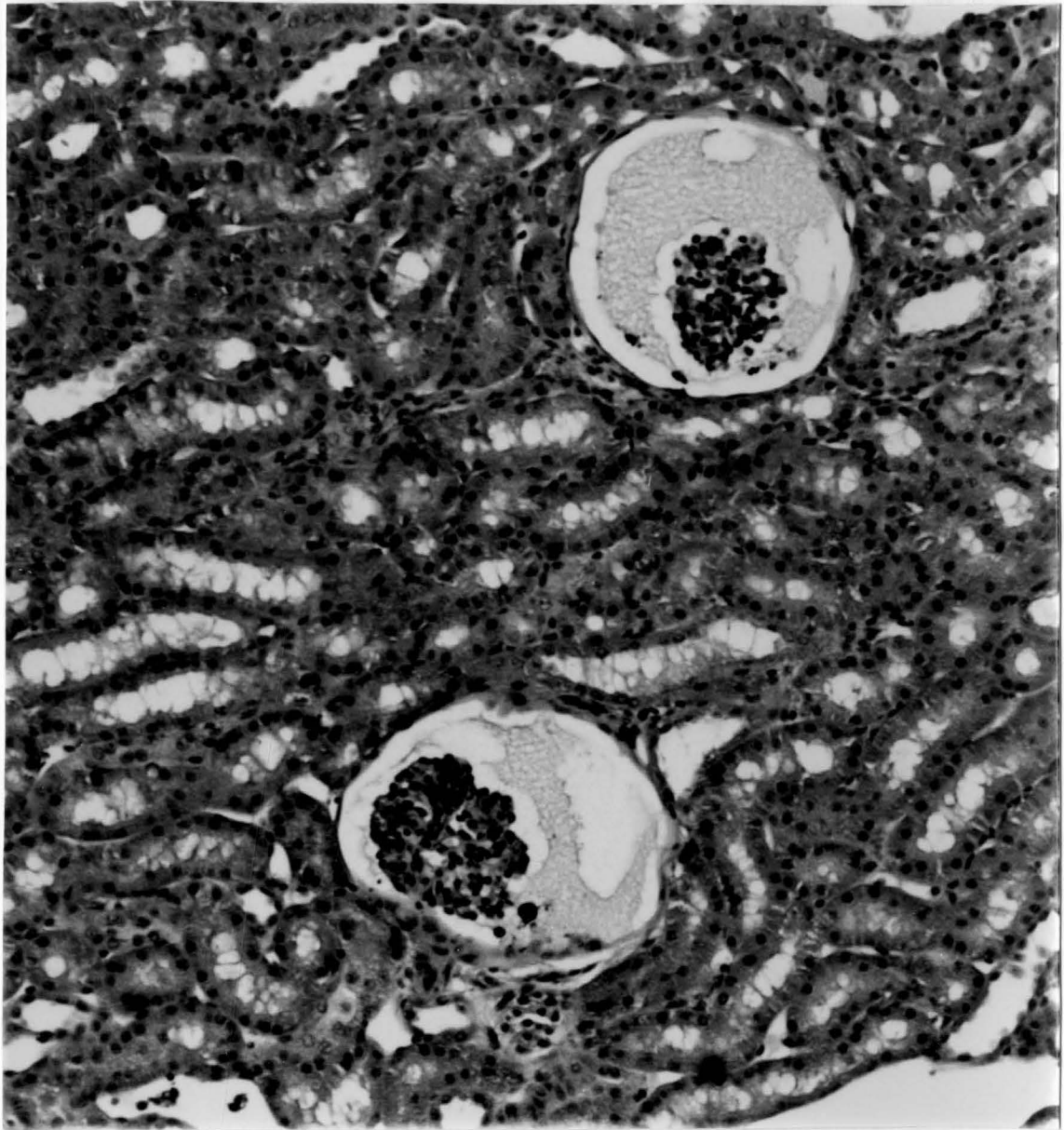
A renal corpuscle 14 days after injection. There is a marked periglomerular mononuclear cell infiltrate. Some of these cells are plasma cells (arrows). The renal tubules appear normal. x750.





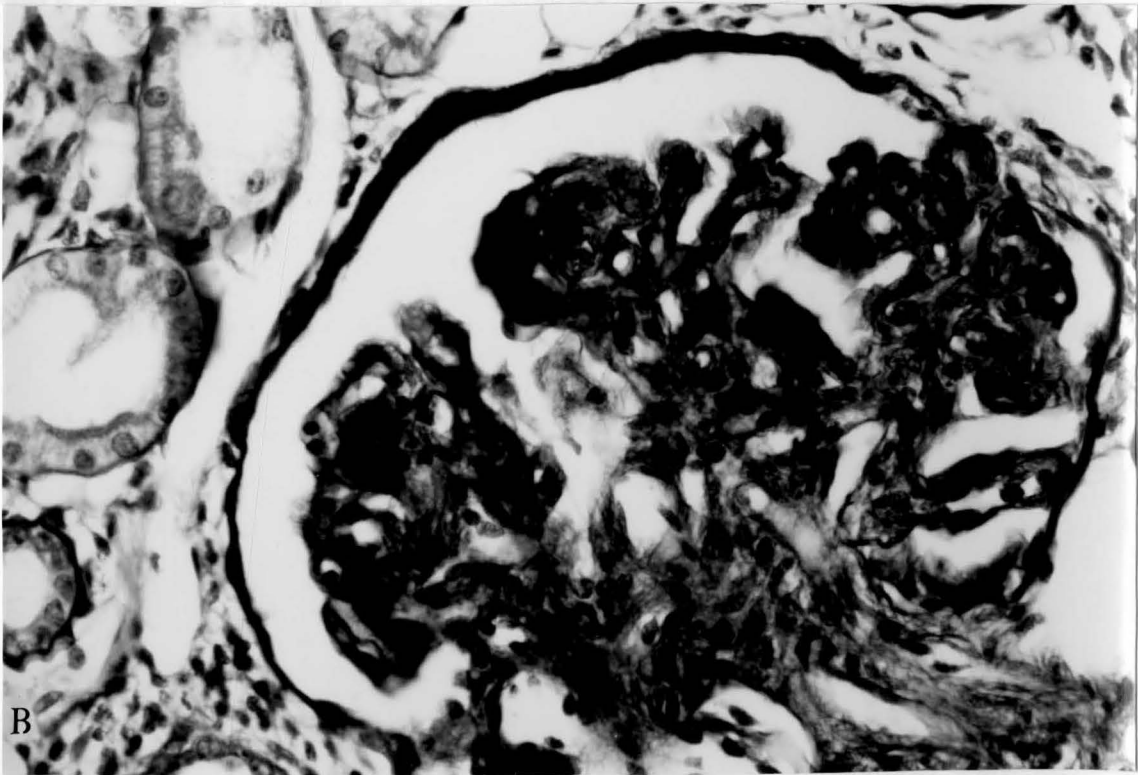
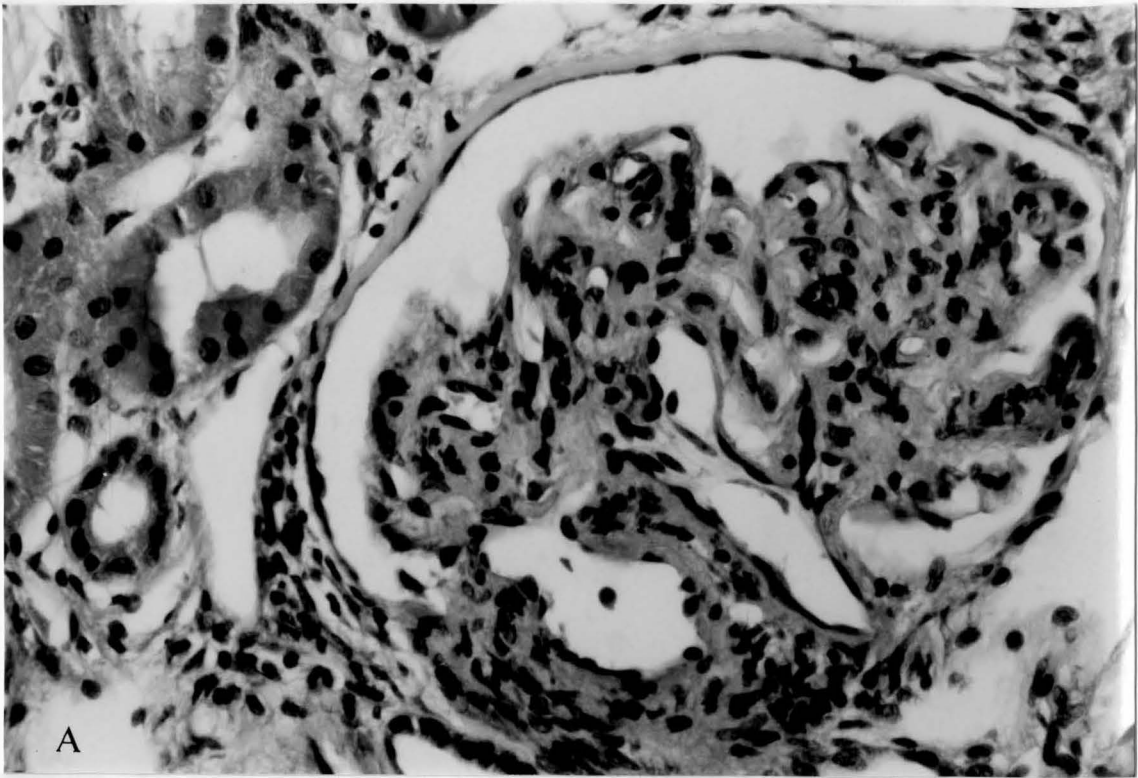
## FIGURE 11

Renal cortex 7 days after the injection of antiserum. Some capillary tufts are shrunken with decreased luminal size and the deposition of proteinaceous material in Bowman's space. x220.



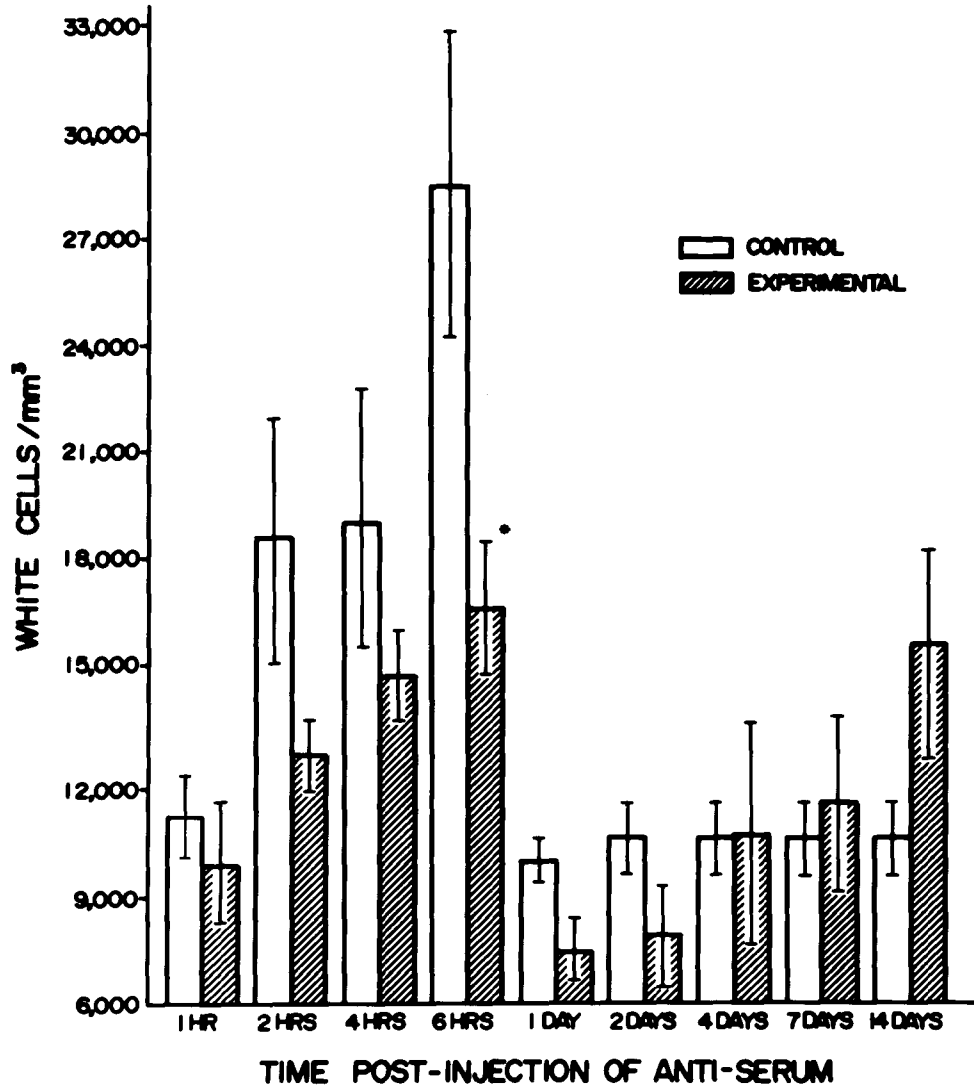
## FIGURE 12

Renal corpuscle 7 days after the injection of antiserum.  
A) There is an increased mesangial cellularity and some lobulation of the glomerular tuft. Hematoxylin and eosin x500. B) A comparable glomerulus stained to demonstrate the deposition of PAS-positive material. PAS x500.



## FIGURE 13

Total blood white cell counts of the control and experimental dogs. The stimulated animals are contrasted to a group of control animals anesthetized for a comparable period of time. \* indicates statistical significance at the .05 level.



terminated on the 14th day. The changes in the total white cell counts were not reflective of a change in any one cell type, with the exception of a neutrophilia due to anesthesia. The changes in lymphocyte diameter are depicted in Figure 14. The tendency toward an increase in cell size observed in the later time periods was due to an increase in the frequency of the medium-sized (10-13  $\mu\text{m}$ ) cells accompanied by a decrease in the number of small ( $< 10 \mu\text{m}$ ) cells present (Fig. 15). An increase in the nuclear-cytoplasmic ratio (N/C) was also observed (Fig. 16).

#### Thoracic Duct Lymph

The total white cell counts were not significantly different from the control values, although over the total experimental period there was a tendency for the cell count to be slightly decreased (Control 7,709 S.E.  $\pm$  1,226 cf. Experimental 5,752 S.E.  $\pm$  698). This was due to an absolute decrease in the number of lymphocytes. Differential counts were comparable to controls. By the 14th day there was a marked reduction in the average size of the lymphocytes (Fig. 14), which was attributable to an increase in the number of small lymphocytes (Fig. 15). The N/C ratio also increased considerably with time (Fig. 16).

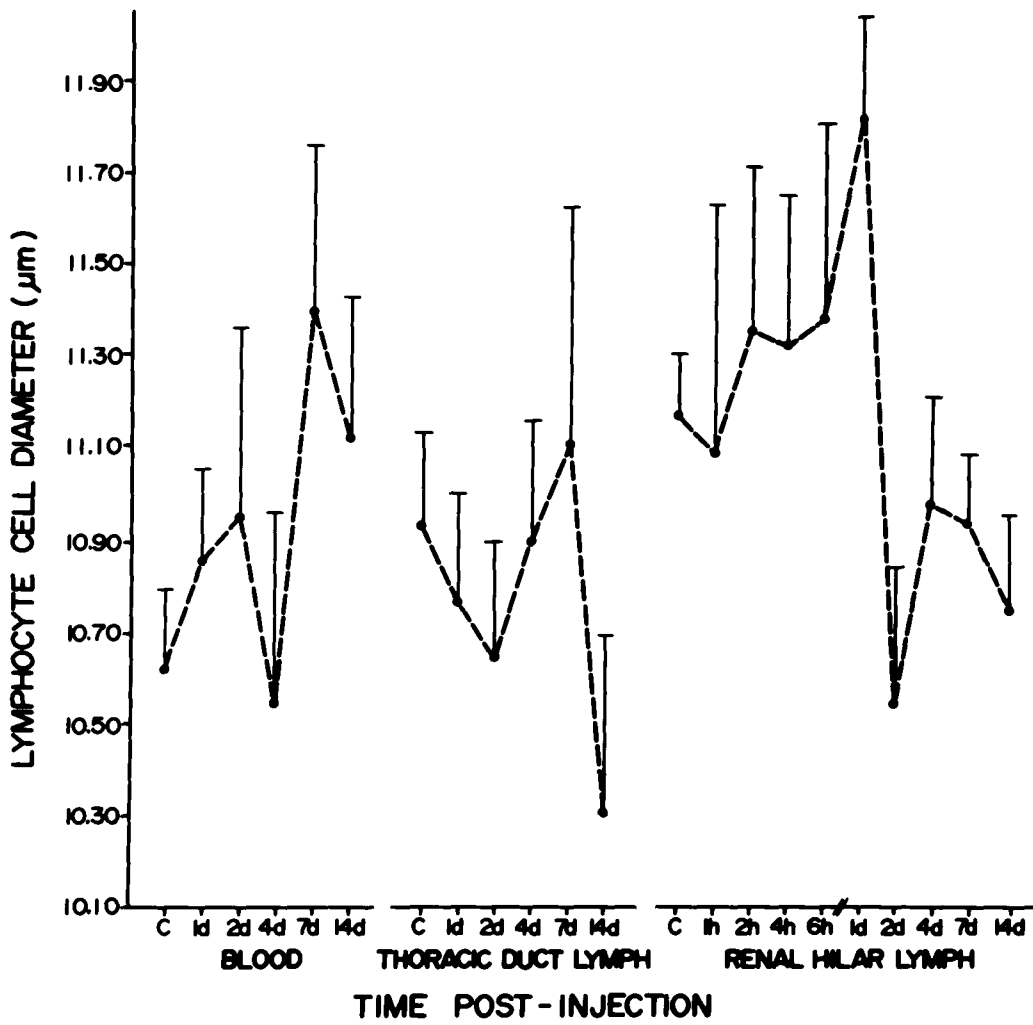
#### Renal Lymph

By far the most dramatic changes resulting from the injection of anti-glomerular serum were seen in the renal lymph. The white



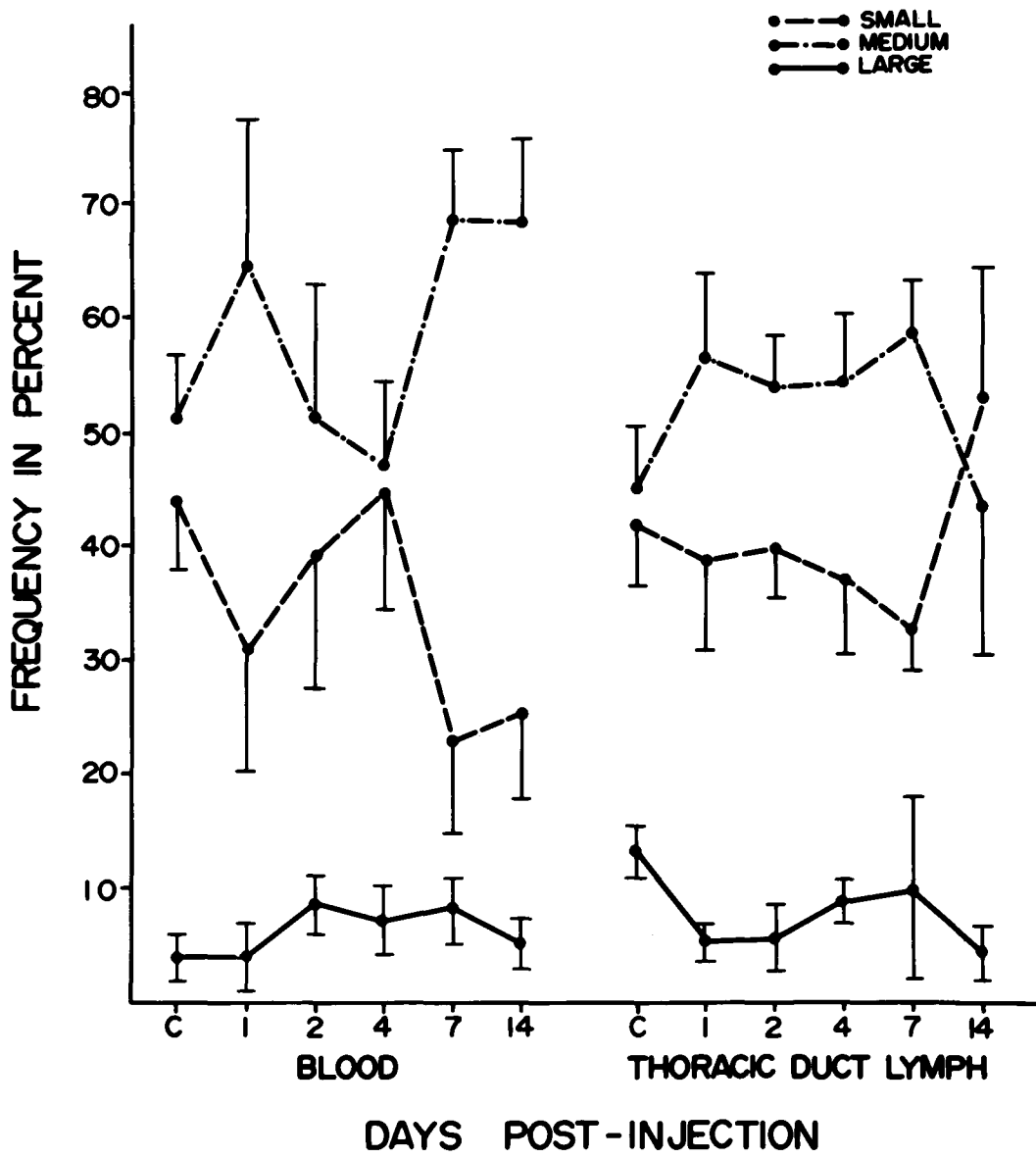
## FIGURE 14

Lymphocyte cell diameters. The mean diameters of the lymphocytes in the three compartments sampled are shown as a function of time after the injection of antiserum.



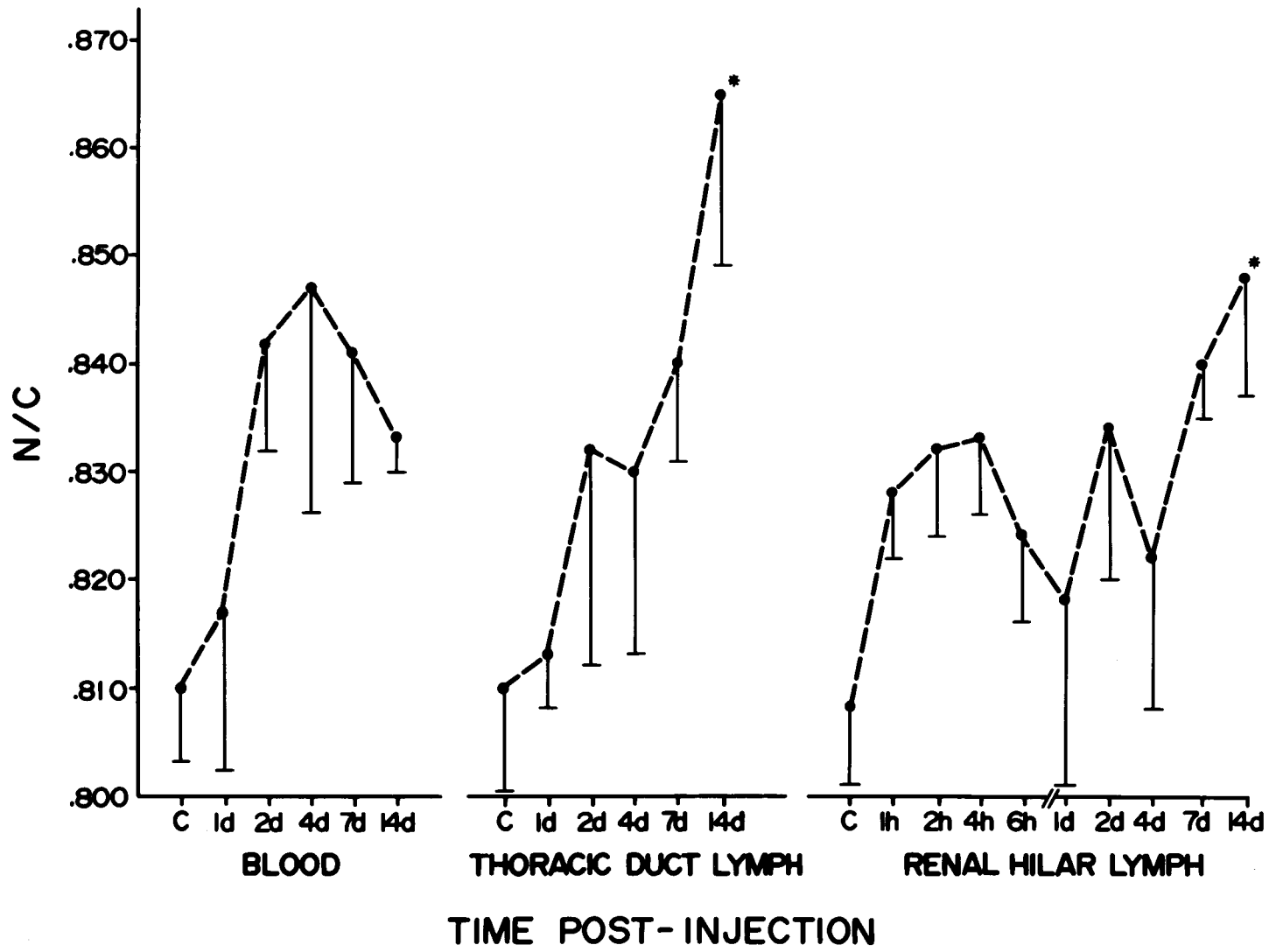
## FIGURE 15

The frequency of blood and thoracic duct lymphocytes in given size ranges is shown as a function of time after the injection of antiserum. Small =  $<10 \mu\text{m}$ ; Medium =  $10-13 \mu\text{m}$ ; Large =  $>13\mu\text{m}$ .



## FIGURE 16

Nuclear-cytoplasmic ratios (N/C) for the three compartments sampled during the time after the injection of antiserum. \* indicates statistical significance at the .05 level.



cell count trippled within the first two hours, but had returned to normal levels 6 hours later (Fig. 17). There followed a secondary peak at 10 hours after which the number of cells began to decline, and subsequently remained at a low level. The initial response and the peak at 10 hours was composed mainly of neutrophils: however at 12 hours and thereafter, the lymphocyte was again the predominant cell present. The character of the lymphocyte population also changed. Whereas in the control animals less than 10% of the lymphocytes were of the large ( $> 13 \mu\text{m}$ ) type (Fig. 18), during the first 24-hours following injection of antiserum 15-20% of the lymphocytes fell into this category. Cells in various stages of mitosis were also frequently seen (Fig. 19). This increase in large cells subsided by the second day, after which small ( $< 10 \mu\text{m}$ ) and medium ( $10\text{-}13 \mu\text{m}$ ) cells comprised the majority of the lymphocyte population. By the fourteenth day the N/C ratio of the lymphocytes was significantly increased above the control level (Fig. 16).

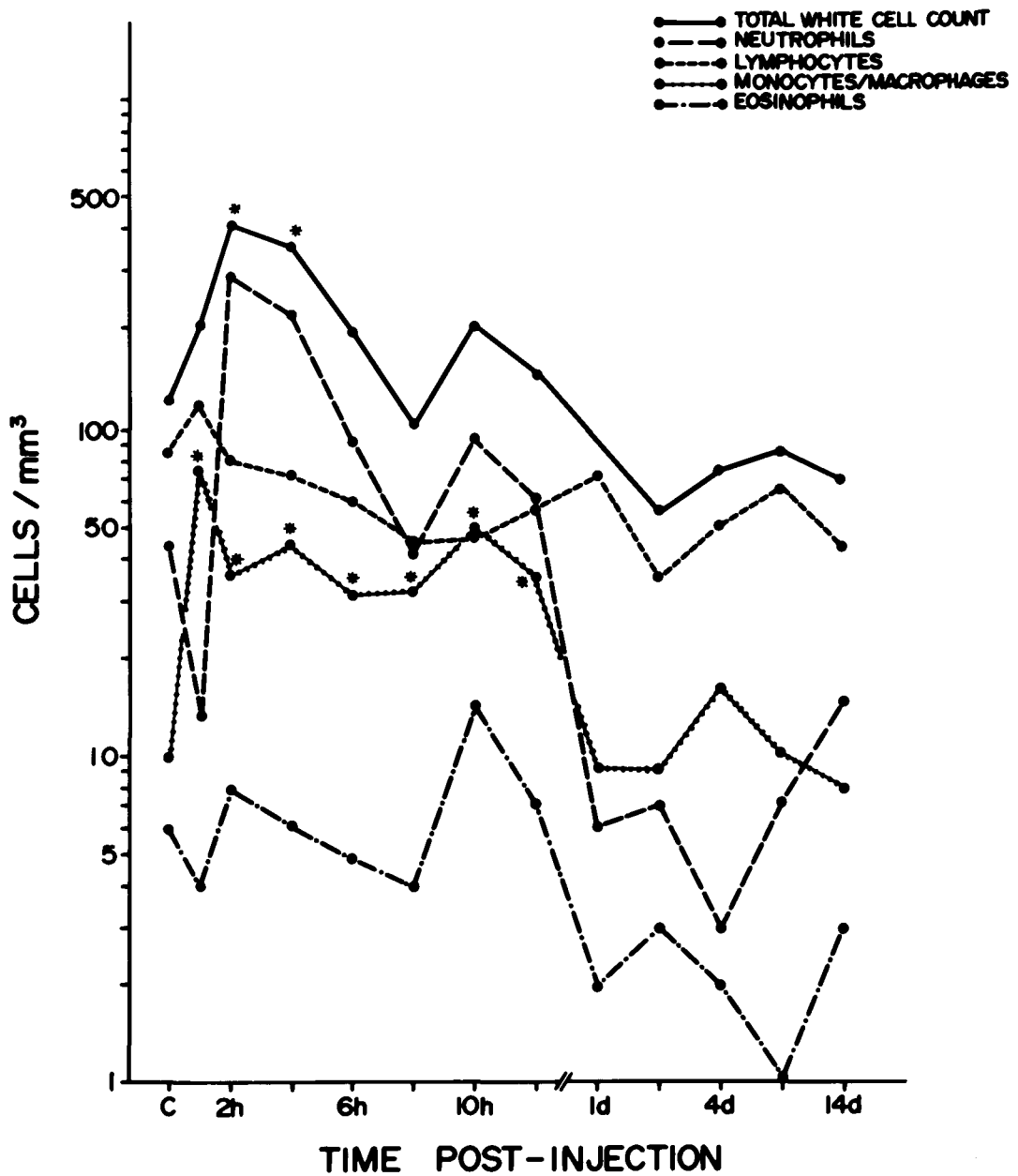
Following antiserum administration there was a sudden increase in the number of monocytes present and this elevation lasted for the first 12 hours. Monocyte numbers then returned to pre-injection levels and remained there except for a small increase on the fourth day. Statistical analysis failed to reveal any significant correlation ( $p > .05$ ) between the length of time the cannula had been in place and the number of any one cell type present.

By the end of the first day and for the duration of the time

## FIGURE 17

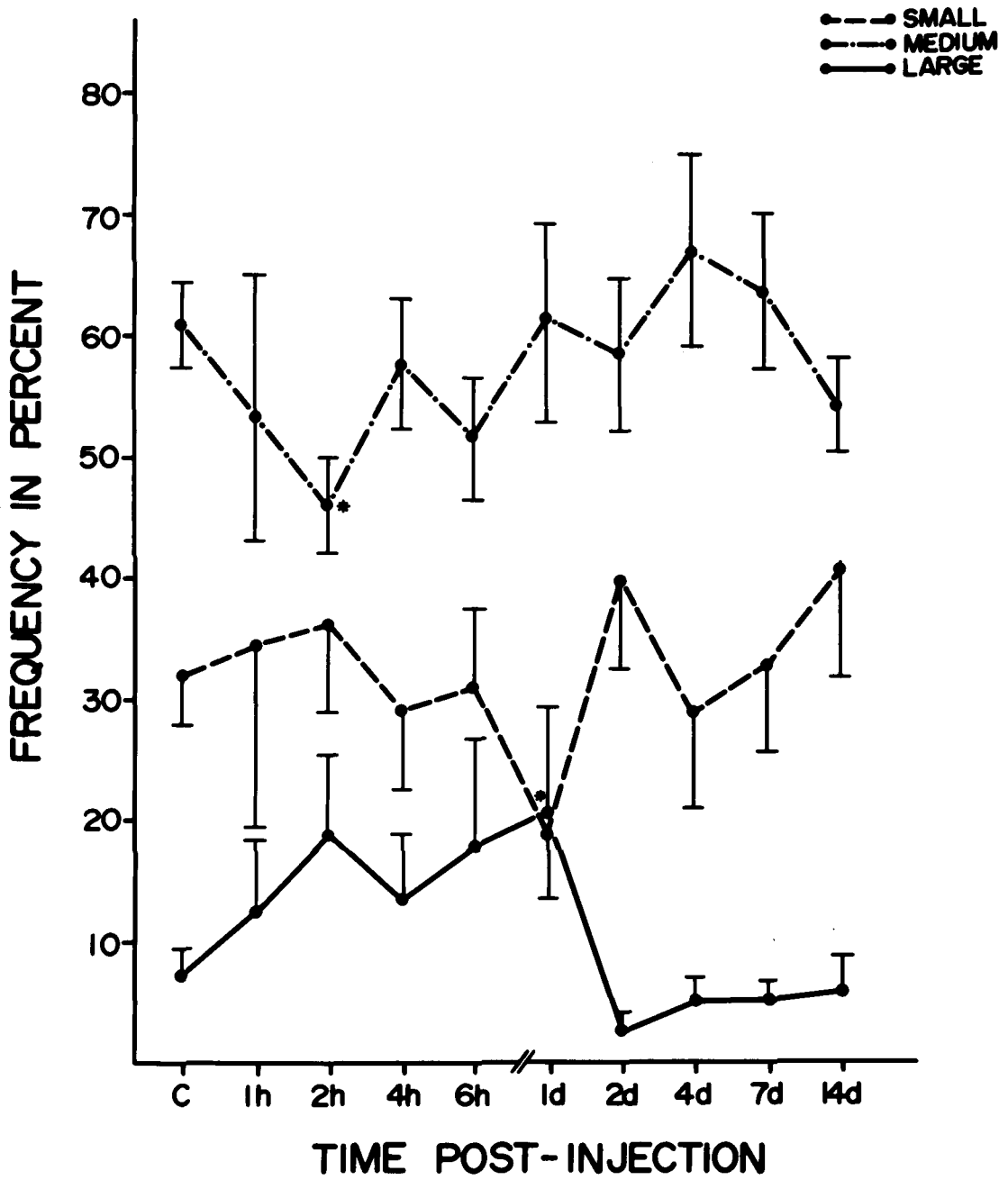
Total white cell count and differential for renal hilar lymph. The counts are expressed in absolute numbers during the time after the injection of anti-serum. A logarithmic scale is used on the ordinate. \* indicates statistical significance at the .05 level.





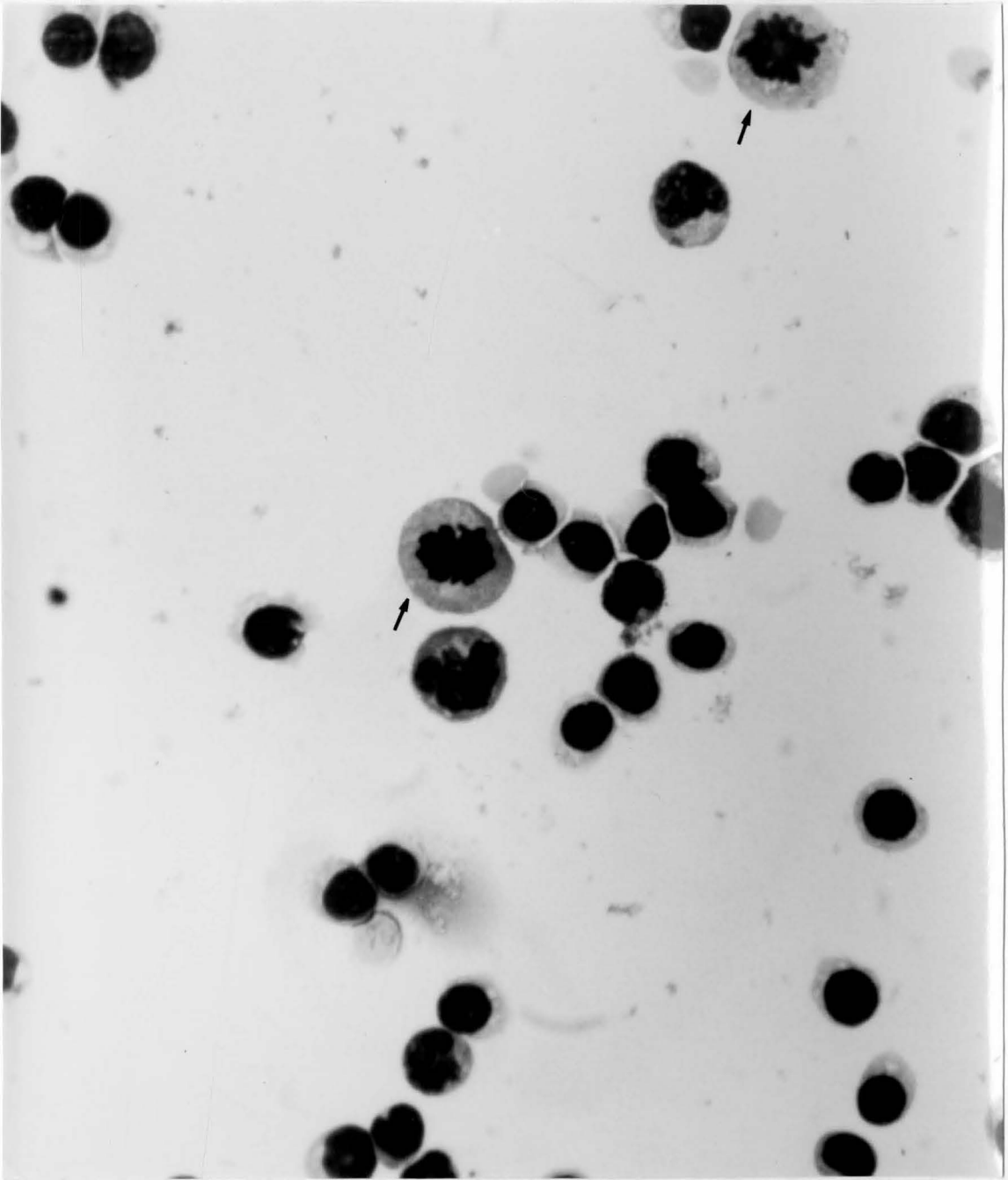
## FIGURE 18

Frequency distribution of lymphocyte size in renal hilar lymph. The frequency of lymphocytes in a given size range is shown during the time after the injection of antiserum. Small =  $< 10\mu\text{m}$ ; Medium =  $10-13\ \mu\text{m}$ ; Large =  $>13\ \mu\text{m}$ . \* indicates statistical significance at the .05 level.



## FIGURE 19

The cells of renal hilar lymph 24 hours after the injection of anti-glomerular serum. There is a decrease in the number of neutrophils and an increase in the number and size of the lymphocytes. Cells undergoing mitosis are frequently seen (arrows). xl,100.

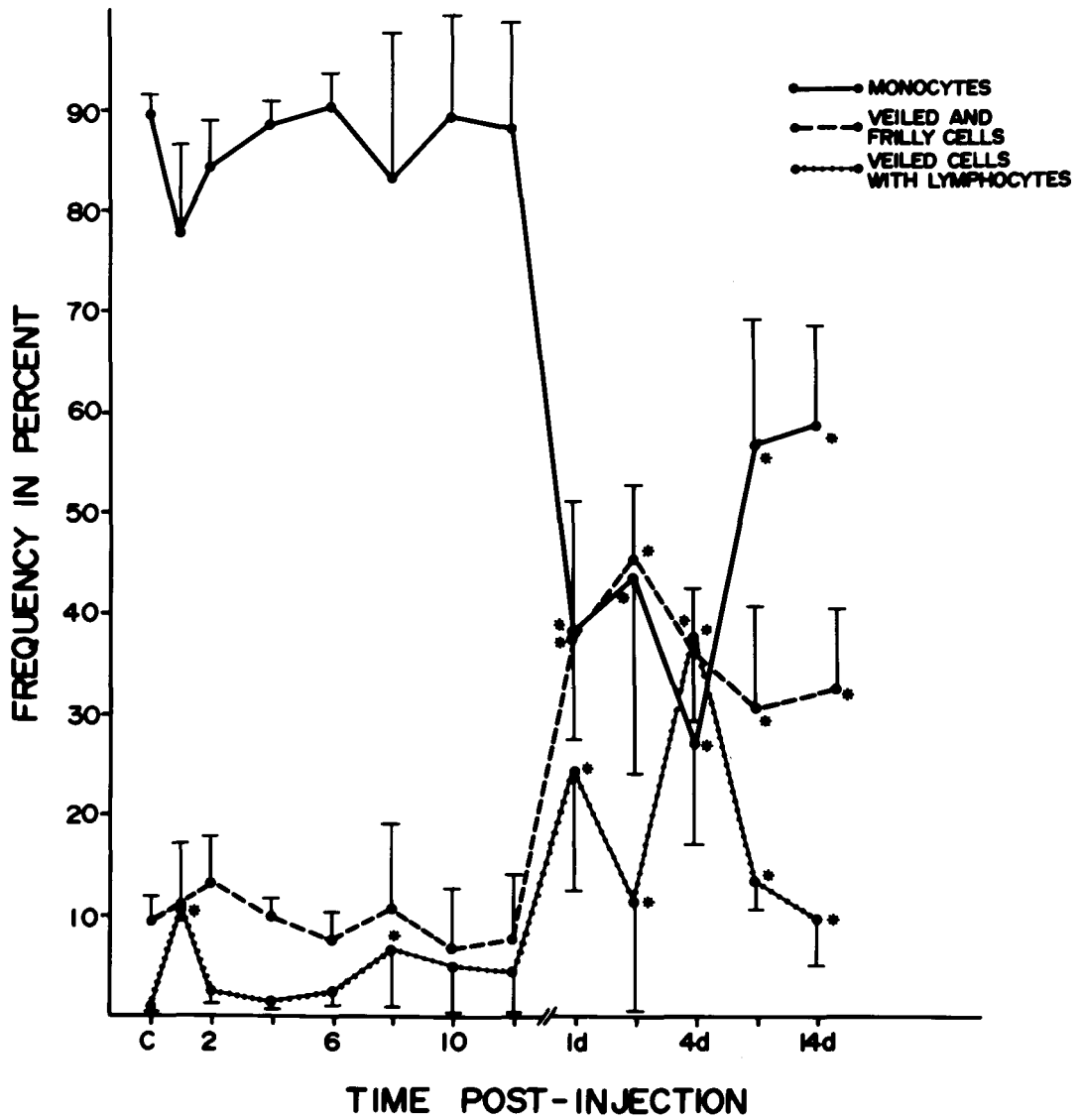


span studied, cells resembling both "veiled" and "frilly" cells accounted for an increased percent of the non-lymphoid mononuclear cells present (Fig. 20). The "frilly" cells had villous cytoplasmic projections around the entire circumference of the cell, whereas the "veiled" cell was characterized by randomly spaced cytoplasmic projections, some of which were equal in length to the diameter of the cell itself (Fig. 21). The nuclei of these cells resembled that of the unidentified cell previously noted in normal renal lymph. Both "veiled" and "frilly" cells were occasionally seen to contain 2-3 latex particles in their cytoplasm, and many of them were also seen forming rosettes with lymphocytes (Fig. 22).

No evidence of circadian variability in blood, lymph or renal response to immunologic challenge was noted. However, due to the small sample size and large interanimal variability, the possibility of a chronoinmunesusceptibility cannot be excluded.

## FIGURE 20

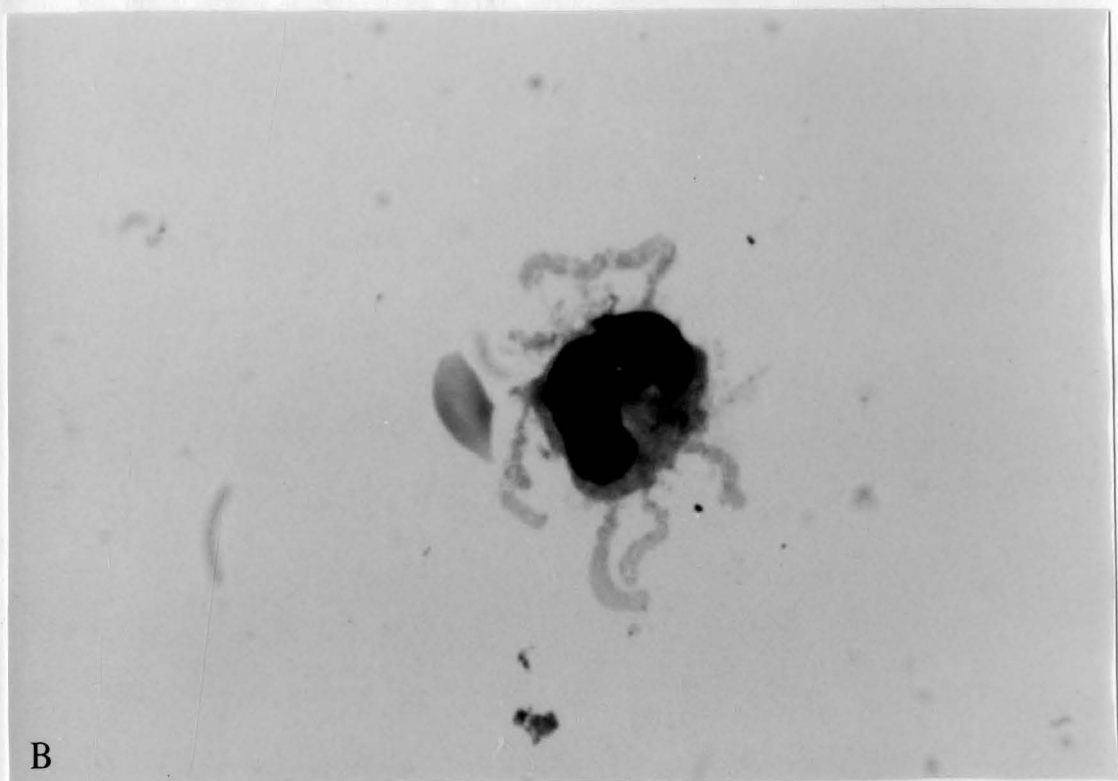
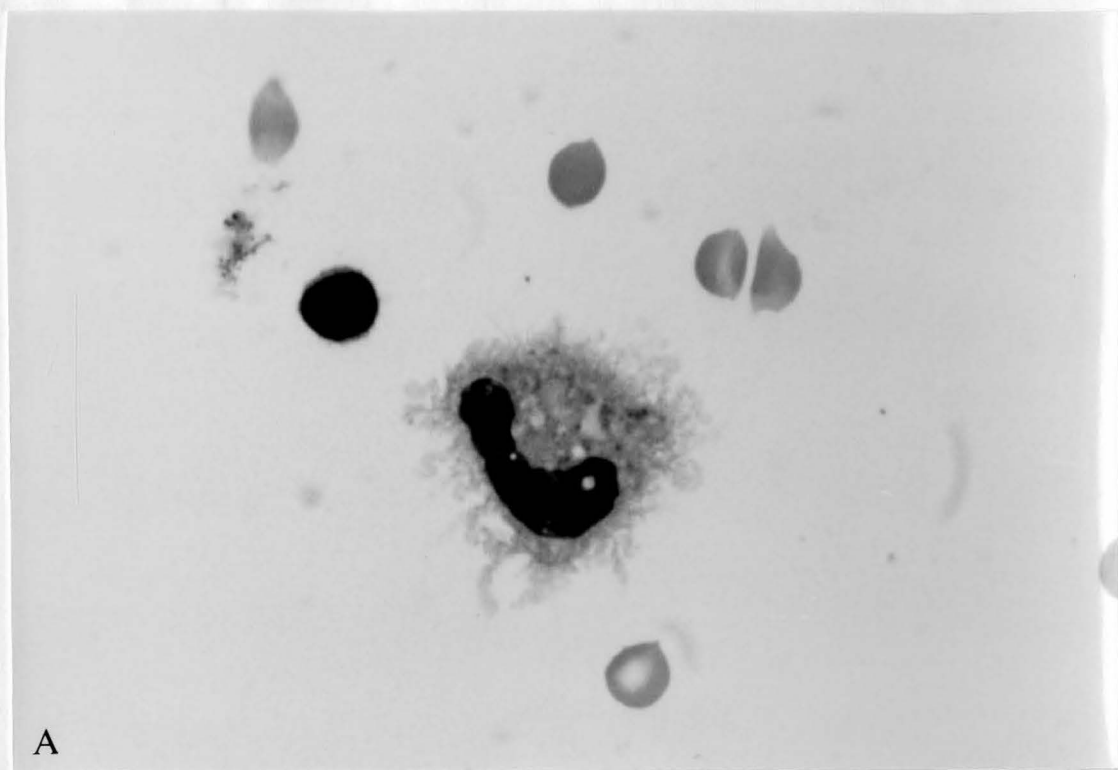
Distribution of non-lymphocytic mononuclear cells in renal lymph. The percentage of each cell type contributing to the total non-lymphoid mononuclear cell population is shown during the time after the injection of antiserum. \* indicates statistical significance at the .05 level.





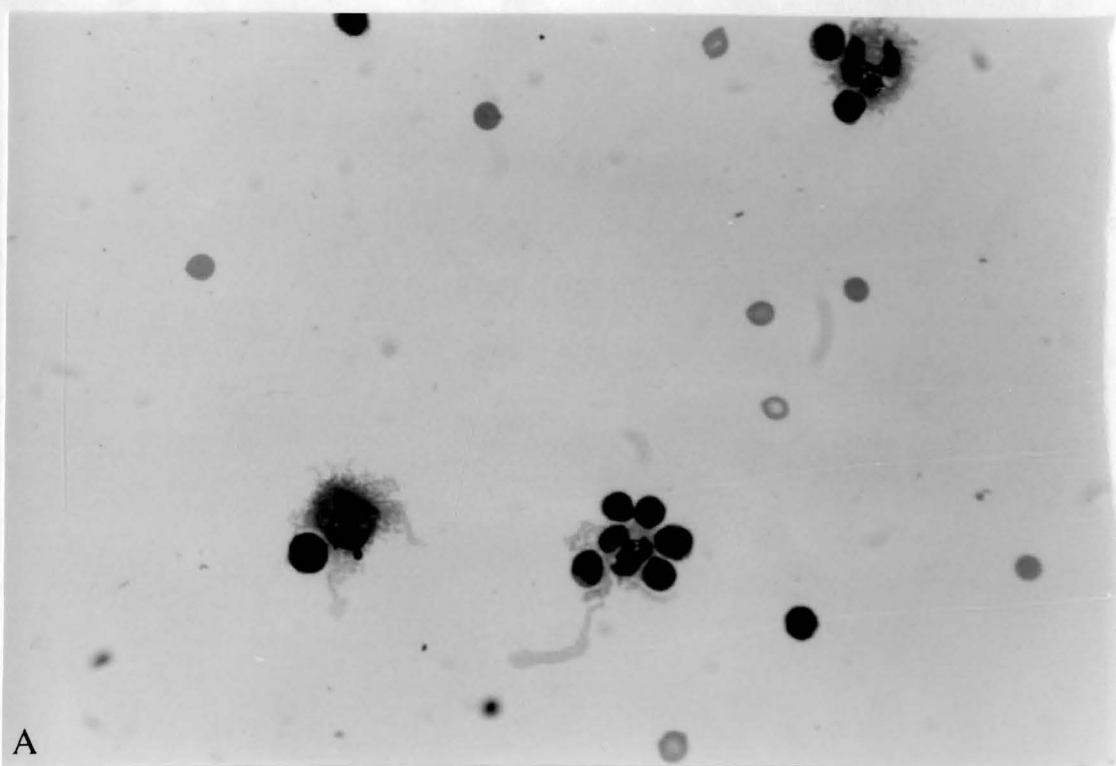
## FIGURE 21

Non-lymphoid mononuclear cells of the renal lymph.  
A) "Frimly" cell with evenly distributed villous cytoplasmic projections. 1,300 x. B) "Veiled" cell with randomly spaced projections that at times are equal to the diameter of the cell itself in length. 1,650 x.

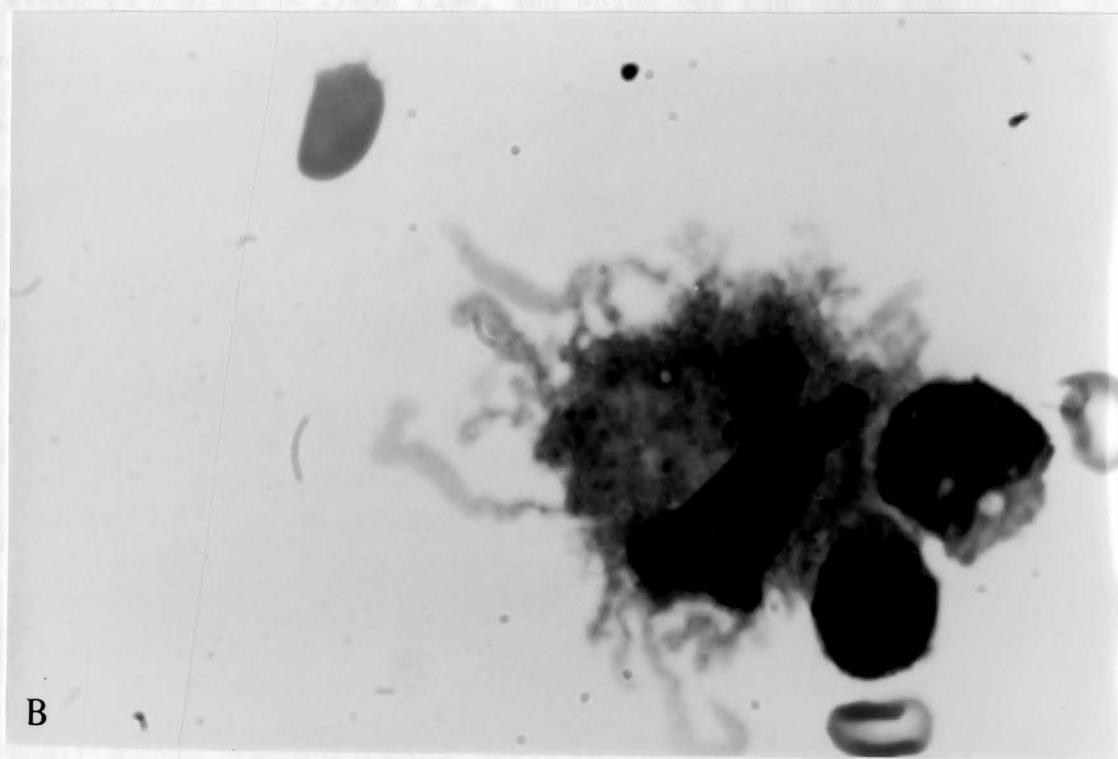


## FIGURE 22

"Veiled" cell-lymphocyte rosettes. A) an overview of the renal lymph 7 days after the injection of antiserum with several aggregates of "veiled" cell with associated lymphocytes. x 600. B) a "veiled" cell with two associated lymphocytes. The cytoplasmic extensions appear to be embracing the lymphocytes. x 2,200.



A



B

## DISCUSSION

In the absence of specific antigenic stimulation, the cellular content of peripheral (prenodal) lymph is extraordinarily low when compared to blood or central lymph. The values presented here for canine renal lymph ( $133 \pm \text{S.E. } 25 \text{ cells/mm}^3$ ) appear to be even lower than those that have been reported for other regions and in other species. Kelly (1970) reported a count of 300-400 cells/mm<sup>3</sup> for lymph afferent to the rabbit popliteal node, Yoffey and Drinker (1939) a mean of 550 cells/mm<sup>3</sup> for prenodal lymph in the dog limb, and Morris and Courtice (1977) 100-700 cells/mm<sup>3</sup> for renal lymph in the sheep. The explanation for these differences may be in the experimental design, which for popliteal lymph requires external massage in order to stimulate lymph flow, and which for the sheep has involved chronic cannulation. Undoubtedly both procedures might be expected to augment the cellular traffic. In any event, such low counts have led some investigators to the conclusion that functionally, at least, this cellular traffic is unimportant, and only attains significance in abnormal circumstances.

In contrast to the work of Smith et al. (1970) who examined ovine renal lymph, we did not find any noticeable erythrocyte contamination in canine renal lymph. Smith et al. (1970) also found a higher percentage of macrophages in renal lymph than in afferent

lymph from other organs, but we did not find a high number of these cells in normal renal lymph. Whether this is a species difference or not is difficult to tell since there are few reports in the literature on afferent lymph from normal dogs with which we can compare our results. We did find that under normal circumstances there is a higher percentage of neutrophils ( $27\% \pm \text{S.E. } 5.6$ ) in canine renal lymph than that reported for sheep renal lymph (2-4%). Yoffey and Drinker (1939) also reported approximately 24% neutrophils in canine afferent Popliteal lymph. However they also noted 22% monocytes/macrophages in this peripheral lymph. While the differences in the number of neutrophils can possibly be attributed to species differences, this does not explain the discrepancies in monocyte numbers. Yoffey and Drinker (1939) used supravitaly stained preparations for cell identification. While it is possible to differentiate between polymorphonuclear and mononuclear cells using this technique, it is difficult to distinguish between two types of mononuclear cells such as lymphocytes and monocytes. Smith et al. (1970) performed differential counts on stained smears, and as an aid to monocyte/macrophage identification they injected labeled particles intravenously. This alone may act as a stimulus for an increased movement of macrophages into lymph.

One of the technical problems that we encountered was the difficulty in obtaining good quality smears because of the low protein

content of peripheral lymph. Even when this difficulty was overcome by the addition of normal plasma, some distortion in cell morphology and uneven distribution were present. Accordingly, we used the cytocentrifuge, which in our hands has proven to be a useful tool for making reproducible preparations of the cells in lymph. In view of the enhanced nuclear detail realized in cytocentrifuged preparations of lymphocytes compared to conventional films, we anticipated an increase in the cell diameters. However this was not the case. The measurements were less on the cytocentrifuged specimens. Thus these preparations are closer to the in vivo state. It appears that the effect of air drying, which would cause the cells to shrink, does not overcome the distortion of mechanical spreading of the cells.

It is evident from Table 1 that under non-stimulated conditions the cellular content of peripheral lymph is not reflective of that in the blood or thoracic duct lymph. This is the case even when lymphocytes alone are considered. Comparison of differential cell counts (Table 2) demonstrates significant differences among blood, peripheral lymph and central lymph. Thus it would appear that the movement of cells from blood through the interstitium to lymphatics is not random and preferentially favors cells of the lymphocytic series.

A comparison of peripheral and central lymph revealed that cells of the monocyte/macrophage series, as well as neutrophilic

leukocytes, are largely absent from central lymph. This absence of macrophages is in accordance with published observations (Reinhardt and Yoffey, 1957). A reduction of the neutrophil:lymphocyte ratio from 1:3 in renal lymph to 1:100 in thoracic duct lymph could be attributed to a dilution of the neutrophil population by the addition of lymphocytes from the lymph node. However, the presence of eosinophils in both limbs of the lymphatic system lends credence to the hypothesis that neutrophils, like macrophages, are sequestered in the lymph node. This also casts doubt on Rous's theory (1908) that eosinophils of the thoracic duct lymph have their proximal origin solely in the gut.

Normal renal lymph has a higher proportion of medium-large lymphocytes and fewer lymphocytes under 10  $\mu\text{m}$  than the blood (Fig.5). The morphology of the large lymphocytes ( $>13 \mu\text{m}$ ) is characteristic of cells subjected to antigenic stimulation. These cells have been reported to occur on a relatively massive scale following the placement of a renal allograft (Pederson and Morris, 1970; Cockett et al., 1973). The finding that they occur under unstimulated conditions suggests that the functional importance of the cellular traffic from blood through tissues and afferent lymphatic to the regional lymph node is out of proportion to the paucity of cell numbers. It is possible that it is this population of cells that is responsible for immune surveillance and that the blast cells are responding to low levels of antigenic stimulation.



The effect of a major surgical procedure on the number of leukocytes in the blood of the human subject is a well-known if not well-understood phenomenon (Miller et al., 1976). Our findings in the dog during 20 hours of anesthesia complement those seen in human patients on the first post-operative day. In our animals the marked rise in the blood leukocyte count is unlikely to be attributable to sepsis, although this is a tempting speculation, since a sterile technique was used in most instances. In fact, those cases where sterility was not maintained are indistinguishable from the other animals. Unlike Kaufman et al. (1980), who found a significant increase in dog blood white cell counts during the first three hours of experimentation, we did not observe an increase until eight hours after the induction of anesthesia. During the first three hours the count dropped in comparison to pre-anesthetic control counts. However, in agreement with Kaufman et al. (1980), we also found that anesthesia did not affect the cell count of the thoracic duct lymph. Likewise the renal lymph was also unaffected. This reemphasizes the lack of any correlation between the numbers and types of cells circulating in the blood and in the lymph.

The lack of detection of a circadian rhythm in the total white cell count of the blood could be due to a damping effect of the anesthesia, or to the nonexistence of such a rhythm in the dog. Since counts were not established over a 24-hour period on

unanesthetized animals, it is not possible to address this question in the present study. However, since cell counts in the thoracic duct and renal lymph were apparently unaffected by anesthesia, the question of a possible circadian influence in these compartments is more readily discussed. The variability seen (Fig. 7) can be attributed to chance when appropriate statistics are applied. While it is known that the rhythmic fluctuations in glucocorticoids influence the rhythm in the blood white cell count (Brown, 1962), it is not known what effect these compounds have on the cellular content of the lymph under normal conditions. Following canine renal allotransplantation, prednisolone, a corticosteroid used in immunosuppressive therapy, does not affect the lymphocyte count of thoracic duct lymph (Evans et al., 1978). It seems unlikely, therefore, that even if anesthesia causes a perturbation in the rhythmic release of corticosterones, that this would affect the cell count of the thoracic duct lymph. The absence of an environmental signal such as light and dark cycling is not likely to be responsible for the lack of a noticeable rhythm, because when animals are rapidly shifted from a standardized light-dark schedule to constant dark, a period of several days is required before changes in some rhythms are observable (Sharp, 1960). Further studies, specifically designed to investigate rhythms in blood and lymph white cell counts will be necessary before the question of a circadian rhythm in this variable can be addressed adequately.

It is difficult to assess the amount of nephrotoxic antibody used in other studies since the doses are usually expressed only as the total volume of rabbit serum given. For example, Wagnild (1977) administered 15-25ml of rabbit serum to each dog, whereas Wright et al. (1973) gave 2ml/kg body weight to their animals. In neither case was the activity of the serum given. Our pooled antiserum had a titer of 1:32 when tested against normal dog kidney by immunofluorescence. The gamma globulin content was 5.5mg/ml. The 1mg/kg dose that we gave was approximately equivalent to 0.2ml of whole rabbit serum. It was our aim to deliver a minimal antigenic challenge to the kidney rather than a large dose that would also initiate a massive inflammatory response. Our success in achieving this is evident from the 24-hour urinary protein values as well as from the renal morphology. There was a slow rise in the protein level that peaked on the 10th day and then began to decline. There were few neutrophils in the glomeruli, and though the protein values and urinary sediment indicated impaired renal function, the amount of damage to the kidney was small in the early heterologous phase. We suggest that this experimental model resembles the insidious beginnings of naturally-occurring glomerulonephritis.

This model also offers a unique approach to the study of the role of cells in the afferent lymph in the induction of the immune response. Previous investigators have utilized such antigens as

foreign erythrocytes and gamma globulin, both of which cannot be confined to a specific area. For example, when using subcutaneously injected  $^{131}\text{I}$ -labeled human globulin as an antigen, 75% of the dose is recovered in the postnodal lymph at the end of 24 hours. Similarly, soon after the injection of erythrocytes into a lymphatic bed the postnodal lymph becomes colored with hemoglobin (Hall and Morris, 1963). The antigen may also become localized within the lymph node as is the case with Salmonella typhi (Hall et al., 1967). In our model the antigen is fixed to the glomerular basement membrane and is not free to migrate to the lymph node. We were able to show this by using immunofluorescence where we were unable to detect any rabbit Ig in the lymph node regional to the kidney.

In order to evaluate critically the timing of the sequential events of the cellular response in the afferent lymph, it is necessary for the antigen to be immediately and constantly exposed to immunocompetent cells. Because of the high blood flow to the kidney, and the rapid and reproducible fixation of antibody, the timing of the response to antigen is not dependent on the variable lag time needed for diffusible antigen and cells to interact in the tissue. In our experiments the injection of antiserum was followed within one hour by a transient increase in the white cell count of the afferent renal lymph. Hall and Morris (1962) noted a similar change in the lymph afferent to the popliteal node following the subcutaneous

injection of serum globulin but the rise in the count occurred later and was of greater magnitude. Kelly (1970) similarly reported an elevation in the white cell traffic beginning four days after the injection of bovine erythrocytes into rabbits. Drexhage et al. (1979) noted a similar rise ten hours after painting the skin with 1-fluoro-2,4-dinitrobenzene. Therefore, antigenic stimulation causes an increase in the number of cells in the afferent lymph, but the timing of the response appears to be related to the method of antigen presentation as well as to the nature of the antigen itself.

Injection of antiserum appeared to suppress the rise in the blood white cell count that was seen during prolonged anesthesia. This was accompanied by a slight decrease in the cell count of the thoracic duct lymph. Although the latter decrease is not statistically significant, it is worthy of comment. It has been reported (Sprent et al., 1971) that following secondary antigenic stimulation, there is a selective recruitment of antigen-sensitive cells out of the recirculating pool in the thoracic duct and a retention of these cells in the lymph node. At this time the efferent lymph may be virtually free of all cells (Morris and Courtice, 1977). The absence of detectable antigen-sensitive cells in the thoracic duct lasts one to two days. The ability of the cells to respond specifically to the antigen is normal by the third day and considerably enhanced by the fifth (Sprent et al.,

1971). These findings on thoracic duct lymphocyte function paralleled in time, our findings on the thoracic duct cell count. This does not however, explain the depression of the white cell count in the peripheral blood, which was a total cellular depression and not one limited to lymphocytes.

In our study the most obvious immediate manifestation of a response to the injection of antiserum, as seen in the peripheral renal lymph, was an increase in the number of neutrophils and macrophages. In apparent contrast Hay et al. (1973) in their work on the afferent popliteal lymph in the sheep did not find an increase in these cell types following the subcutaneous injection of purified protein derivative (PPD) of the tubercule bacillus. They did however comment that classification of intermediate (mononuclear) cell types was difficult. This is not unexpected since they did not use an identifying label as an aid. Drexhage et al. (1979) noted an 18-fold increase in neutrophils and an associated increase in small mononuclear cells, which they presumed to be phagocytes, after painting the skin with 1-fluoro-2,4-dinitrobenzene. Kelly (1970) also noted a slight increase in neutrophils and monocytes during the first 24 hours after the intradermal injection of diphtheria toxoid into rabbit hindfeet. It appears therefore, that the initial response to most antigens is a non-specific inflammatory one. The neutrophilia was not a generalized response since the

increase in the blood in response to anesthesia was seen much later and persisted for a longer period of time. Additionally, our control studies have shown no correlation between cellular events in these two compartments.

After 1-2 days the cellular response had taken on a different character. Eighty percent of the cells were lymphocytes, compared with 63% in the non-stimulated animal and 21% two hours after the injection of antiserum. Our results fail to confirm those of Kelly (1970) who did not observe any significant changes in the proportions of small, medium and large lymphocytes after the treatment with either bovine erythrocytes or diphtheria toxoid. We noted a significant increase in the number of large ( $> 13 \mu\text{m}$ ) lymphocytes with a concomitant decrease in the number of small ( $< 10 \mu\text{m}$ ) cells. Mitotic figures, which were not seen in the control samples of afferent lymph, became obvious. There was also a small increase in the number of blast cells present. The total cell count at this time was not elevated but rather tended to be below the normal level. Kelly et al. (1972) reported a similar depression in the white cell count of the afferent popliteal lymph following application of 10% DNFB. Drexhage et al. (1979) using the same concentration of DNFB noted a 25-fold increase in the cell count in the afferent lymph draining pig skin. Although the concentration of antigen was the same in both cases, the dosages

varied considerably. Kelly et al. used  $20 \mu\text{l}$  to sensitize their animals whereas Drexhage et al. administered five times this amount. Similarly, Morris et al. (1968) noted few changes in the afferent lymph following stimulation with a total concentration of  $5 \times 10^7$  erythrocytes whereas Kelly (1970) found a significant increase in the number of lymphocytes in the afferent lymph after administration of  $1.0 \times 10^9$  erythrocytes. It appears, therefore, that the degree of lymphocytosis following stimulation is dose dependent, and it is the qualitative change in the cells rather than the quantity of cells that is the key factor in the induction of the immune response.

Hall and Morris (1963) reported that even with a low primary dose of antigen, secondary stimulation caused not only a marked increase in the white cell count, but also an increase in the magnitude of the blast cell response and the subsequent antibody production. Our results suggest that following the non-specific inflammatory response, there was a recruitment of lymphocytes to the kidney. The cause of this is unknown but it could be the result of the release of chemotactic factors produced by the neutrophils and macrophages that initially respond to the insult.

In addition to the preponderance of lymphocytes, this second stage, or immune induction phase of the response, was characterized by the presence in the renal lymph of two additional



types of mononuclear cells, one possessing long cytoplasmic veils and another whose cytoplasm has a villiform or frilled appearance. These cells were similar to the "veiled" or "frilly" cells described by others (Kelly, 1970; Kelly et al., 1978; Drexhage et al., 1979; Spry et al., 1980), which have been assumed to be related to the Langerhan's cell of the skin and the interdigitating cells of the thymus and lymph node. Kelly et al. (1978) based this relationship on the presence of Birbeck granules, unusual rod-shaped, membrane limited structures seen by electron microscopy and considered to be characteristic of the Langerhan cell, in 3% of the "veiled" cells of rabbit leg lymph. Sokolowski et al. (1978) also described Birbeck granules in 1.5% of the mononuclear cells in normal human afferent lymph. Although Sokolowski et al. described these cells as having many cytoplasmic extensions, it was not reported that they had cytoplasmic "veils". These findings, plus the presence of 'Ia-like' antigens on human "veiled" cells in lymph led Spry et al. (1980) to the conclusion that "Langerhans' cells leave the skin to become the large mononuclear (veiled) cells in afferent lymph". If indeed, this cell is specific to the skin, then the presence of an increased number of "veiled" cells in the renal afferent lymph following antigenic stimulation is very difficult to explain.

We propose, rather, that the "veiled" cell represents a functional transformation of a cell that is normally present in all peripheral lymph, but which develops its characteristic cytoplasmic extensions in response to stimulation. Normal renal lymph contains a large mononuclear cell typified by a horseshoe-shaped nucleus with a smooth inner margin and a convoluted or segmented outer edge. The cytoplasm possesses the "ground-glass" appearance of the typical blood monocyte, but like the "veiled" cell this cell rarely has ingested latex in its cytoplasm (Fig. 4). Sokolowski et al. (1978) reported similar cells with radial nuclear segmentation in the leg lymph of normal men, but they considered them an artifact since the cells had been collected at room temperature. Following the injection of diphtheria toxoid, an agent producing a mixed cellular and humoral response, Kelly (1970) noted an increase in cells with a "frilly" cytoplasmic edge. As the response progressed there was an increased tendency toward nuclear fragmentation in these cells. Drexhage et al. (1979) induced a cell-mediated response by painting the skin of pigs with DNFB, and found an increase in the number of "veiled" cells and over a 100% increase in the number of these that formed aggregates with other lymph-borne cells, mainly lymphocytes. They also reported an increase in "veiled" cells and aggregates following frostbite, a non-immunologic stimulus. This response however was short-lived and the number of "veiled" cells returned to normal

within seven hours. It appears therefore that there is a relationship between the degree of stimulation and the development and persistence of the "veiled" cell in the response.

We believe that the monocytoïd cell of normal lymph, the "frilly" cell with its ruffled cytoplasm and the "veiled" cell are the same cell. We base this on the similarity of the nucleus, the appearance of the cytoplasm and the common properties of limited phagocytic function and apposition to lymphocytes. Drexhage et al. (1979) reported that in vitro using phase microscopy, the "veiled" cell could be seen to extend and withdraw its cytoplasmic veils, indicating that the veils are not a fixed structure. The report of Spry et al. (1980) of the presence of 'Ia-like' membrane antigens on the "veiled" cells in human afferent lymph adds to the evidence that this cell may be part of the reticuloendothelial (mononuclear phagocyte) system, since there is a subset of macrophages that also possess this membrane marker (Schwartz et al., 1976). The presence of the "veiled" cell in renal lymph following antigen presentation, as in this work, and following allogeneic renal transplantation (Pederson and Morris, 1970) argues against their being derived from the Langerhan cell of the skin. However, recent work (Tamaki and Katz, 1980) has shown that the Langerhans cell may be of bone marrow origin and a common lineage for this cell and the "veiled" cell is not unlikely.

It has been proposed that the "veiled" cell is engaged in antigen processing and presentation to immunocompetent cells (Spry et al., 1980). The fact that these cells are persistently seen in peripheral lymph in large numbers only following stimulation with activators of the T-cell system is consistent with this theory. It appears, therefore, that the initial immune response, that of presentation of antigen, can take place in non-lymphoid tissues, at least in those cases where the antigen itself is not free to reach the regional lymph node.

The cellular changes seen in the kidneys of our animals are consistent with a minimal change membranoproliferative disease. This condition is not known to be mediated by T-cells or hypersensitivity. Additionally there is no obvious mononuclear cell infiltrate of the kidney in the early phase. Yet, following the very early transient inflammatory response, the cells leaving the kidney by way of the peripheral lymphatics, especially the "veiled" cells, are in keeping with a cell-mediated or T-cell response. It is possible that in this experimental model we are dealing with a mixed reaction, since after 7-14 days plasma cells and autologous antibody can be seen in the kidney.

Kelly et al. (1978) have shown that the mononuclear cells of the afferent lymph preferentially localize in the thymus-dependent areas of the regional lymph node. We postulate that in the kidney,

"veiled" cells, a specialized subset of the mononuclear phagocyte system, interact with antigen and present it in some form to policing lymphocytes, probably T-cells. These cells then migrate to the paracortical areas of the regional lymph node where the interaction between T and B cells takes place and the immune response is propagated.

## CONCLUSIONS

1.) In the normal dog there are relatively few cells in the renal hilar lymph. The mean white blood cell count is 133 cells/mm<sup>3</sup>. Of these cells, 63% are lymphocytes, 27% neutrophils, 7% monocytes, and 3% eosinophils. "Veiled" and "frilly" cells account for less than 1% of the cells present. The average diameter of the lymphocytes is 11.17  $\mu$ m, making these cells larger than the lymphocytes of either the blood or the thoracic duct lymph. The renal lymph has a higher proportion of medium to large lymphocytes and fewer lymphocytes in the under 10  $\mu$ m range. The mean nuclear-cytoplasmic ratio of the lymphocytes is 0.810. Blast-like cells account for approximately 5% of the cell population, indicating a continual low level of antigenic stimulation.

2.) The distribution of cells in the renal lymph is unrelated to the simultaneous cellular distribution in either the peripheral blood or thoracic duct lymph. A comparison of prenodal and central lymph reveals that cells of the monocyte-macrophage series as well as neutrophilic leukocytes are absent from central lymph. Eosinophils are present in both limbs of the lymphatic system. It appears that the movement of cells from the blood through the interstitium of the kidney to the afferent lymphatics is not a random process but preferentially favors cells of the lymphocytic series.

3.) There is no apparent circadian rhythm in the total white cell count of the peripheral blood, renal hilar or thoracic duct lymph of the anesthetized dog. Anesthesia has a marked effect on the white cell count of the blood. The intravenous injection of sodium pentobarbital (32.5mg/kg) causes an approximate 22% drop in the white cell count after one hour followed 10 hours later by a 2-3 fold increase in the number of cells. This increase is due to a rise in the absolute number of neutrophils. There is no effect of anesthesia on the cell counts of the thoracic duct or renal hilar lymph.

4.) Measurements of lymphocyte cell diameters from whole blood smears and cytocentrifuged preparations of the same sample reveal that the cells in the smears are larger. Erythrocyte diameters are the same in both preparations. Thus the cytocentrifuge preparations are closer to the in vivo state. Cytocentrifugation also enhances nuclear detail.

5.) The induction of an immune response by the administration of rabbit anti-canine whole glomerular serum suppresses the rise in the blood white cell count that is seen during prolonged anesthesia. There is also a slight decrease in the cellular content of the thoracic duct lymph. Within one hour of the administration of antiserum, there is a rise in the number of cells in the afferent renal lymph. The first 16 hours of the response in the renal lymph may be characterized as a response of phagocytic cells. Following

this non-specific inflammatory response, there is a recruitment of lymphocytes to the kidney. The cell count of the hilar lymph is below normal and there is an increase in the number of blast cells and cells in mitosis. "Veiled" and "frilly" cells account for the majority of the non-lymphoid mononuclear cells present. Many of these form rosettes with the lymphocytes. This association of "veiled" cells and lymphocytes is typical of that seen in response to an inducer of cell-mediated immunity.



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APPROVAL SHEET

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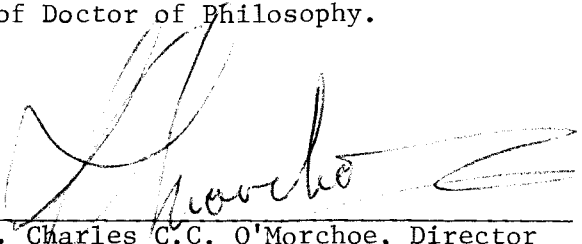
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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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