CANINE ADENOVIRUS NEPHROPATHY

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by

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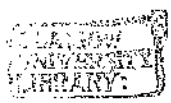

"There is nothing final nor absolute in the description of disease. ..... Pathology cannot reach beyond the limits of knowledge in the basic sciences, and current ideas cannot escape the scientific climate of the time, nor exceed the bounds imposed by the immediate rule of philosophy or even of language."

W.N. MANN.
(Guy's Hospital Reports, 1958).

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GENERAL INTRODUCTION

The immunological response against an infectious agent is not always beneficial to the host and indeed, may sometimes be instrumental in exacerbating the disease process. A good example of this type of immunologically mediated disease is the immune complex glomerulonephritis which develops during a number of persistent virus infections. In recent years, the prevalence of immune complex mediated glomerulonephritis in the dog has been increasingly recognised; however, the antigenic components of such complexes have yet to be identified.

The purpose of the present investigation was to carry out a detailed study of the renal lesions which occur during canine adenovirus (CAV) infection and to determine the influence of the immune response on the development of these lesions.

THE ROLE OF VIRUSES IN RENAL DISEASE: A REVIEW

VIRUSES WHICH INDUCE LESIONS AS A RESULT OF REPLICATION IN RENAL TISSUE

VIRUSES ASSOCIATED WITH IMMUNE COMPLEX MEDIATED RENAL LESIONS

Tables 1 and 2.

In recent years, a great deal of work has been devoted to investigation of the role of viruses in renal disease of man and animals. Considerable progress has been made towards identifying the viruses involved (see earlier reviews by Smith and Aquino, 1971, Jensen, 1967, and Oldstone and Dixon, 1971a), but much has still to be elucidated concerning the mechanisms by which viruses induce nephron damage.

Broadly speaking, there are two mechanisms whereby a virus may induce renal lesions. Firstly, direct infection of the cellular components of the kidney may result in lytic damage. If virus persists within tubular epithelial cells, then the lesions may become augmented by lymphoid cellular infiltration into the interstitium surrounding those cells. Secondly, a systemic virus infection may result in the deposition of virus antigen-antibody immune complexes within the renal glomeruli, thus inducing immune complex glomerulonephritis. This requires the simultaneous presence of virus or viral antigen and anti-viral antibody within the circulation and is not dependent on direct infection of the kidney by virus. These two mechanisms, however, are not mutually exclusive and both may operate simultaneously during the same virus infection.

The following discussion reviews the current knowledge of the role of viruses in renal disease of man and animals, with particular reference to the pathogenetic mechanisms involved.

VIRUSES WHICH INDUCE LESIONS AS A RESULT OF REPLICATION IN RENAL TISSUE

Despite the numerous reports in the literature implicating viruses in renal disease, there are very few well documented accounts of renal pathology associated with direct infection of kidney tissue. Utz (1974), in his review of viruria in man, reported that, to date, 16 different viruses had been isolated from human urine. These included coxsackie and ECHO viruses (Utz, 1960), measles (Gresser and Katz, 1960; Llanes-Rodas and Levi, 1966), mumps (Utz et al., 1958; Utz and Szwed, 1962), adenoviruses (Gresser and Kibrick, 1960; Gelfkunst and Heggae, 1961), cytomegalovirus (Weller and Hanshaw, 1962; Reynolds et al., 1973) and vaccinia virus (Blattner et al., 1964). Measles, adenoviruses and varicella have also occasionally been isolated, in tissue culture, from apparently normal human kidneys, always from children less than 1 year of age (Klein and Huang, 1963; Benyesh-Melnick and Rosenberg, 1974; Melnick et al., 1965). However, of all these human viruses, only the enteroviruses (i.e. coxsackie and ECHO viruses) and cytomegalovirus have been demonstrated to replicate in renal tissue. Moreover, demonstration of a virus in kidney tissue is, in itself, insufficient evidence to implicate that virus in renal disease. Thus, human cytomegalovirus may often be detected in distal tubular epithelium and, although mild interstitial infiltrates of lymphoid cells have occasionally been recorded (Medearis, 1957), histological changes are usually confined to the presence of intranuclear inclusion bodies in tubular cells (Evans and Williams, 1968). A number of viruses of other animals, however, do produce severe pathological changes due to direct infection of renal tissue.

Canine herpesvirus is associated with a sporadic acute systemic disease which is confined to neonatal puppies (Carmichael et al., 1964; Wright and Cornwell, 1968). The reason for the restriction of the disease to this age-group is thought to be partly due to the inability of neonatal puppies to regulate their own body temperature (Crighton, 1968), allied to the fact that, in vitro, canine herpesvirus appears to replicate less efficiently at temperatures above 37°C (Cornwell and Wright, 1969). Although adult dogs are not susceptible to the systemic disease, localised infections of the respiratory and genital tracts have been reported (Wright et al., 1970; Poste and King, 1971). Systemic infection of neonatal puppies causes a generalised disease in which there is infection of a wide range of organs resulting in widespread focal necrotising lesions. The kidneys contain focal areas of tubular necrosis in which large numbers of virus-infected cells can be detected by means of immunofluorescence. These foci are found mainly in the cortical regions of the kidneys and give rise to the characteristic macroscopic appearance of multiple haemorrhagic foci on the kidney surfaces.

Two subtypes of adenovirus occur in the dog. The first of these subtypes (CAV1) is associated with an acute systemic disease in dogs, characterised by severe hepatitis and widespread vascular damage. The second subtype (CAV2) has never been shown to cause systemic disease. Both viruses can locally infect the respiratory tract and are thought to be important in the aetiology of "kennel cough" in dogs (Wright et al., 1972).

Acute CAV1 infection was first described in detail by Rubarth (1947). Since then, it has been extensively studied and a number of reviews have comprehensively dealt with the virology, epidemiology and pathology of the disease (Cabasso, 1962; Cabasso and Wilner, 1969).

In the kidney, virus replication occurs mainly in the glomeruli where it produces lytic cellular damage. Histologically, numerous characteristic basophilic intranuclear inclusion bodies may be observed in glomerular cells and, by immunofluorescence, specific CAV antigen is observed as discrete fluorescing cells mainly confined to the glomeruli (Wright, 1967a).

In a proportion of dogs which recover from CAV1 infection, virus persists within foci of tubular epithelial cells and this is associated with the development of focal interstitial nephritis (Hamilton et al., 1966). These interstitial lesions consist of infiltration of a mixed population of lymphoid cells, plasma cells and macrophages into the interstitium surrounding virus-infected tubules (Wright et al., 1971). Persistence of infection in this way results in intermittent excretion of virus in the urine, thus providing a source of infection for other dogs. Although the duration of persistence of the interstitial lesions is unknown, virus has been detected in the urine of dogs up to 9 months after infection (Poppensiek and Baker, 1961; Baker et al., 1954).

Canine distemper virus may also be demonstrated in the kidneys of dogs infected with the virus. Although infection of the kidney is not a prominent feature of the disease, the presence of inclusion bodies in renal tubular epithelium has occasionally been recorded (Jubb and Kennedy, 1970; Lauder et al., 1954). Appel (1969), using the immunofluorescence technique to study the distribution of distemper virus in experimentally-infected dogs, was able to detect virus only in the pelvic epithelium of the kidney. Using the same technique, the author and his colleagues have found large numbers of virus infected cells in the kidneys of a small percentage of natural cases of distemper (Wright et al., unpublished data). In these animals, virus was present in tubular epithelial cells and in cells lying in the interstitium.

Histologically, this was sometimes accompanied by mild interstitial infiltrates consisting of lymphoid and plasma cells. The clinical significance of these findings has yet to be fully investigated.

Papadimitriou (1969) has studied the histological and ultrastructural changes in the kidneys of neonatal mice following experimental infection with reovirus type 3. Virus was shown to multiply within distal and collecting tubules and, histologically, affected tubules showed mild degenerative changes with occasional cellular necrosis. However, since mice were only studied up until 9 days after infection, it was not known if virus persisted in the kidney beyond the acute systemic phase of infection. Glomerular hypercellularity and capillary loop thickening were also detected from 4 days onwards and ultrastructural studies demonstrated the presence of mesangial and subendothelial electron-dense deposits. Although Papadimitriou did not consider an immune complex mechanism of glomerular damage, the fact that virus was not detected in the glomeruli and the finding of electron-dense deposits suggest that these glomerular lesions may have resulted from immune complex deposition.

A number of other viruses, which are associated with immune complex mediated glomerular changes, may also replicate in renal tissue and produce direct lytic lesions. These infections, which are discussed in the second part of this review, include swine fever, coxsackie and ECHO virus infections in mice and man and polyoma virus infection in mice.

VIRUSES ASSOCIATED WITH IMMUNE COMPLEX MEDIATED RENAL LESIONS

Extensive studies of experimentally induced and naturally occurring glomerulonephritis of man and animals, have shown that deposition of antigen-antibody immune complexes plays an important role in the pathogenesis of the disease (Dixon et al 1969; McCluskey 1971; Murray and Wright 1974). Recent studies have, therefore, been aimed at trying to identify the antigenic components of such complexes. Since many infectious agents, including some viruses, were known to produce persistent infections, it seemed probable that these might provide a long term source of circulating antigen from which immune complexes could be formed and subsequently deposited in the renal glomeruli. Thus, studies in recent years have provided convincing evidence that such a mechanism of renal disease occurs during a number of virus infections, (Table 1).

(a) Viruses known to be associated with immune complex glomerulonephritis

It is well established that the glomerulonephritis accompanying lymphocytic choriomeningitis, lactic dehydrogenase, and leukaemia virus infections in mice, aleutian disease in mink and equine infectious anaemia, is associated with deposition of virus antigen-antibody immune complexes. Similarly, there are numerous well documented accounts of glomerulonephritis in man accompanying infection with hepatitis B virus, which is thought to be the causal agent of various forms of hepatitis in man (Table 2). The histological nature of the glomerular lesions observed in all of these virus infections, with the exception of hepatitis B virus infection, is predominantly a proliferative glomerulonephritis characterised by mesangial expansion and hypercellularity and varying degrees of capillary loop thickening. The glomerulonephritis observed in association with hepatitis B virus is usually of the membranous or

Table 1:- Viruses associated with immune complex glomerulonephritis

Viruses known to be associated with Immune Complex Glomerulonephritis		Host	References
Lymphocytic Choriomeningitis Virus		Mouse	Hotchin & Colling, 1964
			Oldstone & Dixon, 1967
Lactic Dehydrogenase Virus		Mouse	Oldstone & Dixon, 1971
			Porter & Porter, 1971
Murine Leukaemia Viruses:	Gross	Mouse	Recker et al., 1966
	Friend		Hirsch et al., 1969
	Raucher		Oldstone & Dixon, 1972b
	Moloney		
Aleutian Disease Virus		Mink	Henson et al., 1969
			Porter et al., 1969
Equine Infectious Anaemia Virus		Horse	Banks et al., 1972
Hepatitis B Virus		Man	Combes et al., 1971
			Nowoslawski et al., 1971
Viruses suspected of producing Immune Complex Glomerulonephritis			
Polyoma Virus		Mouse	Tonietti et al., 1970
Enteroviruses (Coxsackie B and ECHO)		Mouse	Sun et al., 1967 and 1971
		Man	Mary & Swallow, 1970
			Yuceoglo et al., 1966
			Bayatpour et al., 1973
Swine Fever Virus		Pig	Cheville et al., 1970
Other Leukaemia Viruses		Cat	Anderson & Jarrett, 1971
		Man	Dathan et al., 1974
			Sutherland & Mardiney, 1977
			Sutherland et al., 1974
Measles Virus		Man	Dayan & Stokes, 1972
Mumps Virus		Man	Utz et al., 1964
			Hughes et al., 1966
			Thomas et al., 1968

Table 2:- Evidence for the role of immune complexes in glomerulonephritis associated with virus infections.

Virus	Host	Glomerular deposits of immunoglobulin, viral antigen and complement	Anti-viral antibody eluted from kidney	Circulating virus-antibody complexes
L.C.M.	Mouse	+	+	+
L.D.V.	Mouse	+	+	+
Mu.L.Vs.	Mouse	+	+	+
A.D.	Mink	+	+	+
E.I.A.	Horse	+	+	+
Hepatitis B virus.	Man	+	NR	+

NR = Elution studies not reported

membranoproliferative type, although a few cases of proliferative and one case of focal sclerosing glomerulonephritis have also been reported (Brzosko et al 1974; Knieser et al 1974). In all of these virus infections, immunofluorescence studies have demonstrated granular deposits of immunoglobulin, complement (C3) and viral antigen within the glomeruli. Kidney tissue from all, excepting hepatitis B infection in man, has been subjected to elution procedures and, in each case, anti-viral antibody has been demonstrated in the renal eluates, thus providing direct evidence that the deposited immunoglobulin is directed against viral antigens.

Lymphocytic Choriomeningitis (LCM):- Although LCM virus can infect a wide range of mammals including man, experimental studies have been carried out almost exclusively in mice. When adult mice are inoculated with LCM virus, they quickly die from an acute disease characterised by widespread tissue damage (Hotchin and Weigard, 1961). However, infection of neonatal mice produces a carrier state in which virus can be demonstrated in a range of tissues, particularly the liver, brain and kidney (Traub, 1935; Oldstone and Dixon, 1969). Once infected, these animals remain viraemic for life. In a number of strains of mice, this persistent infection results in a characteristic chronic disease consisting of glomerulonephritis, focal hepatic necrosis and generalised proliferation of lymphoid tissues with widespread focal infiltration of lymphoid cells into many tissues (Oldstone and Dixon, 1969). The onset and severity of these lesions varies markedly between different strains of mice, some strains having histological evidence of glomerulonephritis as early as 2 months of age, whereas others show virtually no abnormalities up to 2 years of age (Oldstone and Dixon, 1968). Since anti-LCM antibodies could not be detected in the serum of chronically infected mice, it was initially considered that neonatal

mice were tolerant to LCM virus infection (Burnet and Fenner, 1949). However, subsequent studies have shown that antibody is present in the form of circulating infectious virus-antibody complexes (Oldstone and Dixon, 1969). Furthermore, there is good evidence that the glomerulonephritis observed in chronically infected mice is due to deposition of circulating immune complexes containing virus antigen and anti-viral antibody (Hotchin and Colling, 1964; Oldstone and Dixon, 1967). In contrast, tissue injury other than glomerulonephritis appears to be, at least partly, due to interaction of free antibody with virus infected cells (Oldstone and Dixon, 1970a). Further studies carried out by Oldstone and Dixon (1970b) showed that mice transplacentally infected with LCM virus develop more severe glomerulonephritis which, in some animals, is lethal within two weeks of age. This was shown to be due to anti-LCM antibody acquired from the mother's milk, thus increasing the circulating antibody in the neonate and leading to formation and subsequent deposition of greater quantities of virus antigen-antibody complexes (Oldstone and Dixon, 1972).

Lactic Dehydrogenase Virus (LDV):- When inoculated into mice of any age, LDV produces a lifelong asymptomatic infection; the virus multiplies in macrophages and produces widespread lymphoid hyperplasia and splenomegaly (Riley, 1964). As with LCM infection, there is persistent viraemia, in which infectious virus circulates as a complex with host IgG. Neutralising antibody to LDV can only be detected in serum which has been pretreated with either ether or ultraviolet light (Notkins et al., 1966), although, by use of an indirect immunofluorescence test, Porter and Porter (1971) were able to demonstrate anti-LDV antibody in untreated serum. From an early age, deposits of IgG, C3 and occasionally LDV antigen are present in the glomeruli of infected mice and are associated with a mild proliferative glomerulonephritis. Furthermore,

elution studies have shown that deposited IgG contains anti-IDV antibody (Oldstone and Dixon, 1971b; Porter and Porter, 1971).

Murine Leukaemia Viruses (MuLVs):- Wild type murine leukaemia virus (Gross virus) and the laboratory strains, Moloney, Friend and Raucher, all produce prolonged infection and viraemia when inoculated into neonatal mice and, in later life, a proportion of infected mice develop leukaemia. All of these viruses have been associated with glomerulonephritis, in which IgG, C3 and sometimes virus antigen may be detected in the glomeruli by immunofluorescence (Becker et al., 1966; Hirsch et al., 1969; Oldstone et al., 1972a). However, most of the detailed studies of the renal lesions have been carried out in AKR mice which are endemically infected with Gross leukaemia virus. Although it is not possible to detect circulating antibody to Gross virus, eluates obtained from kidneys of infected mice have yielded anti-viral antibodies. These eluted antibodies have been shown to be directed against a number of Gross virus antigens; thus, antibody can readily be detected to group specific and infected cell surface antigens (Markham et al., 1972; Oldstone et al., 1972b), while the detection of low levels of antibody directed against viral envelope (Yoshika et al., 1974) and against RNA-dependent DNA polymerase (Hollis et al., 1974) has been reported. Oldstone et al., (1972b) suggested that the inability to demonstrate antibodies in the serum was probably due to circulating antibodies being bound to virus or viral antigens and that deposition of these complexes resulted in the glomerulonephritis observed in infected mice. This concept is supported by the fact that infectious virus-antibody complexes have been demonstrated in the serum of mice neonatally infected with Moloney leukaemia virus (Hirsch et al., 1969). Ultrastructural studies of glomerular lesions associated with Gross virus infection have demonstrated the presence of intact virus particles

within some of the electron dense deposits observed in the glomeruli (Pascal et al., 1973; Batzing and Hanna 1973). Similar virus particles have been observed within and budding from the surface of mesangial cells. Batzing and Hanna suggested that the glomerulonephritis may have resulted from circulating antibody to viral envelope fixing to intact virus particles already present within the glomeruli. However, Yoshika et al., (1974) have shown that only some deposits contain intact virus particles and only a small proportion of antibody eluted from glomeruli is directed against viral envelope.

Gross leukaemia virus has also been implicated in the glomerular lesions of New Zealand black (NZB) mice and their F1 hybrid NZB/W mice. These mice suffer from a spontaneous disease which shows many similarities to systemic lupus erythematosus (SLE) in man and characterised by immune complex glomerulonephritis, haemolytic anaemia and the presence of a number of auto-antibodies (Mellors et al., 1965). NZB mice are also endemically infected with Gross leukaemia virus but, unlike other strains of mice, they produce detectable circulating antibody (Mellors et al., 1971). Extensive immunofluorescence and elution studies have shown that both antinuclear auto-antibodies and antibodies to Gross virus, along with their corresponding antigens, are present in the glomeruli of NZB and NZB/W mice (Mellors et al., 1971; Lambert and Dixon, 1968). In contrast to other strains of mice, the anti-Gross virus antibodies detected in the glomeruli of NZB and NZB/W mice contained large amounts of anti-viral envelope antibody (Yoshiki et al., 1974). The results of quantitative studies on renal eluates, carried out by Dixon et al., (1971) have indicated that a large proportion of deposited immunoglobulin consists of antinuclear antibody and it was therefore considered that, while the deposition of Gross antigen-antibody complexes contributed to the glomerular lesions, it was not the major contributary factor. Whether or not other immunological abnormalities, including

the production of antinuclear antibodies, observed in NZB and NZB/W mice, are related to the presence of Gross leukaemia virus or another unidentified virus, is currently the subject of investigation. Thus, Croker et al., (1974) recently described a systemic lupus erythematosus - like syndrome induced in immunologically normal (BALB/C X NZB)F1 mice by infection of neonates with a murine leukaemia virus (Scripps leukaemia virus) isolated from NZB lymphoblasts.

Aleutian Disease Virus (ADV):- The virus which causes aleutian disease in mink has only recently been isolated (Kenyon et al., 1973; Cho and Ingram, 1973a), although it has been recognized as a filterable, transmissable agent since 1962 (Karstad and Pridham, 1962). Vertical or horizontal transmission of the virus can occur and, once mink become infected, they remain so for life (Ingram and Cho, 1974). Virus multiplies within macrophages in the lymph nodes and spleen and in the Kuppfer cells of the liver. The disease, which is slowly progressive, is characterised by widespread plasmocytosis, hepatitis, glomerulonephritis and arteritis, and death may occur any time from 2 to 24 months after infection (Henson et al., 1966; Henson et al., 1968). Genetic factors appear to influence the severity of disease; thus, mink which are homozygous recessive for the aleutian coat colour gene (aa) develop a particularly severe disease. The serum of infected mink shows massive hypergammaglobulinaemia which has been shown to be due mainly to increased IgG production (Porter et al., 1965). High levels of anti-ADV antibody have been detected in serum by the indirect immunofluorescence test, although this antibody was found to be incapable of neutralisation (Porter et al., 1969). Since Porter and Larsen (1967) demonstrated infectious virus - antibody complexes in the serum of ADV infected mink, it has been considered that some of the lesions observed in aleutian desease might be immune complex mediated. Subsequent work has shown that both the glomerular and arterial wall lesions contain

deposits of IgG and C3; ADV antigen has been demonstrated in arterial walls following acid elution studies and anti-ADV antibody has been detected in eluates obtained from affected kidneys (Henson et al., 1969; Porter et al., 1969; Porter et al., 1973). Furthermore, the finding by Cho and Ingram (1973b) of IgG and C3 on the surface of erythrocytes, from which anti-ADV could be eluted, suggests that circulating ADV antigen-antibody-complement complexes are absorbed on to the surface of erythrocytes.

Equine Infectious Anaemia (EIA):- EIA virus produces persistent viraemia in horses (Henson and McGuire, 1971). Most infected horses develop an acute illness 1-2 weeks after infection and, although some animals die early in the course of disease, a large number recover but subsequently suffer from recurring episodes of clinical illness from which death can result months or years later. Furthermore, a few infected horses do not develop clinical disease but become asymptomatic carriers. Virus replication is thought to occur mainly in macrophages and, by the immunofluorescence technique, virus can be detected in a wide range of tissues including the kidneys (McGuire et al., 1971). The disease is characterised by severe haemolytic anaemia, glomerulonephritis, hepatitis, widespread lymphoproliferative lesions and hypergammaglobulinaemia (Henson and McGuire, 1971). Despite the persistence of viraemia, neutralising and complement fixing antibodies have been detected in the serum of infected animals (Kobayashi et al., 1969; McGuire et al., 1971) and some of this antibody is present in the form of circulating infectious virus-antibody complexes (McGuire et al., 1972). Recent studies by Pono et al., (1973) indicate that persistence of viraemia in the presence of circulating antibody may be related to changes in virus surface antigens (antigenic drift), with different antibody responses being mounted to each successive antigenic variant. There is good evidence that the glomerulonephritis is due to deposition

of virus antigen-antibody complexes, as deposits of IgG and C3 have been demonstrated in the glomeruli of EIA infected horses and anti-EIA antibody has been detected in eluates obtained from affected renal tissue (Banks et al., 1972). Furthermore, the haemolytic anaemia may also have an immunological mechanism, as hypocomplementaemia (Perryman et al., 1971) and C3 bound to the surface of erythrocytes (McGuire et al., 1969) have been demonstrated in EIA infected horses.

Hepatitis B virus:- Hepatitis B virus antigen has been demonstrated in the serum and liver tissue from patients suffering from acute and various forms of chronic hepatitis (Wright et al., 1969; Gitnick et al., 1969; Nowoslawski et al., 1972). In addition, however, a small number of apparently healthy humans carry the virus in their serum and, although virus antigen may also be present in hepatic tissue, only in a small proportion of cases is this associated with histological abnormalities (Reinicke et al., 1970; Hadziyannis et al., 1972). The antibody response to hepatitis B virus appears to be extremely variable; thus, little or no antibody is found in asymptomatic carriers (Reed et al., 1974), whilst, in hepatitis patients, antibody appears to be bound to antigen in the form of antigen-antibody immune complexes and accompanied by varying amounts of either free antigen or free antibody (Almeida and Watersan, 1969; Millman et al., 1970). The presence of circulating immune complexes was first suspected when Schulman and Barker (1969) found that 95 per cent of serum samples from patients with acute hepatitis contained anti-complement activity. Subsequently, complexes have been demonstrated in the serum of patients with acute and chronic hepatitis using a number of different techniques (Almeida and Waterson, 1969; Millman et al., 1970; Brzosko et al., 1971; Nydegger et al., 1974). Glomerulonephritis, associated with hepatitis B virus infection, was first reported by Combes et al., (1971) in a patient who had suffered from acute hepatitis. Subsequently, there has been a number of other reports

describing glomerulonephritis in association with various forms of acute and chronic hepatitis (Nowoslawski et al., 1972; Myers et al., 1973; Knieser et al., 1974; Kohler et al., 1974; Blaker and Thoenes, 1974). In all of these cases, deposits of IgG, C3 and hepatitis B antigen were demonstrated in the glomeruli by immunofluorescence. Furthermore, glomerulonephritis associated with deposition of hepatitis B antigen, IgG and C3 has also been reported in virus carriers showing no evidence of liver disease (Brzosko et al., 1974).

All of the above virus infections have a number of general features in common which are of relevance to their ability to induce glomerulonephritis.

(i) They are all persistent infections and, despite the production of antiviral antibodies by their hosts, they all exhibit long-standing viraemia. This persistence of circulating virus is the crucial factor which ensures a constant source of antigen from which circulating antigen-antibody complexes can form and subsequently become deposited in the glomeruli.

(ii) One distinctive property of viruses in this group is that they do not kill the cells in which they replicate (Allison, 1972) and virus can, therefore, replicate to high titres without causing severe tissue injury and acute disease. Tissue lesions, however, do occur, although they are not considered to be due to direct virus induced damage but to an immunological response against virus infected cells. This, it has been shown that in LCM, AD and EIA, immunosuppression leads to a marked decrease in severity of disease (Hoffsten and Dixon, 1973; Cheema et al., 1972; Henson and McGuire, 1971).

(iii) Circulating virus-antibody immune complexes have been detected in all of these diseases and, with the exception of hepatitis B virus infection in man, these complexes have been shown to retain virus

infectivity. Whether or not the presence of these complexes is directly related to the pathogenesis of the glomerulonephritis is uncertain. However, since intact virus particles are usually not found in glomerular deposits in these diseases, it would seem more likely that the deposited complexes contain viral component antigens rather than intact virus particles. The circulating infectious virus-antibody complexes, therefore, may merely indicate the hosts inability to eliminate the virus. In LCM, Gross leukaemia and in some cases of hepatitis B infection, circulating antibody can only be detected after dissociation of virus-antibody complexes, whilst, in AD and EIA, high levels of free anti-viral antibody can be detected as well as virus-antibody complexes. This variation in antibody production between the different diseases suggests that there may either be a quantitative or a qualitative defect in the immune response or perhaps a combination of both. Detailed studies on the fate of preformed immune complexes when inoculated into experimental animals have shown that soluble nephritogenic complexes are only formed when there is relative antigen excess and that complexes formed in antibody excess are insoluble and readily removed by the mononuclear phagocytic system. However, if the antibody has a low affinity for antigen, then soluble immune complexes may be formed even if there is an excess of antibody (Germuth and Rodrignez, 1973). These experimental situations may have a parallel in some persistent virus infection. The glomerular lesions observed in LCM, LDV infection and AD all show variation in severity between genetically different strains of mice and mink respectively. Southill and Steward (1971) have shown that those strains of mice most susceptible to nephritis during LCM virus infection were the strains which, under normal circumstances, produced antibody of lowest affinity. Another factor of importance may be the ability of some of the viruses in question (LCM, LDV, AD and EIA) to replicate in macrophages. Thus, the normal process of removal of virus-antibody

complexes by the mononuclear phagocytic system, in these infections, may lead to further replication and release of virus. Moreover, since macrophages play an important role in the immune response and since inter-strain variation of antibody affinity in mice is thought to be related to macrophage function (Passwell et al., 1974), it is quite concievable that infection of these cells by virus could not only lead to a quantitative, but also qualitative defect in the immune response. Under such circumstances, virus could persist in the circulation and lead to the formation of nephritogenic virus antigen-antibody immune complexes.

(b) Viruses suspected of producing immune complex glomerulonephritis

The glomerulonephritis observed during the course of several other virus infections is also thought to be mediated by the deposition of virus antigen-antibody immune complexes, although final proof is still lacking. Chronic swine fever and long-standing polyoma, coxsackie B and ECHO virus infections in mice are accompanied by a proliferative glomerulonephritis in which deposits of immunoglobulin can be detected in the glomeruli by the immunofluorescence technique (Tonietti et al., 1970; Sun et al., 1967; Sun et al; 1971; Cheville et al., 1970). Although foci of viral antigen have also been found in the glomeruli during Coxsackie B, ECHO, and swine fever virus infections, they appear to represent descrete virus-infected cells rather than granular immune deposits. The finding of glomerular deposits of immunoglobulin in this group of virus infections clearly suggests an immune complex mechanism; however, until elution studies are carried out on affected renal tissue and attempts are made to detect circulating immune complexes, it remains uncertain whether or not these deposits contain virus antigen-antibody complexes. In addition, these viruses have all been shown to replicate in renal tissue resulting in direct virus induced renal lesions.

Swine fever virus may infect pigs of all ages and infection usually results inrn acute disease leading to death within 1-2 weeks. However, a small proportion of animals survive the acute phase of the disease, remain chronically infected and succumb at a later stage. During the acute disease, there is widespread infection of lymphoid tissue and vascular endothelium which, in the kidney, is associated with multiple small interstitial haemorrhages (Marcato and Bacchi, 1963). Furthermore, in the later stages of the acute disease, virus invades tubular epithelial cells resulting in foci of tubular necrosis. In animals which become chronically infected, there is extensive invasion of tubular epithelial cells with subsequent development of focal interstitial nephritis characterised by accumulations of lymphocytes and plasma cells (Cheville and Mengeling, 1969). During this chronic phase, glomerulonephritis also develops and, with increased duration of infection, there is a progressive increase in severity of both glomerular and interstitial lesions.

Levinthal (1962) studied the kidney lesions following experimental infection of mice with polyoma virus. A proportion of mice died from an acute systemic disease during which the virus infected renal tubular epithelial cells resulting in focal areas of tubular degeneration. In those infected animals which did not develop the acute fatal disease, there was persistence of virus within foci of tubular epithelium and this was accompanied by infiltration of mononuclear and plasma cells into the surrounding interstitium. Recent studies have also demonstrated that mice chronically infected with polyoma virus develop a mild glomerulonephritis associated with the presence of immunoglobulin deposits in the glomeruli (Tonietti et al., 1970).

Mice experimentally infected with Coxsackie B1, B4 and ECHO9 viruses develop glomerulonephritis associated with infection of glomerular cells and deposition of immunoglobulin within the glomeruli

(Sun et al., 1967 and 1971). Whether or not direct virus induced damage contributes significantly to these glomerular lesions, however, remains uncertain. Coxsackie B4 and ECHO9 viruses have also been shown to infect tubular epithelial and interstitial cells giving rise to tubular necrosis and, in some instances, focal interstitial infiltrates of lymphoid cells (Burch and Sun, 1968; Burch et al., 1968).

There are numerous reports in the literature associating Coxsackie B and ECHO viruses with renal lesions in man. These viruses have been demonstrated in human urine, both by virus isolation (Utz, 1960; Utz and Shelokov, 1958) and by using immunofluorescence techniques on urinary sediment (Hinuma et al., 1962). Furthermore, Mary and Swallow (1970) showed that, during an outbreak of ECHO type 9 infection, 50 per cent of patients developed haematuria and 10 per cent had low levels of proteinuria. In a series of routine autopsies, Burch et al., (1969) using immunofluorescence, demonstrated intracellular Coxsackie B antigen in the kidneys of eleven patients; viral antigen was found in glomerular, tubular and interstitial cells. Although most of these patients did not die as a result of renal disease, they all showed histological renal abnormalities consisting of varying degrees of pyelonephritis and, in one case, glomerulonephritis. In addition, Burch and Colcolough (1969) reported a case of Coxsackie virus-associated pancarditis with concurrent pyelonephritis and focal glomerulonephritis in which Coxsackie B antigen was detected at autopsy in glomerular and interstitial cells. Isolated cases of glomerulonephritis have also been reported in association with these virus infection (Yuceoglo et al., 1966; Mary and Swallow, 1970; Bayatpour et al., 1973), although in none of these cases have immunofluorescence or virological studies been performed on renal tissue. The possibility, therefore, still remains that infection of man by these viruses may occasionally result in glomerulonephritis, either due to direct infection of

glomerular cells or, as results of experiments in mice might suggest, through the deposition of virus antigen-antibody immune complexes.

Glomerulonephritis has also been noted in association with feline and human leukaemia. Anderson and Jarrett (1971) described three cases of membranous glomerulonephritis in a series of twenty-seven naturally-occurring cases of feline leukaemia. Glomerulonephritis and nephrotic syndrome have been reported in three human patients suffering from lymphocytic leukaemia and, in two of these cases, immunoglobulin and C3 were detected in the glomeruli (Brodovsky et al., 1968; Dathan et al., 1974). Glomerular deposits of immunoglobulin and C3 have also been detected at necropsy in the kidneys of patients with lymphoma and leukaemia without any evidence of clinical renal disease (Sutherland and Mardiney, 1973; Sutherland et al., 1974). In two of these patients, Sutherland and Mardiney (1973), using immunofluorescence and elution techniques, also demonstrated the presence of interspecies group specific leukaemia virus antigen (gs-3) within the glomeruli. Although as yet a human leukaemia virus has not been identified, it is tempting to draw a parallel with the situation in murine leukaemia and speculate that glomerulonephritis may occur in other species due to deposition of leukaemia virus antigen-antibody complexes.

Evidence of glomerular immune complex deposition during measles infection has been obtained in a single patient who died from subacute sclerosing panencephalitis (Dayan and Stokes, 1972). Granular deposits of immunoglobulin, measles antigen and complement were detected in the glomeruli of this patient by immunofluorescence; however no histological or clinical renal abnormalities were found.

Although there are no reports of histological or immunofluorescence studies being carried out on kidney tissue from patients with mumps, a proportion of infected individuals have been shown to develop renal functional abnormalities consisting of mild proteinuria, haematuria

and slightly decreased rates of creatinine clearance (Utz et al., 1964; Thomas et al., 1968). Indeed, severe fatal mumps has been described in 2 cases in which mumps was diagnosed clinically (Hughes et al., 1966). However, in neither case was renal tissue subjected to immunofluorescence or virological studies and evidence for their association with mumps infection is therefore only circumstantial.

MATERIALS AND METHODS

THE VIRUS

EXPERIMENTAL ANIMALS

NECROPSY PROCEDURES

HISTOLOGICAL PROCEDURES

ULTRASTRUCTURAL PROCEDURES

IMMUNOFLUORESCENCE PROCEDURES

ELUTION PROCEDURES

DETECTION OF ANTIBODY TO CAV

Tables 3 and 4; Figure 1.

THE VIRUS

The virus used throughout the experimental studies was a strain of canine adenovirus (CAV) isolated from the kidney of a dog with viral interstitial nephritis. Virus pools were prepared by infecting monolayers of a continuous dog kidney cell line (MDCK, Madin and Darby Canine Kidney, Flow Laboratory Ltd., Ayrshire) (Cornwell et al., 1970). Infected cells were incubated at 37°C until the cytopathic effects were complete, at which time the virus was harvested by repeated freezing and thawing. The virus suspension was then stored in 1ml aliquots at -70°C until required. The titre of virus was 10^7 tissue culture infectious doses (TCID50).

EXPERIMENTAL ANIMALS

For experimental studies, farm-bred unvaccinated puppies, ranging from 8 to 14 weeks of age, were obtained from a commercial source. On arrival, the puppies were isolated and serum samples were obtained in order to measure antibody levels to CAV. All puppies were housed indoors at an ambient temperature of 20°C and were fed on a commercial dog food ("Lassie", Pet Foods Ltd., Melton Mowbray, Leic.) and reconstituted dried milk.

During the study, all experimental procedures involving the inoculation of dogs by the intravenous route were carried out under "Immobilon"-induced neuroleptanalgesia ("Immobilon", Reckitt and Colman Pharmaceutical Division, Hull.).

NECROPSY PROCEDURES

Those animals which did not succumb spontaneously to infection were anaesthetised by rapid intravenous injection of pentabarbitone sodium ("Euthatal", May and Baker Ltd., Dagenham) and exsanguinated by severing the jugular veins. All animals were subjected to a comprehensive postmortem examination. Immediately on opening the abdominal cavity, the kidneys were removed and small pieces of kidney tissue were taken for ultrastructural studies (see below). Multiple blocks of kidney were also selected for histological and immunofluorescence studies. In addition, a range of other tissues, including liver, spleen, lymph nodes, thymus, tonsil, urinary bladder and brain, were subjected to routine histological examination.

HISTOLOGICAL PROCEDURES

Tissues were fixed in mercuric chloride-formol for 72 hours, dehydrated in an alcohol-amyl acetate-chloroform series and embedded in paraffin wax. Sections were cut at 6 microns and stained with Mayer's haemalum and eosin; selected sections were also stained with Martius Scarlet Blue (collagen and fibrin) and periodic acid-Schiff stains (basement membranes).

ULTRASTRUCTURAL PROCEDURES

(a) Fixation

Small pieces of kidney were exised as soon as possible after euthanasia and placed in drops of chilled fixative on a block of dental wax. Using a grease-free razor blade, the tissue was finely sliced into small blocks approximately 0.5mm in thickness and transferred to glass vials containing chilled fixative. Tissue was fixed at $4^{\circ}C$, either in a 1 per cent solution of osmium tetroxide

for 1½ hours or in a paraformaldehyde/glutaraldehyde mixture (Karnovsky, 1975) for 4½ hours. Tissue fixed in the paraformaldehyde/glutaraldehyde mixture was rinsed overnight in a cacodylate rinsing solution and then postfixed for one hour in osmium tetroxide.

The fixatives were prepared as follows:-

(i) Osmium tetroxide: 1 per cent osmic acid (BCH Chemicals Ltd., Poole, England.) was made up in Millonigs buffer at pH 7.2-7.4.

(ii) Paraformaldehyde/glutaraldehyde: A mixture of 1.3 per cent paraformaldehyde and 1.6 per cent glutaraldehyde was prepared in cacodylate buffer at pH 7.2-7.4.

The proportions were:-

Paraformaldehyde	2g
Distilled water	25ml
1 N Sodium hydroxide	2-3 drops
25 per cent glutaraldehyde	10ml
Cacodylate buffer	115ml
Anhydrous calcium chloride	25mg

The buffers were prepared as follows:-

(i) Millonigs phosphate buffer:

Sodium dihydrogen phosphate	(2.26 per cent)	83ml
Sodium hydroxide	(2.52 per cent)	17ml
Distilled water		10ml
Sucrose		0.54g

Final pH 7.2-7.4.

(ii) Cacodylate buffer: this was prepared as a 0.1 M solution of sodium cacodylate (21.4g/litre) and adjusted to pH 7.2-7.6 by addition of a few drops of concentrated hydrochloric acid.

Cacodylate rinsing solution: sucrose was added to cacodylate buffer (34.2g/litre) resulting in a 0.1 M solution of sucrose and the pH was adjusted to 7.2-7.4.

(b) Embedding

Fixed tissue was dehydrated in an ascending series of 70 per cent, 90 per cent, and absolute alcohol, followed by rinsing in propylene oxide. The tissues were then soaked for 1 hour in an equal parts mixture of propylene oxide and Araldite and left overnight in a mixture containing 80 per cent Araldite. Tissue blocks were then individually embedded in Araldite contained in gelatin capsules and the resin allowed to polymerise at 57°C for 48 hours.

Araldite (CIBA-Geigy (U.K.)Ltd., Cambridge) was prepared as follows:-

Equal parts of Araldite resin (CY212) and Araldite hardener (HY964) were mixed by stirring overnight and stored at 4°C. Before use for embedding, 0.6ml of accelerator (DY064) and 2.4ml of di-n-butyl phthalate were added to 57ml of the resin/hardener mixture and the whole stirred thoroughly for 30 minutes.

(c) Staining

Sections approximately 1 micron in thickness were cut on an LKB Mark III ultratome using glass knives and mounted on glass slides. They were then stained with Mallory's borax methylene blue or toluidine blue according to the methods of Richardson et al., (1960) and Trump et al., (1961). These sections were used to locate lesions and select particular fields for electron microscopical examination.

Ultrathin sections were cut on the ultratome using glass knives and mounted on uncoated Athene 483 copper specimen grids (Smethurst High-Light Ltd., Bolton, Lancs.). Sections were stained for 20 minutes

with uranyl acetate (Watson, 1958), rinsed successively in methanol, 50 per cent methyl alcohol and distilled water and dried on filter paper. They were then stained for 10 minutes with lead citrate (Reynolds, 1963) and finally rinsed with 0.02 N sodium hydroxide and distilled water before drying on filter paper. The sections were examined using an AEI 6B electron microscope.

The stains were prepared as follows:-

(i) Uranyl acetate: a 20 per cent solution of uranyl acetate (May and Baker Ltd., Dagenham, Essex) was made up in absolute methanol.

(ii) Lead citrate:

Lead nitrate 1.33g

Sodium citrate 1.76g

Each salt was dissolved in 15ml of distilled water and, when dissolved completely, mixed together. The resultant precipitate of lead citrate was shaken for 1 minute and left to stand for 30 minutes with periodic stirring. The precipitate was solubilised by adding 8ml of 1 N sodium hydroxide and diluted to 50ml with distilled water.

The resultant lead citrate solution had a pH of 12 ± 0.1.

IMMUNOFLUORESCENCE PROCEDURES

(a) Preparation of tissue

Small blocks of tissue approximately 1cm x 1cm x 0.5cm were placed in individual Pyrex containers and snap-frozen by placing the containers into a freezing mixture of solid carbon dioxide and 2-methylbutane. The blocks were stored at -20°C until required for examination.

Sections 3-4 microns in thickness were cut at -20°C on a Slee cryostat.

(b) Preparation of antisera

Fluorescein isothiocyanate (FITC) labelled and unlabelled antisera,

prepared in rabbits against canine immunoglobulin (Ig), IgG and B1C globulin (C3), and mouse IgG and B1C globulin were obtained from commercial sources (Anti-dog IgG:- Cappel Laboratories, Downingtown, Pennsylvania, USA; Others:- Sera Services Ltd., Maidenhead, Berks).

FITC-labelled antiserum to CAV was prepared as follows:-

(i) Production of antiserum:- A 14-week-old dog was inoculated subcutaneously with 1ml of virus suspension emulsified in an equal volume of Freund's complete adjuvant. A further 1ml dose of virus was administered intravenously 6 weeks later and, 10 days after the second inoculation, the dog was bled under general anaesthesia. The serum was found to have an neutralising antibody titre of 1:10,000 and an indirect immunofluorescence titre of 1:4,096.

(ii) Fractionation:- Equal volumes of serum and saturated ammonium sulphate were thoroughly mixed and left for 10 minutes at 4°C. The resultant precipitate containing the globulin fraction was separated by centrifugation at 4°C and resuspended in phosphate buffered saline (PBS) pH 7.2 to one third of the original volume of serum. The protein solution was then dialysed for 48 hours at 4°C against PBS to remove ammonium ions.

(iii) Conjugation:- The globulin fraction was conjugated at 4°C with 10 per cent FITC on celite powder (Calbiochem, California, USA) as recommended by Rinderknecht, (1962). Ten mg of dry FITC was added to a mixture of 2ml of the globulin solution and 2ml of carbonate/bicarbonate buffer pH 9 and the mixture was gently stirred in an ice bath for 5 minutes. Free dye remaining in the solution after this

time was removed by passing the conjugate through a column of Sephadex G25 fine grade, (Pharmacia, Uppsala, Sweden.). The conjugate was further purified by absorption for 3 hours at 4°C with 100mg of dog tissue powder per 1ml of conjugate.

Tissue powder was prepared from liver and kidney tissue from young healthy dogs. The tissues were homogenised in a Waring blender and repeatedly washed in isotonic saline before washing several times in large volumes of acetone. The supernatant was removed and the material spread on filter paper to be dried overnight at 37°C. It was then ground in a mortar, passed through a sieve to remove course fibrous material and stored in air-tight containers at 4°C.

The optimal working dilution of each conjugated antiserum was determined by staining known positive material with a range of dilutions of the appropriate antiserum. The dilution at which there was a sufficiently low level of background staining without adversely affecting the intensity of specific fluorescence was then adopted as the working dilution.

Consequently, the FITC-labelled antisera were used at the following dilutions:-

Rab/Dog Ig	1:20
Rab/Dog IgG	1:10
Rab/Dog B1c	1:5
Anti CAV	1:5

(c) Staining Procedures

In order to determine which fixation and washing procedures gave optimal results, an initial trial was carried out using known positive material. This involved the use of 7 different fixation regimes,

either on unwashed sections or on sections pre-washed for 30 minutes in PBS or isotonic sucrose. The influence of these different procedures on the results obtained when staining for CAV antigen and IgG was then assessed. Serial sections of kidney known to contain large numbers of virus infected cells were used in staining for CAV antigen and sections of kidney in which the glomeruli were known to contain diffuse deposits of IgG were used for the detection of IgG. As control material, sections of kidney from young healthy dogs were subjected to the same treatment. A comparative assessment of the intensity of specific fluorescence and the degree of non-specific background staining was made for the different regimes. The results are summarised in Table 3.

(i) Fixation: The most satisfactory results were obtained with unfixed and acetone fixed sections. In comparison, the other fixatives all caused varying degrees of reduction in intensity of specific staining.

(ii) Washing: Unwashed sections stained for IgG showed a high level of nonspecific background staining. This background was markedly reduced by pre-washing, particularly with PBS, although there was also a slight decrease in the intensity of specific staining. In general terms, the distribution of IgG within the glomeruli was also more readily defined in washed than in unwashed sections.

In contrast, unwashed sections stained for CAV antigen exhibited relatively low non-specific background staining and, while pre-washing caused further reduction in background, it also produced a marked decrease in the intensity of specific staining.

In view of these findings the following procedures were decided upon.

Air dried cryostat sections were fixed in acetone for 10 minutes

before staining for CAV antigen. Sections being stained for the presence of serum proteins (Ig, IgG and B1C globulin) were washed in PBS for 30 minutes before fixing in acetone.

In all cases, fluorescent stain was layered on to fixed sections for 30 minutes, during which time the sections were placed in a moist chamber at room temperature. After thorough washing in PBS for 30 minutes, sections were mounted in PBS and examined by means of a Leitz "Orthoplan" fluorescence microscope equipped for incident light fluorescence.

Specificity of staining was demonstrated on duplicate sections by blocking with unlabelled antiserum prior to staining with the corresponding FITC-labelled antiserum.

Selected sections of kidney, in which there was in interstitial infiltrate containing plasma cells, were examined using an indirect "sandwich" immunofluorescence techinque in order to determine whether or not the plasma cells contained anti-CAV antibody. This consisted of layering a drop of virus suspension concentrated by ultracentrifugation, on to cryostat sections of kidney for 30 minutes and, after thorough washing in PBS, the sections were stained with FITC labelled anti-CAV serum for a further 30 minutes. A positive reaction was indicated by the presence of characteristic cytoplasmic fluorescence. As control material, duplicate sections of kidney were exposed to uninfected tissue culture fluid and followed by staining with FITC labelled anti-CAV serum.

Table 3: The influence of different washing and fixation procedures on the results obtained following immunofluorescence staining for canine IgG and CAV antigen.

WASHING AND FIXATION PROCEDURES	IMMUNOFLUORESCENCE RESULTS			
	IgG		CAV	
	Intensity of staining	Nonspecific background	Intensity of staining	Nonspecific background
A. SECTIONS UNWASHED PRIOR TO FIXATION				
1) Unfixed	++++	High	++++	Low
2) Acetone 10min	++++	"	++++	"
3) 95% Ethanol 10min	++	"	+	"
4) Ether-Ethanol 1:1 6min	++	"	++	"
5) Ether-Ethanol 4min Then 95% Ethanol 4min	+	"	+	"
6) 2% Formalin 5min	++	"	+	"
7) 2% Glutaraldehyde 6min	-	"	-	"
B. SECTIONS PREWASHED IN PBS FOR 20 MINUTES				
1) Unfixed	+++	Very low	++	Very low
2) Acetone 10min	+++	"	+	"
3) 95% Ethanol 10min	++	"	Trace	"
4) Ether-Ethanol 1:1 6min	++	"	Trace	"
5) Ether-Ethanol 4min Then 95% Ethanol 4min				
6) 2% Formalin 6min	+	"	-	"
7) 2% Glutaraldehyde 6min	-	"	-	"
C. SECTIONS PREWASHED IN ISOTONIC SUCROSE FOR 20 MINUTES.				
1) Unfixed	+++	Moderate	+++	Moderate
2) Acetone 10min	+++	"	++	"
3) 95% Ethanol 10min	++	"	Trace	"
4) Ether-Ethanol 1:1 6min	++	"	Trace	"
5) Ether-Ethanol 4min Then 95% Ethanol 4min	+	"	Trace	"
6) 2% Formalin 6min	+	"	-	"
7) 2% Glutaraldehyde 6min	-	"	-	"

ELUTION PROCEDURES

In an attempt to extract antibody deposited in the kidneys, kidney tissue from dogs used in sections III and V of this study was subjected to an elution procedure similar to that described by Lambert and Dixon, (1968). A whole kidney from each dog was minced, homogenised using a Waring blender and washed repeatedly with PBS (pH 7.2) to remove any serum proteins. The kidney tissue was then eluted with 0.02 M citrate buffer (pH 3.2) for 90 minutes at room temperature with constant stirring; ten volumes of buffer were used to elute one volume of sedimented kidney. At the end of this time, the kidney tissue was removed by centrifugation and the resultant supernatant dialysed against large volumes of PBS (pH 7.2) for 48 hours at 2°C; the pH was then adjusted to 7.2 by dropwise addition of 0.1 N NaOH. At this stage, the eluates were concentrated twenty-fold using Carbowax and any precipitate present at the end of this procedure was removed by centrifugation.

DETECTION OF ANTIBODY TO CAV

Throughout the studies, serum samples and renal eluates were examined for the presence of antibody to CAV using an indirect immunofluorescence test. Cryostat sections of known positive infected liver from cases of acute systemic CAV infection were used. Acetone-fixed sections were exposed to serial two-fold dilutions of serum for 30 minutes and, after thorough washing in PBS for 1 hour, they were stained with FITC labelled rabbit anti-dog globulin. The sections were then washed and mounted in PBS before examination with the fluorescence microscope. A positive result was indicated by the presence of characteristic fluorescence of hepatic cell nuceli (Fig. 1) and the antibody titre was read as the reciprocal of the highest dilution at which this specific fluorescence was still present.

As control material, duplicate sections of liver were stained with FITC labelled anti-dog globulin,(negative control) and anti-CAV (positive control).

In order to assess the sensitivity of the indirect immunofluorescence method for detecting antibody to CAV, a comparison was made with the haemagglutination inhibition test (HA-I test). A series of serum samples obtained from dogs used in section II of the present study were subjected to anti-CAV antibody estimations using both tests. These sera had antibody titres ranging from zero to greater than 512 as measured by the indirect immunofluorescence test.

The HA-I test was based on procedures described by Espmark and Salenstedt, (1961). Serum samples were inactivated at 56°C for 30 minutes and absorbed at room temperature with equal volumes of 25 per cent kaolin (Flow Laboratories Ltd., Ayrshire). Serial two-fold dilutions were added to the wells of perspex haemagglutination plates and to each well was added 4 haemagglutinating units of virus. Serum and virus controls were also used to ensure that the serum alone did not agglutinate red blood cells (RBCs) and that the amount of virus used in the test was indeed 4 haemagglutinating units. The serum-virus mixtures were allowed to stand at room temperature for 1 hour before adding a 1 per cent suspension of human type O erythrocytes. To exclude the possibility of autoagglutination, an erythrocyte plus saline control was also used. The results of the test were read after 2 hours. The highest dilution of serum at which complete inhibition of agglutination occurred (100 per cent end-point) was recorded as the antibody titre.

The indirect immunofluorescence (IF) and HA-I titres of the serum samples are given in Table 4. From these results,it is apparent that

the IF test was much more sensitive than the HA-I test in detecting antibody to CAV. Whereas 23 of the 28 serum samples had antibody titres ranging from 1 to greater than 512 as measured by the IF test, only 10 sera gave positive reactions (titres of 4 or more) with the HA-I test. This perhaps is not surprising, since, presumably only antibody directed against the antigenic components of the virus which are responsible for haemagglutination is measured by the HA-I test, whereas the IF test probably measures antibody to all antigenic components of the virus.

The positive HA-I titres ranged from 4 to 96 and, in general terms, these sera also had high titres of antibody by the IF test. There was, however, a relatively poor correlation between the titres obtained by the two tests. In retrospect, a comparison of the relative sensitivities of the IF test and the serum neutralisation test might have been more valid; however, in comparison to the latter, the IF test is much quicker and less expensive to perform.

Table 4: Anti-CAV antibody titres of sera measured by the indirect immunofluorescence test (indirect IF) and the haemagglutination inhibition test (HA-I).

Serum Sample	Indirect IF	HA-I
42/1	0	< 4
42/2	0	< 4
44/1	0	< 4
44/2	0	< 4
48/2	0	< 4
29/1	1	< 4
34/1	1	< 4
31/1	2	< 4
32/2	4	< 4
33/3	8	< 4
32/1	16	< 4
28/1	32	< 4
27/1	32	4
28/2	32	8
27/2	64	< 4
43/3	64	< 4
48/3	64	< 4
45/4	256	< 4
48/5	256	48
48/6	256	96
42/3	512	4
48/4	512	4
30/2	512	32
31/2	< 512	4
34/2	< 512	4
45/5	< 512	12
48/7	< 512	48

Fig. 14- Anti-CAV antibody estimation using the indirect immunofluorescence test: Numerous hepatic cell nuclei show granular fluorescence, indicative of the presence of antibody in the test serum.

(Immunofluorescence X 500)

SECTION I : EXPERIMENTAL CAV NEPHRITIS : A STUDY OF RENAL CHANGES DURING ACUTE SYSTEMIC INFECTION

INTRODUCTION

In 1947, Rubarth described in detail a naturally occurring acute fatal disease of dogs, characterised by hepatitis, widespread petechial haemorrages and serous effusion into the body cavities. Under experimental conditions, the disease could be transmitted to other dogs using bacteria-free filtrates obtained from affected hepatic or splenic tissue; Rubarth therefore postulated a viral aetiology. Subsequently, other workers have confirmed Rubarth's observations and have shown the causative agent to be a virus belonging to the adenovirus group (Siedentopf and Carlson, 1949; Cabasso et al., 1954). Extensive studies on the pathogenesis of the disease have demonstrated that the main target sites for virus replication are hepatic tissue and vascular endothelium and, in these sites, characteristic basophilic intranuclear inclusion bodies are often found (Rubarth, 1947; Stünzi and Poppensiek, 1952). One of the extra-hepatic sites where inclusion bodies are most consistently observed is the renal glomerulus and, associated with this, viruria and proteinuria may be detected (Hamilton et al., 1966; Persson et al., 1961). The immunofluorescence technique was first applied to the study of the kidney during acute CAV infection by Wright, (1967a) who showed that virus replication was indeed mainly confined to the glomeruli. As yet, however, only one brief account of the renal ultrastructural changes associated with acute CAV infection has appeared in the literature (Givan, 1968).

The purpose of the first section of this dissertation is to describe a detailed study of the histological and ultrastructural alterations in the kidneys of dogs suffering from acute CAV infection. Since the excretion of renal antigens in the urine has been demonstrated in a variety of natural and experimentally induced renal diseases (Rosemann et al., 1971), the present study included an examination of the urine of

dogs with acute CAV infection for the presence of glomerular basement membrane (GBM) and tubular epithelial (TE) antigens.

MATERIALS AND METHODS

Experimental Procedures

Twenty-six 12-week-old, CAV antibody-free dogs weighing approximately 6kg and comprising four litters were used. Twenty-one of these animals were inoculated intravenously with 1ml of CAV suspension; the remaining 5 dogs received 1ml of uninfected tissue culture suspension intravenously and were housed in separate accommodation.

Two of the dogs were killed one day after receiving virus. With the exception of 6 animals which died, the remaining infected dogs were killed when they became clinically ill on the second, third and fourth days after inoculation of virus (see Table 5). The 5 control dogs were killed on day 3.

Histological Ultrastructural and Immunofluorescence Procedures

These were carried out as described in the section on "materials and methods". As part of the histological examination, an attempt was made to assess the degree of virus infection in the kidneys by calculating the percentage of glomeruli which contained inclusion bodies; this was done by examining 100glomeruli from each animal for the presence of one or more inclusions. Liver and kidney from all animals were examined by immunofluorescence for the presence of CAV antigen. With the exception of the 6 dogs which died, the kidneys of all animals were subjected to ultrastructural examination.

Preparation of GBM and TE Antigens

GBM was prepared from the kidneys of normal dogs by the method of Krakower and Greenspon, (1951). The cortices were minced, washed repeatedly in cold PBS and forced through a fine stainless steel sieve

(mesh-size - 150). The resultant suspension was collected in an ice bath, centrifuged at 500g for 3 minutes and both the supernatant fluid (containing crude TE antigen) and the sediment collected. The sediment which contained intact glomeruli was washed repeatedly with cold PBS, allowed to settle after each wash and the supernatant, containing cellular debris, discarded. After the final washing, the glomerular fraction was centrifuged at 500g for 2 minutes and samples of the sediment stained with methylene blue to assess the number of intact glomeruli; only fractions containing large numbers of glomeruli were used for further study. The harvested glomeruli were then ultrasonicated for 1 minute to disrupt Bowman's capsule and to release glomerular cells from the GBM. After centrifugation at 500g for 2 minutes, the resuspended sediment was further ultrasonicated for 5 minutes to disrupt the GBM. The resultant suspension was then lyophilised and stored at -25°C. The crude TE antigen was further centrifuged at 78,000g for 45 minutes and the resultant sediment, which contained the nephritogenic tubular epithelial (TE) antigen (Edgington et al., 1967), was then washed repeatedly with PBS, lyophilised and stored at -25°C.

Preparation of Anti-GBM and Anti-TE Antibodies

Rabbits were inoculated subcutaneously with 60mg of GBM or TE powder suspended in saline and emulsified in an equal volume of Freund's complete adjuvant. After a period of 8 weeks, further inoculations of antigen without adjuvant were administered subcutaneously and intramuscularly at monthly intervals for 3 months and the rabbits were bled 10 days after the final inoculation. The pooled anti-GBM serum was inactivated at 56°C for 30 minutes, absorbed twice with freshly harvested dog red blood cells and finally twice with lyophilised TE antigen (60mg per ml).

The specificity of the anti-GBM serum was tested by means of an indirect immunofluorescence test. Frozen sections of normal dog kidney were exposed to absorbed anti-GBM serum for 30 minutes and then, after washing for 1 hour in PBS, were further stained with sheep anti-rabbit globulin conjugated with FITC. The sharp linear fluorescence of glomerular and tubular basement membranes showed that the anti-GBM serum did not contain antibodies which reacted with other parts of renal tissue.

In a similar fashion, pooled anti-TE serum was heat inactivated, absorbed twice with dog red blood cells and twice with GBM powder (60mg/ml). The specificity of the anti-TE serum was also tested by indirect fluorescence. In contrast to anti-GBM serum, specific fluorescence was confined to the apical portions of the proximal tubules; there was no staining of basement membranes. Having established the specificity of anti-GBM and anti-TE sera, both sera were finally absorbed twice with lyophilised normal dog liver homogenate (60mg/ml), normal dog serum (60mg per ml) and normal dog urine protein (10mg per ml): these absorptions were carried out to remove any non-specific reactions which might occur with serum or liver antigens leaking through damaged glomeruli. There was no specific staining when the absorbed anti-GBM or anti-TE serum conjugated with FITC was applied to normal canine liver, heart muscle, skeletal muscle or spleen in the direct immunofluorescence test.

Detection of Renal Antigens in Urine

Where possible, urine samples from infected and control dogs were collected at necropsy by aspiration from the bladder and centrifuged to remove any cellular debris; in some animals, in particular those which had died, insufficient urine was present in the bladder to permit

examination for renal antigens. Both unconcentrated urine and urine concentrated 10 times using Carbowax (Searle, High Wycombe, Bucks.) were tested by double diffusion in agar gel against anti-GBM and anti-TE serum. The diffusion plates were incubated at room temperature and were retained up to 6 days before being discarded as negative.

RESULTS

Clinical Findings

The onset of clinical illness occurred in all infected dogs from 2 to 4 days after inoculation of virus. Typically, an infected animal would become anorexic, dull and reluctant to move and, when these signs were first noticed, the animal was killed; despite regular observation, however, 6 of the dogs were found dead without clinical illness having been detected.

Macroscopic Findings

All animals examined on the second, third and fourth days after infection showed macroscopic changes characteristic of acute CAV infection. There was excess serosanguinous fluid in the peritoneal cavity and strands of fibrin were commonly found on the surface of the liver and between the intestinal loops. The liver was enlarged, pale, and finely mottled in appearance and oedematous thickening of the gall bladder wall was frequently observed. All the lymph nodes were slightly enlarged and haemorrhagic and petechial haemorrhages were observed in a range of organs, being most frequently encountered in the thymus. Subcutaneous oedema of the submandibular and neck regions was occasionally found. The kidneys showed no macroscopic abnormalities at this time.

Histological Findings

The control animals and those examined on the first day after infection showed no histological abnormalities. However, the remaining animals all had histological lesions characteristic of acute CAV infection. There was focal hepatic necrosis accompanied by varying numbers of basophilic intranuclear inclusion bodies in hepatic and Kupffer cells (Fig. 2). In many of the animals examined on days 2 and 3, hepatic necrosis was confined to small clusters of cells (graded + Table 5) and, indeed, sometimes foci of only 2 or 3 cells were involved; however, in the later stages, particularly in those animals examined on day 4, larger foci of necrosis were observed. In all of these dogs, occasional inclusion bodies were also found in vascular endothelial cells in a wide variety of organs.

In the kidneys, the most striking finding was swelling of the glomerular tufts in which swollen and finely vacuolated cytoplasm of glomerular endothelial cells was frequently observed partly occluding the capillary loops (Fig. 4). Polymorphonuclear leukocytes were commonly observed lodged within capillary loops and granular cell debris was sometimes found in the urinary spaces. Intranuclear CAV inclusion bodies were found in endothelial and mesangial cells in all animals examined 2 to 4 days after infection (Fig. 3). The number of inclusions observed varied, being most numerous in those animals showing themost severe glomerular cytological changes. The number of glomeruli in which inclusions were observed in the present series of dogs ranged from 2 per cent to 76 per cent (see Table 5) and no more than 4 inclusions were observed in a single glomerulus.

Elsewhere in the kidney, in both cortex and medulla, occasional small interstitial haemorrhages were found. In all animals examined 2 to 4 days after infection, inclusion bodies were found in endothelial cells of interlobar and arcuate veins and occasional interstitial capillaries (Figs. 5 and 6).

The tubular epithelial cytoplasm, especially of the proximal tubules, appeared swollen and finely vacuolated and, in some cases, the swollen proximal cells appeared to occlude the tubular lumina. Granular cell debris was frequently observed within the tubules and, in some cases, desquamated cells were also found in occasional collecting tubules. In one animal, (No. 9) there was necrosis of a few scattered proximal tubules.

Immunofluorescence Findings

CAV antigen was detected as discrete granular fluorescing nuclei in the liver of all infected dogs. Whereas in those animals which were examined on the first day after inoculation, only occasional infected cells were found, numerous infected hepatic cells were present in dogs 2, 3 and 4 days after infection. In these animals, discrete virus-infected cells were also found in the renal glomeruli (Fig. 7) and in the vascular endothelium of large renal vessels and occasional interstitial capillaries. Many more fluorescing cells were present in the glomeruli than was suggested by the number of inclusions seen with light microscopy; in many animals, virtually every glomerulus was found to contain at least 1 infected cell and as many as 6 fluorescing nuclei were observed in some individual glomeruli. Antigen was not detected in the kidneys of animals one day after infection nor in any of the controls.

Table 5: Acute CAV infection: Histological and immunofluorescence findings in the liver and kidneys

Dog Number	Day Examined	Histology			Immunofluorescence
		Liver		Kidney	
		Necrosis	Inclusions	Percentage glomeruli containing inclusions*	Glomerular CAV antigen
1	1	-	-	0	-
2	1	-	-	0	-
3	2	+	++	10	+
4	2	+	+	2	+
5	2	+	+	12	+
6	2	++	++++	36	++
7	2	+	++	56	+++
8	2	+	+++	40	++
9	2	++	++++	62	+++
10	2	+	++	50	+
11	2 (D)	+	+++	42	+
12	3	+	++	46	++
13	3	+	++	40	++
14	3	++	+++	52	+++
15	3	++	+++	46	+++

Continued on next page.....

Table 5/continued

Dog Number	Day Examined	Histology			Immunofluorescence
		Liver		Kidney	
		Necrosis	Inclusions	Percentage glomeruli containing inclusions *	Glomerular CAV antigen
16	3 (D).	+++	++++	76	+++
17	3 (D)	++	++++	60	+++
18	4 (D)	++	+++	52	+++
19	4	++	++++	76	+++
20	4 (D)	++	+++	40	++
21	4 (D)	+++	+++	40	+++
22	3 control	-	-	0	-
23	3 control	-	-	0	-
24	3 control	-	-	0	-
25	3 control	-	-	0	-
26	3 control	-	-	0	-

Various parameters graded + to ++++ according to severity

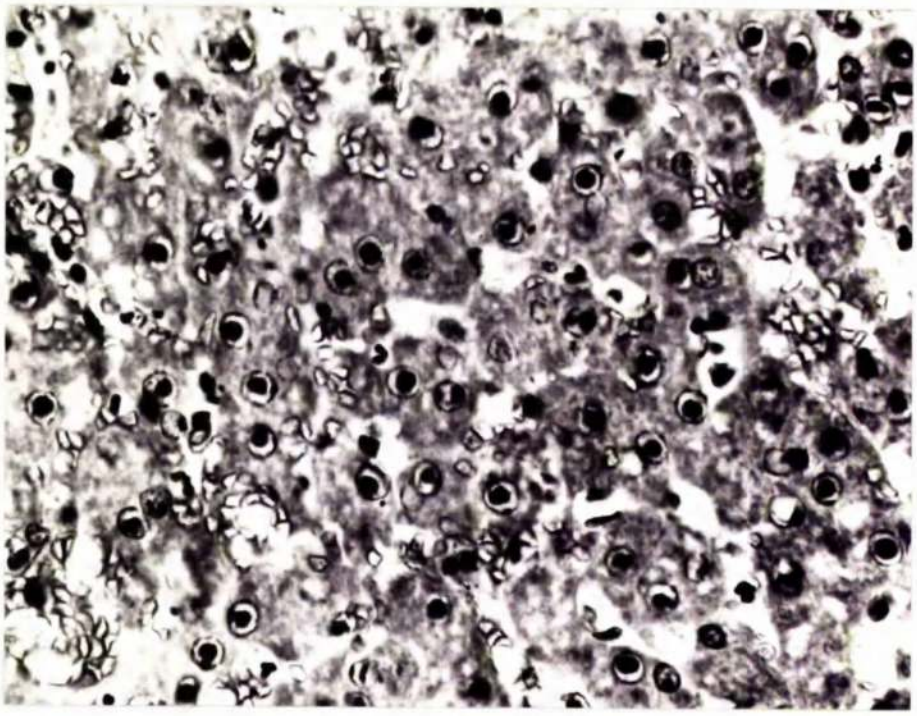
* = 100 glomeruli from each animal examined for the presence of one or more inclusions

Fig. 2:- Acute CAV infection: Liver from a dog examined 3 days after infection, showing large numbers of intranuclear inclusions in hepatic cells.

(HE X 400)

Fig. 3:- Acute CAV infection: Glomerulus, 2 days after infection, showing 3 intranuclear inclusion bodies.

(HE X 500)



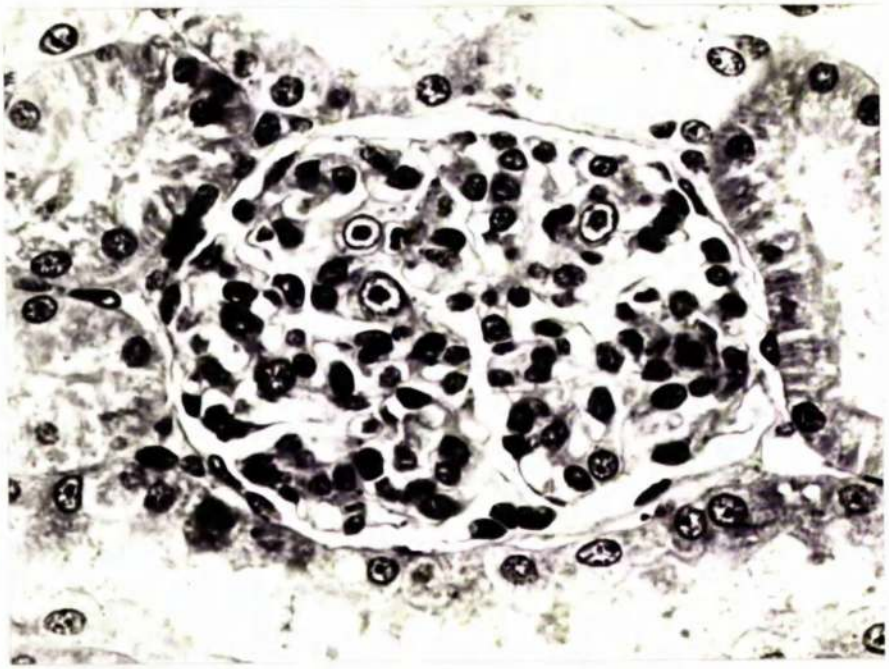


Fig. 4:- Acute CAV infection: Glomerulus, 3 days after infection, showing swelling and vacuolation of endothelial cells with occlusion of capillary lumina. Polymorphonuclear leukocytes are also present in the capillary loops (large arrows). Two intranuclear inclusion bodies (small arrows) can be seen in mesangial cells.

(Mallory's borax methylene blue, 1μ section X 1200)

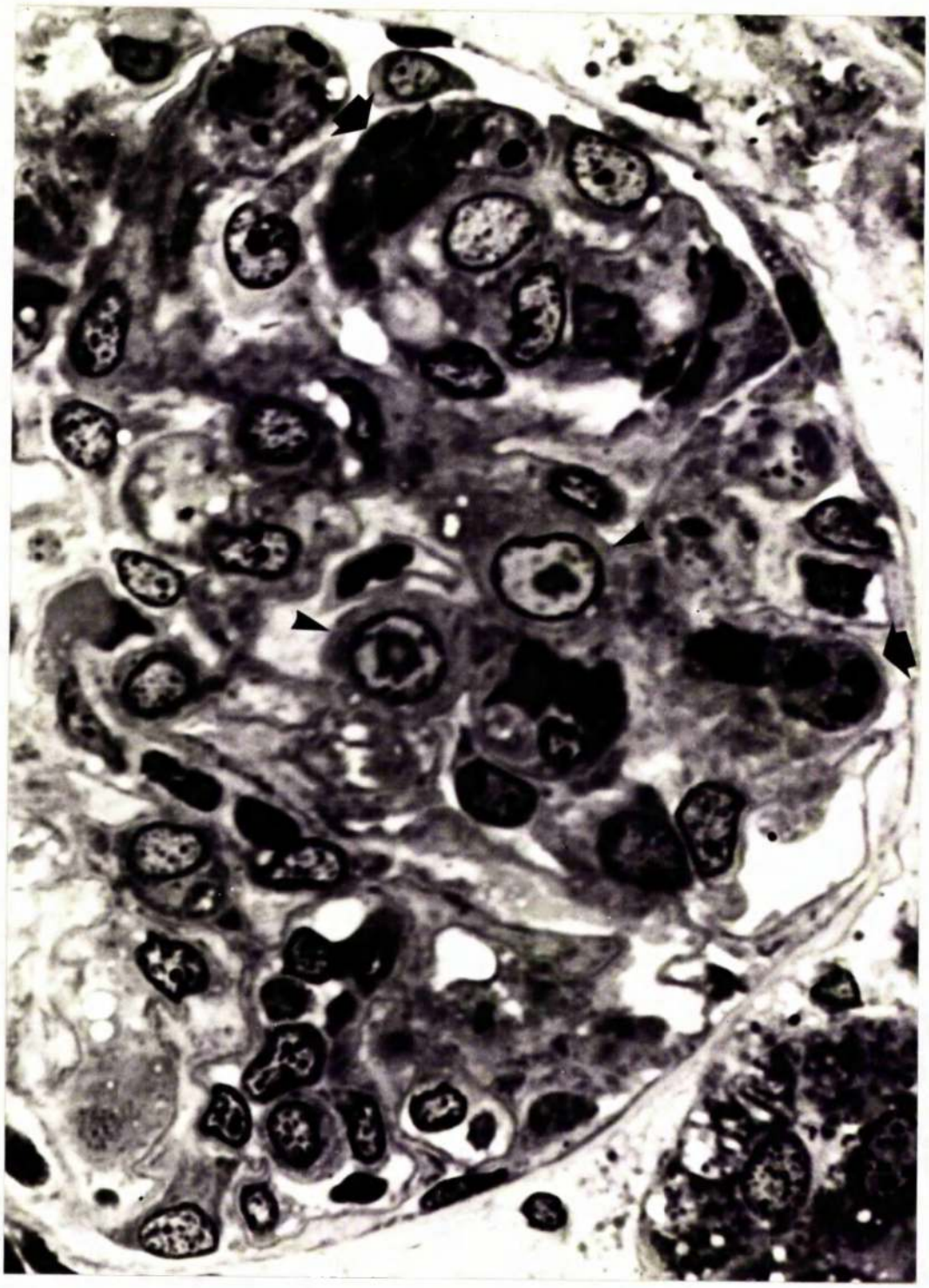
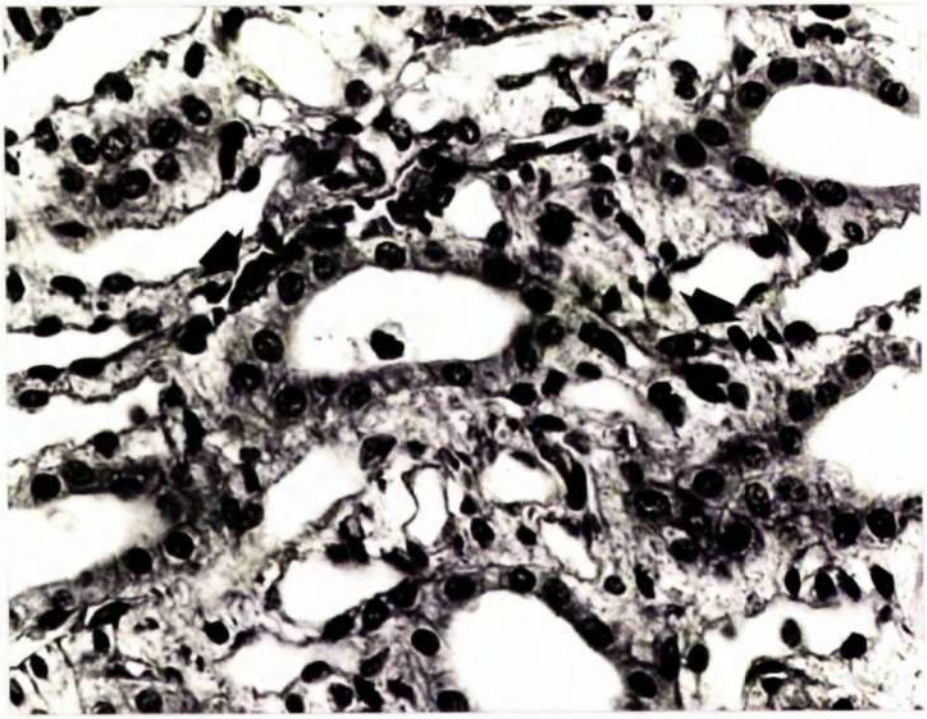


Fig. 5:- Acute CAV infection, 2 days: Section of medulla, showing inclusion bodies in interstitial capillary endothelial cells (arrows).

(HE X 400)

Fig. 6:- Acute CAV infection, 3 days: Inclusion bodies (arrows) can be seen in the endothelium of an interlobar vein.

(HE X 400)



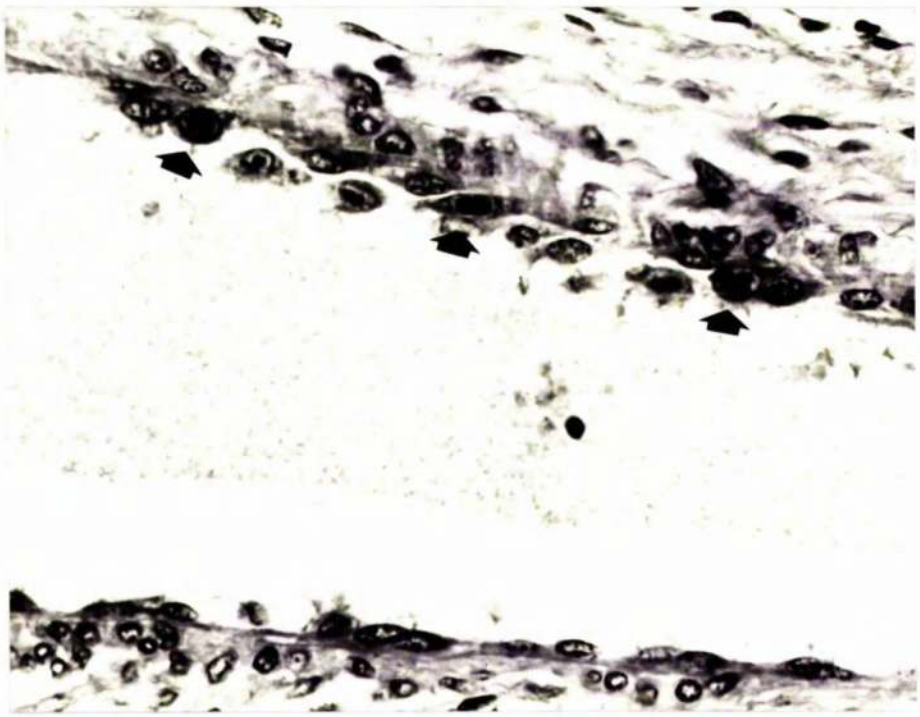
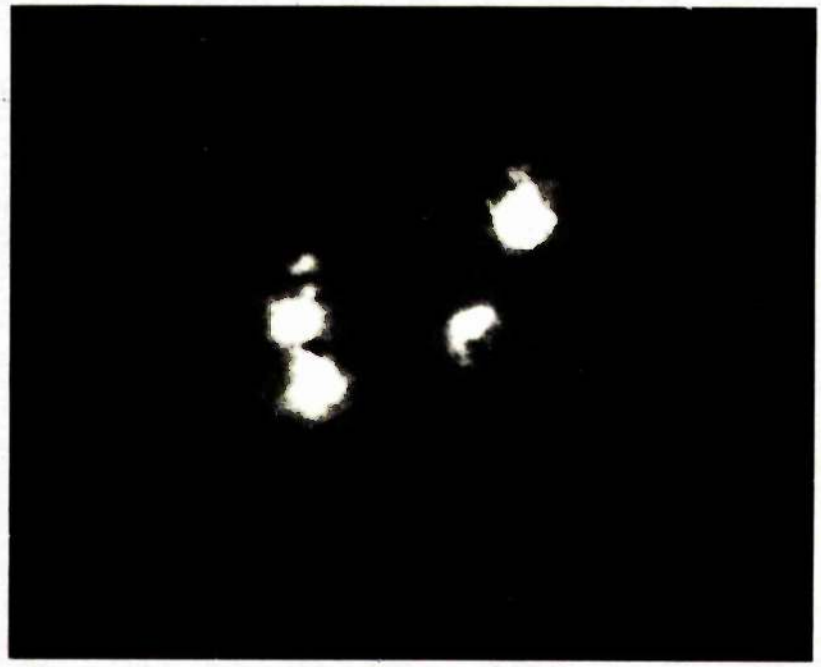


Fig. 7:- Acute CAV infection: CAV antigen in a glomerulus 2 days after administration of virus.

(Immunofluorescence X 400)



Ultrastructural Findings

The kidneys of the control animals and dogs killed on the first day after infection did not show any ultrastructural changes(Fig. 8). Infected dogs examined on the second, third and fourth days after inoculation of virus showed marked degenerative changes in both glomeruli and tubules. The severity of these changes varied between different animals, being particularly severe in those animals whose glomeruli contained large numbers of CAV infected cells. The most striking effect in the damaged glomeruli was partial occlusion of capillary lumina by swollen endothelial cells (Fig.9); the cytoplasm of these cells contained numerous large vacuoles filled with fine loose granular material while their nuclei showed varying degrees of pyknosis.

The thin cytoplasmic lining of the peripheral portions of the capillaries was often frayed and, in some cases, fragments of endothelial cytoplasm had detached from the basement membrane and were found free in the capillary lumina. The GBM was normal in thickness and density in the majority of cases but, in a few instances, focal areas of swelling and splitting of the membrane were observed. In many areas, however, the endothelial cytoplasm was elevated from the underlying GBM and these subendothelial sites often contained pale finely granular material which probably represented oedema fluid. Occasional polymorphonuclear leukocytes were found lodged in the glomerular capillaries (Fig. 10). The contents of the capillaries showed a marked increase in granularity which was considered as evidence of some degree of vascular stasis.

Mesangial cells appeared more prominent and bulged out into the axial region of the capillaries; small cytoplasmic vacuoles were often seen within mesangial cytoplasm. The nuclei of both visceral and parietal epithelial cells showed varying degrees of pyknosis and

their cytoplasm was sometimes vacuolated and swollen resulting in partial occlusion of the urinary spaces. In the most severely affected glomeruli, fusion of epithelial cell foot processes was observed. Fragments of epithelial cell debris were often found within the urinary spaces (Fig. 11). There was no evidence of cell proliferation of any of the cellular components of the glomeruli.

CAV particles were found in the nuclei of mesangial and endothelial cells (Figs. 12 and 13) and occasionally in cells lying free within the capillary loops. At no time was virus detected in epithelial cells or within the GBM. Virus infected cells were extremely swollen with abundant pale and often vacuolated cytoplasm. In many instances, the nucleus contained a central area composed of fine granular moderately electron-dense material surrounded by a pale zone, outside which there was distinct nuclear margination of chromatin. Virus particles were usually found scattered throughout the nucleus, although in a few instances they were largely confined either to the central granular matrix or the pale peripheral zone. In animals examined 3 and 4 days after inoculation, disruption of infected cells was occasionally observed; in such cells it was no longer possible to distinguish a discrete cytoplasmic membrane, there was focal disintegration of the nuclear membrane and the nuclear chromatin had coalesced into large electron-dense clumps (Fig. 14). Neighbouring intact mesangial cells were occasionally observed phagocytosing fragments of these degenerating infected cells.

Degenerative changes were also detected elsewhere in the kidney. Cytoplasmic swelling and nuclear pyknosis were constant features of tubular epithelial cells at all levels of the nephron. Proximal and distal tubules showed the most severe damage with, in many cases, marked intracellular oedema and dispersion of mitochondria (Fig. 15).

The apical portions of these cells showed partial loss of microvilli and bulged out into the tubular lumen. Particles of cellular debris and sometimes whole desquamated cells were noted in many tubules. Although the tubular cytological changes were severe, virus particles were not detected in any tubular epithelial cells.

In the interstitium, the endothelium of capillaries was frayed and fragments had often detached into the lumen. Distinct pericapillary oedema was a common finding and virus particles were occasionally found in the nuclei of swollen endothelial cells. Polymorphonuclear leukocytes were occasionally lodged within the lumen of the interstitial capillaries.

Detection of Urinary Renal Antigens

The results of detection of renal antigens in the urine are summarised in Table 6.

Neither GBM nor TE antigens were detected by gel diffusion in unconcentrated urine from any of the inoculated animals. When the urine was concentrated 10 times, however, lines of precipitation indicative of the presence of GBM and TE antigens, were found in urine of infected dogs examined on the second, third and fourth days after the administration of virus (Figs. 16 and 17). In no instance could antigen be detected in the urine of control animals, even when concentrated 17 times. The precipitation reactions were abolished when the anti-GBM serum was absorbed with GBM antigen or when anti-TE serum was absorbed with TE antigen.

Table 6:- Acute CAV infection: Detection of renal antigens in the urine.

Dog Number	Day Examined	Kidney Antigens in the urine GBM	TE
1	1	-	-
2	1	-	-
3	2	+	+
4	2	+	+
5	2	+	+
6	2	+	+
7	2	+	+
8	2	+	+
9	2	ND	ND
10	2	+	+
11	2 (D)	ND	ND
12	3	+	+
13	3	+	+
14	3	+	+
15	3	ND	ND
16	3 (D)	ND	ND
17	3 (D)	ND	ND
18	4 (D)	ND	ND
19	4	+	+
20	4 (D)	ND	ND
21	4 (D)	ND	ND
22	3 control	-	-
23	3 control	-	-
24	3 control	-	-
25	3 control	-	-
26	3 control	-	-

D = Died ND = Not done

Fig. 8:- Ultrastructural appearance of normal glomerulus: Two capillary loops can be seen (*); the capillary wall is made up of a 3 layered structure, consisting of a basement membrane sandwiched between an inner thin layer of endothelial cytoplasm and, on the outer aspect, the foot processes of visceral epithelial cells (Ep).

(Electron microscopy X 10,000)

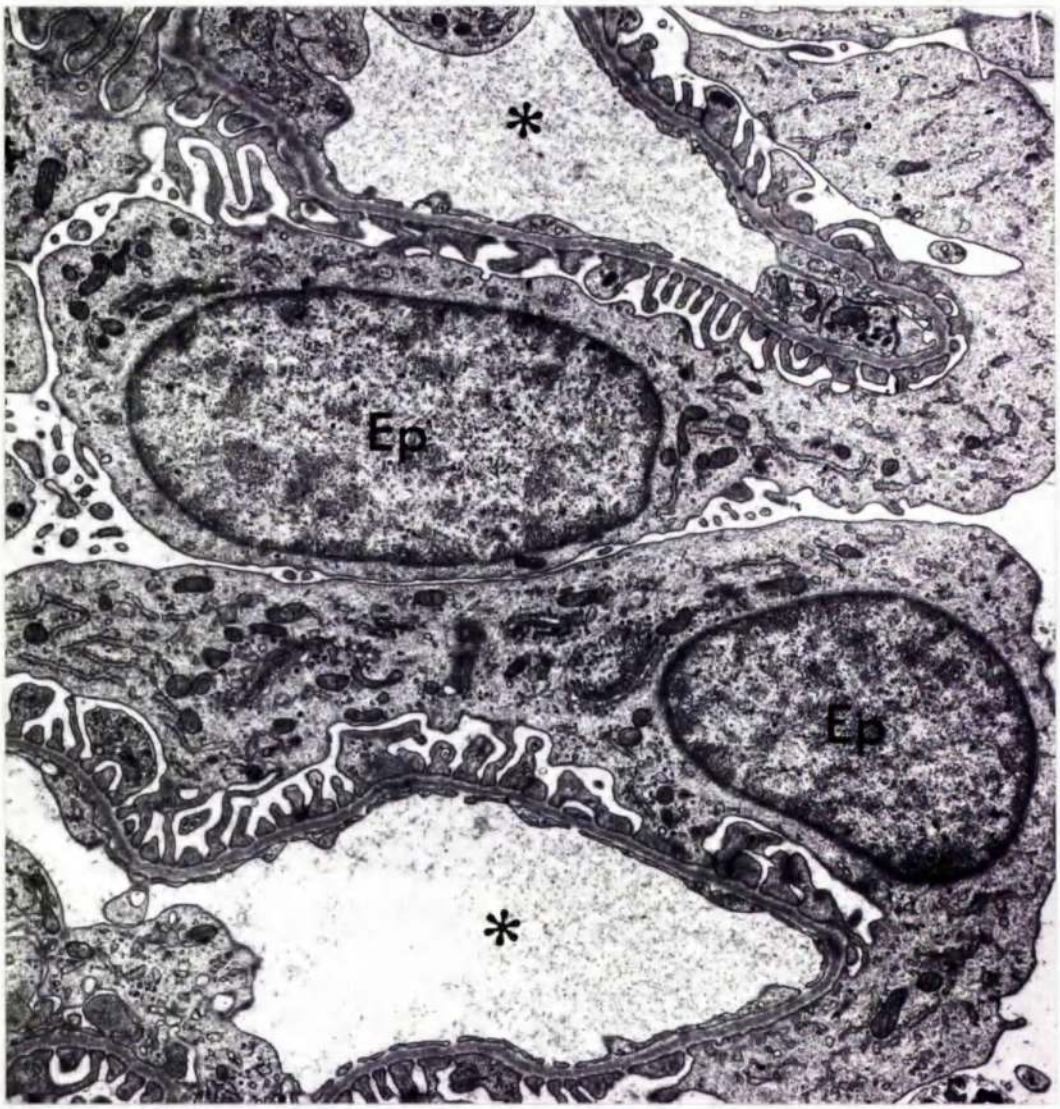

Ep
Ep
*
*

Fig. 9 (a):- Acute CAV infection, 3 days: Section of glomerulus, showing swelling and vacuolation of endothelial cytoplasm with resultant occlusion of capillary loops. E = Endothelial cell; Ep = Epithelial cell; M = Mesangial cell.

(Electron microscopy X 6,000)

(b) (Insert):- A higher power view of a capillary wall from the same glomerulus, showing areas of sub-epithelial oedema (*).

(Electron microscopy X 12,000)

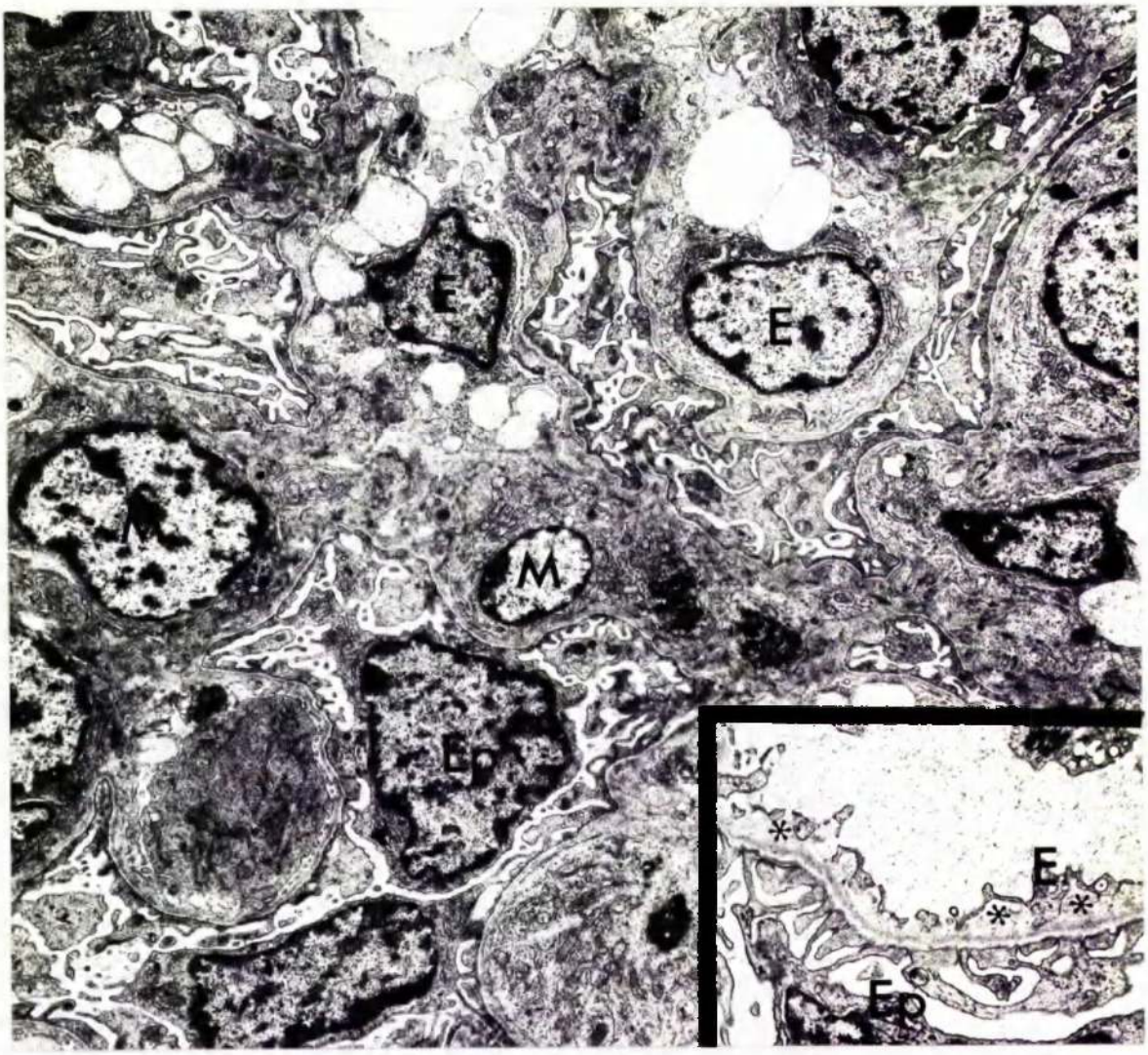
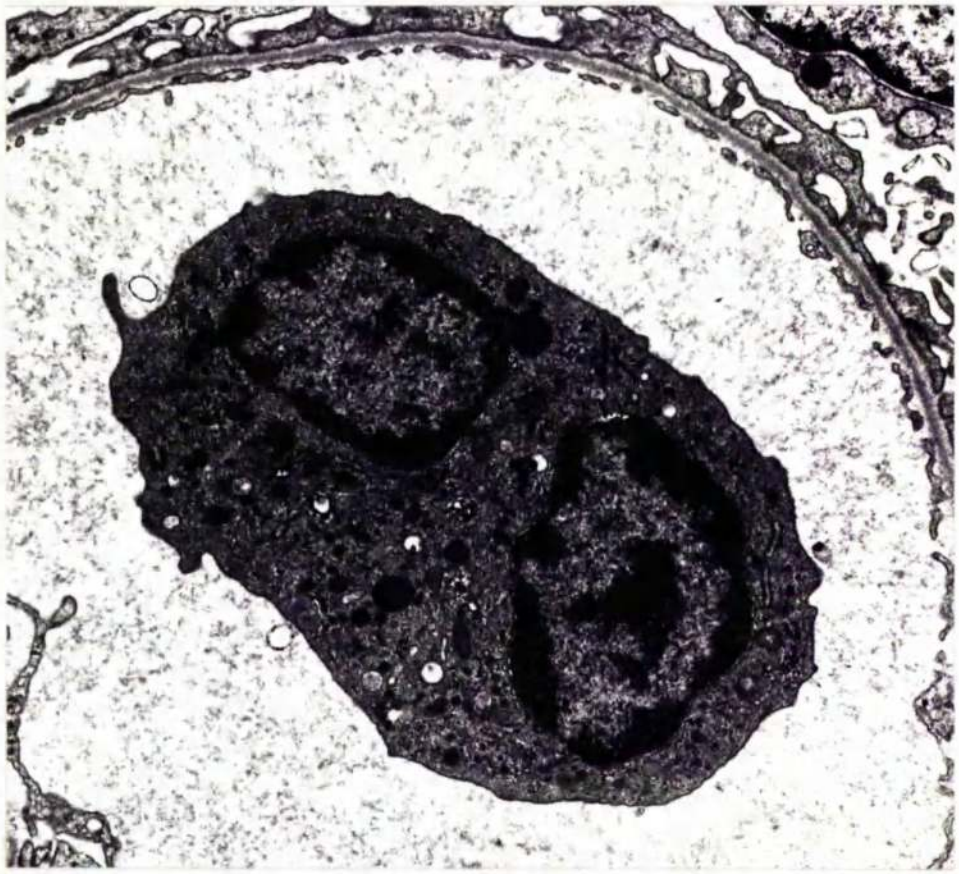

E
E
M
M
Ep
E
Ep

Fig. 10:-. Acute CAV infection, 3 days: Section of a glomerulus, showing a polymorphonuclear leukocyte in a capillary loop.

(Electron microscopy X 15,000)

Fig. 11:- Acute CAV infection, 3 days: Section of glomerulus, showing fragments of cytoplasmic debris (*) in the filtration space. B = Basement membrane of Bowman's capsule; Ep = Visceral epithelial cell.

(Electron microscopy X 10,000)



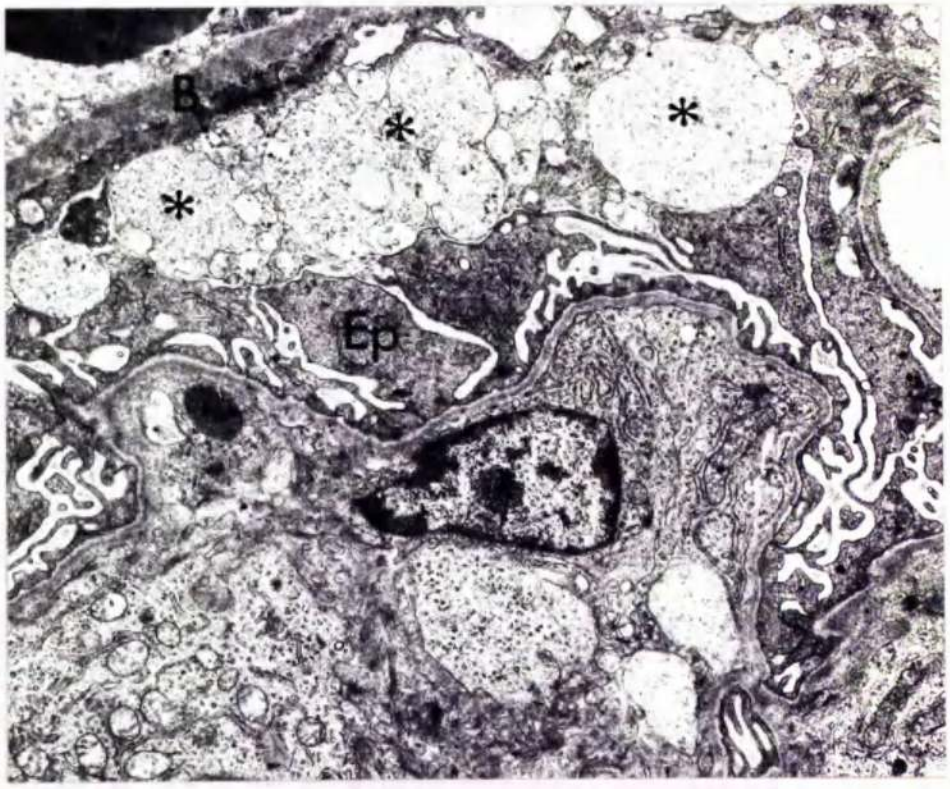
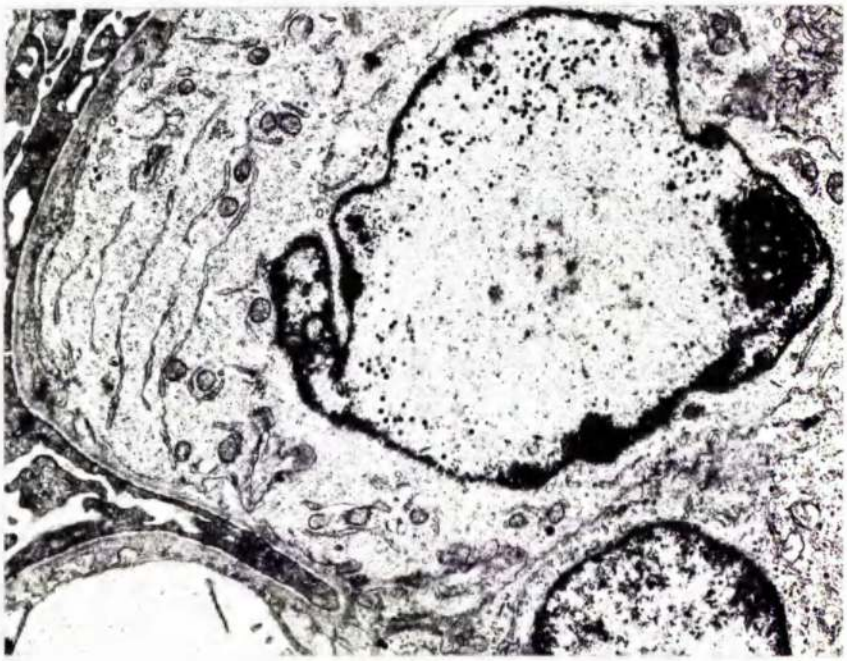

B
*
*
*
Ep

Fig. 12:- Acute CAV infection, 2 days: Section of glomerulus, showing a virus-infected endothelial cell. Numerous virus particles can be seen within the nucleus which is swollen and shows distinct margination of chromatin; the cytoplasm is also markedly swollen.

(Electron microscopy X 10,000)

Fig. 13:- Acute CAV infection, 3 days: Section of glomerulus, showing an infected mesangial cell. The nucleus is swollen and contains a central fine granular matrix surrounded by a paler zone in which large numbers of virus particles can be seen.

(Electron microscopy X 10,000)



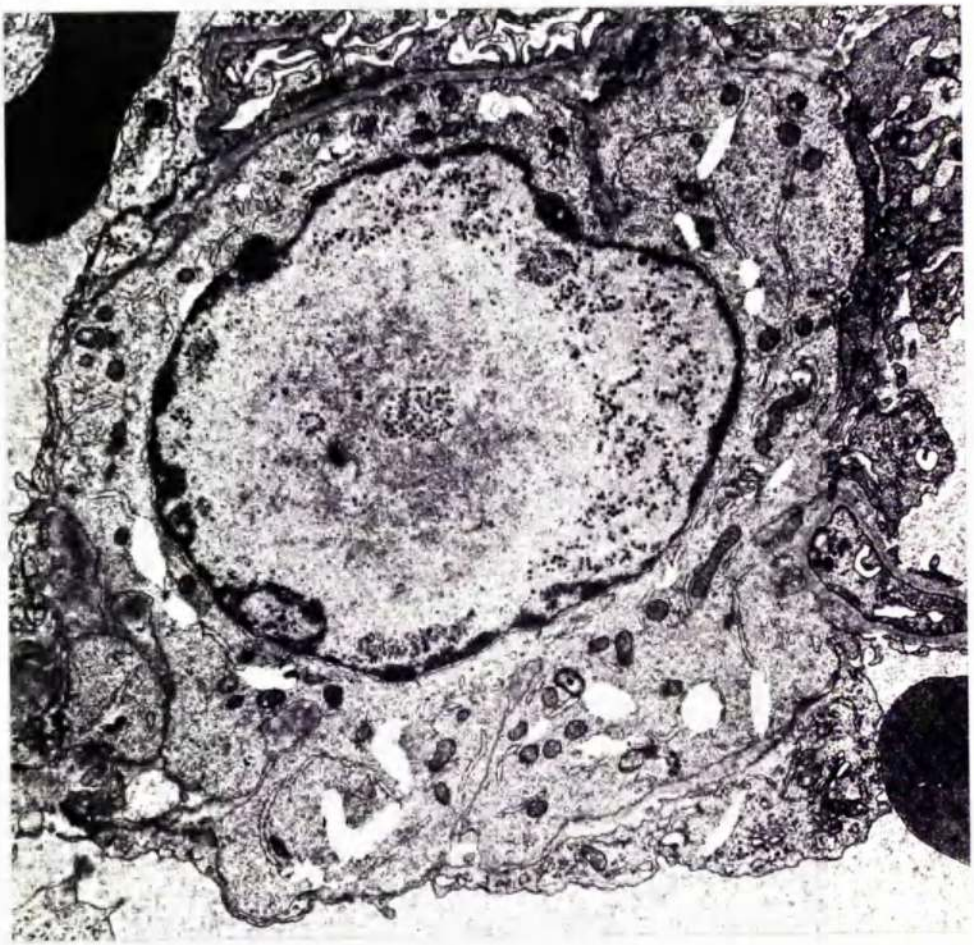


Fig. 14:- Acute CAV infection, 4 days: Section of glomerulus, showing a degenerating virus-infected mesangial cell. The cytoplasmic and nuclear membranes are disrupted and large numbers of virus particles can be seen lying among clumps of chromatin (arrows). E = Endothelial cel

(Electron microscopy X 18,00

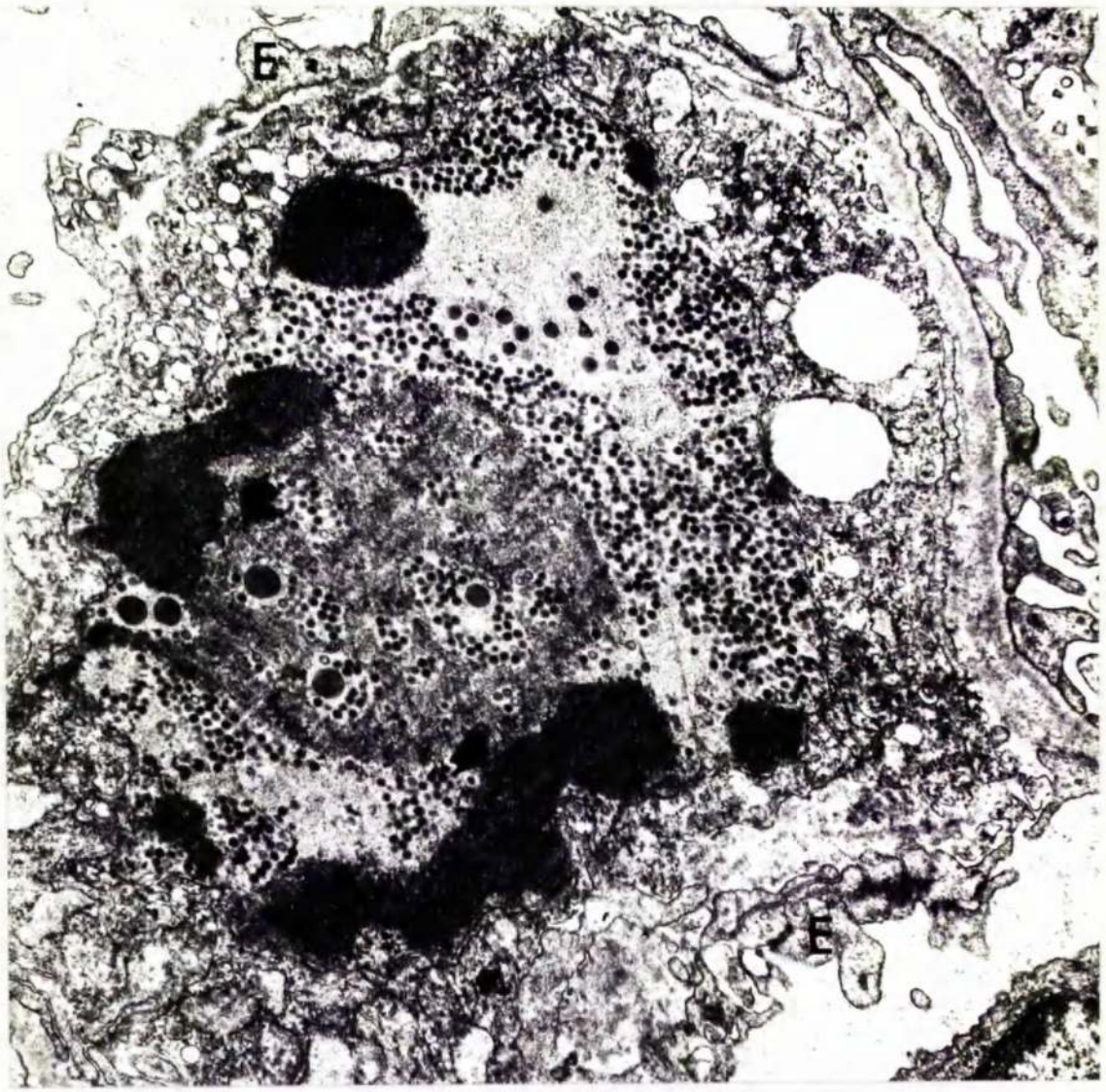

Б
Е

Fig. 15:- Acute CAV infection, 3 days: section of proximal tubule, showing marked intracellular oedema (*), loss of microvilli (Mv) and bulging of cytoplasm (arrows) into the tubular lumen.

(Electron microscopy X 10,000)

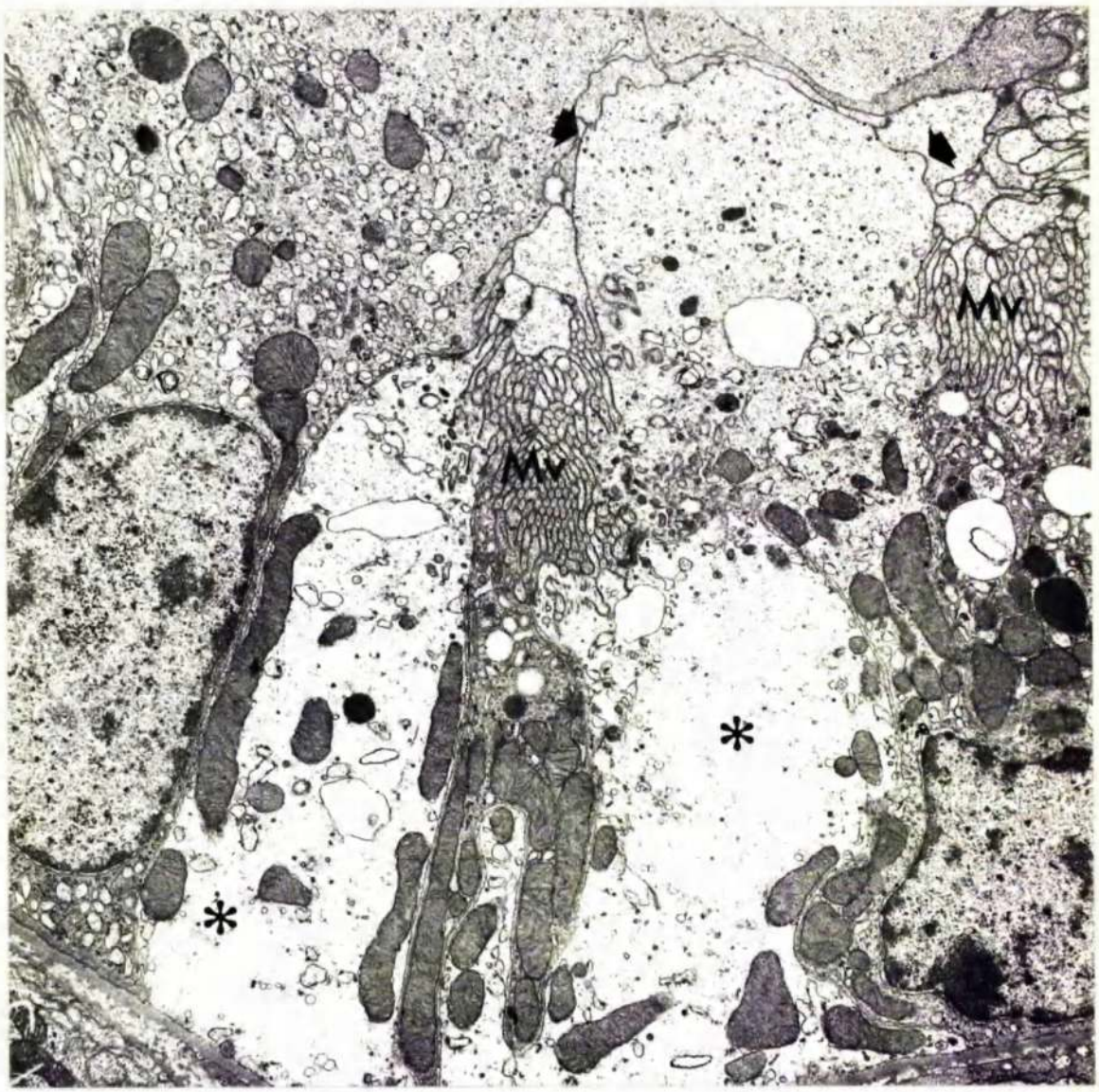
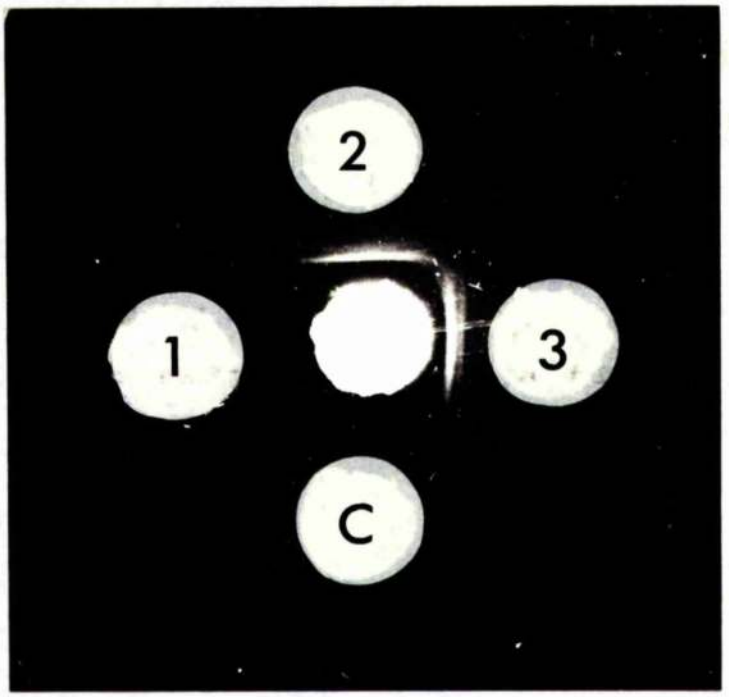
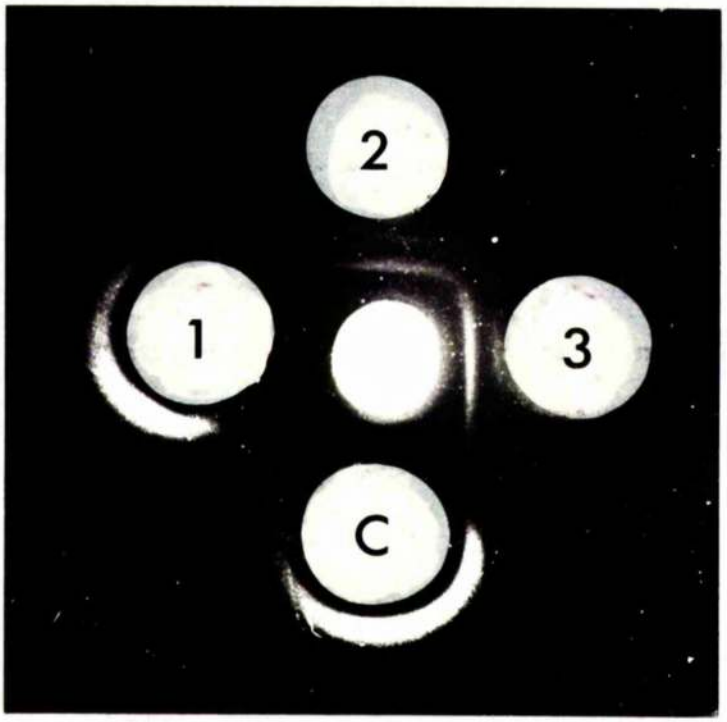

Mv
Mv
*
*

Fig. 16:- Detection of GBM antigens in the urine by gel diffusion: The central well contains anti-GBM serum and the outer wells contain concentrated urine from 1 control and 3 infected dogs. Numbers correspond to days after inoculation of virus; C = Control. Lines of precipiation can be seen with urine from dogs examined on days 2 and 3.

Fig. 17: Detection of TE antigen in the urine by gel diffusion: The central well contains anti-GBM serum and the outer wells contain concentrated urine from the same animals as shown in Fig. 16. Lines of precipitation can be seen with urine from dogs examined on days 2 and 3.


2
1
3
C


2
1
3
C

DISCUSSION

The histological and immunofluorescence findings in the present study confirm and expand the results obtained by other workers (Rubarth, 1947; Stinzi and Poppensiek, 1952; Wright, 1967a). In the kidney, virus replication occurred mainly within the glomeruli where characteristic basophilic intranuclear inclusion bodies were observed in all animals from the second day onwards. The number of glomeruli in which inclusions were observed varied between individual animals; in general terms, however, those animals in which hepatic inclusions were most numerous also had the greatest number of glomerular inclusions. As far as evidence of replication of virus in glomerular cells was concerned, there was a good correlation between the presence of inclusions and the detection of infected cells by immunofluorescence, although in all animals larger numbers of virus infected cells were detected by immunofluorescence than could be determined by the presence of inclusion bodies.

With the aid of electron microscopy, degenerative cellular changes were observed at all levels of the nephron. There was marked swelling of all the cellular components of the glomeruli resulting in varying degrees of occlusion of the glomerular capillaries and urinary spaces. The GBM remained relatively normal in thickness and density, although areas of separation from the overlying endothelium were observed which were interpreted as oedema of the capillary walls. Virus was found within glomerular endothelial and mesangial cells and occasionally in cells lying free within the capillary lumina. Although virus was not found in tubular epithelial cells, degenerative changes were present in the tubules at all levels but being particularly severe in the proximal tubules.

From the present study, it is clear that not only does CAV

produce lysis of infected glomerular cells but also a range of non-specific cytological changes in parts of the nephron where virus replication does not occur. These non-specific lesions may simply result from anoxia due to glomerular swelling or they may be related to the build-up of cytotoxic substances in the bloodstream as a result of severe hepatic damage. Papadimitriou, (1969) described similar cellular changes and sub-endothelial lesions in the glomeruli of neonatal mice experimentally infected with reovirus 3, although he was unable to demonstrate virus particles within glomerular cells.

Urinary excretion of renal antigens has been demonstrated in a variety of natural and experimentally induced renal diseases (Rosenmann et al., 1971). In the present study, both GBM and TE antigens were detected in the urine of CAV infected dogs on the second, third and fourth days following inoculation of virus. Thus, despite the relatively minor ultrastructural changes observed in the GBM, sufficient damage apparently occurred to result in the release of detectable levels of GBM antigens in the urine. The source of TE antigens found in the urine was probably related to damage to the apical portions of the proximal tubular epithelium.

In the detection of renal antigens, it has previously been pointed out that, because of antigenic cross-reactions with other tissues, care must be taken in the interpretation of immunodiffusion precipitation lines (Rosenmann et al., 1971). This is particularly so in an acute systemic disease such as that resulting from CAV infection, since, antigens derived from other organs, in particular the liver, may also be excreted in the urine. However, in planning the present experiment, care was taken to ensure specificity of the antisera used in the immunodiffusion test; these antisera were absorbed with liver tissue and were checked by indirect immunofluorescence for non-specific staining

of a range of other tissues.

Previous work has shown that both GBM and TE antigen are potentially nephritogenic. Thus, inoculation of sheep with heterologous GBM in adjuvant results in a severe acute exudative glomerulonephritis due to the production and subsequent glomerular fixation of anti-GBM antibody (Steblay, 1962). Immunisation of rabbits with homologous TE antigen in adjuvant has also been shown to induce a chronic membranous glomerulonephritis due to glomerular deposition of TE antigen-antibody immune complexes (Edgington et al., 1967). Lerner and Dixon (1968) have pointed out the possible significance of urinary excretion of such nephritogenic renal antigens. They have suggested that the reabsorption, by the tubules into the circulation, of sufficient quantities of renal antigen might lead to an antibody response by the host with the subsequent development of glomerulonephritis. In the present experiment, all the dogs died or were killed before any such autoantibody could develope. However, in the subsequent sections of this study, it is hoped to resolve whether or not such a sequence of events might occur in dogs following recovery from CAV infection.

SECTION II : EXPERIMENTAL CAV NEPHRITIS : THE INFLUENCE OF PASSIVE HUMORAL IMMUNITY

INTRODUCTION

MATERIALS AND METHODS

RESULTS

Tables 7 and 8 ; Figures 18-21.

DISCUSSION

INTRODUCTION

Studies carried out in recent years have shown that immune complex glomerulonephritis is a relatively common disease in the dog (Kurtz et al., 1972; Murray and Wright, 1974). Although gramular deposits of immunoglobulin (mainly IgG) have been demonstrated in the glomeruli of affected animals, as yet, none of the component antigens involved in the complexes have been identified. A number of animal virus infections, including lymphocytic choriomeningitis in mice, aleutian disease of mink and equine infectious anaemia, are associated with glomerulonephritis due to deposition of virus antigen-antibody immune complexes (Oldstone and Dixon, 1967; Henson et al., 1969; Banks et al., 1972). In the first section of this thesis it was shown that, during systemic CAV infection and prior to production of circulating antibody, the virus is capable of inducing direct lytic damage in the kidney. However, little is known about the way in which dogs posessing low levels of circulating antibody might respond to heavy challenge with CAV and whether or not, under such circumstances, circulating immune complexes might be produced and result in immunologically-mediated glomerular disease.

The purpose of the second part of this study was to subject dogs, posessing low levels of anti-CAV antibody, to challenge with CAV and thereafter to examine their kidneys at varying intervals. Two groups of dogs were used in this study; the first group consisted of puppies with low levels of maternally derived antibody while the second were CAV antibody free dogs which recieved a single dose of CAV hyperimmune serum prior to challenge.

MATERIALS AND METHODS

Experimental Procedures

Experiment 1: For the first experiment, 15 8-week-old collie-cross dogs were used. Utilising the indirect immunofluorescence technique, these animals were all found to have low levels of circulating anti-CAV antibody, with titres ranging from 1 to 32. Nine dogs recieved 1ml of of CAV suspension intravenously and were killed serially from 1 to 15 days after inoculation (see Table 7). Four animals recieved a similar inoculum of uninfected tissue culture suspension and were killed 3, 6, 9 and 12 days later. The remaining 2 dogs were killed as normal controls in order to compare histological, immunofluorescence and ultrastructural findings.

Experiment 2: For the second experiment, 13 12-week-old CAV antibody-free dogs were used. Seven of these animals recieved 10ml of CAV hyperimmune serum (indirect immunofluorescence antibody titre >4096) by slow intravenous injection. On the 1st, 3rd and 7th days after recieving hyperimmune serum and at weekly intervals thereafter, each of these animals recieved intravenously 1ml of CAV suspension containing 10^4 TCID50. The dogs were then killed serially from 15 to 43 days after the initial virus inoculation, each animal being killed 24 hours after recieving the last dose of virus (see Table 8). As controls, 4 dogs which recieved similar initial doses of hyperimmune serum were given repeated inoculations of uninfected tissue culture fluid and killed at 15, 22, 36 and 43 days. The remaining 2 dogs were killed as normal controls.

Histological Ultrastructural and Immunofluorescence Procedures

All animals were subjected to a comprehensive macroscopic and histopathological examination. Kidney tissue was examined by electron microscopy and by immunofluorescence for the presence of CAV antigen,

IgG and B1C globulin as described in the section on "materials and methods".

Serology

Serum samples were obtained from all animals at the onset of the experiment, from those in experiment 1 at the time of death and from animals in experiment 2 at weekly intervals just prior to each virus inoculation. Antibody to CAV in these sera was measured using the indirect immunofluorescence test.

RESULTS

Clinical and Macroscopic Findings

Throughout both experiments none of the inoculated or control animals showed any clinical abnormalities. At necropsy, apart from a few small white foci 1-2mm in diameter, observed in both cortex and medulla of kidneys from 2 dogs in experiment 2 (Nos. 44 and 47), all animals appeared macroscopically normal.

Histological Findings

The kidneys of 2 animals in experiment 2 (Nos. 44 and 47) contained a few scattered foci of tubular necrosis in both cortical and medullary regions. In dog 44 these necrotic foci were surrounded by accumulations of ploymorphonuclear leukocytes whilst in dog 47 interstitial infiltrates consisting of a mixed population of lymphocytes and macrophages were present. In neither animal were inclusion bodies observed. The glomeruli in both groups of dogs and the controls showed a normal histological appearance. However, in comparison with the glomeruli of dogs in experiment 2, many of which were 18 weeks of age when examined, the glomeruli of the 8 week old dogs in experiment 1 were smaller and the visceral epithelial cells around the periphery of of the tufts often appeared more prominent (Figs. 18 and 19).

Immunofluorescence Findings

Neither CAV antigen nor host complement were detected in the kidneys of any of the inoculated or control animals. Small segmental deposits of IgG were, however, found in approximatetely 20-40 per cent of the glomeruli in 11 of the 16 inoculated animals, being of equal incidence in both groups of dogs (Fig. 20). However, identical deposits were also found in a similar proportion of inoculated and uninoculated control animals from both groups. These deposits were always found in the hilar regions of the glomeruli.

Ultrastructural Findings

The kidneys of all inoculated and control animals showed a normal ultrastructural appearance (Fig. 21). Evidence of glomerular immune complex deposition, such as increased mesangial activity and the presence of electron-dense deposits, was not found in any of the dogs. Pieces of kidney examined from dogs 44 and 47 did not contain any foci of tubular necrosis or cellular infiltration.

Serological Findings

The results of anti-CAV antibody estimations are presented in Tables 7 and 8.

Experiment 1: Of the 9 animals which received virus, 5 showed an antibody response to CAV. Three of these animals had antibody titres of 512 or more when they were killed 7, 9 and 13 days after inoculation. The remaining 4 animals showed no significant rise in titre; 2 of these dogs were examined 1 and 3 days after inoculation and therefore had insufficient time to mount an antibody response.

Experiment 2: All of the animals which received hyperimmune serum followed by repeated inoculation of virus, developed an antibody response to the virus. However, antibody was not detected until 2 weeks after the initial dose of virus at which time some dogs had developed high

levels of antibody (e.g. dog 40 with a titre of 512), whereas others had extremely low levels (e.g. dog 42 with a titre of 1) By 3 weeks, all animals had titres of 32 or more and from 4 weeks onwards all had titres of 128 or more.

Table 7:- Experiment 1: Challenge of dogs, posessing low levels of maternal antibody, with CAV. Anti-CAV antibody titres, measured at the onset of the experiment and at the time of death, are given for each animal.

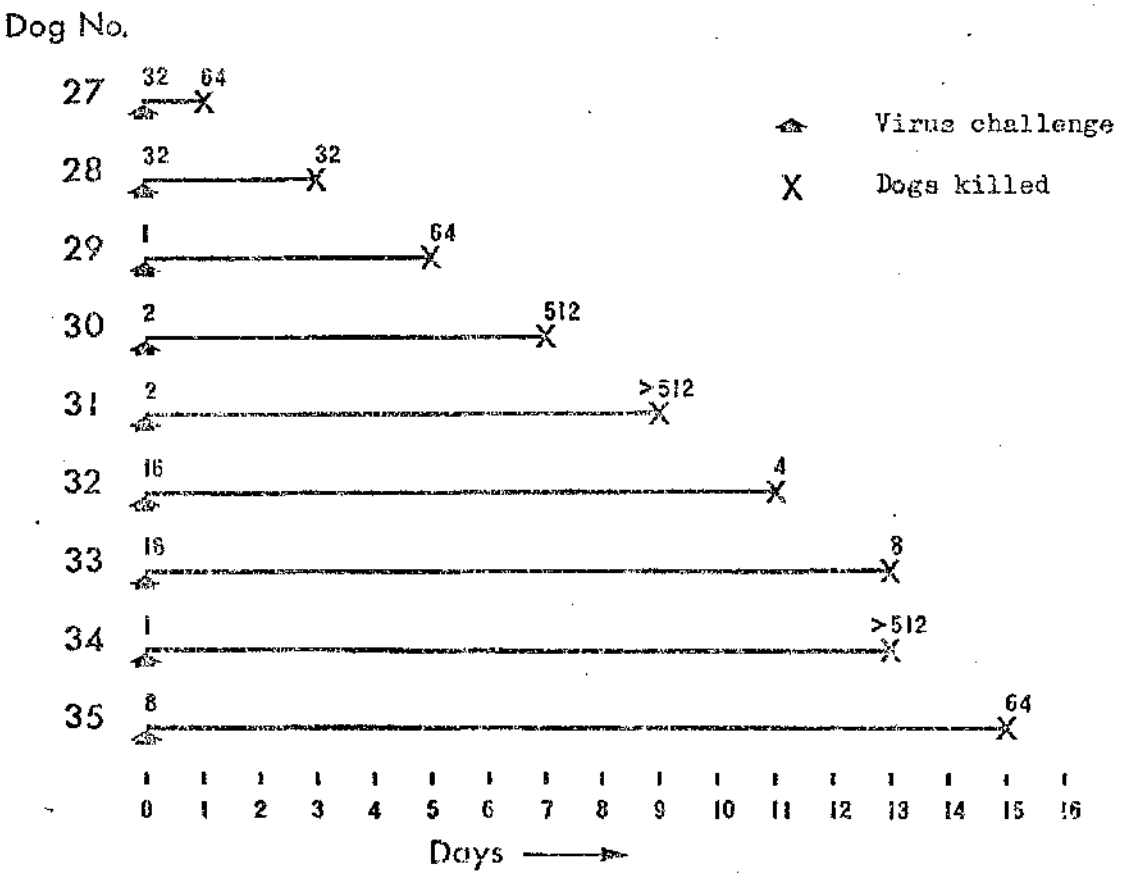


Table 8:- Experiment 2: Antibody-free dogs given hyperimmune serum prior to repeated challenge with CAV. Anti-CAV antibody titres, measured at the onset of the experiment and at weekly intervals thereafter, are given for each animal.

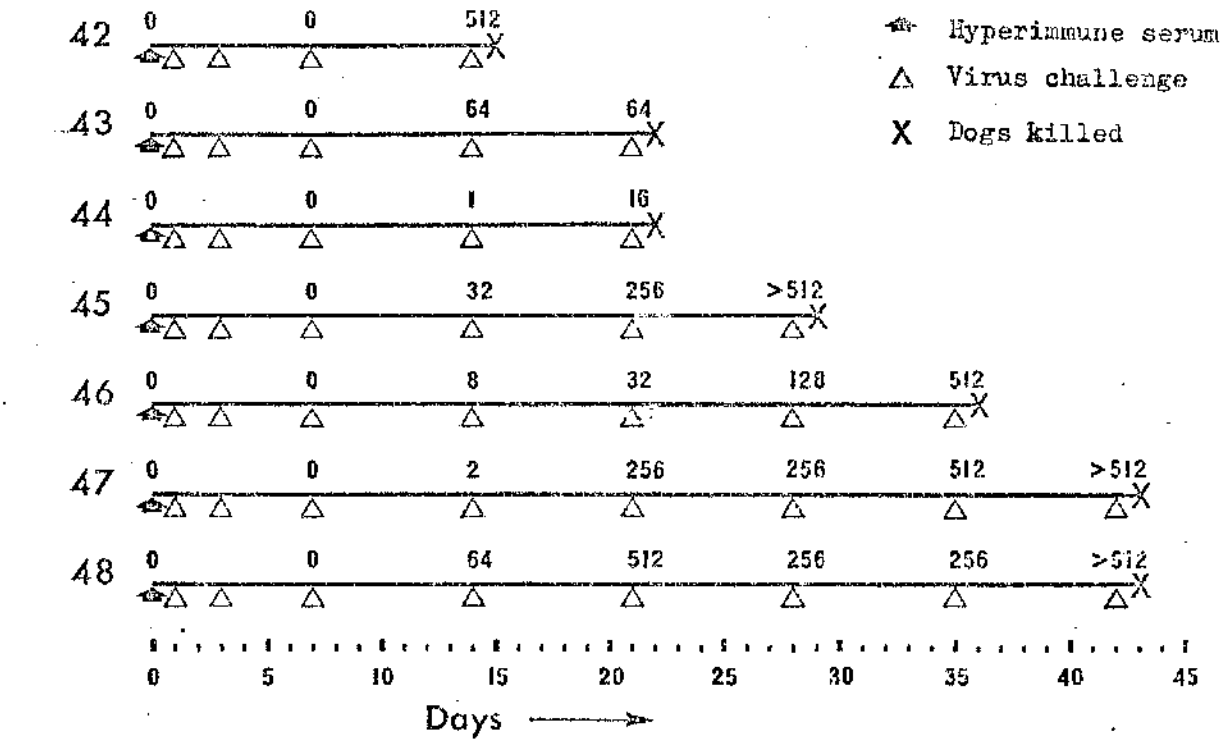
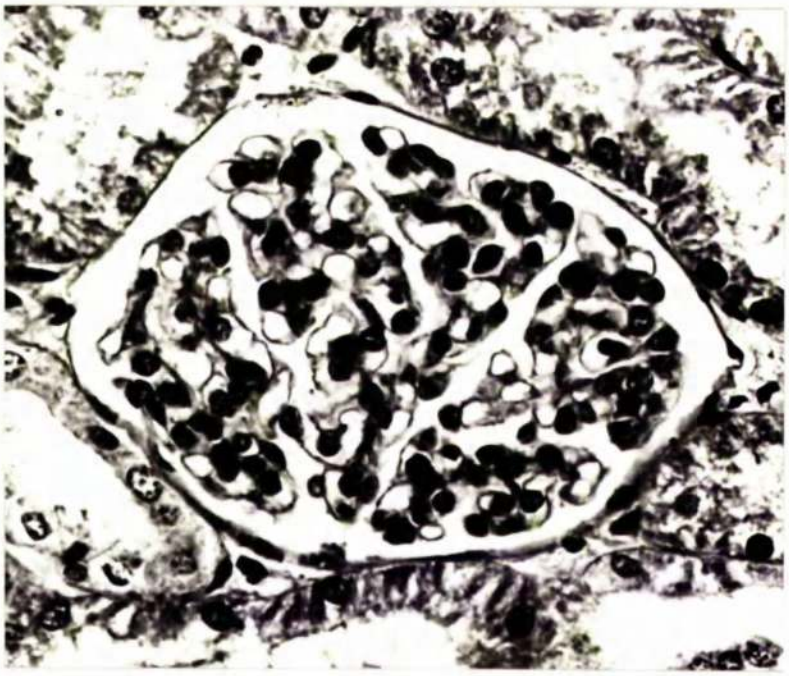


Fig. 18:- Glomerulus from 8 week old dog in experiment 1, showing normal cellularity and numerous patent thin-walled capillary loops.

(HE X 500)

Fig. 19:- Glomerulus from an 18 week old dog in experiment 2: The glomerulus appears normal, although it is larger and the nuclei are less prominent than in the younger animal.

(HE X 400)



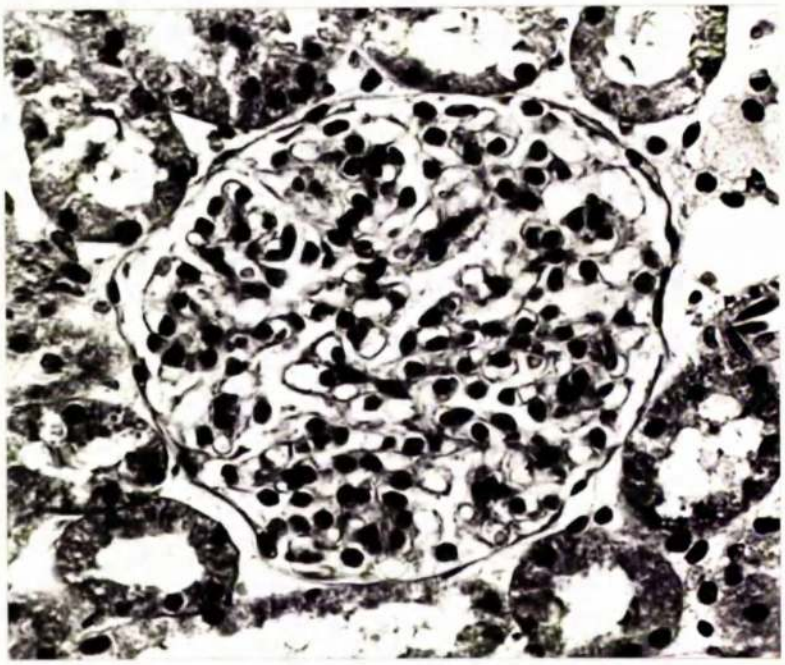
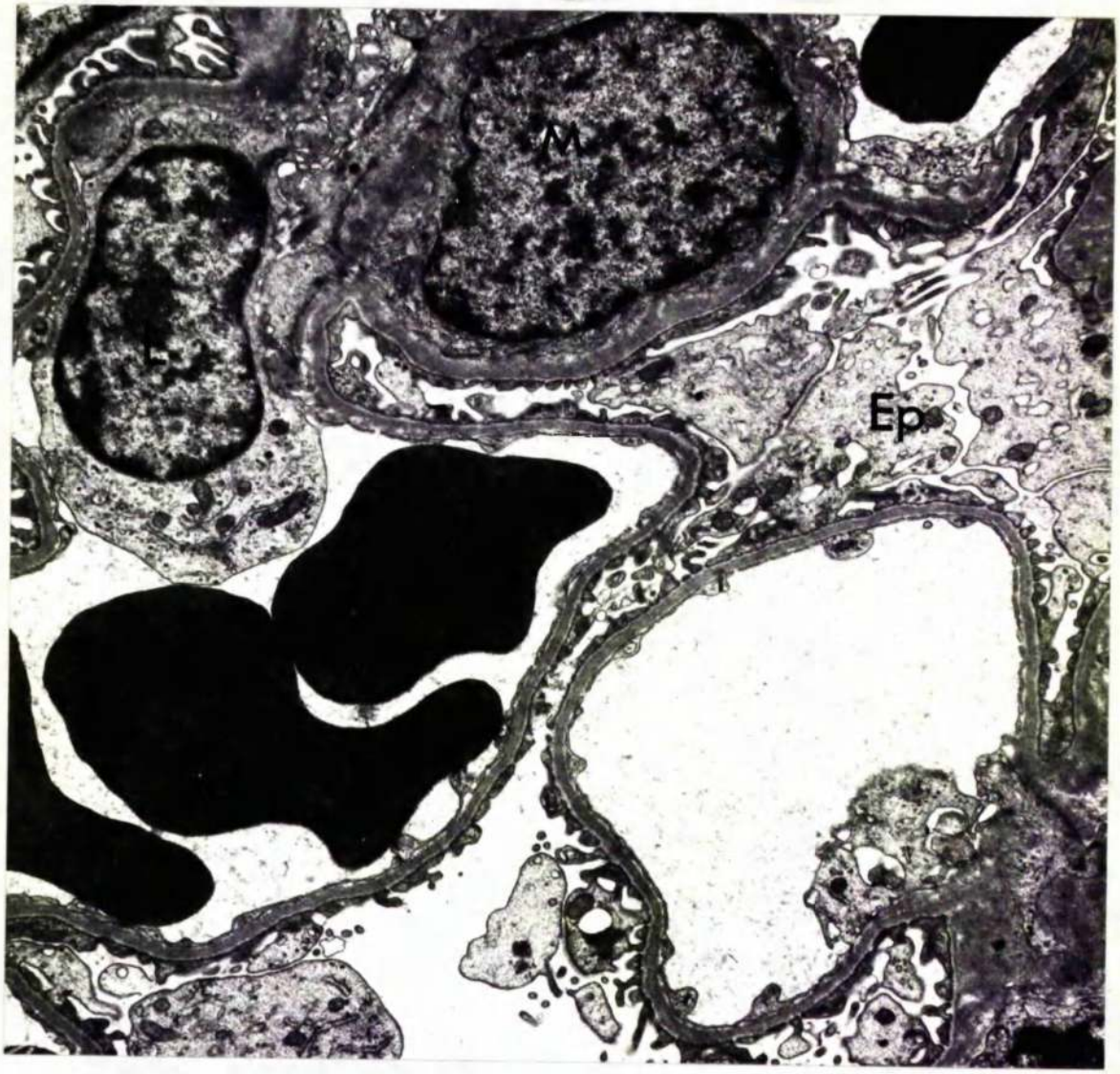


Fig. 20:- IgG in glomerulus of a dog examined 7 days after administration of virus: segmental granular fluorescence for IgG can be seen in the hilar region of the glomerulus.

(Immunofluorescence X 400)

Fig. 21:- Electron micrograph of glomerulus from a dog which received hyperimmune serum followed by repeated inoculations of CAV: Note the normal appearance of the capillary walls and mesangium. E = Endothelial cell; Ep = Epithelial cell; M = Mesangial cell.

(Electron microscopy X 10,000)


M
Ep

DISCUSSION

In all inoculated and control dogs examined in the above experiments the glomeruli showed a normal histological and ultrastructural appearance. Small segmental deposits of IgG were detected in the glomeruli of both inoculated and control dogs. These deposits were considered not to be of significance in relation to the experimental procedures carried out; they may represent the trapping of small amounts of immune complexes formed in the circulation during the normal processes of eliminating foriegn antigens. Results obtained in the present study, therefore, indicate that challenge of dogs, posessing low levels of anti-CAV antibody, with virus does not result in significant glomerular deposition of virus antigen-antibody immune complexes. However, it must be pointed out that, since none of the dogs in experiment 2 were examined in the first 14 days of the experiment, it remains a possibility that a transient deposition of immune complexes may have occurred in these animals.

It has been shown that the formation of potentially nephritogenic soluble immune complexes in the circulation requires the presence of an excess of antigen in relation to antibody (Germuth and Rodriguez, 1973). Since many of the present series of dogs mounted an antibody response to CAV, it is probable that the administered virus was not immediately overwhelmed by circulating antibody. However, it is unlikely, even if there was an excess of viral antigen, that the quantities of virus which the dogs recieved were sufficient to result in the formation of significant amounts of immune complexes. Other viruses which are associated with immune complex glomerulonephritis (e.g. lymphocytic choriomeningitis virus and aleutian disease virus) produce chronic infections with prolonged high levels of viraemia. In this way, a continual source of circulating antigen is present from which immune

complexes can be formed and deposited in the glomeruli. Consequently, it is unlikely that CAV could result in immune complex mediated glomerular disease without active virus replication in the host in the presence of circulating antibody.

A number of interesting features arose from the results of anti-CAV antibody estimation carried out prior to and following administration of virus. In experiment 1 (i.e. those with low levels of maternal antibody), all except 2 of the dogs examined from day 5 onwards developed a rising antibody response to CAV. It is of note that the 2 animals (Nos. 32 and 33) which failed to respond had the highest initial pre-infection antibody titres. In the second group of dogs, which were initially antibody-free, administration of CAV hyperimmune serum successfully protected them against subsequent repeated challenge with virus. All of these dogs, however, mounted an antibody response to CAV although antibody was not detected until the second week after the initial dose of virus. It is of note that all dogs were negative by the immunofluorescence test for anti-CAV antibody 7 days after recieving hyperimmune serum and just prior to their third virus challenge. Indeed some of these dogs were notably slow to produce high titres of anti-CAV antibody. This was particularly evident in dog Nos. 44 and 46 whose titres were 16 and 32 respectively 3 weeks after initial challenge; in contrast, dog No. 40 had an antibody titre of 512 at 2 weeks. This could be explained by antibody complexing with inoculated virus, thus reducing the levels of detectable circulating antibody. In this situation of antibody excess, large insoluble complexes would be formed and these would be taken up by the mononuclear phagocytic system as opposed to being deposited in the glomeruli.

Although many of the present series of dogs developed an antibody response to CAV, it remains uncertain whether or not virus replication

occurred in the tissues of these animals. Indeed, foci of interstitial nephritis, which commonly occur in dogs following recovery from systemic CAV infection (Wright, 1967b), were not found in the kidneys of any of the animals in either experiment. The kidneys of 2 dogs in experiment 2 contained occasional foci of tubular necrosis with surrounding interstitial cellular infiltrates; however, in neither of these dogs were CAV inclusions observed nor was CAV antigen detected in the kidneys by immunofluorescence.

These results, therefore, suggest that dogs posessing extremely low levels of circulating anti-CAV antibody may be resistant to systemic infection with CAV but are still capable of mounting an antibody response. Moreover, with such low levels of antibody, it is probable that the levels of virus challenge are important in determining whether or not an antibody response occurs.

SECTION III : EXPERIMENTAL CAV NEPHRITIS : THE INFLUENCE OF IMMUNITY ACTIVELY INDUCED DURING CAV INFECTION

INTRODUCTION

MATERIALS AND METHODS

(a) CAV infected dogs

(b) Passive transfer of serum to mice

RESULTS

(a) CAV infected dogs

Tables 9-12 ; Figures 22-74.

(b) Passive transfer of serum to mice

Table 13 ; Figure 75.

DISCUSSION

INTRODUCTION

It is well established that, following recovery from acute CAV infection, dogs may continue to excrete virus in the urine for periods up to 9 months (Poppensiek and Baker, 1951; Baker et al., 1954). This has been shown by a number of workers to result from localisation of virus in renal tubular epithelium as a sequel to the acute systemic infection. Persistence of virus is associated with cellular infiltration into the renal interstitium with resultant focal interstitial nephritis (Hartley, 1958; Wright et al., 1971). Wright (1967a) demonstrated that 70 per cent of dogs recovering from experimental CAV infection develop such lesions and the same author described similar lesions in 29 per cent of naturally occurring cases of CAV infection (Wright, 1967b).

Although the histological picture of CAV-associated interstitial nephritis is well documented, there are no detailed accounts of the ultrastructural changes within the lesions. Similarly, the literature contains no information concerning any glomerular changes during the early stages of recovery from acute CAV infection. As CAV antigen is, presumably, being released into the circulation in large quantities from infected hepatic and endothelial cells, this would, theoretically, appear to provide an ideal situation of antigen excess in which potentially nephritogenic soluble immune complexes might be formed in the circulation with subsequent deposition in the renal glomeruli.

The purpose of this part of the study was twofold. Firstly, a detailed histological, immunofluorescence and ultrastructural study of the renal lesions which occurred in dogs during and following recovery from acute CAV infection was carried out. Particular attention was paid to the study of glomerular and interstitial lesions, with special emphasis placed on attempting to elucidate the pathogenetic mechanisms. Secondly, an attempt was made to demonstrate the presence

of circulating virus antigen-antibody complexes in infected dogs by passive transfer of serum to mice.

MATERIALS AND METHODS

(a) CAV infected dogs

Experimental Procedures

Thirty-six 16-week-old, antibody-free dogs weighing approximately 6kg were used. In an attempt to produce severe clinical CAV infection with subsequent recovery, 25 of these dogs were inoculated with diluted CAV suspension; each animal recieved, intravenously, 1ml containing 10^3 TCID50 (i.e. the original stock virus suspension diluted 1:10,000). The times of examination of these animals, which ranged from 4 to 27 days after inoculation, are given in Table 10. A further 3 dogs were kept in the same accommodation, in contact with the infected dogs and were killed at 10 days. The remaining 8 dogs were each inoculated intravenously with 1ml of uninfected tissue culture suspension, housed in separate accommodation and killed on days 4, 6, 8, 10, 12, 14, 18 and 22 after inoculation.

Histological, Ultrastructural and Immunofluorescence Procedures

All dogs were subjected to a comprehensive macroscopic and histopathological examination as described in the section on "materials and methods". As part of the histological examination of the kidneys, 100 glomeruli from each animal were examined in detail and the number of glomeruli in which one or more intranuclear inclusion bodies were found was recorded. Samples of kidney were obtained for ultrastructural studies from all except 9 dogs which died and whose tissue was therefore unsuitable for electron microscopical examination. The kidneys of all animals were examined by immunofluorescence for the presence of canine IgG and B1C globulin (C3) and CAV antigen. Liver tissue was also

examined for CAV antigen. In addition, the kidneys of 4 dogs (Nos. 74, 76, 77 and 78), in which there was an interstitial infiltrate containing plasma cells, were examined using the indirect "sandwich" fluorescence technique, as described in the section on "materials and methods", to determine if the immunoglobulin (IgG) produced by these cells was anti-viral antibody.

Biochemistry

Where possible, blood and serum samples were obtained at necropsy. Urine was examined for the presence of protein by the turbidometric method using standard sulpho-salicylic acid; blood urea nitrogen levels were measured by the Standard Technicon AA II-1 method (Technicon Instruments Corp., Torrytown, New York).

Elution Procedures

One whole kidney from each of 16 infected and 6 control dogs was subjected to elution procedures as described in the general "materials and methods". A further 6 control kidneys were obtained from dogs used in the experiments described in section II and subjected to the same elution procudure; these animals (Nos. 30, 31, 34, 42, 45 and 46) all had high levels of circulating anti-CAV antibody (see Tables 7 and 8).

Serology

Serum samples taken before commencement of the experiment and at necropsy and also kidney eluates were examined for the presence of anti-CAV antibody by the indirect immunofluorescence test. In addition, eluates were tested for antibody directed against kidney tissue antigens by an indirect immunofluorescence test. Cryostat sections of normal dog kidney were exposed to the eluate for 30 minutes and, after washing

in PBS, stained with FITC conjugated rabbit anti-dog globulin for a further 30 minutes.

(b) Passive transfer of serum to mice

Experimental Procedures

Serum from each of 6 CAV infected dogs was inoculated into groups of 4 8-10 week old albino mice (Porton strain). The 6 serum samples were obtained from dogs examined 4, 5, 8, 9, 10 and 14 days after inoculation of virus (Nos. 55, 57, 65, 70, 72 and 73). Each mouse recieved 4 doses of 0.4ml of serum by slow intravenous injection at 12 hour intervals and was killed 6 hours after the 4th dose. A further 4 mice recieved similar inoculations of serum from an uninfected control dog; 4 mice were killed as normal uninoculated controls in order to compare the histological, immunofluorescence and ultrastructural features of mice of the Porton strain.

Histological Ultrastructural and Immunofluorescence Procedures

Postmortem examination of all inoculated and control mice was confined to the kidneys. General anaesthesia was induced using Trichloroethylene ("Trilene", Imperial Chemical Industries Ltd., Cheshire), the animals exsanguinated by severing the major vessels in the neck and the kidneys removed from the abdomen. Pieces of kidney taken for histological, immunofluorescence and ultrastructural studies were treated in the same manner as described for canine renal tissue. The kidneys of all mice were examined by immunofluorescence for the presence of canine IgG, mouse IgG and B1C globulin and CAV antigen.

RESULTS

(a) CAV infected dogs

Clinical Findings

The 25 inoculated animals remained clinically normal until the 3rd day after administration of virus when all except Nos. 74, 75 and 79 developed pyrexia in the region of 104-106° F. On the following day the temperature of most animals was below 104° F and throughout the remainder of the experiment Nos. 74-79 showed no further obvious clinical abnormalities. However, the remaining 19 dogs, on the 4th day, were depressed, anorexic and showed considerable reluctance to move about. There was congestion of the mucosae, slight enlargement of superficial lymph nodes and, on palpation, signs of anterior abdominal pain were noted. Over the next 5 days, the affected dogs remained extremely depressed and anorexic and jaundice became apparent on the 6th day. On the 7th, 8th and 9th days jaundice became more pronounced, the dogs were extremely dehydrated and there was more obvious enlargement of superficial lymph nodes. An additional feature at this stage was the presence of petechial haemorrhages on the gums, buccal mucosa and on the abdominal skin in 4 dogs. Deaths occurred from 4 to 9 days after infection (see Table 9) and during this time a number of dogs were also killed while in a moribund state. Only 2 dogs which were severely ill survived beyond 9 days; on the 10th day they showed marked clinical improvement with return of appetite and gradual reduction of jaundice which in dog 73 had disappeared by day 14.

Liver function tests were carried out and albumen and globulin levels measured in serum samples obtained from 2 dogs (Nos. 67 and 72) on day 6. The results obtained serve to illustrate the severity of the disease. Both animals showed marked elevation in the levels of serum

transaminases, alkaline phosphatase and bilirubin while albumen and globulin levels were both depressed (see Table 8).

	No. 67	No. 72
Glutamic oxalo-acetic transaminase	620 SF units	515 SF units
Glutamic pyruvic transaminase	510 SF units	705 SF units
Alkaline phosphatase	62 KA units	96 KA units
Bilirubin	5.1mg/100ml	2.8mg/100ml
Albumen	1.6g/100ml	1.9g/100ml
Globulin	2.1g/100ml	2.8g/100ml

Table 9: Results of liver function tests carried out on serum from 2 CAV infected dogs 6 days after inoculation of virus.

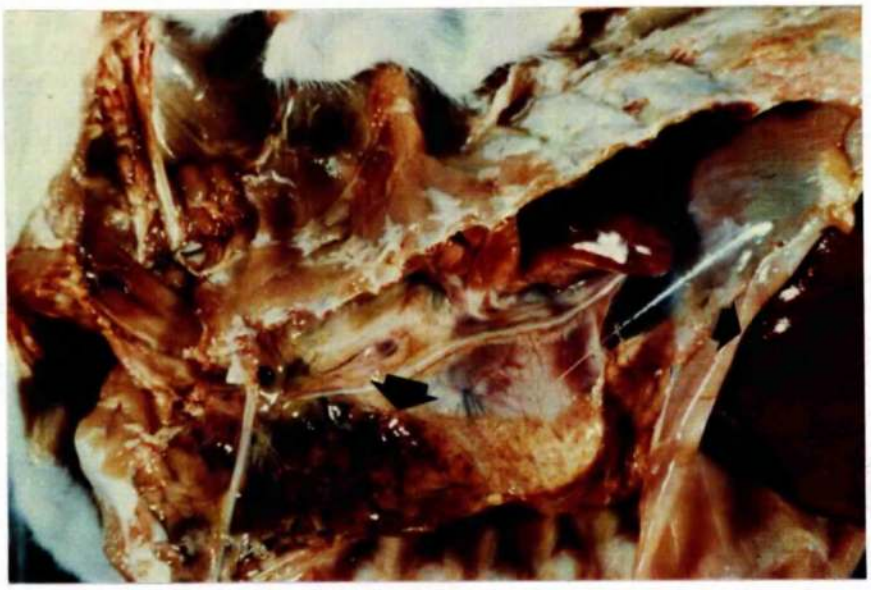
The 3 animals which were kept in contact with the infected dogs remained clinically normal for the first 8 days. On day 9, No. 81 became depressed and refused food; the following day, that is the day on which they were killed, all 3 dogs were dull and anorexic. Throughout the study, the 8 control dogs, which were housed in separate accommodation, showed no clinical abnormalities.

Macroscopic Findings

The 17 dogs which died or were killed up to and including day 9 all showed macroscopic changes characteristic of acute systemic adenovirus infection, similar to those described in section I; hepatomegaly, gall bladder wall oedema, serofibrinous peritonitis, petechial thymic haemorrhages (Fig. 22) and haemorrhagic lymphadenitis were the main findings. In those animals examined 7, 8 and 9 days after infection, serous effusion was not so apparent, although strands of fibrin were present in the abdominal cavity and the surface of the intestines took on a distinctive fine granular appearance. At this stage, the lymph

Fig. 22:- CAV-infected dog examined 7 days after inoculation of virus: The thoracic cavity has been opened revealing petechial haemorrhage and oedema of the thymus (large arrow). On the right of the picture, part of the liver (small arrow) can be seen; it is somewhat pale and mottled in appearance and the edges of the lobes are distinctly rounded.

Fig. 23:- CAV-infected dog examined 9 days after inoculation of virus: The wall of the aorta shows yellow discoloration, indicative of jaundice.



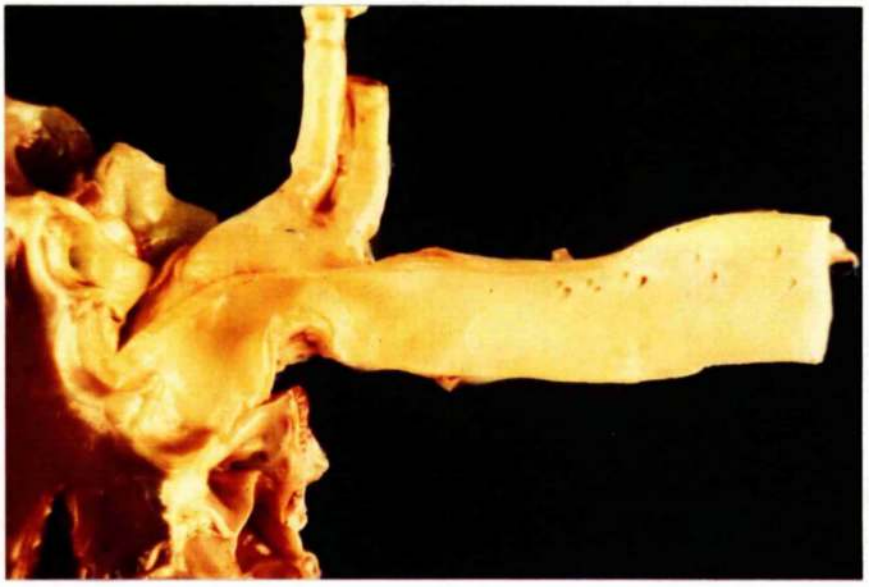
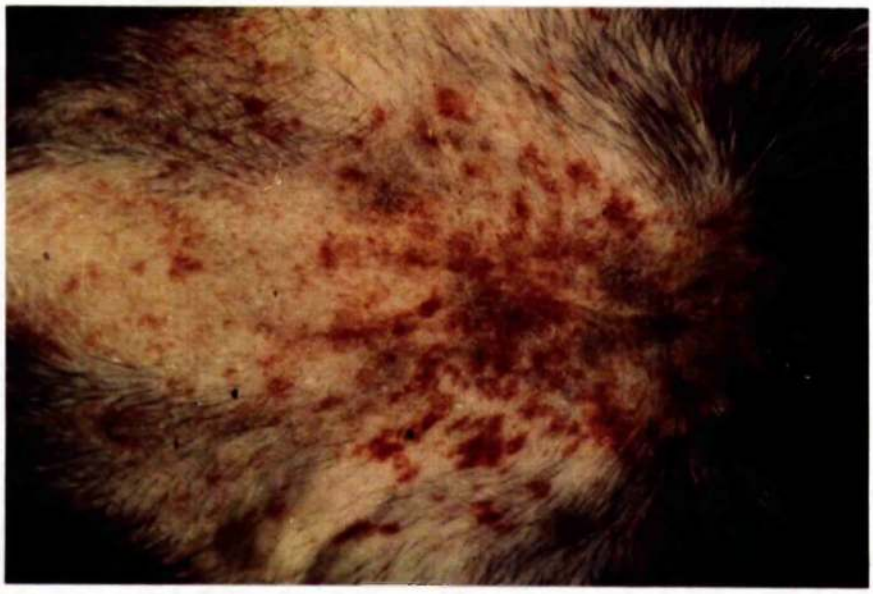


Fig. 24:- CAV infected dog examined 8 days after inoculation of virus: Petechial haemorrhages can be seen in the skin of the inguinal region.

Fig. 25:- CAV infected dog examined 8 days after inoculation of virus: Petechial haemorrhages can be seen in the buccal mucosa.



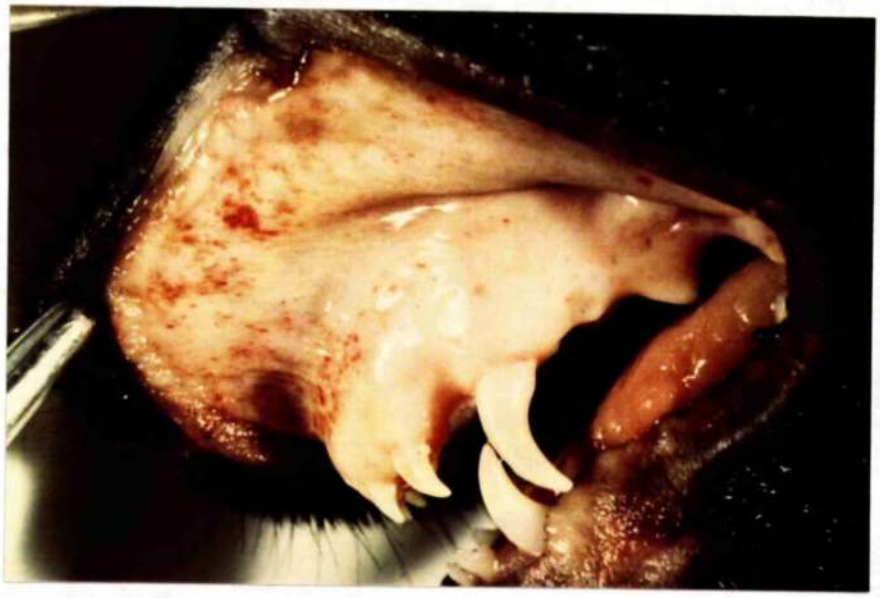
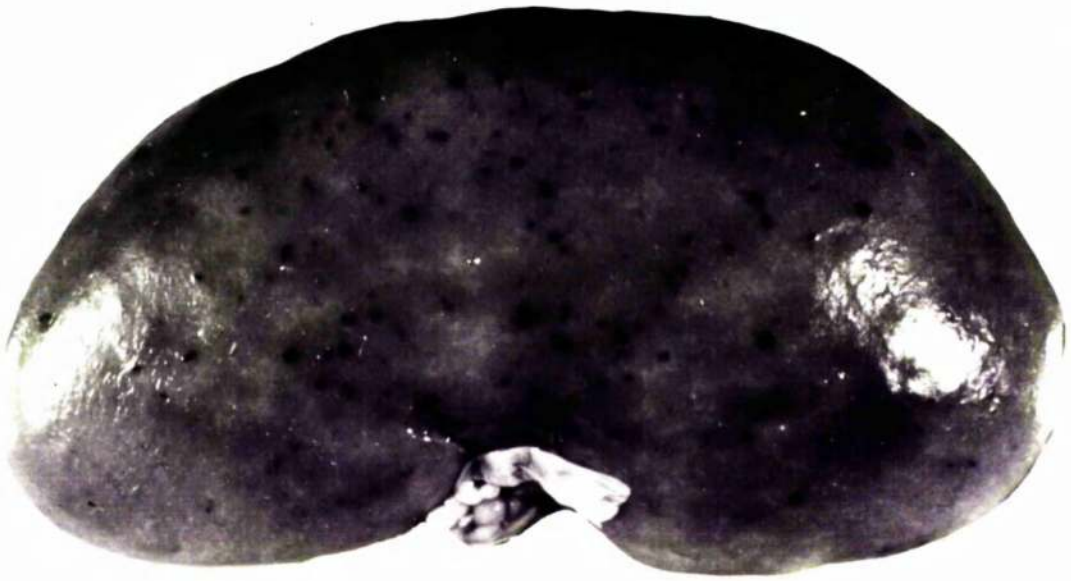


Fig. 26:- Kidney from CAV infected dog examined 8 days after inoculation of virus: Numerous petechial haemorrhages can be seen on the kidney surface.

Fig. 27:- Kidney from CAV infected dog examined 14 days after inoculation of virus: Haemorrhagic foci are particularly numerous in the kidney of this animal.



nodes were extremely enlarged and haemorrhagic and, on occasion, echymotic haemorrhages were found in the cardiac muscle and in the stomach or intestinal walls. In addition, petechial haemorrhages were noted on the abdominal skin and in the oral and vaginal mucosae in 4 dogs (Figs. 24 and 25). Jaundice was macroscopically evident in all animals examined from the 6th to the 10th day after inoculation of virus (Fig. 23). In addition, 11 dogs showed multiple small petechial renal haemorrhages, present mainly in the cortical regions; these lesions were recorded in all animals examined 7 to 14 days after infection, being particularly numerous in the dogs examined on days 10 and 14 (Figs. 26 and 27). In dogs examined from 14 days onwards, macroscopic abnormalities were confined to the kidneys. Multiple small white foci 1-2mm in diameter were found in the kidneys of dogs examined 17 to 27 days after virus inoculation. In the 3 dogs examined on days 17, 18 and 19, these foci had a characteristic haemorrhagic margin. On cut section, the lesions could often be seen as pale streaks extending into the deep cortex and similar small white foci were also observed in the medulla.

The 3 in contact control dogs which were killed on day 10 all showed lesions characteristic of acute systemic CAV infection as described earlier; one animal (No. 81) also had a few scattered petechial haemorrhages in the kidneys. The remaining 8 control dogs showed no macroscopic abnormalities.

Histological Findings

Outwith the kidney, the most striking histological changes were noted in the liver and lymph nodes. All dogs examined from 4 to 9 days after inoculation showed focal hepatic necrosis, the extent of which varied between individual animals. In some dogs, only small clumps of necrosed cells were involved (grade + Table 9) while in others

there were large coalescing foci of necrosis (grade ++++).
In those animals examined on days 7, 8 and 9, small accumulations of lymphoid cells, polymorphonuclear leukocytes and macrophages were observed within the foci of necrosis. Associated with the hepatic necrosis, in the majority of animals examined up to day 7, large numbers of CAV inclusion bodies were found; thereafter, inclusions became less numerous and, in 2 dogs examined on day 9, inclusions were not found despite the presence of severe hepatic necrosis. On days 9 and 10 there was evidence of hepatic regeneration with mitotic figures occasionally encountered in hepatocytes. Hepatic necrosis was not found in any of the dogs examined from 10 days onwards.

The enlargement and haemorrhagic appearance of the lymph nodes at necropsy was mainly due to severe haemorrhage into the lymphoid sinusoids. In the early stages of the experiment (days 4 and 5), erythrocytes were found in the interseptal and outer medullary sinuses; however, all animals examined on days 6, 7, 8 and 9 showed massive haemorrhage into all of the medullary sinuses resulting in distension and enlargement of the lymph nodes. Associated with the haemorrhage, there was extensive erythrophagocytosis by sinusoidal macrophages. From day 10 onwards, the haemorrhage had largely disappeared, although at this stage there was marked immunological activity within the lymph nodes, with numerous large germinal follicles present in the cortices and large numbers of plasmablasts and mature plasma cells appearing in the medullary cords. From day 6 to day 9, small haemorrhages were often found in a number of other organs; they were most consistently observed in the thymic lobules but were also sometimes present in brain, cardiac muscle and submucosa of the gastro-intestinal tract. In dogs examined up to 8 days, inclusion bodies were frequently found in a variety of other organs, particularly in vascular endothelium but sometimes also in

peritoneal mesothelial cells, tonsillar epithelium and occasionally lymph node sinusoidal macrophages.

The main histological changes observed in the kidneys are summarised in Table 10. In all animals examined up to and including day 14, diffuse cytological changes were found in the glomeruli. Early in the course of the experiment (days 4 and 5), the main features were swelling and vacuolation of endothelial and mesangial cells resulting in enlargement of the tuft and partial occlusion of capillary loops (Fig. 29). Occasional polymorphonuclear leukocytes were found lodged in the loops and granular debris was noted in the filtration spaces. From the 6th day onwards, glomerular cytological changes became much more severe. There was marked expansion and increased cellularity of mesangial regions which produced accentuated lobulation of glomerular tufts (Fig. 30). This hypercellularity was further augmented by large scale infiltration of polymorphonuclear leukocytes into the glomeruli. At the height of the disease on days 7, 8 and 9, some of these cells had penetrated the mesangium. At this stage, scattered individual endothelial and mesangial cells were observed in various stages of degeneration and necrosis. In addition, some glomeruli showed capillary thrombosis resulting in segmental necrosis with release of fibrin and erythrocytes into the urinary spaces (Figs. 32 and 33). Haemorrhage into the urinary spaces, in the absence of any apparent glomerular necrosis, also occurred resulting in considerable tubular haemorrhage; this, together with foci of interstitial haemorrhage from damaged capillaries, constituted the haemorrhagic lesions observed at necropsy. In animals examined on days 7, 8 and 9, mitotic configurations of endothelial and mesangial cells were observed in a few glomeruli (Fig. 31).

Inclusion bodies were found in the glomeruli of all animals up to and including day 9. The number of inclusions observed varied

Table 10:- Experimental CAV infection: Histological features of the liver and kidneys.

Dog No.	Day examined	Hepatic necrosis	Hepatic inclusions	Glomerulo-nephritis	Inclusions			Tubular haemorrhage	Tubular necrosis	Interstitial infiltration
					Percentage glomeruli*	Interstitial capillaries	Tubules			
55	4 (D)	+	++	++	66	+	-	-	-	-
56	5	+++	++++	++	76	+	-	-	-	-
57	5	+++	+++	+	36	+	-	-	-	-
58	5	++	++++	++	76	+	-	-	-	-
59	5 (D)	+++	+++	++	96	+	-	+	+	-
60	6	+	++	++	32	+	-	-	-	-
61	6	+++	+++	+++	98	+	-	-	-	-
62	6 (D)	+++	++++	+++	92	+	-	-	-	-
63	7 (D)	++++	+++	++	60	+	-	-	+	+
64	7	++++	++++	++	92	+	-	++	+	+
65	8 (D)	+++	++	+++	78	+	-	+	++	-
66	8 (D)	+++	++	++++	70	+	-	++	++	+
67	8 (D)	+++	++	+++	90	+	-	+	+	+
68	8	+++	+	++++	14	-	-	+	+	+
69	9	++	+	++++	62	+	-	++	-	+
70	9 (D)	++	-	++++	26	-	-	+	+	+
71	9 (D)	++	-	++++	6	-	-	++	+	+

Continued on next page.....

Table 10/continued

Dog No.	Day examined	Hepatic necrosis	Hepatic inclusions	Glomerulo-nephritis	Inclusions			Tubular haemorrhage	Tubular necrosis	Interstitial infiltration
					Percentage glomeruli*	Interstitial capillaries	Tubules			
72	10	-	-	+++	0	-	+	++	++	++
73	14	-	-	++	0	-	-	+++	++++	++
74	17	-	-	+	0	-	++	-	+++	++++
75	18	-	-	+	0	-	-	-	++	+++
76	19	-	-	+	0	-	++	-	+++	++++
77	25	-	-	+	0	-	+	-	+	+++
78	25	-	-	+	0	-	+	-	+	+++
79	27	-	-	+	0	-	-	-	+	++
80	10 c	+++	+++	++	45	+	-	-	-	-
81	10 c	+++	++	+++	34	+	-	+	-	-
82	10 c	+	++	+++	30	+	-	-	-	-

Various parameters graded + to ++++ according to severity

c = In contact controls

* = 100 glomeruli from each animal examined for the presence of one or more inclusions

between individual animals and between different glomeruli in the same animal. In 5 dogs examined between day 5 and day 8, 90 per cent or more of the glomeruli contained inclusions. In contrast, 2 of the dogs examined on day 9 had inclusions in only 6 and 26 per cent of their glomeruli respectively. The number of inclusions per glomerulus usually did not exceed 6, although in a few instances 8 inclusions were observed in a single glomerulus. In these animals, small numbers of inclusions were found elsewhere in the kidney, in the endothelium of interstitial capillaries and larger renal blood vessels.

In dogs 72 and 73, which were examined on days 10 and 14 respectively after inoculation, the glomeruli did not contain any inclusions. Furthermore, polymorphonuclear leukocytes were no longer present in such large numbers and, particularly in dog No. 73, the glomerular tufts contained fewer individual necrotic cells. However, in both animals there was diffuse mesangial expansion and hypercellularity (Fig. 35) and in some instances glomerular loop thrombosis with segmental glomerular necrosis was also observed. Haemorrhage into the urinary spaces and subsequently the tubules was particularly severe in both of these animals (Fig. 34). The remaining 6 dogs which were examined from 17 to 27 days after inoculation with virus showed much less severe glomerular changes; some but not all of the glomeruli showed mild expansion of mesangium accompanied by segmental mesangial hypercellularity.

Focal areas of tubular necrosis were found in the kidneys of one animal examined on day 5 and in all animals examined from the 7th day onwards. In dogs examined 17 days and more after inoculation, these necrotic tubules were always found within foci of interstitial nephritis which were observed randomly in both cortex and medulla and sometimes contained inclusions. However, in the earlier stages of the experiment the foci of necrosis were confined to the cortex, involving mainly

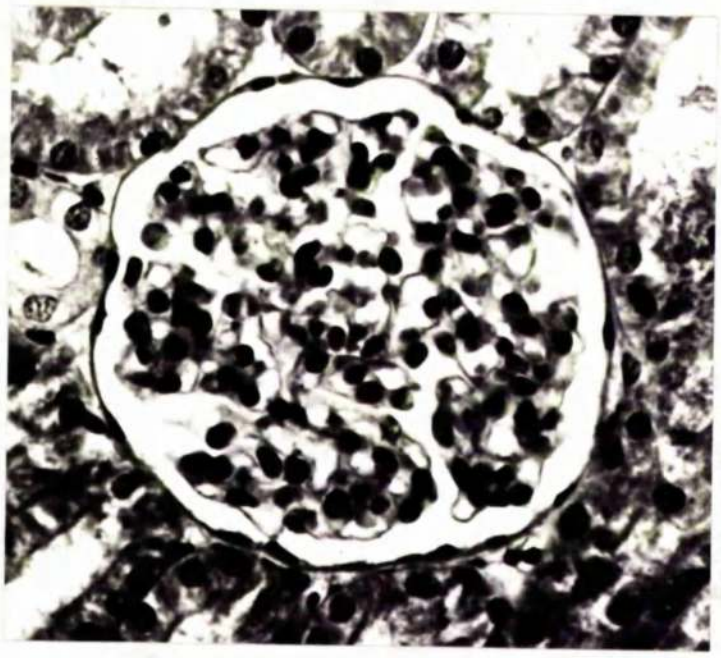
proximal tubules and were apparently unassociated with the presence of virus (Fig. 35). In the 2 dogs examined on days 10 and 14 (Nos. 72 and 73), tubular necrosis was found within and outwith areas of interstitial nephritis; in dog No 73 these foci of necrosis were particularly numerous.

Fig. 28:- Glomerulus from uninfected control dog, showing normal cellularity and patent capillary loops.

(HE X 400)

Fig. 29:- Glomerulus from a CAV-infected dog examined 5 days after administration of virus: There is normal cellularity but glomerular cells are swollen and there is distinct expansion of mesangial regions with occlusion of capillary loops. A single intranuclear inclusion body can be seen in the centre of the glomerulus.

(HE X 400)



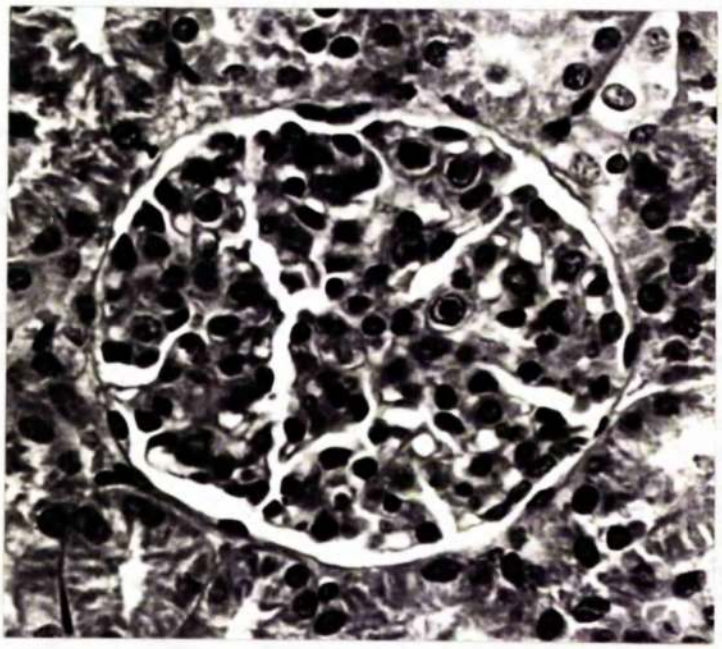
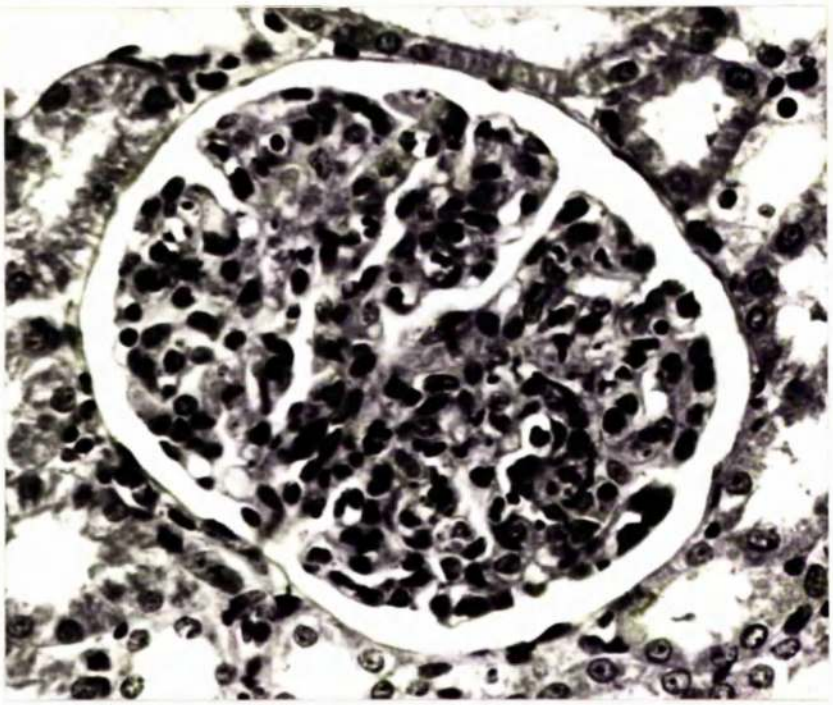


Fig. 30:- Glomerulus from a CAV infected dog examined 9 days after administration of virus: The tuft is swollen and the capillary loops are collapsed; hypercellularity is due to infiltration by polymorphonuclear leukocytes and an increase in the numbers of mesangial cells.

(HE X 400)

Fig. 31:- Glomerulus from CAV infected dog examined 9 days after administration of virus: The glomerulus contains 2 mitotic figures (arrows); an increase in polymorphonuclear leukocytes can also be seen.

(Martius scarlet blue X 400)



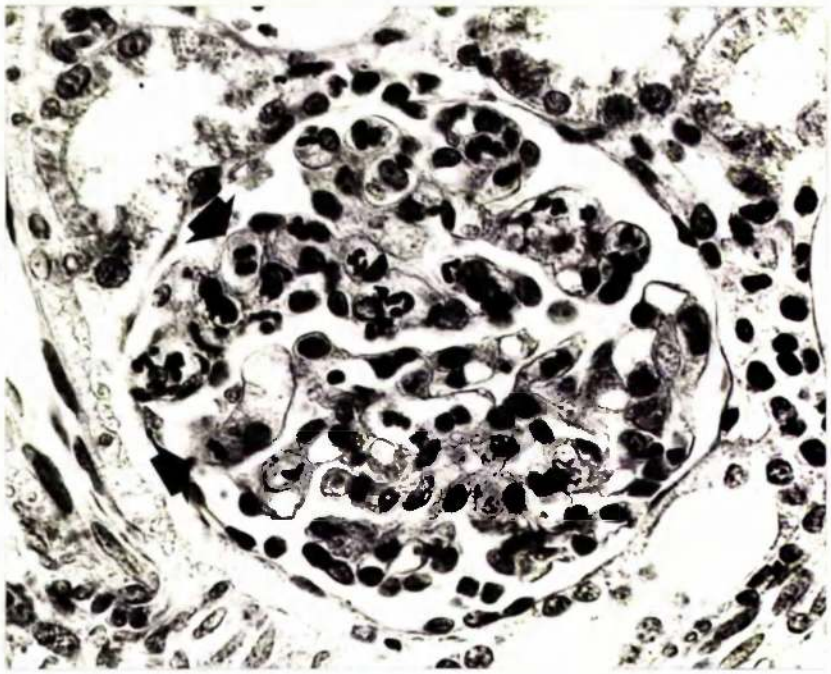
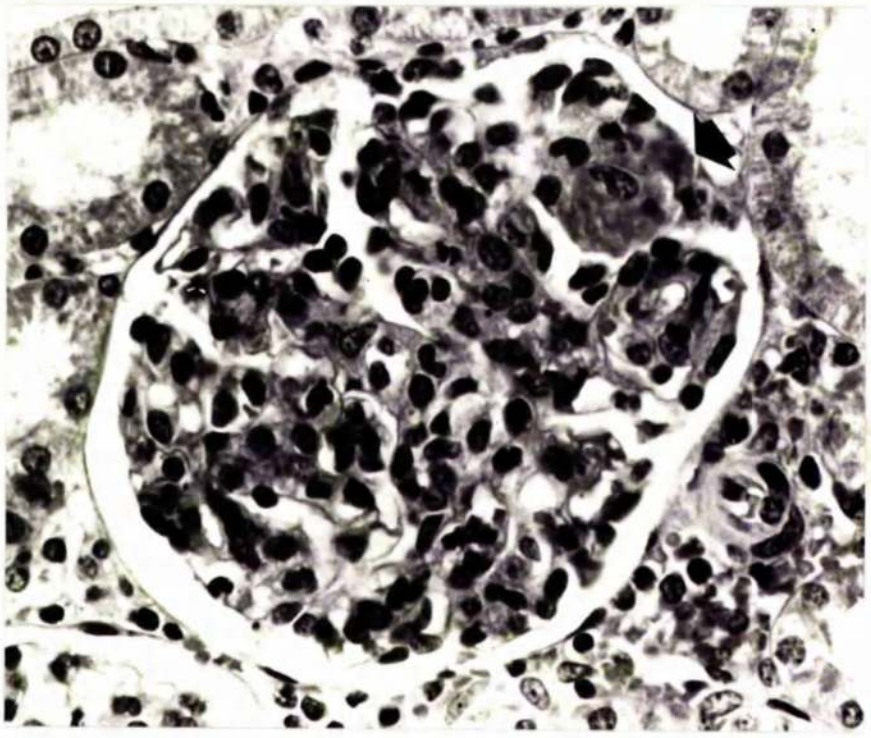


Fig. 32:- Glomerulus from CAV infected dog examined 10 days after administration of virus, showing segmental necrosis (arrow) of a capillary loop.

(HE X 400)

Fig. 33:- Glomerulus from CAV infected dog examined 9 days after administration of virus: Fibrinous exudation (large arrows) and haemorrhage (small arrows) into the filtration space can be seen.

(HE X 400)



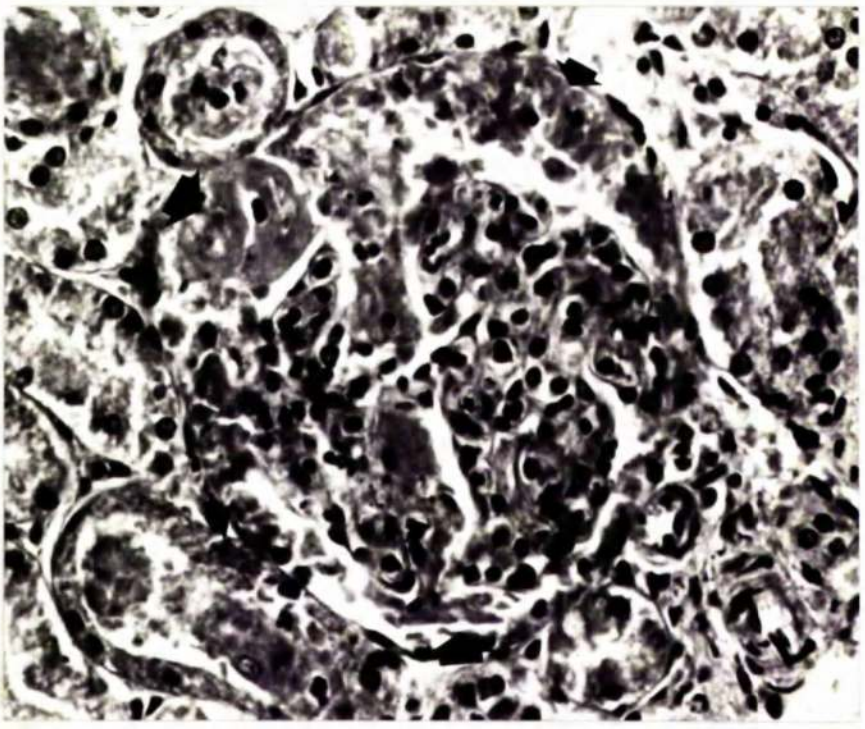
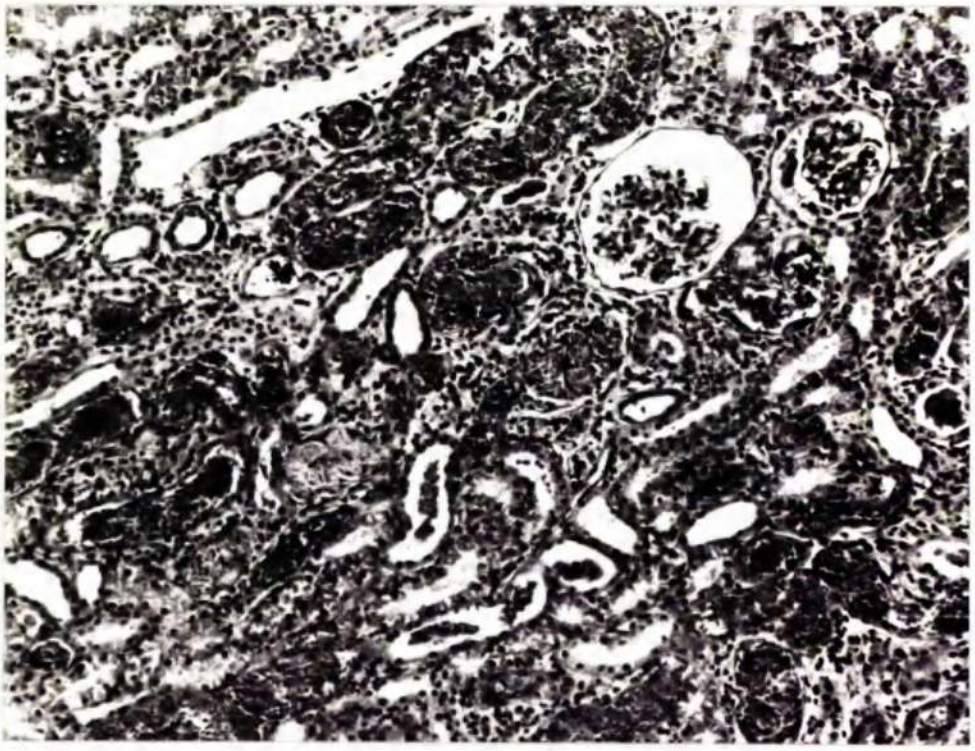


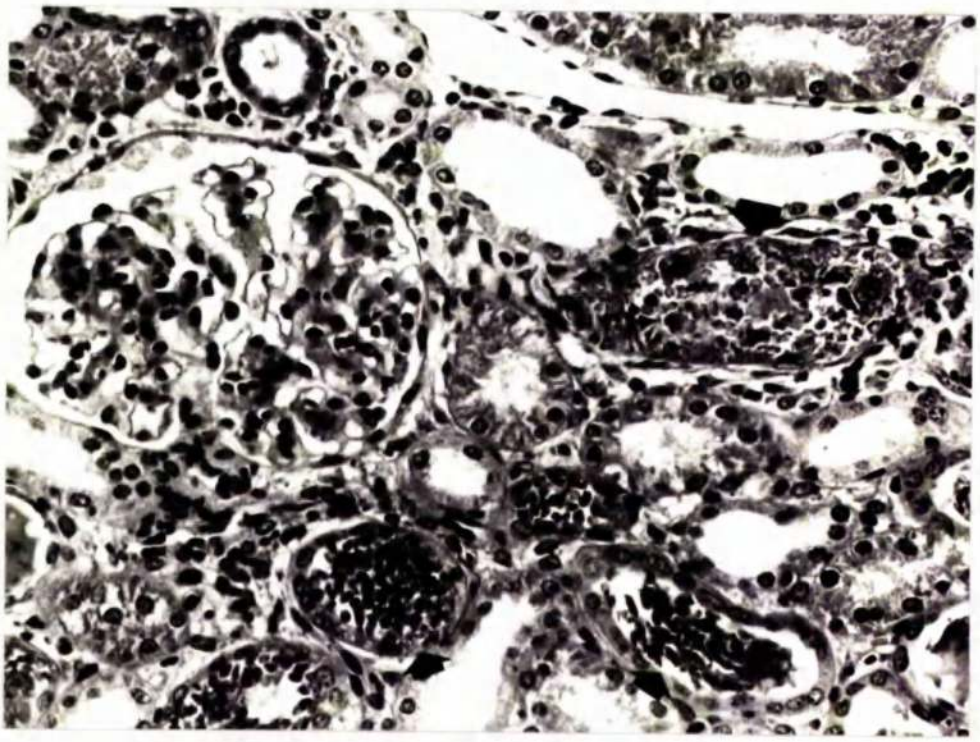
Fig. 34:- Section of kidney cortex from CAV-infected dog examined 14 days after inoculation of virus: Widespread tubular haemorrhage can be seen.

(HE X 100)

Fig. 35:- Glomerulus from CAV infected dog examined 14 days after administration of virus, showing mesangial expansion and segmental hypercellularity. Haemorrhage into the surrounding tubules (small arrows) and tubular necrosis (large arrow) can also be seen.

(HE X 250)





Small poorly-defined focal interstitial infiltrates consisting of macrophages, large lymphoid cells and a few plasma cells were found in the kidneys of dogs examined on days 7, 8 and 9. These were found in the cortex, often in close association with small blood vessels and sometimes around collecting tubules in the outer medulla. Numerous larger foci of interstitial nephritis were present in the kidneys of all dogs examined from the 10th day onwards. Interstitial foci were found in both cortex and medulla (Figs. 36 and 37); the cortical lesions often appeared as streaks of cellular infiltration running from outer cortex towards the medulla, following the course of one or more collecting tubules. The cellular infiltrates consisted mainly of large lymphoid cells, macrophages and plasma cells (Fig. 41) and, particularly in those animals examined on days 17, 18 and 19, these cells showed a high mitotic rate. Tubules lying within the lesions showed varying degrees of degeneration and necrosis and in 5 animals, inclusion bodies were found in tubular epithelial cells (Fig. 38). Completely necrotic tubules appeared as deeply eosinophilic clumps of coalesced cytoplasm containing remnants of pyknotic nuclei; the cellular infiltrates surrounding such tubules contained large numbers of polymorphonuclear leukocytes. In other tubules, the epithelial cytoplasm was swollen, vacuolated and somewhat ragged in appearance and necrotic cellular debris was often found in the lumen. In addition, small lymphocytes and polymorphonuclear leukocytes were sometimes observed migrating through the tubular epithelium and lying free within the lumen (Fig. 39). A small number of mitotic figures were commonly observed in surrounding intact tubules.

In general terms, the medullary cellular lesions contained more numerous necrotic tubules; associated with these, the surrounding cellular infiltrates were composed of greater numbers of polymorphonuclear leukocytes and macrophages but fewer plasma cells (Fig. 40). When the

medullary tubules were observed in transverse section, a distinctive orientation of the cells in the interstitial lesions could often be discerned; this consisted of a central necrotic tubule, in which inclusions were sometimes present, surrounded by an inner zone of polymorphonuclear leukocytes and an outer zone composed largely of macrophages with a few lymphoid cells and plasma cells (Fig. 42). Neighbouring tubules frequently contained abundant necrotic cellular debris and in some cases, inclusions were seen in intact tubular epithelium and in cells lying free within the tubules (Fig. 43).

In the 3 dogs examined 25 and 27 days after inoculation with virus, the cellular infiltrates in both cortex and medulla contained a much higher proportion of plasma cells, there were fewer necrotic tubules and polymorphonuclear leukocytes were not so numerous. In addition, some of the interstitial foci had become more sparsely populated with cells but contained increased numbers of fibroblasts and showed evidence of early interstitial fibrosis. In all dogs examined from day 7 onwards, loose aggregates of lymphocytes, macrophages and polymorphonuclear leukocytes were also found in the sub-pelvic connective tissue; however, inclusions were never found in association with these lesions.

The histological picture presented by the 3 in contact control dogs was similar to that of inoculated animals examined in the early stages of the experiment. There was focal hepatic necrosis with inclusion bodies present in the liver and vascular endothelium of a variety of other organs. In the kidneys, there was swelling of the cellular components of the glomeruli and inclusion bodies were found in mesangial and endothelial cells. In 2 of the dogs, (Nos. 81 and 82) there was also expansion and increased cellularity of the mesangial regions of the glomeruli and moderately increased numbers of polymorphonuclear

leukocytes were present in the capillary loops.

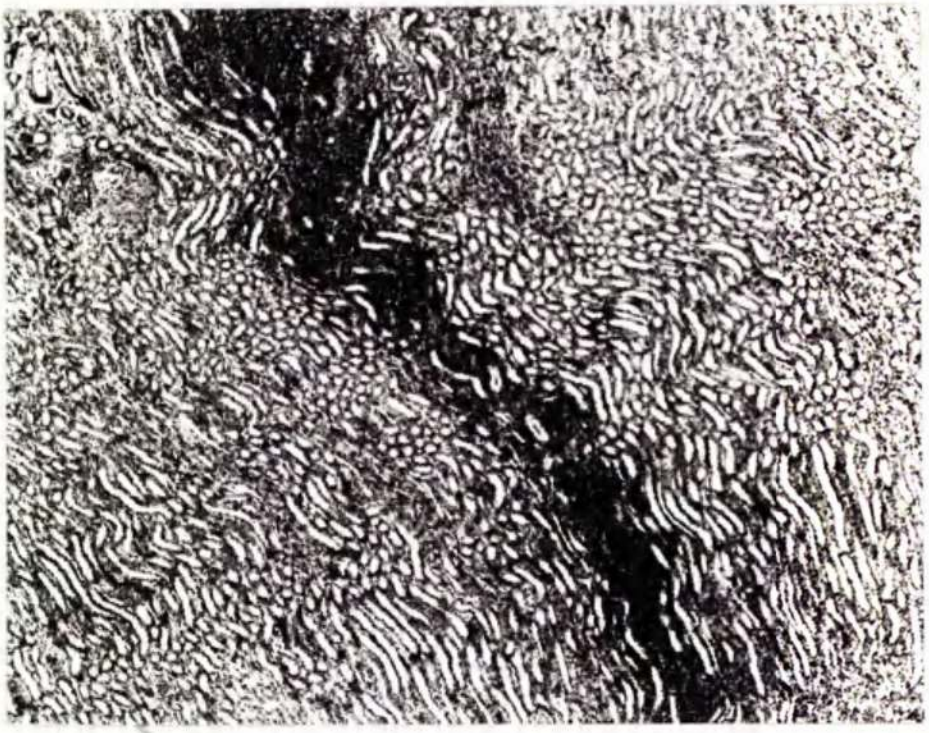
The 8 control dogs which received uninfected tissue culture suspension showed no histological abnormalities in the kidney and elsewhere.

Fig. 36:- Section of kidney from infected dog examined 18 days after administration of virus: A focus of interstitial nephritis can be seen extending from deep cortex into the outer medulla.

(HE X 50)

Fig. 37:- Section of renal medulla from infected dog examined 18 days after administration of virus: The tubules are cut transversely showing numerous foci of interstitial nephritis.

(HE X 60)



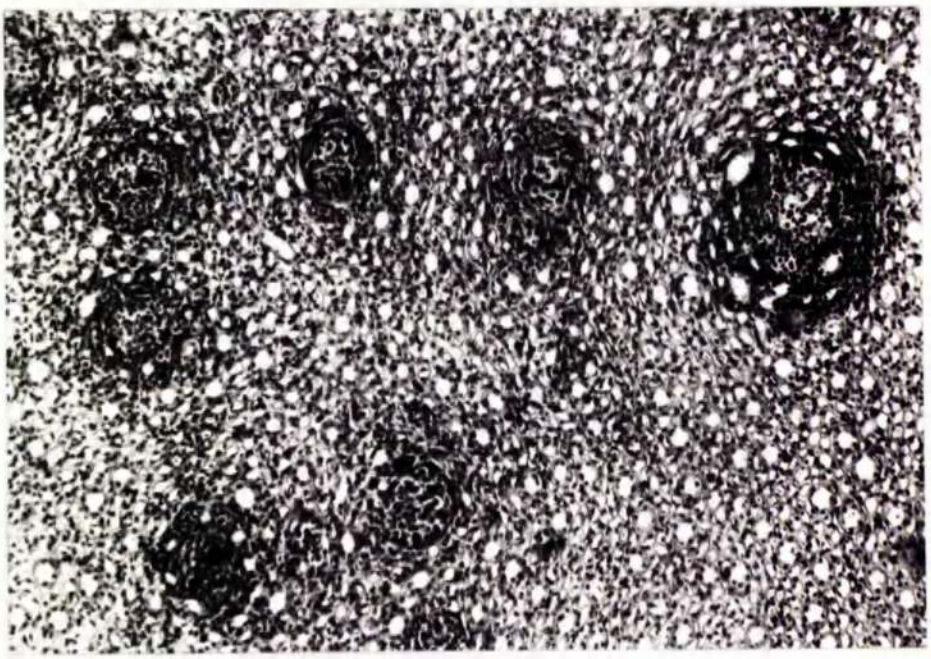
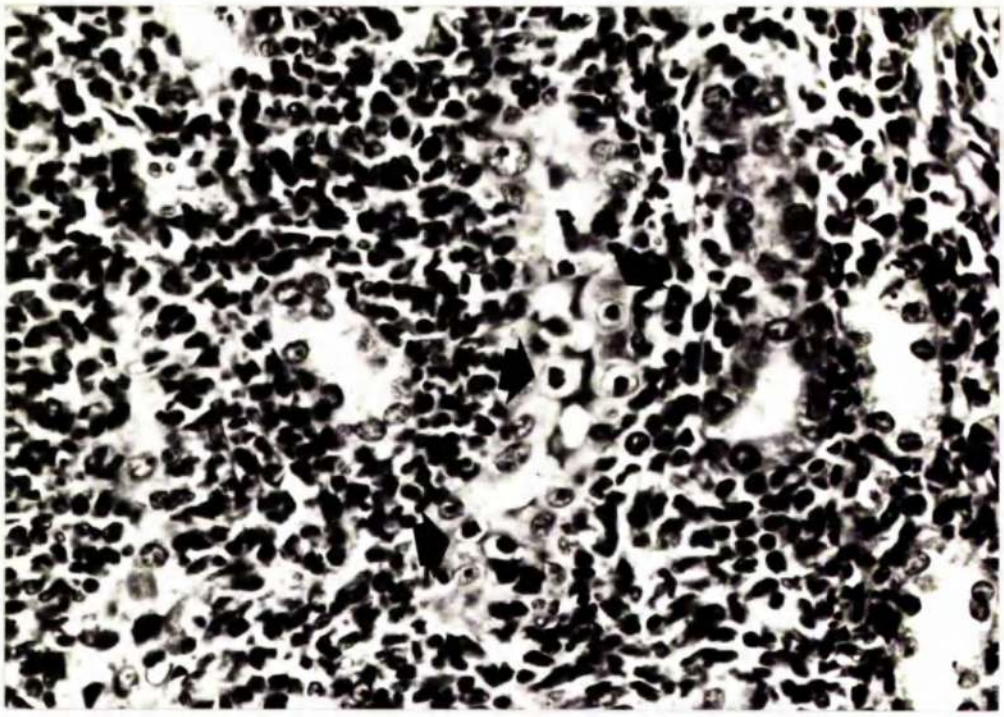


Fig. 38:- CAV interstitial nephritis, 19 days after administration of virus: Within this cortical focus, inclusion bodies (arrows) can be seen in tubular epithelial cells.

(HE X 350)

Fig. 39:- CAV interstitial nephritis, 17 days after administration of virus: In the centre of the photomicrograph, a longitudinal section of part of a medullary tubule can be seen containing lymphocytes and polymorphonuclear leukocytes lying among necrotic cellular debris.

(Mallory's borax methylene blue, 1$_{\mu}$ x 400)



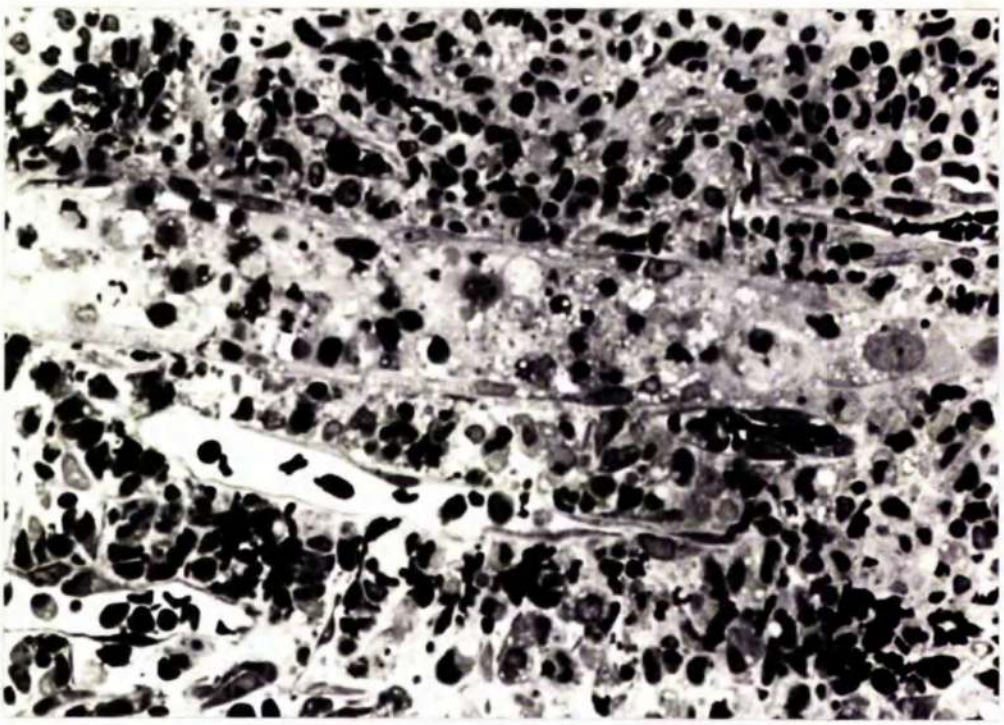
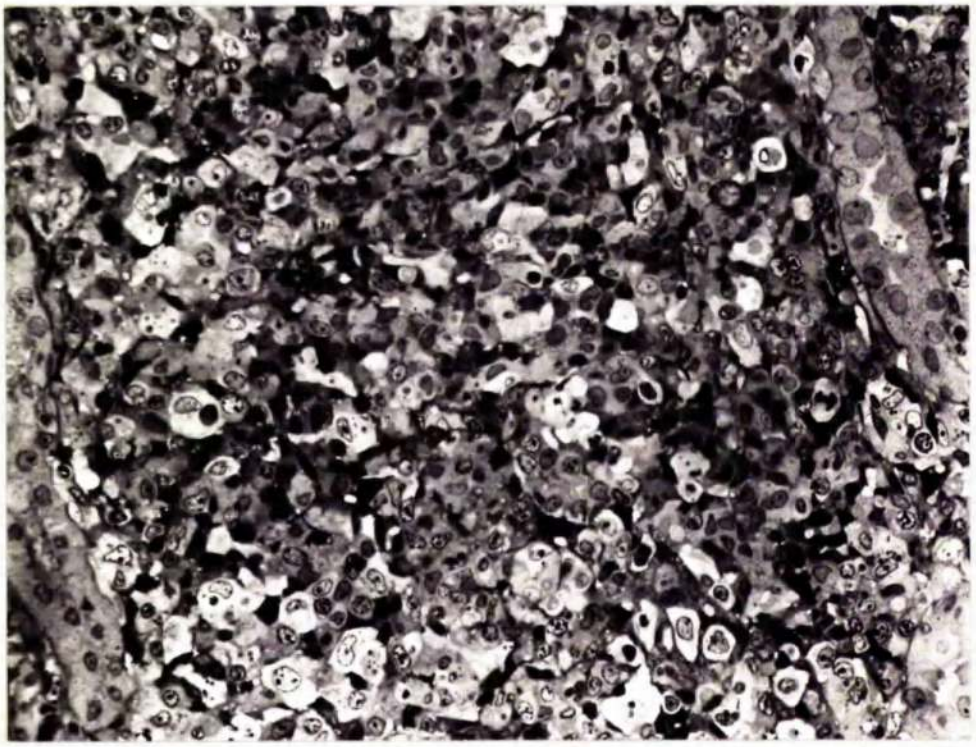


Fig. 40:- CAV interstitial nephritis, 19 days after administration of virus: This focus, which is located in the outer medulla, is composed mainly of macrophages and large lymphoid cells with only a few small lymphocytes and plasma cells.

(Mallory's borax methylene blue, 1μ section X 300)

Fig. 41:- CAV interstitial nephritis, 19 days after administration of virus: In this cortical lesion, the cellular infiltrate is composed mainly of small lymphocytes and plasma cells.

(Mallory's borax methylene blue, 1μsection X 400)



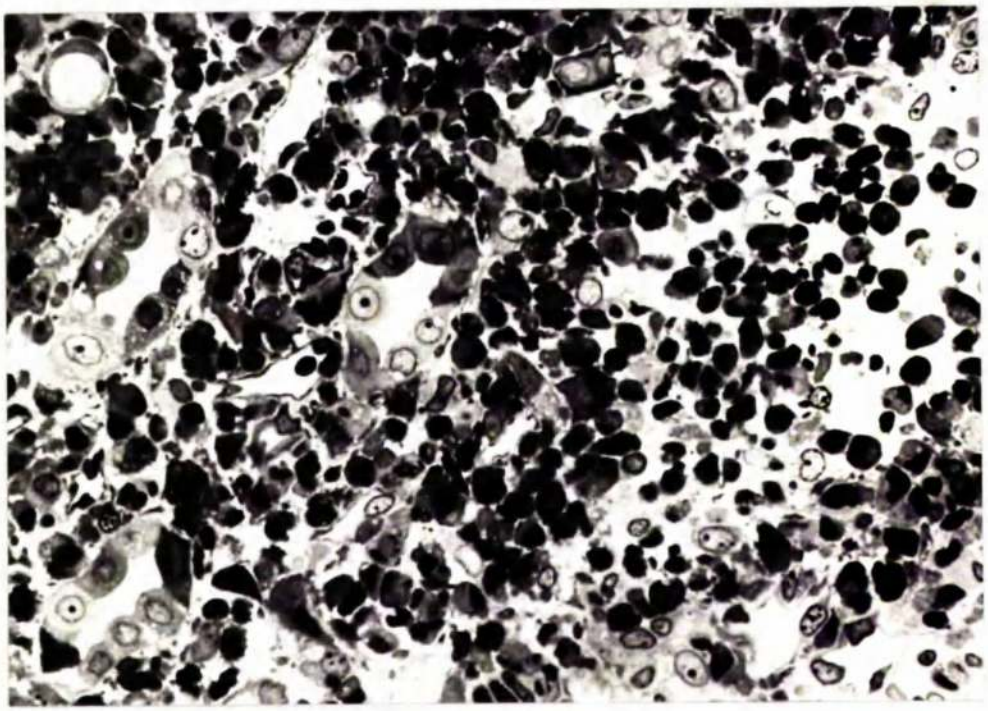
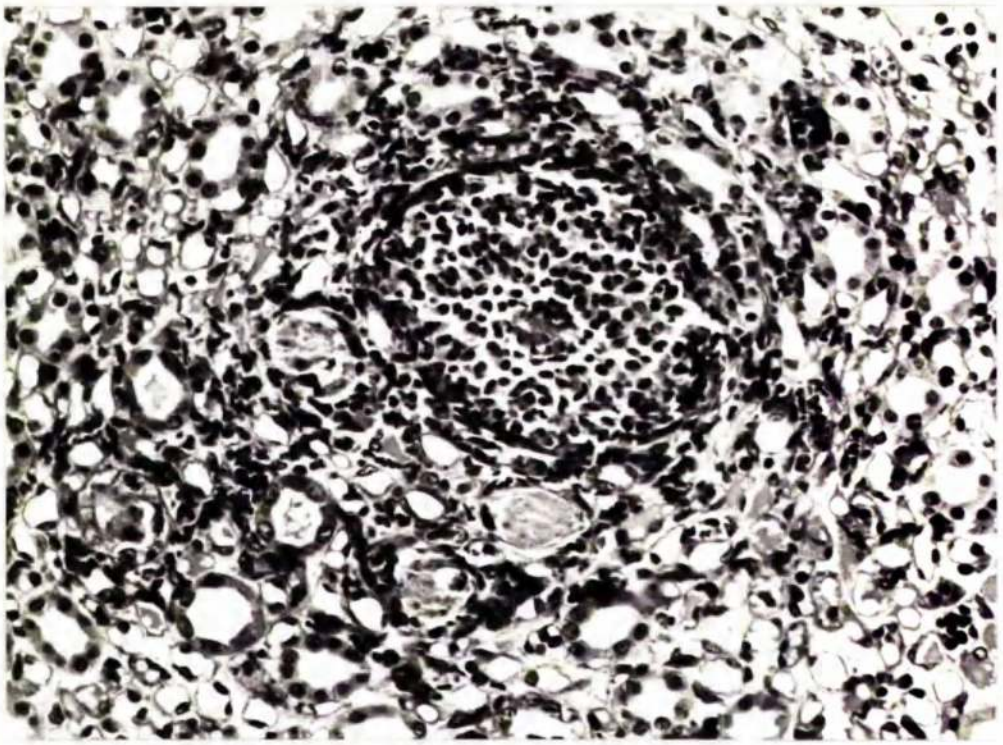


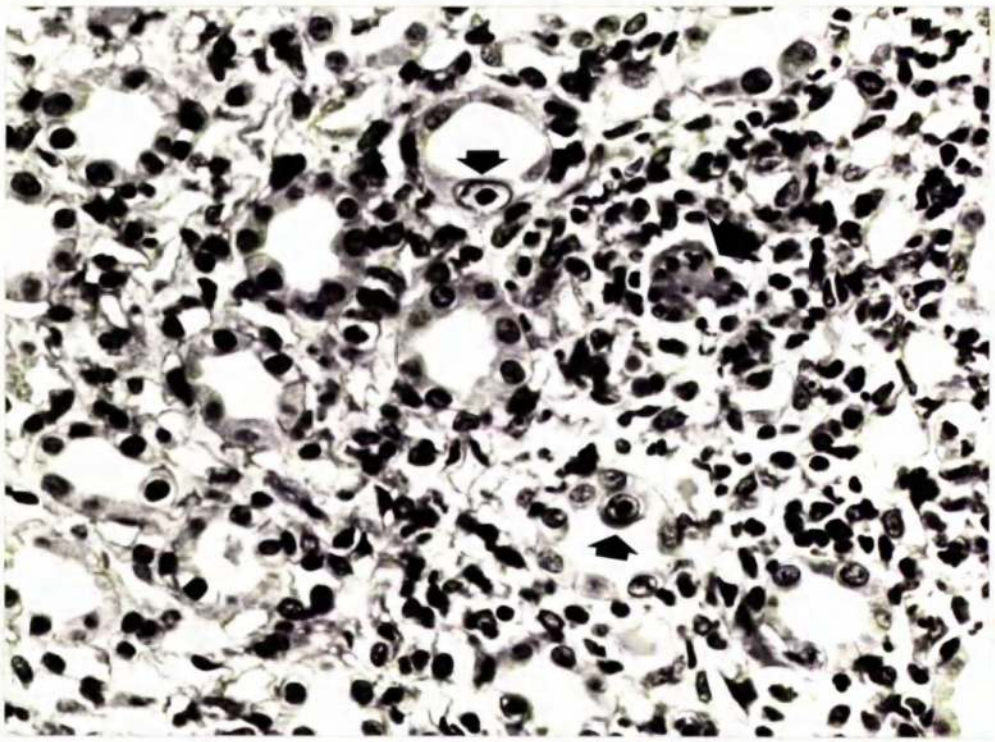
Fig. 42:- CAV interstitial nephritis, 18 days after administration of virus. A characteristic medullary focus of interstitial nephritis, cut in transverse section, shows a central necrotic tubule containing a few polymorphonuclear leukocytes surrounded by large accumulations of macrophages.

(HE X 300)

Fig. 43:- CAV interstitial nephritis, 18 days after administration of virus: Intranuclear inclusion bodies (small arrows) are present in the epithelium of medullary collecting tubules; a necrotic tubule (large arrow) can also be seen.

(HE X 400)





Immunofluorescence Findings

The immunofluorescence patterns are summarised in Table 11. Using FITC labelled anti-CAV serum, numerous fluorescing virus-infected cells were detected in the liver of dogs examined from the 4th to the 8th day following inoculation of virus; occasional infected cells were also found in the liver of 1 animal examined on day 9. When kidneys from infected dogs were stained for the presence of CAV antigen, discrete fluorescing cells were detected in the glomeruli of all dogs up to 9 days after administration of virus (Fig. 44); a small number of fluorescing cells were also detected in extra-glomerular vascular endothelium. This pattern of fluorescence, therefore, corresponded to the distribution of inclusions on histological examination, although, in general terms, larger numbers of virus-infected cells were detected by immunofluorescence. In addition, however, fine granular deposits of CAV antigen were detected in the mesangial regions of the glomeruli of dogs examined on days 7, 8, 9 and 10 (Fig. 45). In 6 of the clinically recovered dogs examined from day 10 onwards, specific antigen fluorescence was detected in occasional scattered foci of tubular epithelial cells; these foci of virus antigen were found in both cortex and medulla (Figs. 50 and 51).

IgG was not detected in the kidneys of the dog examined on day 4. However, in all animals examined from day 5 to day 14 following inoculation of virus, diffuse granular deposits of IgG were detected in the mesangial regions of the glomeruli. In the early stages of the experiment, on days 5 and 6, the fluorescence was finely granular (Fig. 46); however, as the disease progressed, deposits of IgG became more abundant and took on a course clumpy appearance (Figs. 47 and 48). In animals examined on days 10 and 14, there was reduction in the amount of deposited IgG which once again appeared finely granular. Apart from non-specific segmental hilar fluorescence in some cases, deposits of IgG were not

found in the glomeruli of any of the animals examined after day 14. A few interstitial plasma cells also stained for IgG in kidneys of dogs examined 7, 8 and 9 days after inoculation of virus. From 10 days onwards, the kidneys of all dogs were found to contain larger interstitial accumulations of IgG positive plasma cells (Fig. 52). Although these were found mainly in the cortices, a number of small foci were also found in the medulla particularly in the dogs examined on days 25 and 27.

In the 4 animals whose kidneys were examined by the indirect "sandwich" technique, positive fluorescence was observed in many of the interstitial plasma cells, thus indicating the presence of anti-CAV antibody (Fig. 53).

Deposits of C3 were first detected in the glomeruli on day 6 and were found in the kidneys of all animals examined from 7 to 10 days after inoculation (Fig. 49); the deposits, which appeared as fine granular fluorescence in the mesangial regions, were most abundant in those animals with the heaviest deposits of IgG.

Numerous CAV infected cells were detected in the liver of the 3 in contact control dogs killed on day 10. Discrete virus infected cells were also found in the glomeruli of all 3 animals and in 2 animals (Nos. 81 and 82) fine granular deposits of virus antigen were detected in the mesangial regions of the glomeruli. Similar granular deposits of IgG and C3 were found in the glomeruli of these 2 animals.

The kidneys of the remaining 8 control dogs showed no specific fluorescence for CAV antigen, IgG or C3.

Table 11:- Experimental CAV infection: Immunofluorescence patterns in the liver and kidneys

Dog No.	Day Examined	Liver CAV antigen	Glomeruli CAV antigen Discrete cellular	Glomeruli CAV antigen Fine granular	Glomeruli IgG	Glomeruli C3	Tubules CAV antigen	Plasma cells IgG
55	4	++	++	-	-	-	-	-
56	5	+++	++	-	+	-	-	-
57	5	+++	++	-	+	-	-	-
58	5	+++	++	-	+	-	-	-
59	5	+++	+++	-	+	-	-	-
60	6	++	++	-	++	+	-	-
61	6	+++	+++	-	++	-	-	-
62	6	+++	+++	-	++	+	-	-
63	7	+++	++	+	+++	+	-	-
64	7	+++	+++	+	+++	++	-	+
65	8	++	++	+	+++	++	-	+
66	8	++	++	+	++++	++	-	+
67	8	++	++	+	+++	++	-	+
68	8	+	+	+	+++	++	-	+
69	9	+	++	+	++++	++	-	+
70	9	-	+	+	+++	++	-	+
71	9	-	+	+	+++	++	-	+

Continued on next page.....

Table 11/continued

Dog No.	Day Examined	Liver CAV antigen	Glomeruli CAV antigen Discrete cellular	Glomeruli CAV antigen Fine granular	Glomeruli IgG	Glomeruli C3	Tubules CAV antigen	Plasma Cells IgG
72	10	-	-	+	++	+	++	++
73	14	-	-	-	+	-	-	++
74	17	-	-	-	-	-	++	++++
75	18	-	-	-	-	-	+	+++
76	19	-	-	-	-	-	++	++++
77	25	-	-	-	-	-	+	+++
78	25	-	-	-	-	-	+	+++
79	27	-	-	-	-	-	-	++
80	10 c	+	++	-	-	-	-	-
81	10 c	+	++	+	+++	++	-	-
82	10 c	+	+	+	++	+	-	-

Degree of fluorescence graded + to ++++

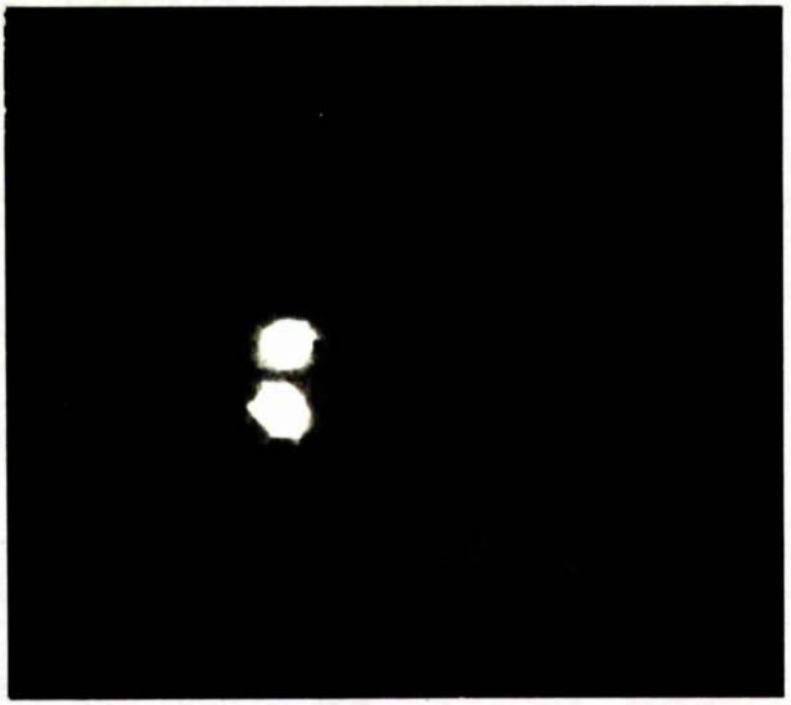
c = In contact controls

Fig. 44:- CAV antigen in glomerulus of dog examined 5 days after inoculation of virus: Two discrete virus-infected cells can be seen in the glomerulus.

(Immunofluorescence X 400)

Fig. 45:- CAV antigen in glomerulus of dog examined 8 days after inoculation of virus: Granular deposits of viral antigen are present throughout the mesangial regions; one discrete fluorescing cell can also be seen.

(Immunofluorescence X 400)



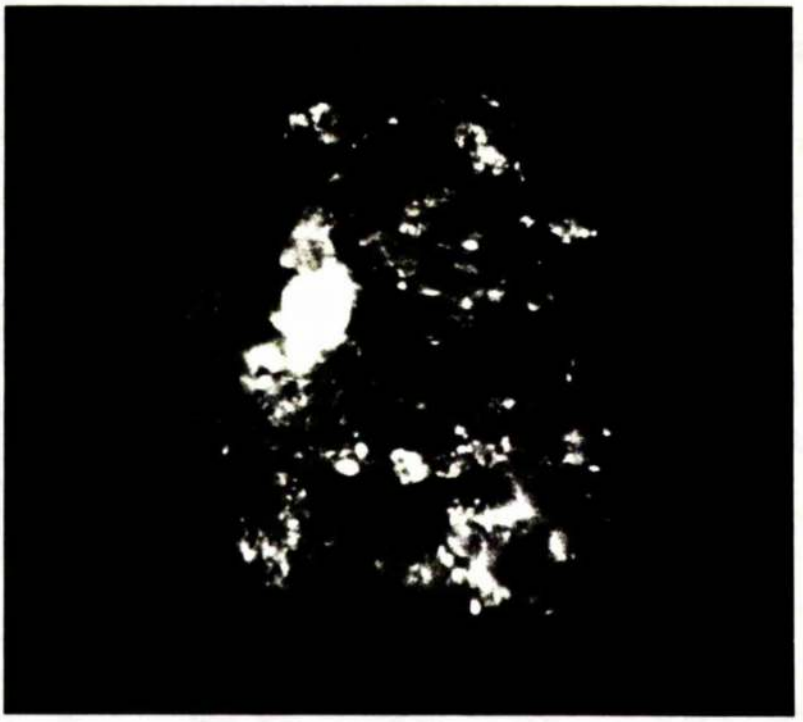


Fig. 46:- IgG in glomerulus of dog examined 5 days after inoculation of virus: fine granules of fluorescence can be seen in the mesangial regions of the glomerulus.

(Immunofluorescence X 400)

Fig. 47:- IgG in glomerulus of dog examined 9 days after inoculation of virus: Coarse granular fluorescence can be seen in the mesangial regions.

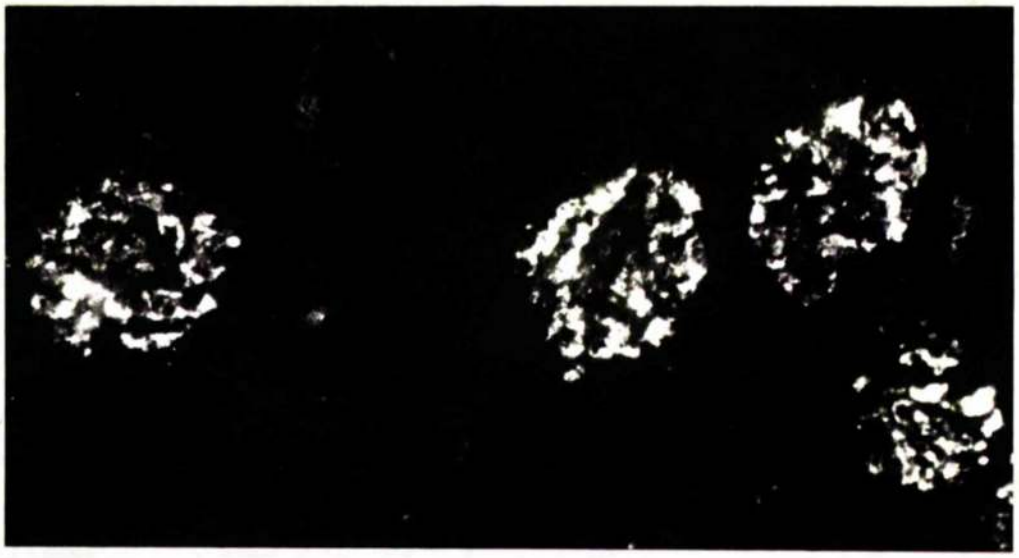
(Immunofluorescence X 400)

Fig. 48:- IgG in glomeruli of dog examined 9 days after inoculation of virus: granular deposits of IgG are found diffusely in all glomeruli.

(Immunofluorescence X 400)

Fig. 49:- Complement (C3) in glomerulus of dog examined 9 days after inoculation of virus: The distribution of granular fluorescence is the same as that observed for IgG.

(Immunofluoresence X 400)



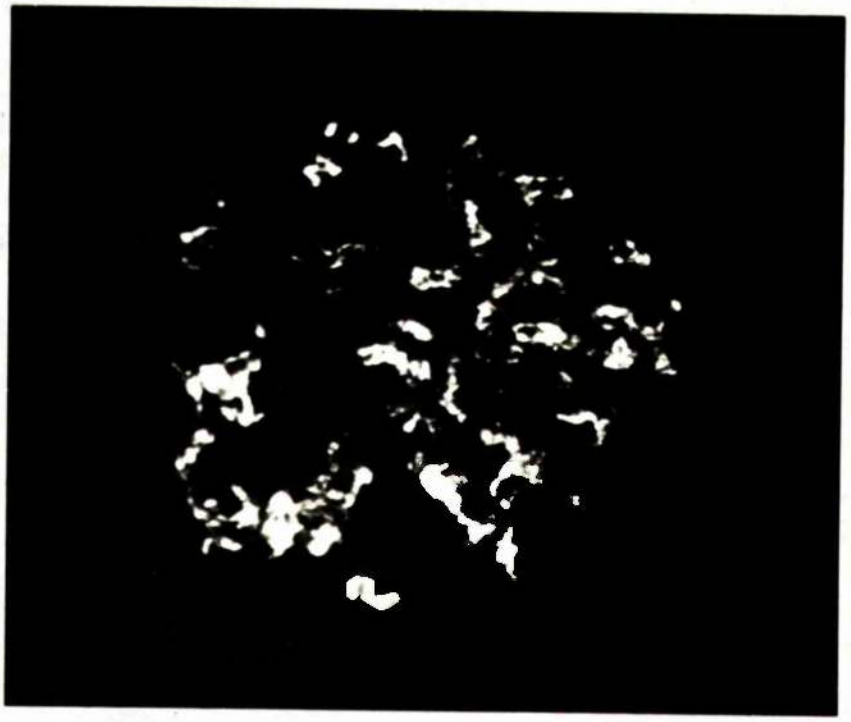


Fig. 50:- CAV antigen in the kidney of a dog examined 19 days after inoculation of virus: A longitudinal section of part of a collecting tubule shows specific fluorescence for virus antigen.

(Immunofluorescence X 400)

Fig. 51:- Transverse section of medullary collecting tubule from the same animal, showing specific fluorescence for virus antigen in the tubular epithelium.

(Immunofluorescence X 600)

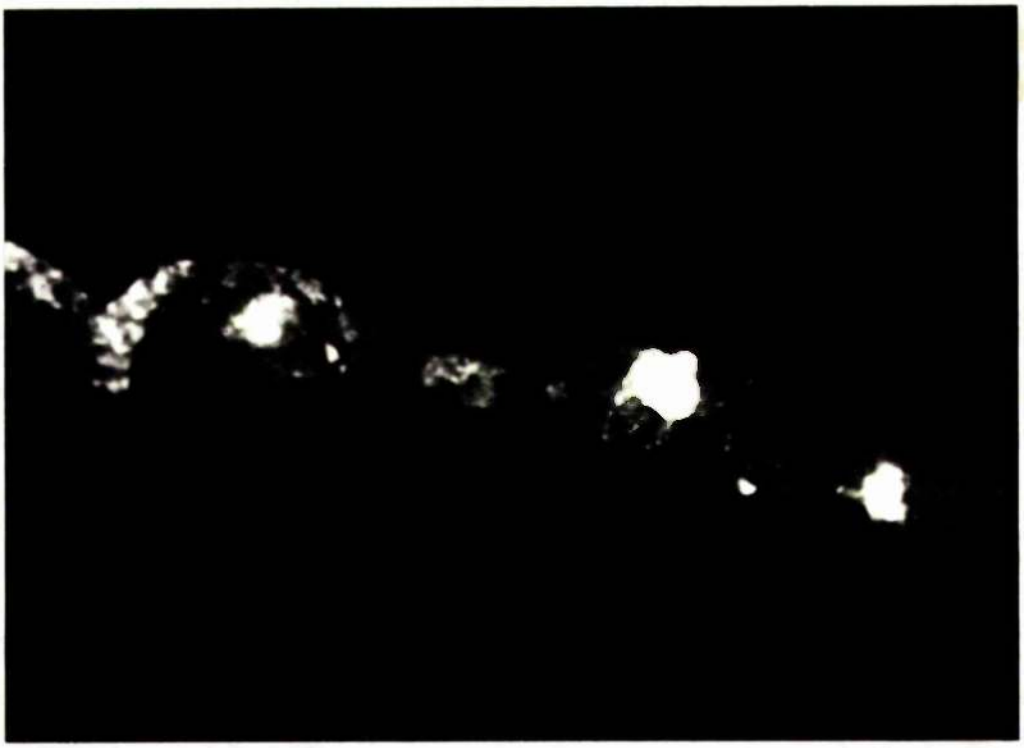
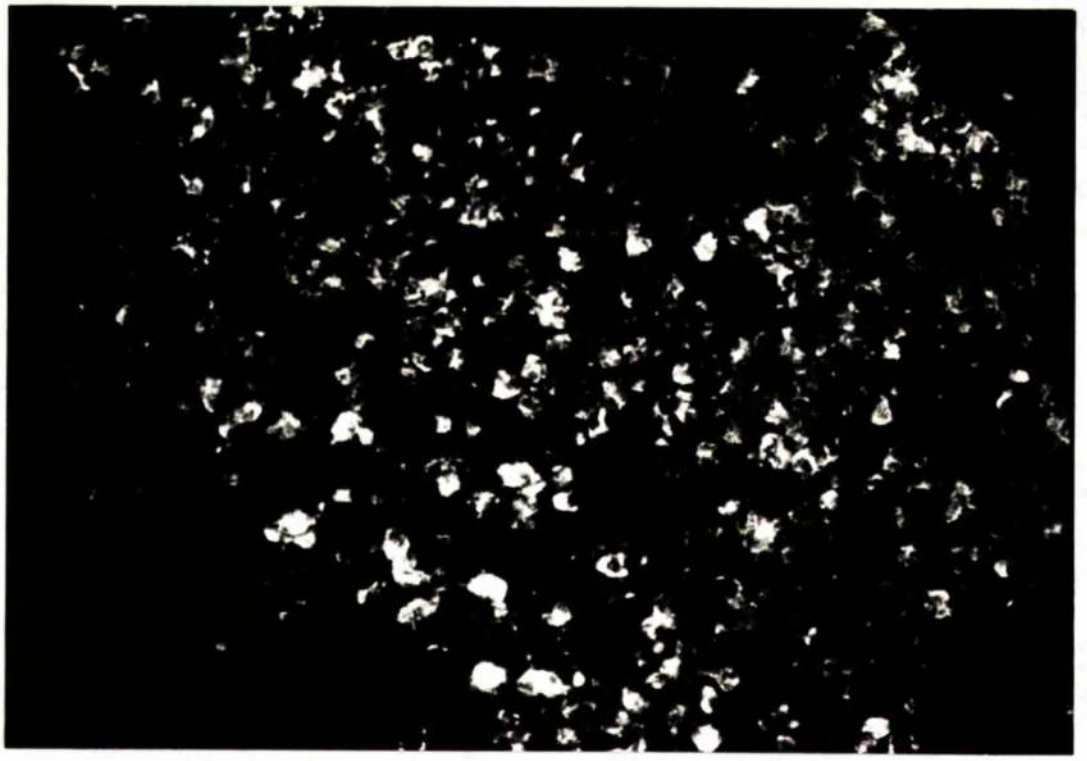


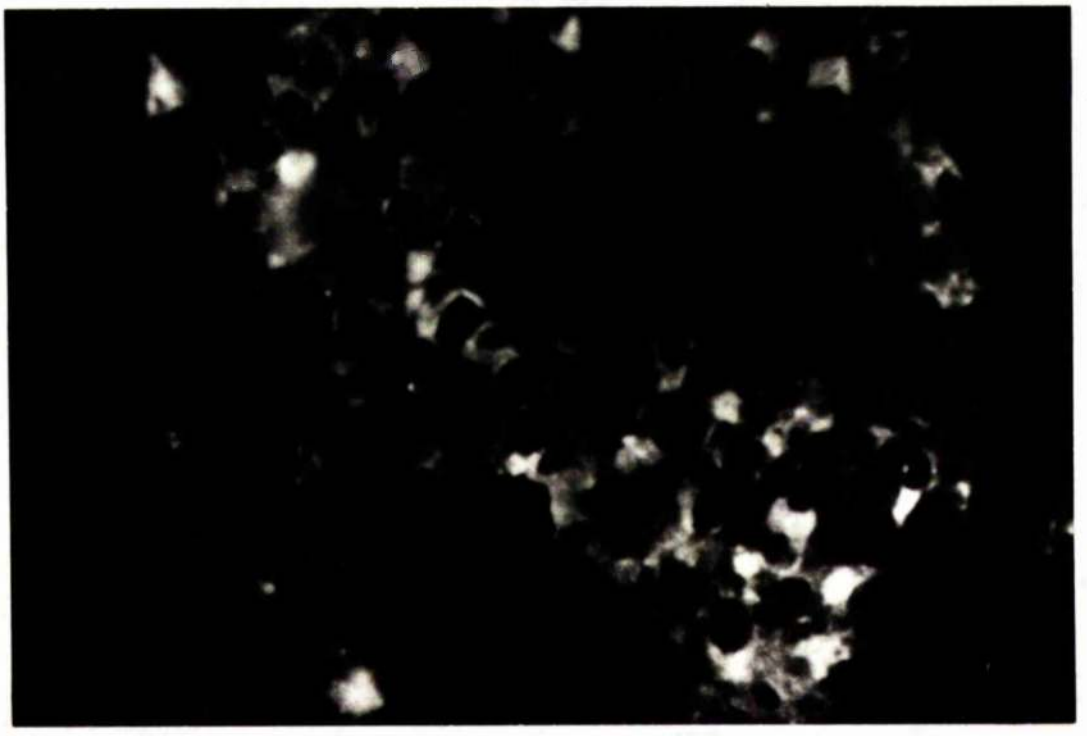
Fig. 52:- CAV interstitial nephritis, 19 days after administration of virus: Numerous IgG staining plasma cells can be seen in cortical cellular infiltrates.

(Immunofluorescence X 250)

Fig. 53:- CAV interstitial nephritis, 19 days after administration of virus: Section of kidney stained by an indirect immunofluorescence test for the presence of anti-viral antibody: Cytoplasmic fluorescence can be seen in a focus of plasma cells.

(Immunofluorescence X 400)





Ultrastructural Findings

Marked ultrastructural changes were observed in the glomeruli of all animals examined up to 14 days after inoculation of virus. In the early stages of the experiment, on days 5 and 6, there was marked swelling and vacuolation of all the glomerular cell types resulting in partial or complete occlusion of the capillary lumina. The thin endothelial lining of capillary loops was frayed and often had partially detached from the GBM. A few capillary loops contained polymorphonuclear leukocytes or large mononuclear cells. Swollen mesangial cells bulged into the axial regions of the loops and, even at this early stage, there was evidence of expansion of mesangial matrix and, in some instances, an increase in the number of mesangial cells. Small electron-dense deposits were found scattered throughout the mesangial matrix; these deposits were not observed in subendothelial or subepithelial situations. The foot processes of swollen epithelial cells showed partial fusion but at this stage the GBM appeared normal.

From day 7 to day 10 the glomerular changes were much more severe. The main feature of affected glomeruli at this stage was the presence of large irregular electron-dense deposits scattered throughout the mesangial regions (Fig. 54). These deposits extended into the axial regions of the capillary loops and at these sites smaller deposits were occasionally observed beneath swollen endothelium. Associated with the deposits, there was a marked increase in mesangial cellularity and expansion of mesangial matrix which resulted in almost complete occlusion of many capillary loops (Fig. 55). A striking variation in the morphology of mesangial cells was noted. Many appeared to be in the process of phagocytosing surrounding deposits; in these cells, the nuclei often showed accentuated lobulation and the cytoplasm contained numerous mitochondria and abundant endoplasmic reticulum. In addition, round homogenous electron dense granules and irregular granular clumps

of necrotic cell debris were frequently observed in mesangial cell cytoplasm. Sometimes, alongside these active mesangial cells, other discrete cells with poorly electron dense cytoplasm containing fewer organelles were found. Many mesangial cells showed evidence of degeneration ranging from mild cytoplasmic swelling and vacuolation to, in a few instances, frank cellular necrosis. At this stage, the glomerular endothelium had also undergone much more severe degenerstive changes; there was marked swelling and vacuolation of endothelial cytoplasm and irregular electron dense necrotic cells were often observed partially detached from the under-lying GBM. At such sites, strands of fibrin were sometimes present within the capillaries and occasionally in the filtration spaces (Fig. 57). In severely affected glomeruli, erythrocytes were found in the mesangial regions and less frequently in the filtration spaces. The number of polymorphonuclear leukocytes found in the glomeruli increased from day 7 onwards, reaching a peak on day 9 at which time they were detected in large numbers lodged in the glomerular capillaries and occasionally in the mesangium (Fig. 56). Large mononuclear leukocytes were also observed with relative frequency in the capillary loops. The polymorhonuclear leukocytes were often altered in appearance; their complement of cytoplasmic granules was markedly reduced and the cytoplasm was sometimes pale, swollen and vacuolated. The glomerular epithelial cells showed an increase in cytoplasmic organelles and marked cytoplasmic swelling often resulted in extensive fusion of foot processes. In animals examined 9 and 10 days after inoculation, degeneration of epithelial cells was sometimes noted; such cells had extremely pyknotic nuclei and their cytoplasm was shrunken and extremely electron dense (Fig. 58). Mitotic figures were occasionally encountered in endothelial and mesangial cells of dogs examined on days 8, 9 and 10 (Fig. 59).

Virus particles were found in the glomeruli of all dogs examined up to and including day 9. On days 5 and 6, virus was detected in the nuclei of mesangial cells and occasionally endothelial cells and in mononuclear cells lying free within the capillary loops. In the later stages, from day 7 onwards, virus particles were confined mainly to mesangial cells (Fig. 60). Infected cell nuclei were swollen and often contained a central granular, moderately electron dense area with a surrounding pale zone, outside which the chromatin had become marginated to a narrow electron dense band on the inner aspect of the nuclear membrane. The cytoplasm was also markedly swollen, poorly electron dense and sometimes vacuolated. In such cells, virus particles were usually found scattered throughout the nucleus. In dogs examined from day 7 onwards, many infected cell nuclei showed a diffuse pale appearance and contained only a very small central matrix; in these cells, virus particles were found mainly in the periphery of the nucleus beneath the nuclear membrane. Infected cells were frequently observed in various stages of degeneration, showing disruption of nuclear and cytoplasmic membranes with release of virus particles into the cytoplasm and surrounding tissue. Small pockets of virus particles were sometimes found within membrane bound vesicles in the cytoplasm of otherwise apparently uninfected mesangial cells (Fig. 61). On a few occasions, an intact degenerating virus infected cell was observed within the cytoplasm of another mesangial cell (Fig. 62). Only on rare occasions were virus particles located within electron dense deposits in the mesangial matrix (Fig. 63).

In dog No. 73, which was examined on day 14, the glomerular ultrastructural changes were much less severe. The cellular components of the glomeruli no longer showed the same degree of swelling and only occasional polymorphonuclear leukocytes were found within the capillary

loops, many of which at this stage were fully patent. The mesangial regions, however, still showed appreciable hypercellularity and increased matrix and occasional small electron dense deposits could still be seen in the mesangial matrix. In addition, electron-dense necrotic endothelial cells were sometimes observed detached from the GBM and being replaced by adjacent healthy endothelial cells. Similar changes were also observed in occasional visceral epithelial cells.

The glomeruli of all dogs examined from day 17 to the end of the experiment, showed only minor ultrastructural changes. There was some expansion of mesangial matrix which impinged on many of the capillary loops, causing slight occlusion of their lumina; occasional foci of increased mesangial cells were also noted. However, in none of these animals were virus particles or electron dense deposits found in the glomeruli.

Fig. 54:- Section of glomerulus from an infected dog examined 9 days after administration of virus. Numerous large, irregular, electron-dense deposits (arrows) can be seen in the mesangial regions. M = Mesangial cell; Ep = Epithelial cell; E = Endothelial cell.

(Electron microscopy X 10,000)

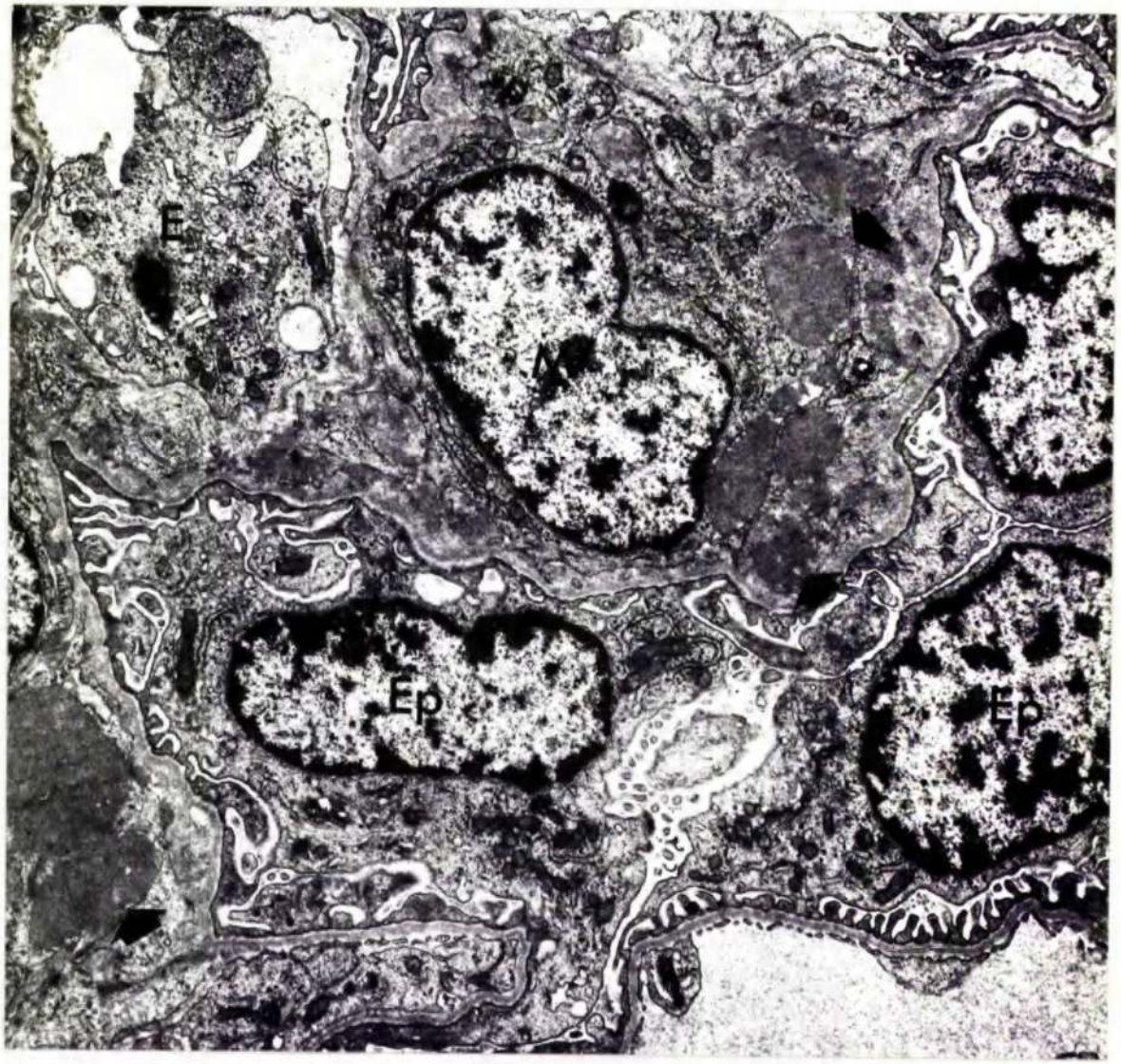

E
M
Ep
Ep

Fig. 55:- Section of glomerulus from an infected dog examined 8 days after administration of virus: Four mesangial cells (M) can be seen in this hypercellular mesangial focus. There is also fusion (arrows) of the epithelial cell (Ep) foot processes.

(Electron microscopy X 10,000)

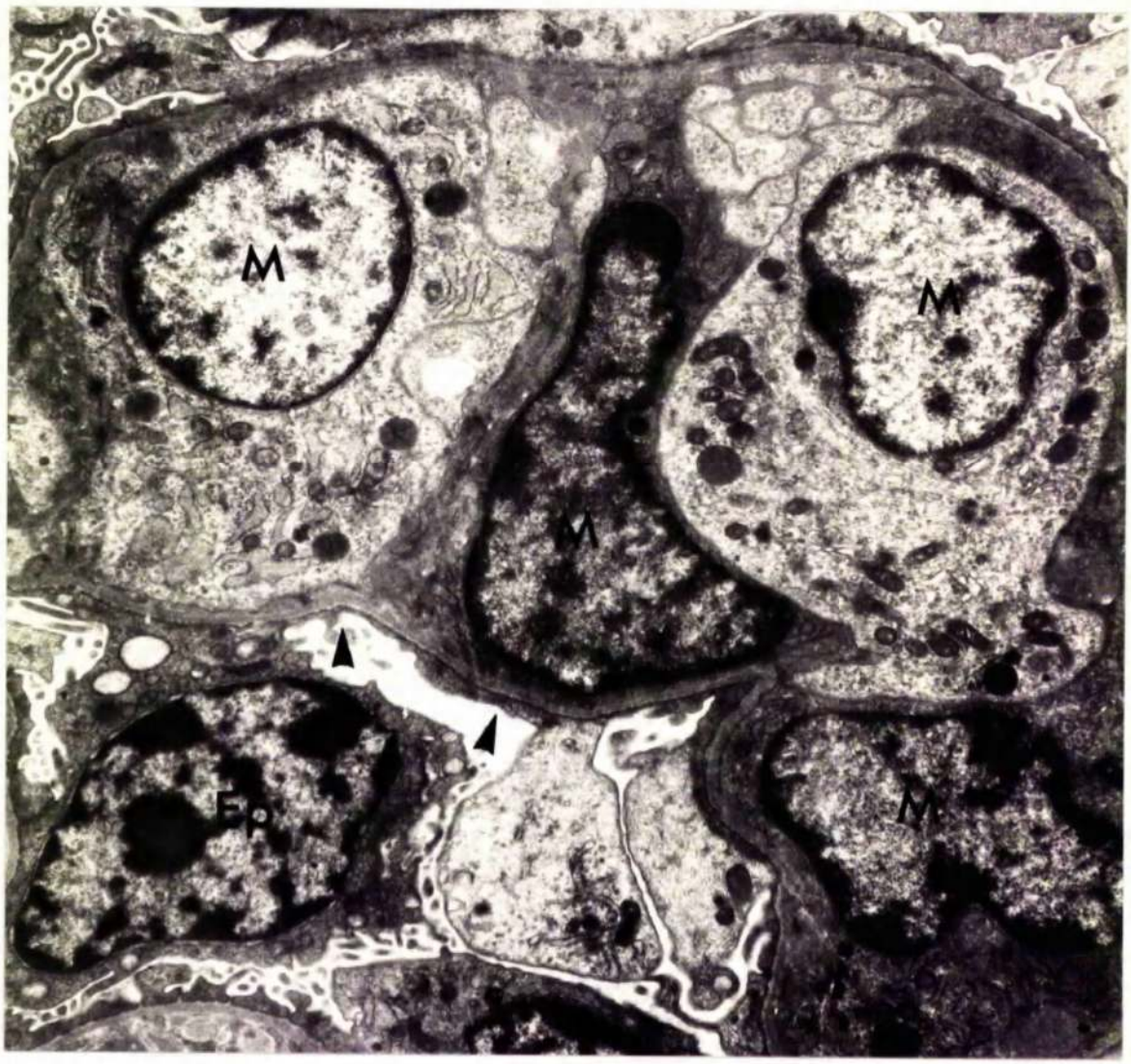
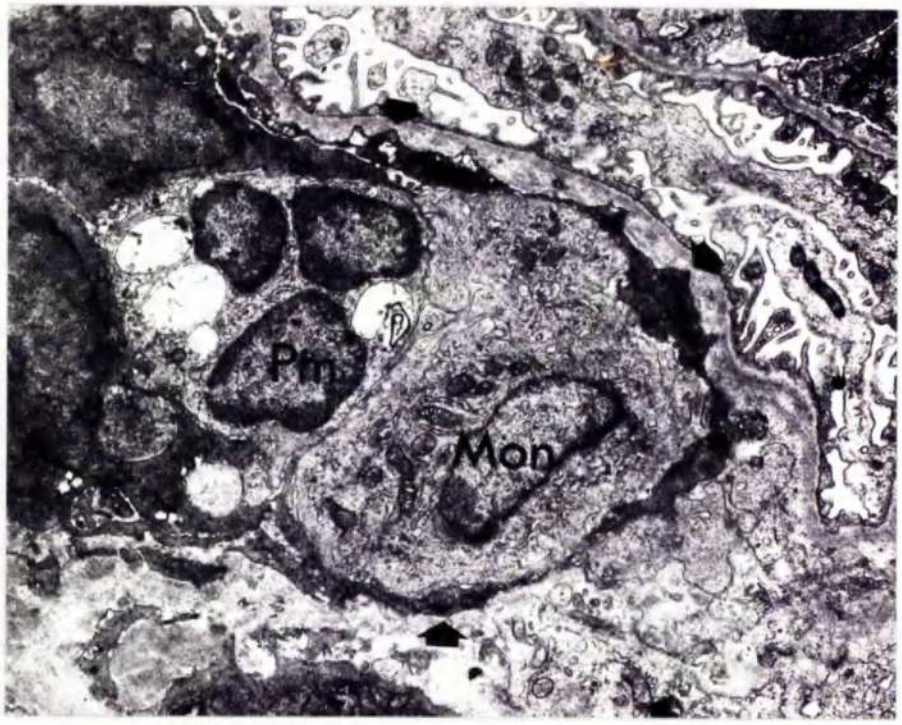

M
M
M
M
ER

Fig. 56:- Section of glomerulus from an infected dog examined 9 days after administration of virus: A capillary loop contains a mononuclear leukocyte (Mon) and a polymorphonuclear leukocyte (Pm);. the latter has discharged and its cytoplasm appears pale and vacuolated. There is also degeneration of the capillary endothelium (arrows) which appears electron-dense and vacuolated.

(Electron microscopy X 6,000)

Fig. 57:- Section of glomerulus from an infected dog examined 10 days after administration of virus, showing strands of fibrin (arrows) in the lumen of a capillary loop.

(Electron microscopy X 10,000)


Pm
Mon

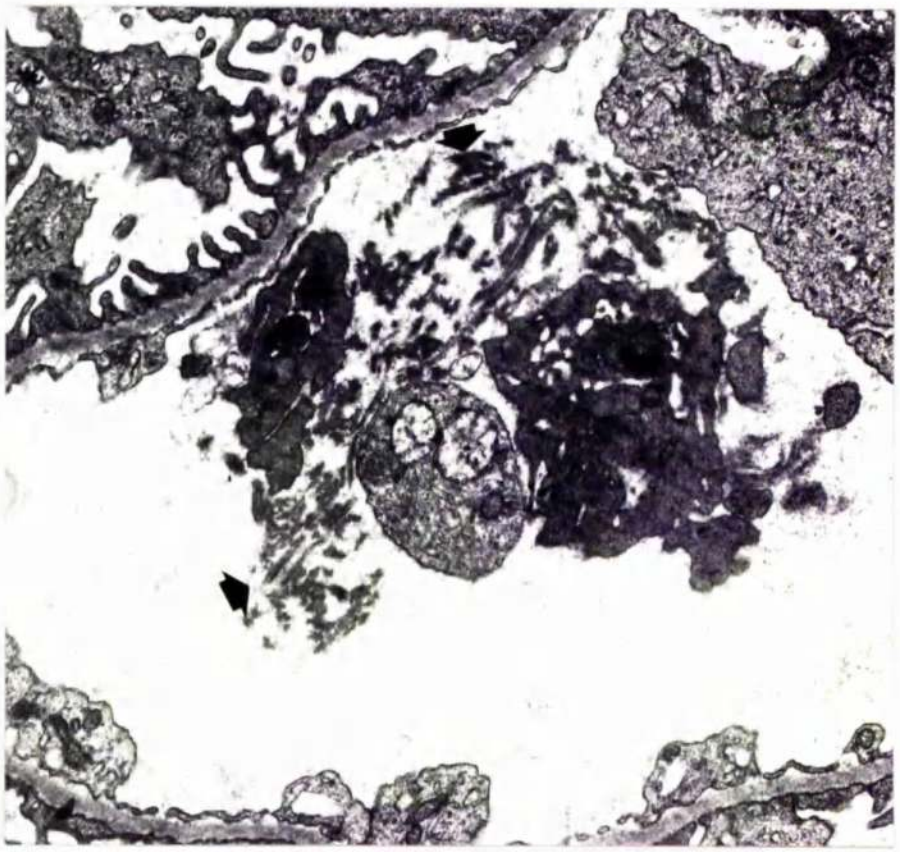
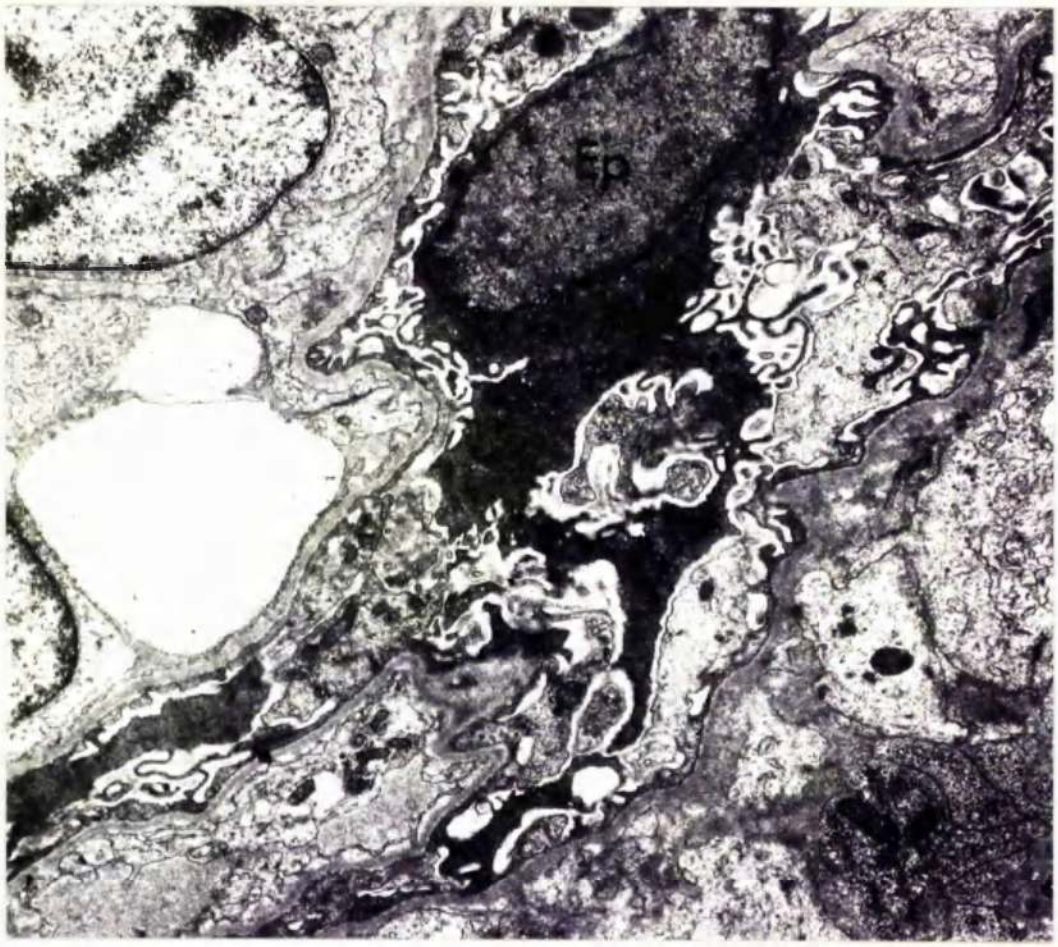


Fig. 58:- Section of glomerulus from an infected dog examined 10 days after administration of virus, showing a degenerating, shrunken, electron-dense epithelial cell (Ep).

(Electron microscopy X 10,000)

Fig. 59:- Section of glomerulus from an infected dog examined 10 days after administration of virus, showing an endothelial cell in the process of mitotic division.

(Electron microscopy X 10,000)


Ep

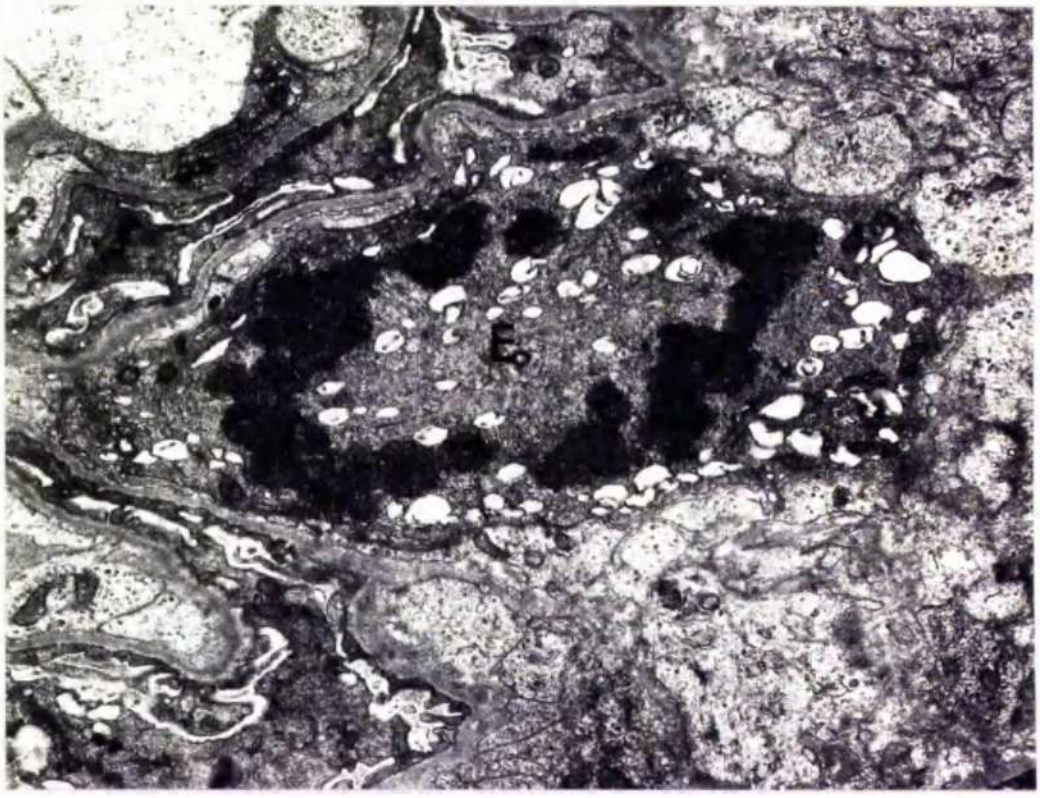
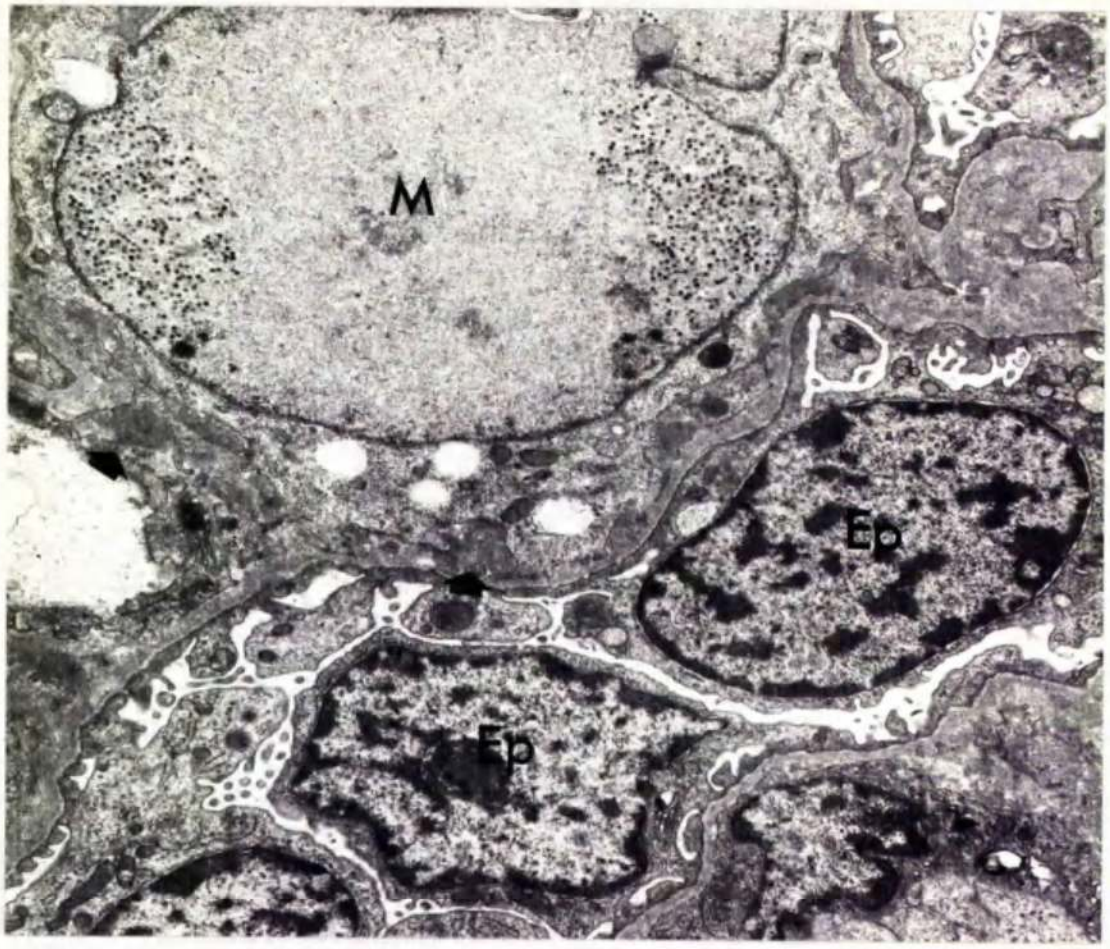

Ep

Fig. 60:- Section of glomerulus from an infected dog examined 7 days after administration of virus: Virus particles can be seen within the pale swollen nucleus of a mesangial cell (M). Small electron-dense deposits (arrows) are also present in the surrounding mesangial matrix. Ep = Epithelial cell.

(Electron microscopy X 10,000)

Fig. 61:- Section of glomerulus from an infected dog examined 8 days after administration of virus: Small clumps of virus particles (arrows) can be seen within membrane-bound vesicles in the cytoplasm of an otherwise uninfected mesangial cell (M).

Electron microscopy X 10,000)


M
Ep
Ep

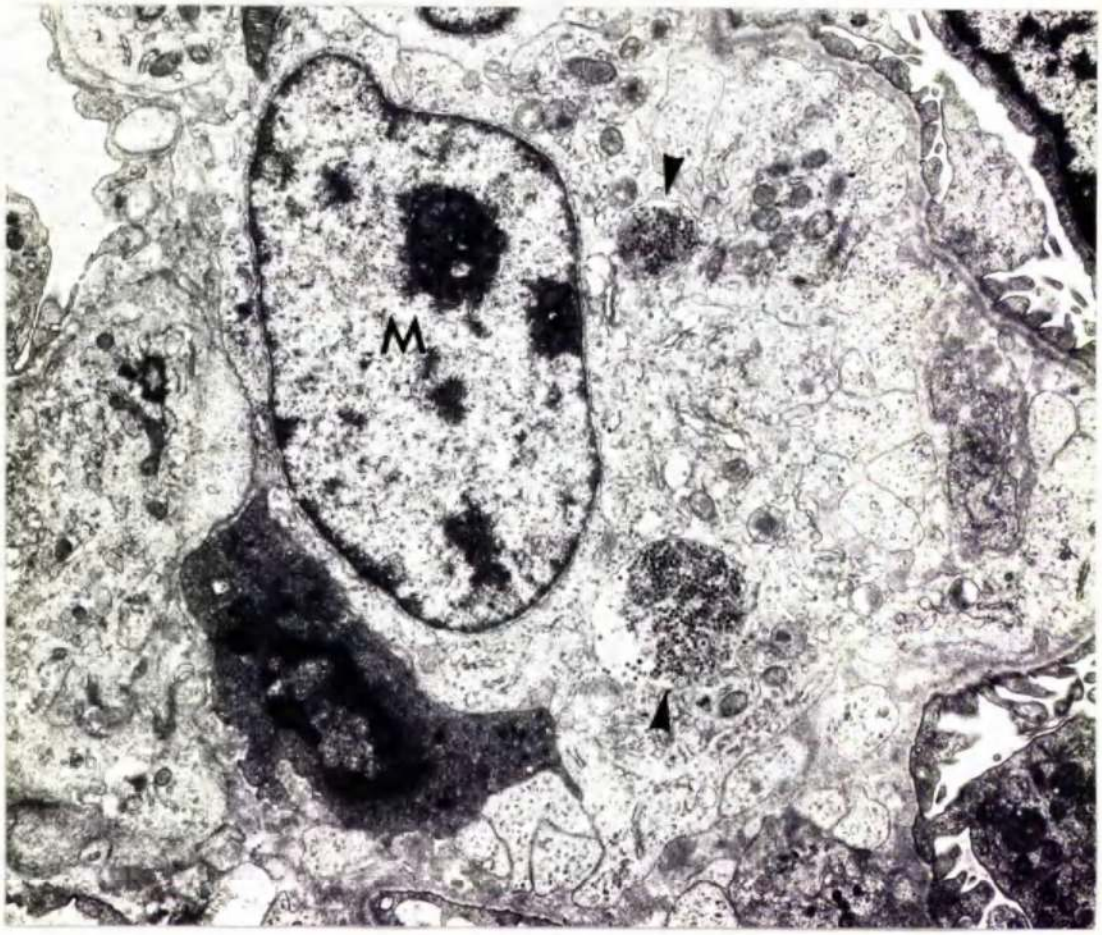
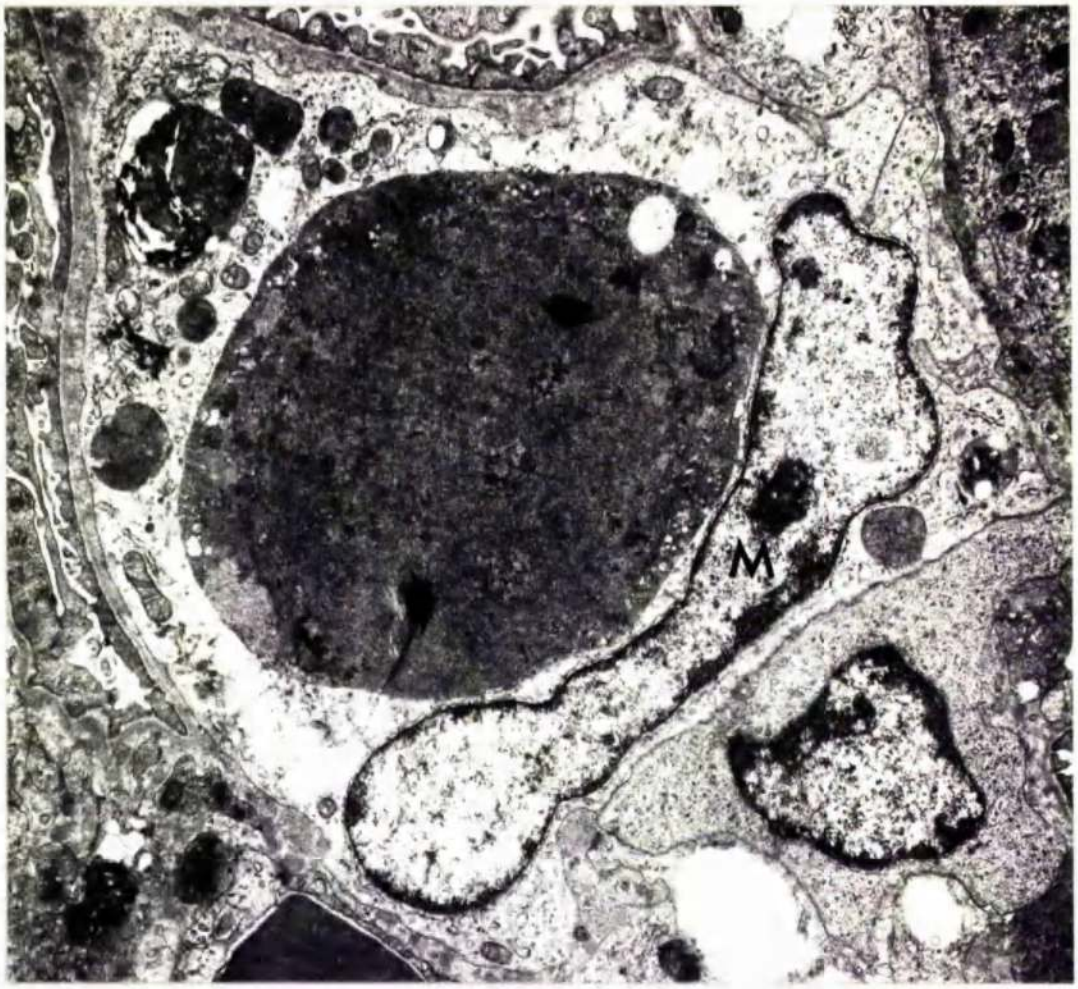

M

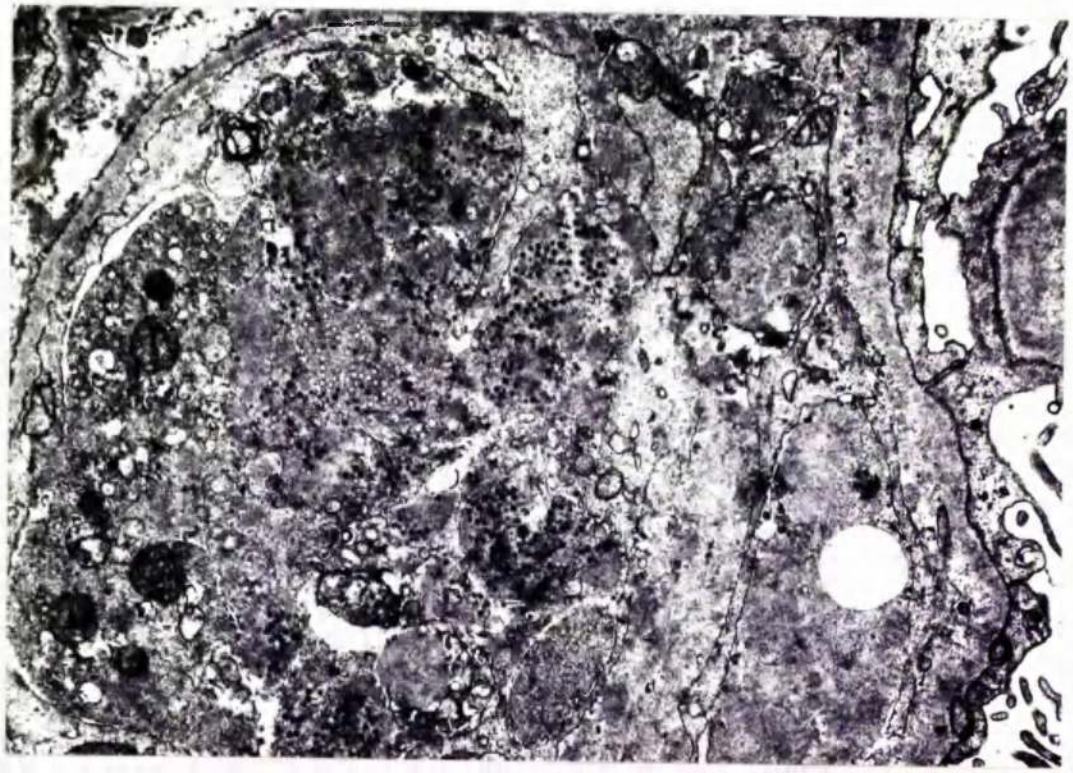
Fig. 62:- Section of glomerulus from an infected dog examined 8 days after administration of virus: An electron-dense, necrotic cell containing virus particles (arrows) can be seen entirely within the cytoplasm of a mesangial cell (M).

(Electron microscopy X 10,000)

Fig. 63:- Section of glomerulus from an infected dog examined 9 days after administration of virus: Numerous virus particles can be seen among electron-dense deposits and necrotic cellular debris.

(Electron microscopy X 15,000)


M



Elsewhere in the kidney, all dogs examined up until 10 days showed swelling and sometimes vacuolation of the tubular epithelial cytoplasm. This resulted in dispersion of mitochondria and protrusion of epithelial cytoplasm into the tubular lumina which often contained fragments of cellular debris. In animals infected 7 to 14 days, occasional necrotic tubules were encountered and numerous red blood cells were sometimes found in the tubular lumina.

Foci of interstitial cellular infiltration were located in samples of kidney from the 6 dogs examined 17 to 27 days after inoculation of virus; these lesions were found in both cortical and medullary tissue. In dogs examined on days 17, 18 and 19, the cortical infiltrates consisted of a mixture of cell types composed mainly of lymphocytes, plasma cells, large monocytes and macrophages (Figs. 64 and 65). Occasional necrotic tubules and remnants of necrotic individual cells were found within the lesions and, in their immediate vicinity, focal accumulations of polymorphonuclear leukocytes and macrophages were usually present. Lymphocytes ranged in appearance, from cells possessing moderate amounts of pale cytoplasm with few organelles, to characteristic small lymphocytes with a round dense nucleus surrounded by small amounts of electron-dense cytoplasm. At this stage, numerous lymphocytes were observed in various stages of mitotic division. Plasma cells of varying maturity were found, ranging from early plasmablasts with a central nucleus and moderate amounts of rough surfaced endoplasmic reticulum, to mature plasma cells with a round eccentrically placed nucleus and cytoplasm packed with distended endoplasmic reticulum.

Lesions found in the medulla at this stage of infection contained more numerous necrotic tubules and the associated cellular infiltrates contained greater numbers of macrophages and polymorphonuclear leukocytes. In many areas, the cellular infiltrates were composed almost entirely of

macrophages (Fig. 66) accompanied by a few polymorphonuclear leukocytes, while at other sites, these cell types were also accompanied by lymphocytes and plasma cells. Macrophages found within these lesions showed evidence of active phagocytosis; they were characteristically irregular in shape with a lobed or bean-shaped nucleus and their cytoplasm was vacuolated and contained varying numbers of round, electron-dense granules and a variety of cellular debris within membrane bound vesicles.

In both cortex and medulla, many intact tubules, lying within the cellular infiltrates, showed evidence of degenerative changes; their cytoplasm appeared ragged, swollen and vacuolated and they often contained necrotic cellular debris (Fig. 67). Virus particles were occasionally found in tubular epithelial cells and sometimes in desquamated cells lying free within the tubular lumen (Figs. 68 and 71). Infected cells found in these sites showed severe degenerative changes, often with disruption of nuclear and cytoplasmic membranes and release of virus particles into the tubular lumen. On numerous occasions, lymphocytes and polymorphonuclear leukocytes were found migrating through the tubular epithelium into the lumen of these tubules (Fig. 69). Polymorphonuclear leukocytes found in such sites sometimes appeared to be phagocytosing necrotic cellular debris and their cytoplasm was often pale and vacuolated and showed a marked reduction in content of lysosomal granules. Phagocytosed clumps of virus particles were also occasionally found within the cytoplasm of polymorphonuclear leukocytes in these sites (Fig. 70). Intact and degenerating virus-infected cells were sometimes found outwith the tubules, in the interstitium (Fig. 72): however, it was not possible to determine whether these were epithelial cells which had been released from disrupted tubules, or phagocytic cells which had become infected by ingestion of virus or virus-infected cellular material. Even at the 17 to 19 day stage of infection, small numbers of fibroblasts

were found in both cortical and medullary lesions; they appeared as irregular or elongated cells, the cytoplasm of which contained abundant distended endoplasmic reticulum. Associated with these fibroblasts, early collagen deposits were found, particularly around the periphery of some of the larger cellular foci (Fig. 73).

In the 3 dogs examined on days 25 and 27, the interstitial lesions showed a number of differences from those examined earlier. The cellular infiltrates contained a much higher proportion of plasma cells (Fig. 74) so that, even in the medulla, large numbers of mature plasma cells were often observed. However, many lymphocytes, macrophages and large mononuclear cells were also present. Fewer necrotic tubules were encountered and polymorphonuclear leukocytes were found in much smaller numbers than in the earlier stages of infection. Another notable feature was the increase in fibroblast activity, with numerous fibroblasts and associated fine bundles of collagen fibres found interwoven among the cellular infiltrates in both cortex and medulla. Despite the finding of small numbers of virus infected cells in Nos. 77 and 78 by immunofluorescence, virus particles were not found in renal tissue from any of the 3 dogs examined at this time.

Fig. 64 (a):- CAV interstitial nephritis, 19 days after administration of virus: A section of cortical cellular infiltrate shows a mixture of lymphocytes (L) and plasmablasts (P).

(b) (Insert):- A lymphocyte from the same lesion can be seen in the process of mitotic division.

(Electron microscopy X 6,000)

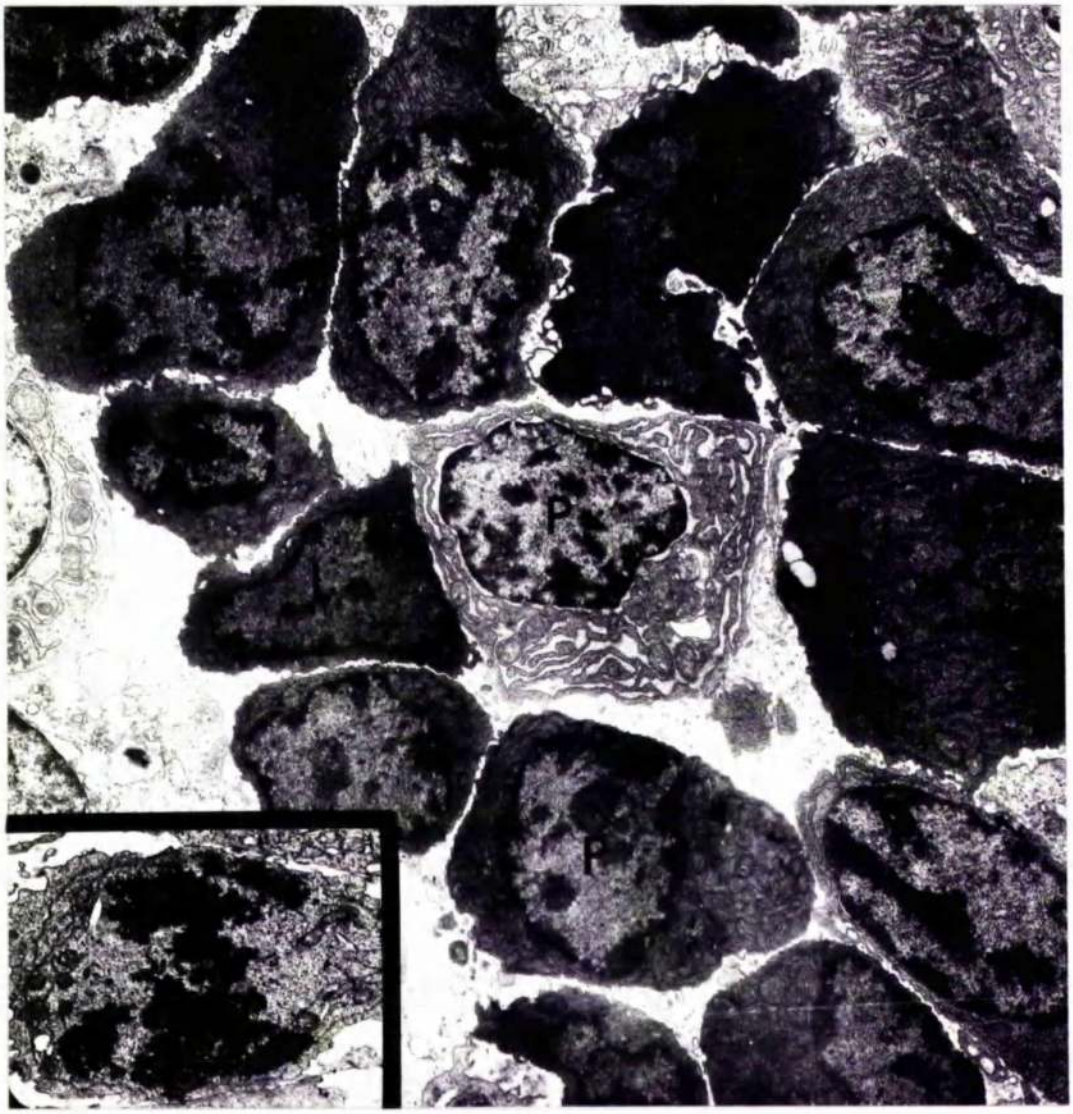

P
P

Fig. 65:- CAV interstitial nephritis, 19 days after administration of virus: A section of cortical cellular infiltrate, showing numerous macrophages (*).

(Electron microscopy X 6,000)

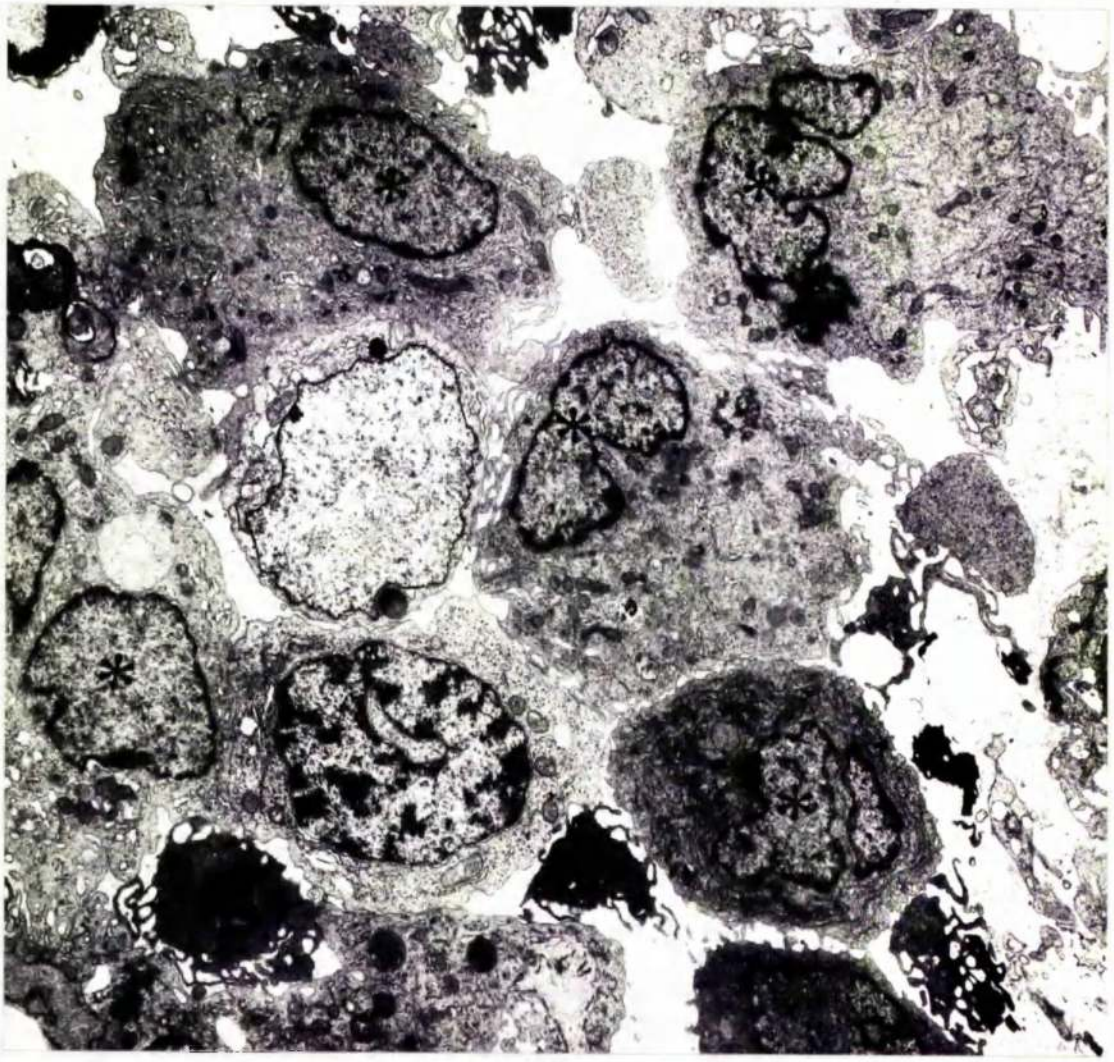


Fig. 66:- CAV interstitial nephritis, 18 days after administration of virus: Section of medullary cellular infiltrate, showing large numbers of macrophages (*); their cytoplasm is vacuolated and contains electron-dense lysosomal granules and irregular clumps of phagocytosed material.

(Electron microscopy X 6,000)

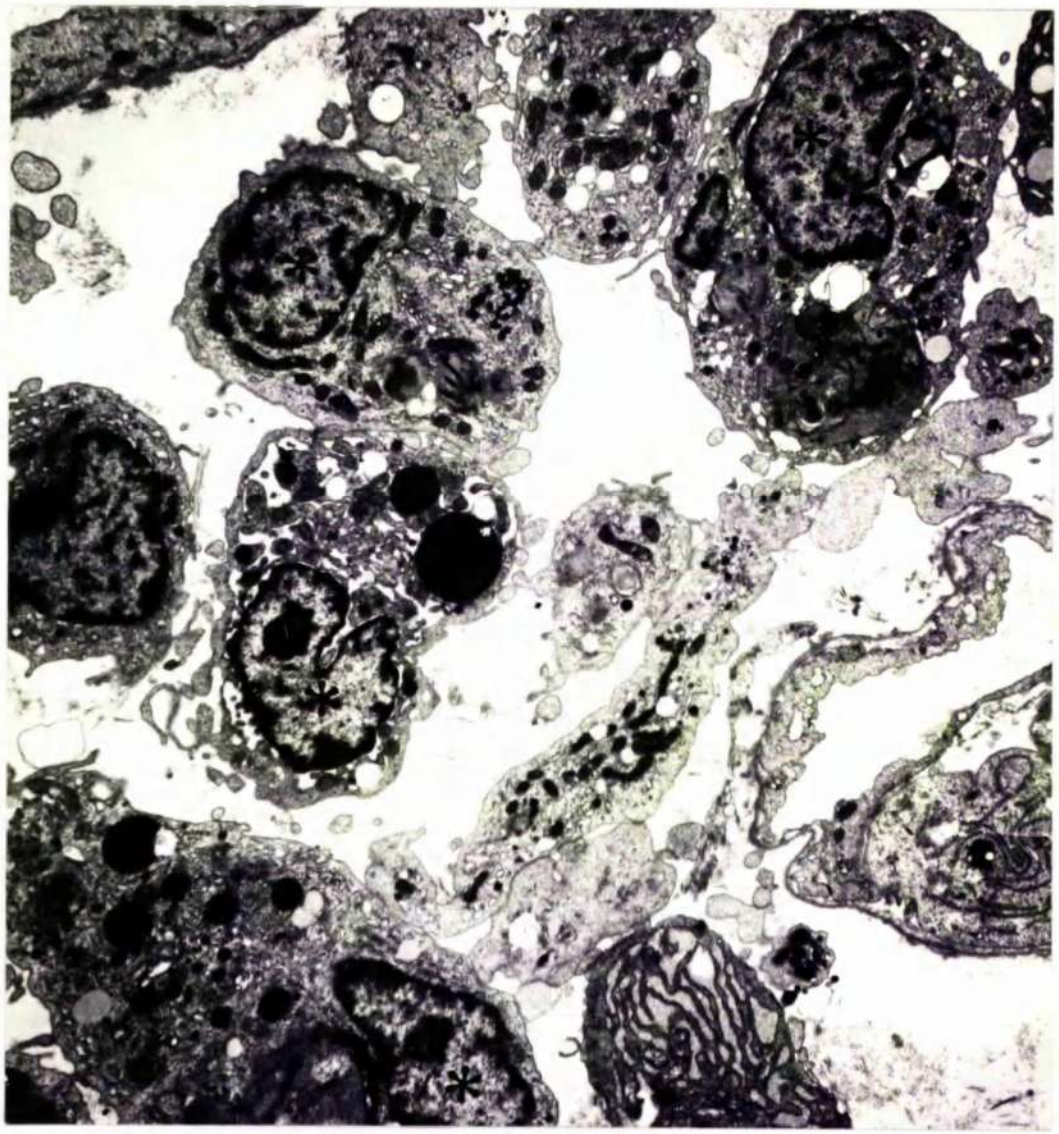
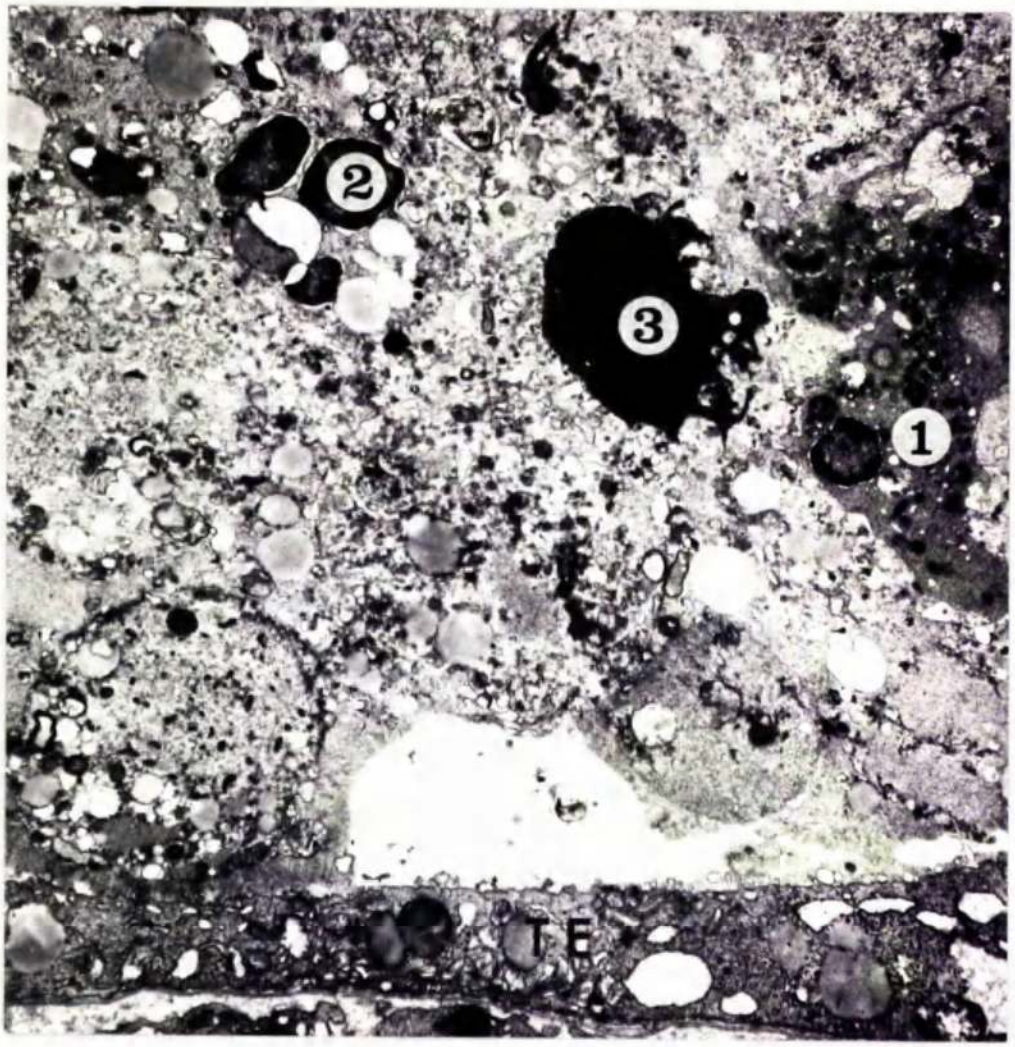


Fig. 67:- CAV interstitial nephritis, 19 days after administration of virus: Section of the medullary tubule shown Figure 39: The tubular lumen contains abundant necrotic cellular debris among which can be seen 3 polymorphonuclear leukocytes; one of these cells (2) has discharged and its cytoplasm is vacuolated while another (3) is necrotic and appears shrunken and electron-dense. TE = Tubular epithelium.

(Electron microscopy X 6,000)

Fig. 68:- Degenerating CAV infected cell found in the lumen of the tubule shown in Figure 67. Large accumulations of virus particles can be seen in the nucleus which shows numerous disruptions (arrows) in its nuclear membrane.

(Electron microscopy X 15,000)


2
3
1
TE

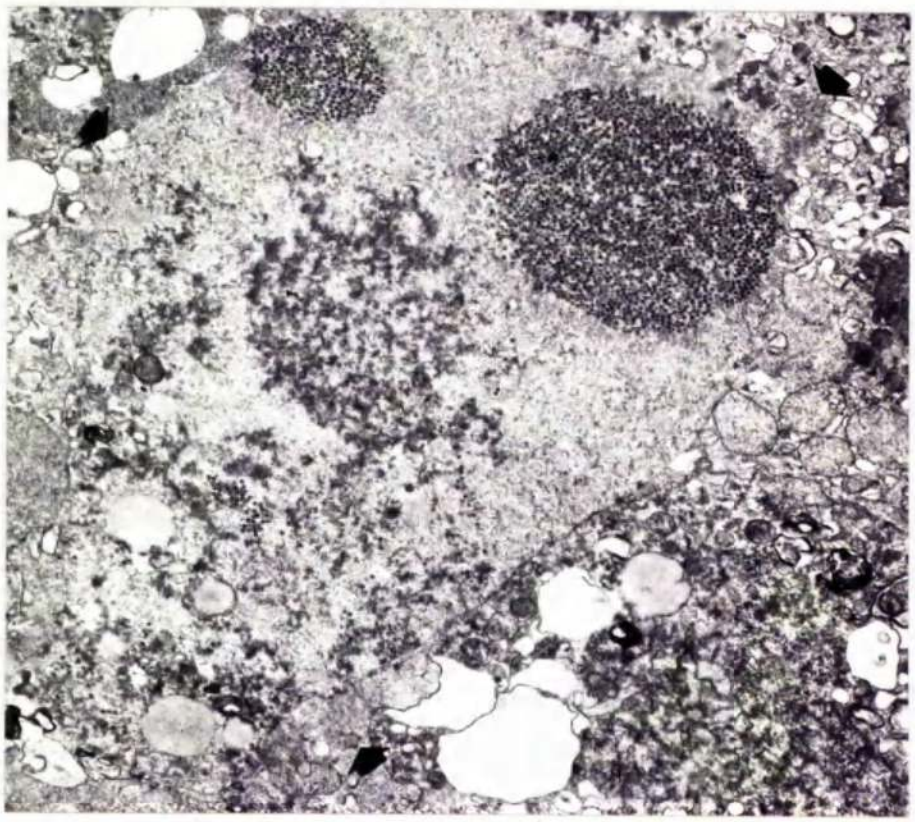


Fig. 69:- CAV interstitial nephritis, 18 days after administration of virus: Section of renal cortex, showing a degenerating virus infected cell (V) in the lumen of a collecting tubule; small lymphocytes (L) can be seen in the interstitium and passing through the tubular epithelium into the lumen. Two larger lymphocytes (Ly) with more abundant pale cytoplasm, can also be seen in the interstitium.

(Electron microscopy X 6,000)

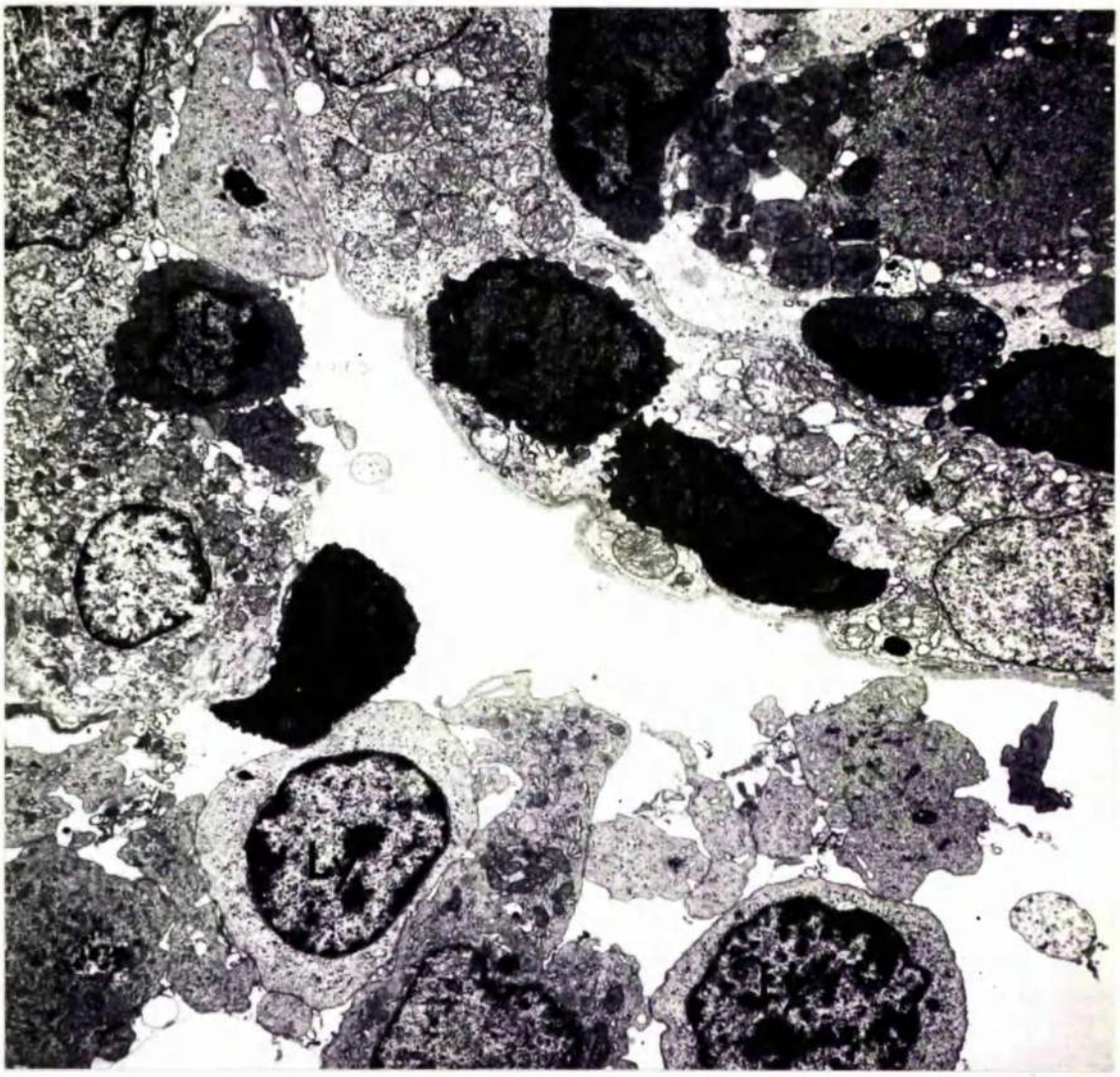
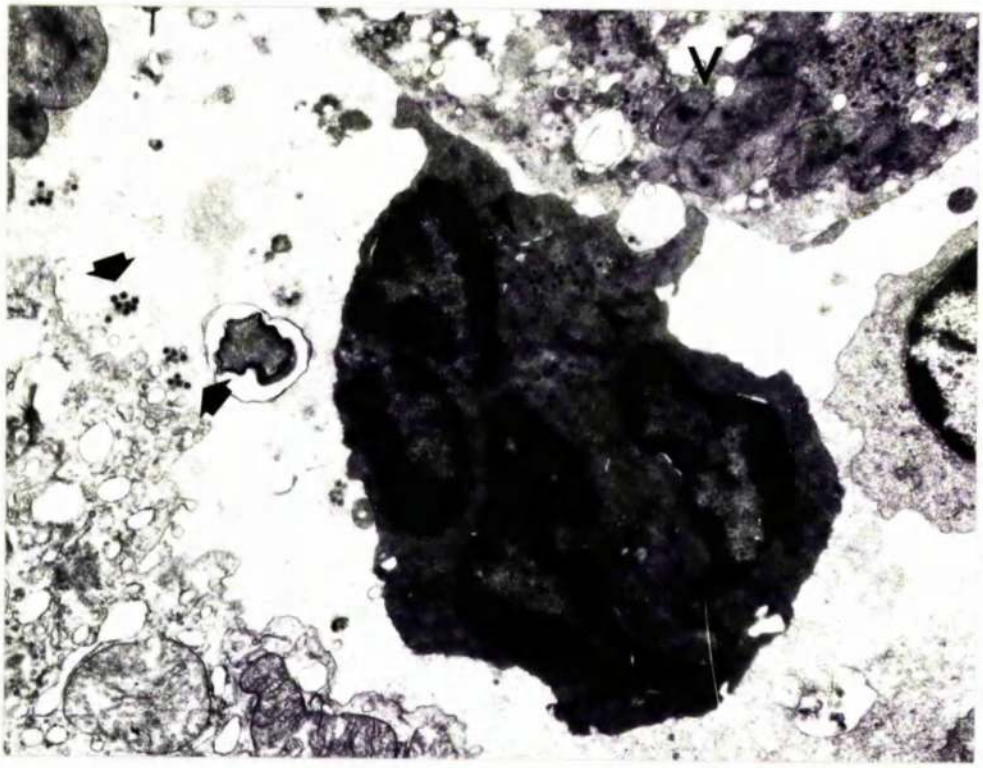

V

Fig. 70:- CAV interstitial nephritis, 19 days after administration of virus: Virus particles (small arrows) can be seen within the cytoplasm of a polymorphonuclear leukocyte lying in the lumen of a collecting tubule; part of a degenerating infected tubular epithelial cell (V) can also be seen and free virus particles (large arrows) are present in the tubular lumen.

(Electron microscopy X 15,000)

Fig. 71:- CAV interstitial nephritis, 17 days after administration of virus: Section of a collecting tubule containing 2 virus-infected epithelial cells, one of which (V1) shows nuclear margination of chromatin and a coarse granular matrix in the centre of the nucleus, while the second (V2) shows disruption of cytoplasmic and nuclear membranes.

(Electron microscopy X 8,000)


T
V

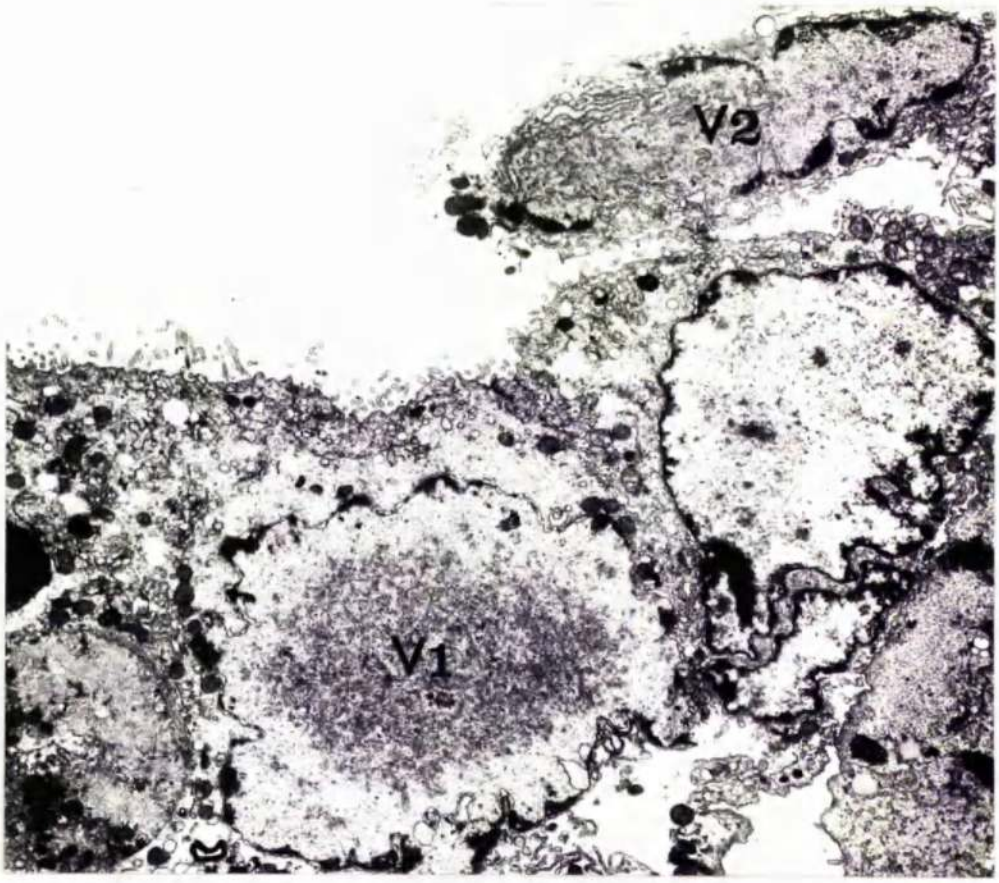

V2
V1

Fig. 72:- CAV interstitial nephritis, 18 days after administration of virus: Section of renal cortex, showing 2 degenerating virus - infected cells (V) in the interstitium; small extracellular clumps of virus particles (arrows) can also be seen.

(Electron microscopy X 10,000)

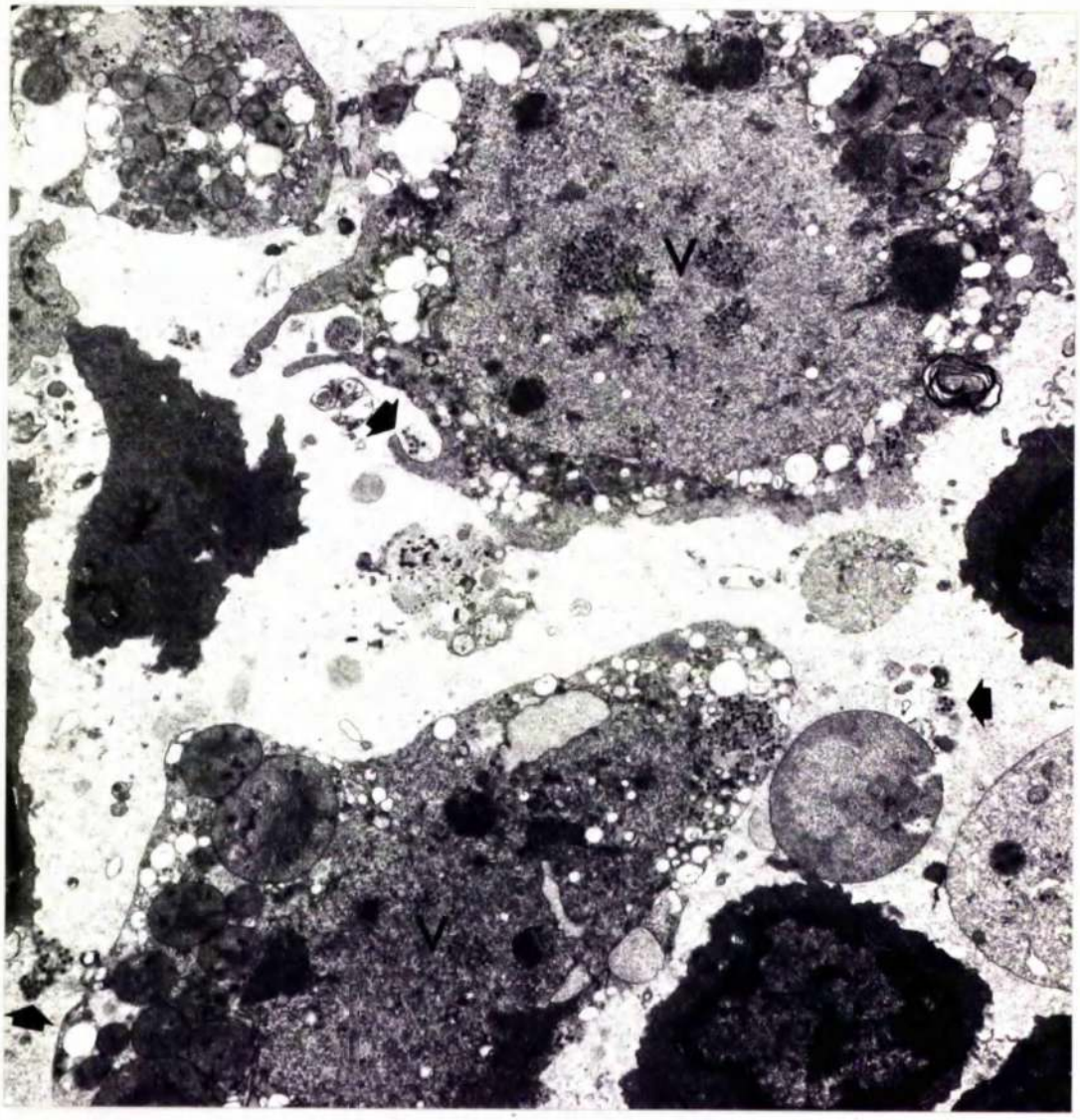

V
V

Fig. 73:- CAV interstitial nephritis, 19 days after administration of virus: Section of renal medulla, showing early collagen deposits (arrows) in close proximity to fibroblasts (F). A polymorphonuclear leukocyte (Pm) and a macrophage (Mac) can also be seen.

(Electron microscopy X 10,000)

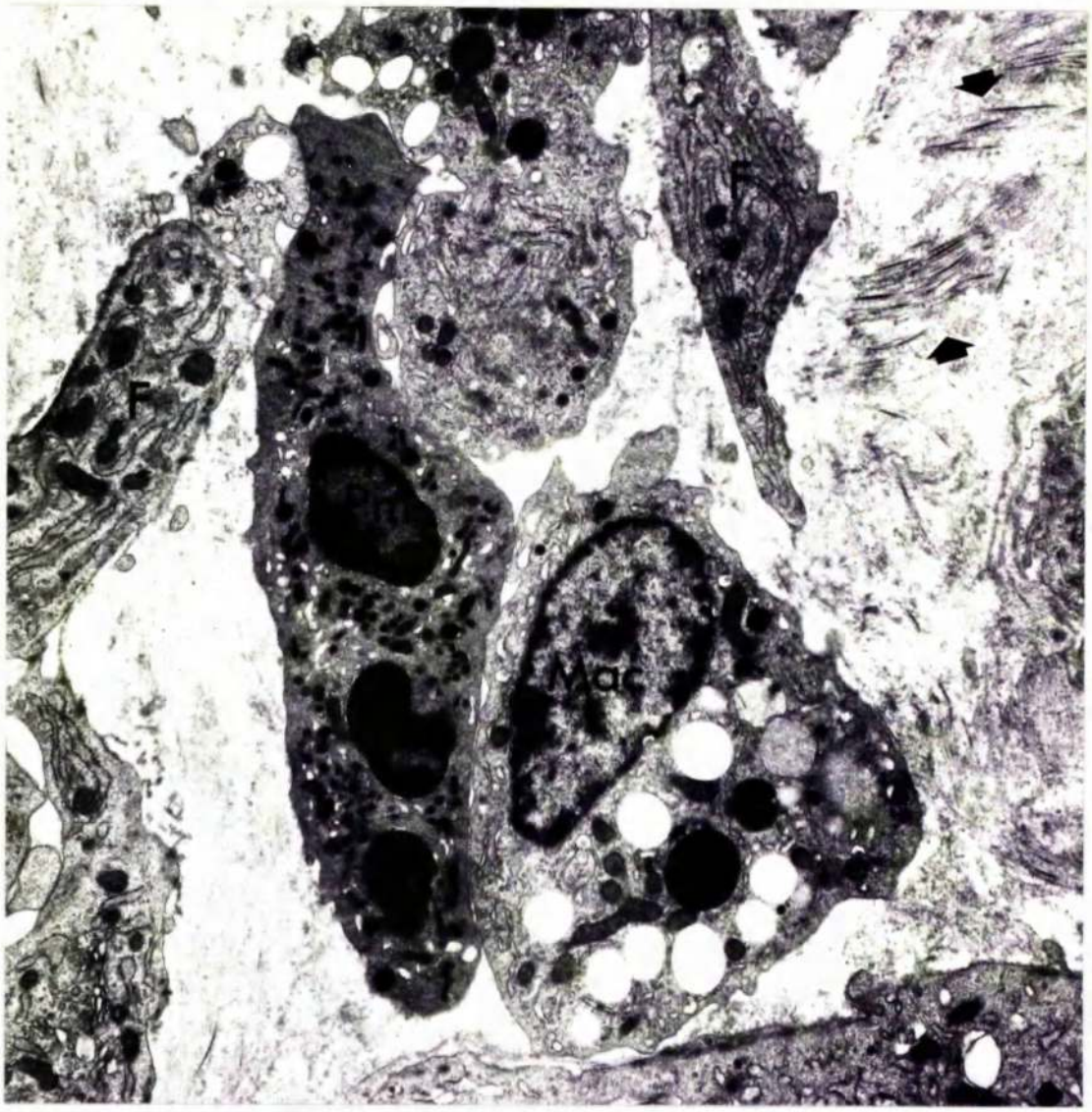
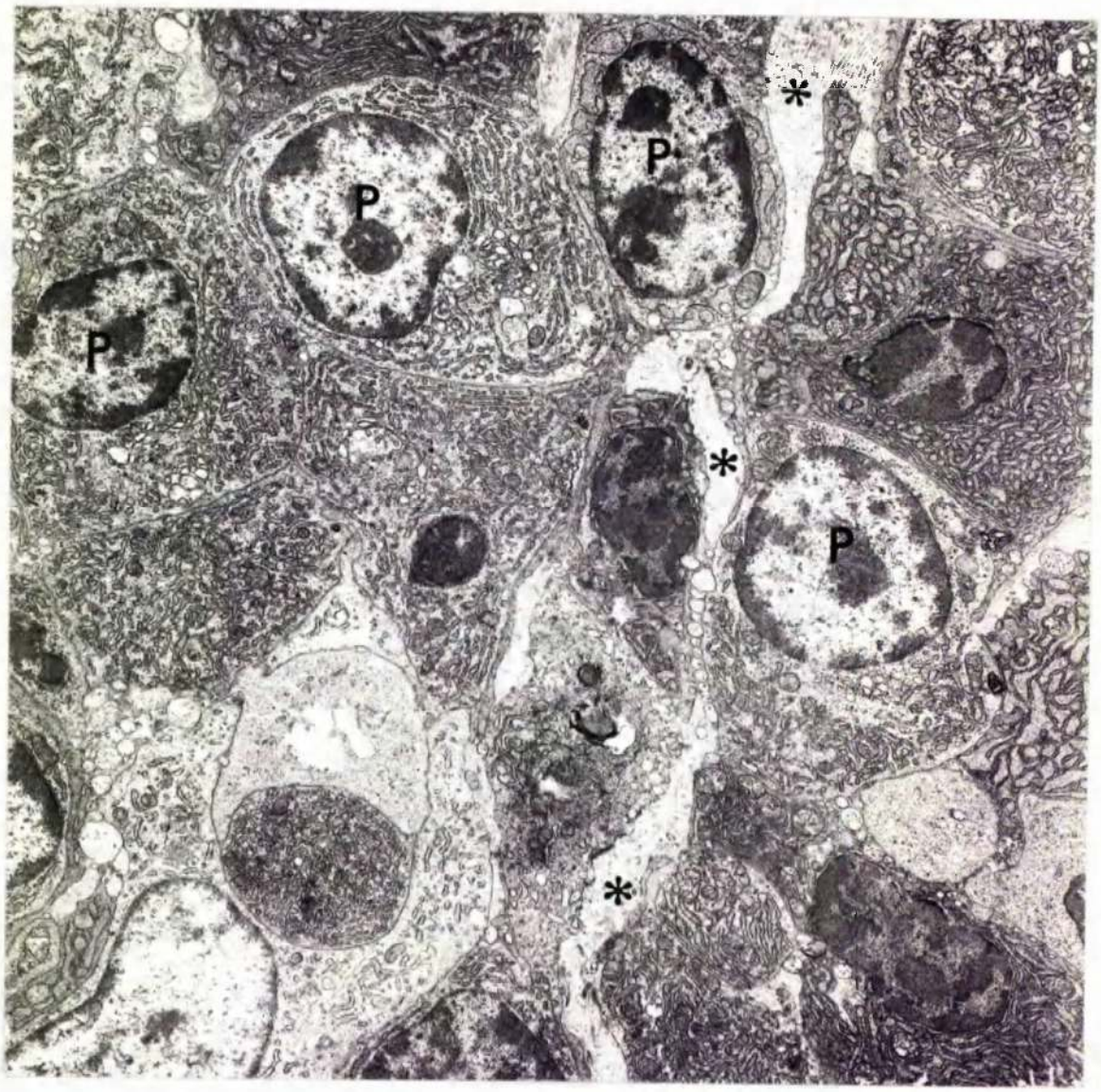

F
F
Mac

Fig. 74:- CAV interstitial nephritis, 25 days after administration of virus: Section of cortical cellular infiltrate, showing numerous plasma cells (P). Bands of collagen (*) can also be seen among the cellular infiltrate.

(Electron microscopy X 6,000)


P
P
P
P
P
*
*
*

Biochemical Findings

The results of urine protein and blood urea nitrogen estimations are presented in Table 12. Urine protein levels were measured in 11 dogs examined up to 9 days following inoculation of virus. Proteinuria in excess of 50mg/100ml was observed in 9 of these animals, with levels up to 500mg/100ml being detected. Significant proteinuria was not found in any of the dogs examined from day 10 onwards. One of the 3 in contact control dogs had a proteinuria level of 270mg/ml.

Obviously elevated levels of blood urea were found in only 3 dogs which were examined on days 5, 7 and 9; however a number of other dogs (Nos. 63, 73 and 76) had marginally elevated levels.

Serological Findings

The results of CAV antibody estimation in serum and renal eluates are presented in Table 12. Although IgG was deposited in the glomeruli as early as 5 days post-infection, circulating anti-CAV antibody was not detected until day 7. From day 7 to day 9 only low levels of antibody were found; however, the animal examined on day 10, which showed clinical recovery, had an antibody titre of 512 and all animals examined thereafter had similarly high titres.

Eleven of the 16 renal eluates examined were found to contain anti-CAV antibody; these were all from dogs examined between day 7 and day 19 and, although one titre of 128 was obtained, the level of antibody detected in the eluates was generally low. Anti-CAV antibody was not detected in any of the eluates of normal control kidneys, either from antibody-free dogs or from dogs with high levels of circulating antibody.

Anti-kidney antibody was not present in any of the eluates.

Table 12:- Experimental CAV infection: A summary of biochemical and serological findings

Dog No.	Day examined	Urine Protein mg/100ml	Blood Urea Nitrogen mg/100ml	CAV antibody Titres	
				Serum	Renal Eluate
55	4	ND	ND	ND	ND
56	5	12	43	-	-
57	5	16	38	-	-
58	5	ND	199	-	ND
59	5	107	ND	ND	ND
60	6	ND	ND	ND	ND
61	6	51	ND	ND	-
62	6	396	56	-	-
63	7	150	ND	4	ND
64	7	61	102	2	4
65	8	ND	40	64	2
66	8	220	ND	8	44
67	8	488	ND	ND	0
68	8	80	ND	ND	2
69	9	500	174	16	32
70	9	ND	ND	ND	16
71	9	ND	ND	2	16

Continued on next page.....

Table 12/continued

Dog No.	Day examined	Urine Protein mg/100ml	Blood Urea Nitrogen mg/100ml	CAV antibody Titres Serum	CAV antibody Titres Renal Eluate
72	10	15	41	512	32
73	14	2	51	512	128
74	17	0	43	512	2
75	18	0	20	512	ND
76	19	17	64	512	4
77	25	0	25	512	ND
78	25	7	34	512	ND
79	27	3	29	512	ND
80	10 c	ND	ND	ND	ND
81	10 c	270	ND	ND	ND
82	10 c	ND	ND	ND	ND

c = In contact controls

(b) Passive transfer of serum to mice

Clinical and Macroscopic Findings

Over the short duration of the experiment, all inoculated and control mice appeared clinically normal. At necropsy, macroscopic abnormalities were not observed in any of the mice.

Histological Findings

No significant difference was noted in the histological appearance of the glomeruli of inoculated and control mice. All glomeruli showed numerous thin walled, patent capillary loops and there was no apparent increase in glomerular cellularity.

Immunofluorescence Findings

The results of immunofluorescence studies are summarised in Table 13. Initially, on staining sections of control mouse kidney with rabbit anti-dog IgG at a 1:10 dilution, there was slight non-specific staining of the glomerular capillary walls and mesangium. However, on using the anti-dog conjugate at a 1:20 dilution, this non-specific staining was no longer present. At this dilution, fine granular deposits of IgG were detected in the mesangial regions of the glomeruli in mice inoculated with serum from dog Nos. 65 and 70 ((i.e. mouse Nos. M9-M16) (Fig. 75).

Deposits of IgG were not found in any of the other inoculated or control mice. Two mice (Nos. M9 and M11) also showed fine granular mesangial deposits of host complement. Neither CAV antigen nor host IgG was detected in the glomeruli of any of the inoculated or control mice.

Ultrastructural Findings

Ultrastructural glomerular alterations were found only in those mice in which deposits of canine IgG were detected by immunofluorescence (Nos. M9-M16). In these animals, there was an appreciable increase in mesangial matrix and mesangial cell cytoplasm often extended into the axial regions of the capillary loops where it impinged on the endothelium and caused partial occlusion of the capillary lumen. A few small, granular, electron-dense deposits were found scattered throughout the mesangial matrix. Occasional necrotic glomerular endothelial cells were observed; these cells had pyknotic nuclei, their cytoplasm was extremely electron-dense, shrunken and sometimes vacuolated and they were often partially separated from the underlying GBM.

Table 13:- Transfer of serum from CAV infected dogs to mice: Immunofluorescence findings.

Mouse Number	Donor Dog Number	Glomeruli		
		Canine IgG	CAV	C3
M1	55	-	-	-
M2	55	-	-	-
M3	55	-	-	-
M4	55	-	-	-
M5	57	-	-	-
M6	57	-	-	-
M7	57	-	-	-
M8	57	-	-	-
M9	65	+	-	+
M10	65	++	-	-
M11	65	++	-	+
M12	65	+	-	-
M13	70	+	-	-
M14	70	+	-	-
M15	70	+	-	-
M16	70	+	-	-
M17	72	-	-	-
M18	72	-	-	-
M19	72	-	-	-
M20	72	-	-	-
M21	73	-	-	-
M22	73	-	-	-
M23	73	-	-	-
M24	73	-	-	-
M25	83	-	-	-
M26	83	-	-	-
M27	83	-	-	-
M28	83	-	-	-

Degree of fluorescence graded + to ++++

Fig. 75:- Passive transfer of complexes to mice: Granular deposits of canine IgG are present in the mesangial regions of a glomerulus from a mouse which received 4 inoculations of serum from dog No. 65.

(Immunofluorescence X 600)

DISCUSSION

The results obtained in this study demonstrate that dogs may develop acute glomerulonephritis as a result of systemic CAV infection. Nineteen dogs examined from 4 to 14 days after inoculation of virus all showed diffuse glomerular lesions of varying severity. The immunofluorescence and ultrastructural findings indicate that 2 mechanisms were involved in the genesis of these lesions. Firstly, virus replication in glomerular endothelial and mesangial cells occurred, resulting in lytic damage to these cell types. Secondly, the finding of granular deposits of immunoglobulin, viral antigen and complement in the glomeruli by immunofluorescence establishes an immune complex mediated glomerular injury. Such a diffuse pattern of granular fluorescence, along with the ultrastructural finding of electron-dense deposits in the glomeruli, suggest deposition of antigen-antibody complexes from the circulation, rather than simply fixation of antibody on to virus already present in the glomeruli. The mesangial distribution of immune deposits is considered characteristic of that produced by the formation of relatively large poorly soluble complexes in the circulation (Germuth and Rodriguez, 1973). The simulataneous presence of diffuse glomerular deposits of IgG and CAV antigen provides strong evidence that the deposited complexes were composed of virus antigen and anti-viral antibody. Further evidence that the immunoglobulin was directed against virus antigen was provided by the detection of anti-CAV antibody in renal eluates. However, the titres of antibody in the eluates were perhaps not as high as might have been expected; this may be explained if, during the elution procedure, virus antigen as well as antibody was extracted from the renal tissue. On subsequently returning to neutral pH, this would have allowed recombination with antibody and thus a reduction in the amount of free antibody in the

eluates. It is of note that anti-kidney antibody was not found in any of the eluates despite the fact that renal antigens are known to be released during acute CAV infection, as shown in section I.

Deposits of IgG were first detected in the glomeruli 5 days after infection, whereas circulating anti-CAV antibody was not detected until day 7, at which time both IgG and virus antigen were found in the glomeruli. However, it is likely that any antibody produced before day 7 would immediately become bound to virus antigen in the circulation and therefore not be detectable as free antibody. This may also account for the relatively low levels of circulating antibody found in all dogs up to day 9.

Evidence for the presence of circulating immune complexes in infected dogs was obtained by passively transferring dog serum to mice. Mesangial localisation of canine IgG occurred in mice receiving serum from 2 dogs; this serum was obtained from 2 dogs examined on days 8 and 9, at which time heavy deposits of IgG and viral antigen were found in the glomeruli and only low levels of circulating antibody were present. Transfer was not achieved with serum taken from dogs examined earlier in the disease when there was only minor deposition of IgG and no circulating antibody, nor from dogs examined in the later stages when there was high levels of circulating antibody. Although the localisation of IgG and complement in the glomeruli of recipient mice suggests that immune complexes were present in the serum, final proof is still lacking that viral antigen was present as the antigenic component of these complexes. However, the absence of staining for antigen may merely indicate that insufficient quantities of complexes were present in order to detect the antigen; since many of the antigenic sites are covered by complexed antibody, the antigenic component of deposited complexes is less readily detected than antibody and, particularly

when relatively small amounts of complexes are deposited, only the antibody will be detectable by immunofluorescence.

The nature of the glomerular lesions was essentially a proliferative glomerulonephritis, characterised by an increase in mesangial cells and infiltration of polymorphonuclear leukocytes and monocytes into the glomerular capillaries. Polymorphonuclear leukocytes were found in greatest numbers on days 8 and 9, at which time the glomerulonephritis was most severe and there was heavy immune complex deposition. The presence of polymorphonuclear leukocytes in the glomeruli at this stage was almost certainly related to complement fixation by deposited complexes, a reaction which is known to be capable of releasing polymorphonuclear leukocyte mediators (Henson, 1971).

At the height of the disease, the mesangial cells showed a striking variation in ultrastructural appearance. Many cells with irregular cytoplasm appeared to be engaged in active phagocytosis of immune deposits while other cells, with discrete pale cytoplasm, resembling circulating monocytes were found. Thus, while there was almost certainly an increase in the number of mesangial cells, this may have been augmented by infiltration into the mesangium of circulating monocytes. Expansion of mesangial regions resulted in occlusion of many of the glomerular capillaries; the endothelial lining showed severe degenerative changes and in some instances, necrosis occurred leading to disruptions in the capillary walls and haemorrhage into the filtration spaces. A number of factors may have contributed to these cytological changes. Firstly, it is likely that local ischaemia occurred within the glomeruli due to occlusion of capillary loops; secondly, severe liver damage over several days may have resulted in the build-up of cytotoxic substances in the circulation; thirdly, fixation of complement by complexes leading to accumulation of polymorphonuclear leukocytes and their subsequent release

of enzymes almost certainly produced local tissue damage within the glomeruli. Fourthly, virus antigen-antibody complexes may have exerted a direct cytotoxic effect independent of complement activation. Although the latter mechanism has not been demonstrated for CAV-antibody complexes, extensive in vitro studies have shown that virus-antibody complexes, prepared from human adenoviruses, are cytotoxic, in the absence of complement, to normal tissue culture cells (Kjellén and Ankerst, 1973a and b; Ankerst and Kjellén, 1973).

Proteinuria in excess of 50mg/100ml was detected in 9 of the 11 dogs whose urine was examined 5-9 days after virus inoculation. All dogs killed from the 10th day onwards had normal levels of less than 20mg/100ml. The protein leak was presumably glomerular in origin and, although the GBM appeared structurally normal, the endothelium in places was frayed and sometimes necrotic and there was patchy fusion of epithelial foot processes. Tubular haemorrhage probably also contributed to the proteinuria.

Although immune complex mediated glomerulonephritis associated with CAV infection has not previously been recognised, careful scrutiny of the literature shows that, even in the early studies of the disease, glomerular lesions were occasionally recorded. Thus Rubarth (1947) described one case, 7 days after experimental infection, in which the "glomeruli showed an accumulation of leukocytes with consequent degenerative changes in their vessels" associated with haemorrhage into the filtration spaces and tubules. Stünzi and Poppensiek (1952) also described focal glomerulonephritis, with albuminuria and haematuria, in one jaundiced case of experimentally induced acute CAV infection.

Despite their severity, the glomerular lesions in the present series of dogs appeared to be transient in nature. Thus, dogs examined from 10 days onwards showed progressively less severe glomerular changes;

however, it must be pointed out that, in comparison with the others, the 6 dogs examined from day 17 onwards suffered a much milder clinical illness and consequently may also initially have had less severe glomerular lesions.

It is now well established that a number of other viruses, including lymphocytic choriomeningitis virus in mice, aleutian disease virus in mink and equine infectious anaemia virus, can induce an immune complex glomerulonephritis in which viral antigen and anti-viral antibody may be demonstrated in the glomerular deposits (Oldstone and Dixon, 1967; Porter et al., 1973; Banks et al., 1972). However, the glomerulonephritis associated with CAV infection in dogs differs from that produced by these viruses, in that it is an acute transient lesion as opposed to being chronic and progressive. In pathogenesis, therefore, it shows a striking resemblence to acute serum sickness where the removal of large quantities of foriegn antigen by host antibody leads to the formation and glomerular deposition of soluble immune complexes, resulting in glomerulonephritis which subsequently resolves following elimination of the antigen.

Foci of tubular necrosis mainly affecting proximal tubules were found in all dogs examined 7-14 days after inoculation with virus. Since these lesions were apparently unassociated with virus infection of the tubules, they may have resulted from local ischaemia due to occlusion of glomerular capillaries. They were particularly widespread in the dog examined on day 14 and, had this animal been allowed to survive, resolution of the lesions could conceivably have resulted in relatively severe interstitial fibrosis.

All dogs examined from day 10 onwards showed focal interstitial nephritis. Virus was first detected in tubular epithelium on day 10 and was found in all except 2 dogs examined thereafter. The extent of

the interstitial nephritis and the numbers of virus infected tubules varied markedly from one animal to another. Infection of tubular epithelium may have resulted either from virus escaping into the urine from infected glomerular cells or via the interstitium from damaged infected interstitial capillaries. Collecting tubules were most often involved and infected tubular cells often appeared to be extruded into the lumen of the tubules, so that degenerating infected cells were frequently seen within apparently intact tubules. In this way, virus released from these cells could infect tubular epithelium at lower levels of the nephron; this accounted for the distribution of many of the interstitial lesions which were observed as streaks extending from outer cortex towards the medulla.

The interstitial infiltrates associated with infected tubules consisted of a mixed population of cells containing lymphocytes, plasma cells, macrophages, monocytes and in some areas, polymorphonuclear leukocytes. The presence of large numbers of IgG-containing plasma cells suggested a local antibody response and indeed, by using an indirect immunofluorescence test, many of the plasma cells were demonstrated to contain anti-viral antibody. Furthermore, renal eluates obtained from 2 dogs in which there were no immunoglobulin deposits in the glomeruli, contained anti-viral antibody. The failure to detect anti-kidney antibodies in these eluated excluded the possibility of a local immune response to tubular antigens.

The morphological appearance of many of the medullary lesions, which showed large accumulations of macrophages surrounding necrotic infected tubules, suggested that cell mediated immunological reactions may also be involved. Furthermore, it is tempting to speculate that the lymphocytes observed migrating into infected tubules might be T cells sensitised to CAV. However, until further detailed immuno-

logical studies are carried out, the role of cell mediated immunity in the pathogenesis of CAV interstitial nephritis remains uncertain.

Previous studies have shown that dogs infected with CAV may continue to excrete virus in the urine for periods up to 9 months after infection (Baker et al., 1954). In the present study, only occasional virus infected cells were found in 2 of the dogs examined in the later stages of the experiment, on days 25 and 27. In these animals, the interstitial lesions were less densely populated with cells than in the earlier stages of infection and there was already mild focal interstitial fibrosis. It is likely that the duration of virus infection in the kidney depends on the initial extent of tubular infection and possibly also on individual variation in the anti-viral immune response. Moreover, since persistence of virus in the kidney represents a focus of foriegn antigen, it is probable that an interstitial infiltrate will continue to be present until the virus is eliminated. Ultimately however, once complete elimination of virus is achieved, repair by fibrosis will occur and whether or not this results in any failure in renal function will probably depend on the extent and duration of the lesions.

SECTION IV : EXPERIMENTAL CAV NEPHRITIS : INOCULATION OF PREFORMED CAV IMMUNE COMPLEXES

INTRODUCTION

MATERIALS AND METHODS

RESULTS

(a) Mice

Table 14 ; Figures 76-85.

(b) Dogs

Table 15 ; Figures 86-90.

DISCUSSION

INTRODUCTION

In the previous section of this thesis, it was shown that dogs suffering from systemic CAV infection, of more that 4 days duration post-inoculation, developed severe glomerular lesions. Two pathogenic components contributed to glomerular injury; firstly, the virus caused direct lytic damage to the cellular components of the glomeruli and secondly, deposition of virus antigen-antibody complexes occurred resulting in proliferative and occlusive changes.

Studies carried out by other workers have shown that glomerulonephritis may develop in experimental animals following the administration of soluble antigen-antibody immune complexes prepared *in vitro* from bovine serum albumen of ovalbumen (McCluskey and Benacerraf, 1959; Okumura *et al.*, 1971). In the present section, an attempt was made to demonstrate that CAV antigen-antibody immune complexes prepared *in vitro* are capable of inducing glomerulonephritis in the absence of active virus replication. The study was carried out in 2 stages. Firstly, because of the large quantities of virus required for the preparation of complexes, a preliminary experiment was performed in which a series of mice received daily intravenous inoculations of soluble complexes. Having established that such a procedure resulted in glomerular lesions in mice, a group of antibody-free dogs were subjected to a similar routine of repeated inoculations of preformed complexes.

MATERIALS AND METHODS

Preparation of CAV protein

Stock CAV suspensions were centrifuged at 2500rev/min for 20 minutes at 4°C, to remove tissue culture debris, and virus was subsequently sedimented by centrifugation at 25,000rev/min for 45 minutes. The pelleted virus was resuspended in small volumes of PBS (pH 7.2) and the virus disrupted, with release of viral protein, by dialysis at 4°C for 5 days against carbonate/bicarbonate buffer pH 10.6 (Wilcox et al., 1963). This resulted in a solution of viral protein, the pH of which was adjusted to 7.2 by dialysis against PBS for a further 2 days. Centrifugation was performed at 4000rev/min for 15 minutes to remove any insoluble fragments and the viral protein solution was then stored at -25°C until required. The protein concentration, as measured by the method of Lowry et al., (1951) was 0.25mg/ml.

Preparation of Complexes

Soluble viral antigen-antibody complexes were prepared according to minor modifications of the method described by Okumura et al., (1971). A preliminary tube precipitation test was performed using fixed volumes of anti-CAV hyperimmune serum, to which was added a range of volumes of viral protein. In this way, the point of equivalence could be determined by visual quantitation of the degree of precipitation. Viral protein and anti-CAV serum were then mixed at equivalence (in this case equal volumes) and left at 4°C for 48 hours. The opalescent complexes so formed were sedimented by centrifugation at 3000rev/min for 20 minutes at 4°C, washed twice in cold PBS and resuspended in 5 times the original volume of viral protein. Solubilisation of the complexed was attained by lowering the pH to 2.4 by slow dropwise addition of 0.1 N HCl at 0°C with constant stirring. The pH was then returned to neutrality by

slowly adding 0.1 N NaOH. Small amounts of precipitate still remaining at the end of this procedure were removed by centrifugation at 3000rev/min for 15 minutes.

Experimental Procedures

(a) Mice

Seventy 8-10 week-old albino mice (Porton strain) weighing 20-25g were used for the first part of the experiment. Forty of these mice received daily inoculations of 0.3ml of soluble CAV protein immune complexes by slow intravenous injection into the tail vein. Groups of at least 3 mice were killed daily at intervals of 1-10 days after the initial inoculation, each mouse being killed 24 hours after its last dose. For the purposes of controls, 2 groups of 10 mice were given similar volumes of viral protein and anti-CAV hyperimmune serum respectively and killed daily from 1 to 10 days. A further 10 mice were killed as normal uninoculated controls for comparison of kidney histology, ultrastructure and immunofluorescence.

(b) Dogs

For the second part of the experiment, 18 12 week-old dogs, which were shown to be anti-CAV antibody-free by the indirect immunofluorescence test, were used. Twelve dogs recieved daily inoculations of 3ml of soluble CAV protein complexes by slow intravenous injection into the jugular vein. The dogs were killed at intervals of 2-7 days after the initial inoculation, each dog being killed 24 hours after its last dose. Three dogs received similar daily intravenous inoculations of CAV protein and were killed 3, 5 and 7 days later. The remaining 3 dogs were killed as normal uninoculated controls.

Histological Immunofluorescence and Ultrastructural Procedures

These were carried out as described in the section on "materials and methods". The kidneys from all dogs and mice were subjected to ultrastructural examination. Sections of mouse kidney were examined by immunofluorescence for the presence of canine IgG, CAV antigen and mouse IgG and B1C globulin (C3); likewise, sections of dog kidney were examined for the presence of canine IgG and B1C globulin and CAV antigen.

RESULTS

(a) Mice

Clinical and Macroscopic Findings

Throughout the course of the experiment, all mice appeared clinically normal. At necropsy, neither inoculated nor control mice showed any macroscopic abnormalities.

Histological Findings

Proliferative glomerulonephritis was found in 14 out of 40 mice (35 per cent) inoculated with viral immune complexes. This change was first detected after 4 daily doses of complexes and was found in 50 per cent of the mice examined from 4 to 10 days after initial inoculation. In all 14 mice, the glomeruli were affected to more or less the same extent. There was expansion and hypercellularity of the mesangial regions and many of the mesangial cell nuclei appeared elongated or bean-shaped (Fig. 77). These changes resulted in distinct swelling of the glomerular tuft and partial occlusion of many capillary loops. Polymorphonuclear leukocytes and large mononuclear cells were sometimes found lodged in the capillary lumina. Occasional proteinaceous casts were found in the tubules. In the remaining inoculated and control animals, the glomeruli appeared histologically normal (Fig. 76).

Immunofluorescence Findings

The results of immunofluorescence studies on inoculated mice are summarised in Table 14. The distribution of specific fluorescence in mouse glomeruli was the same for antigen, antibody and complement; only the frequency of appearance and intensity of fluorescence differed. Fine granular deposits of canine IgG were detected in the mesangial regions of the glomeruli in all mice receiving 3 or more doses of complexes (Fig. 78). The intensity of fluorescence varied from animal to animal but, in general terms, no real increase in intensity was noted in animals receiving more that 4 inoculations. Fluorescence staining for CAV antigen and complement also appeared on the 3rd day, was weaker than that of IgG and was not detected in all animals (Figs. 79 and 80). Twenty-five mice had glomerular deposits of CAV antigen and 18 mice had deposits of complement. All 14 mice which showed proliferative glomerulonephritis were positive for IgG, viral antigen and complement and, generally, there was a good correlation between the degree of antigen fluorescence and the severity of glomerular histological lesions. Host (mouse) IgG was not detected in the glomeruli of any of the inoculated or control mice. Similarly, canine IgG, host complement and CAV antigen were not found in the glomeruli of any of the inoculated or uninoculated control mice.

Ultrastructural Findings

There was a wide range of ultrastructural changes in the glomeruli, whether or not proliferative changes were detected by light microscopy. The earliest change was detected after 3 doses of complexes when there was expansion of mesangial matrix and extension of mesangial cell cytoplasm into the axial regions of the capillaries, often displacing swollen endothelial cytoplasm at these sites. At this time, small electron dense deposits were found in the mesangial matrix and, less

commonly, trapped between endothelial cytoplasm and mesangial cytoplasmic projections (Fig. 81). All of these structural changes occurred to a varying degree in all mice receiving 3 or more doses of complexes. Those mice which showed proliferative glomerulonephritis by light microscopy showed more severe glomerular ultrastructural alterations. In these animals, there was much more pronounced expansion of the mesangial regions and a distinct increase in mesangial cellularity (Fig. 82); the cytoplasm of these active mesangial cells contained increased amounts of endoplasmic reticulum and mitochondria and sometimes numerous large electron dense granules were present. Large mononuclear leukocytes with lobed nuclei and pale cytoplasm were frequently seen lodged within capillary loops and were occasionally observed pushing through capillary endothelium into the mesangium (Fig. 83). Similarly, polymorphonuclear leukocytes were often present in the capillary loops (Fig. 84) and sometimes in the mesangium; in this site, they often showed a marked decrease in content of cytoplasmic granules. The epithelial cells were swollen resulting in focal areas of fusion of their foot processes and there was often an increase in their content of cytoplasmic organelles. Electron-dense, dead or dying endothelial cells were occasionally observed (Fig. 85) while many other endothelial cells showed swelling of their cytoplasm. All these ultrastructural abnormalities persisted to a varying extent up to the close of the experiment on day 10.

The kidneys of inoculated and uninoculated control mice showed no ultrastructural abnormalities.

Table 141- Immunofluorescence patterns and incidence of glomerulonephritis in mice receiving daily doses of preformed CAV immune complexes.

Mouse No.	Day examined	Immunofluorescence				Glomerulonephritis
		Canine IgG	Mouse IgG	CAV	Mouse C3	
M33	1	-	-	-	-	-
M34	1	-	-	-	-	-
M35	1	-	-	-	-	-
M36	1	-	-	-	-	-
M37	2	-	-	-	-	-
M38	2	-	-	-	-	-
M39	2	-	-	-	-	-
M40	3	+	-	-	-	-
M41	3	+	-	-	-	-
M42	3	+	-	-	-	-
M43	3	+	-	+	+	-
M44	3	+	-	+	+	-
M45	4	+	-	-	-	-
M46	4	+	-	-	-	-
M47	4	+	-	+ +	+	+
M48	4	+	-	+ +	+	+
M49	4	+	-	+	-	-
M50	5	+	-	+	-	-
M51	5	+ +	-	+ +	+ +	+
M52	5	+ + +	-	+ +	+ +	+ +
M53	5	+ +	-	+	+	-
M54	6	+	-	+	-	-
M55	6	+ +	-	+	+	-
M56	6	+	-	+	-	-
M57	6	+ + +	-	+ +	+ +	+ +
M58	6	+ +	-	+ +	+	+
M59	7	+	-	-	-	-

Continued on next page.....

Table 14/continued

Mouse No.	Day examined	Immunofluorescence				Glomerulonephritis
		Canine IgG	Mouse IgG	CAV	Mouse C3	
M60	7	+ +	-	+ +	+	+
M70	7	+ + +	-	+ +	+	+ +
M71	7	+ +	-	+	-	-
M72	8	+ + +	-	+ +	+	+ +
M73	8	+ +	-	+ +	+	+
M74	8	+ + +	-	+ +	+	+ +
M75	8	+ + +	-	+ +	+	+
M76	9	+	-	+	-	-
M77	9	+ +	-	-	-	-
M78	9	+ + +	-	+ +	+	+ +
M79	10	+ +	-	+ +	+	+
M80	10	+ +	-	-	-	-
M81	10	+	-	+	-	-

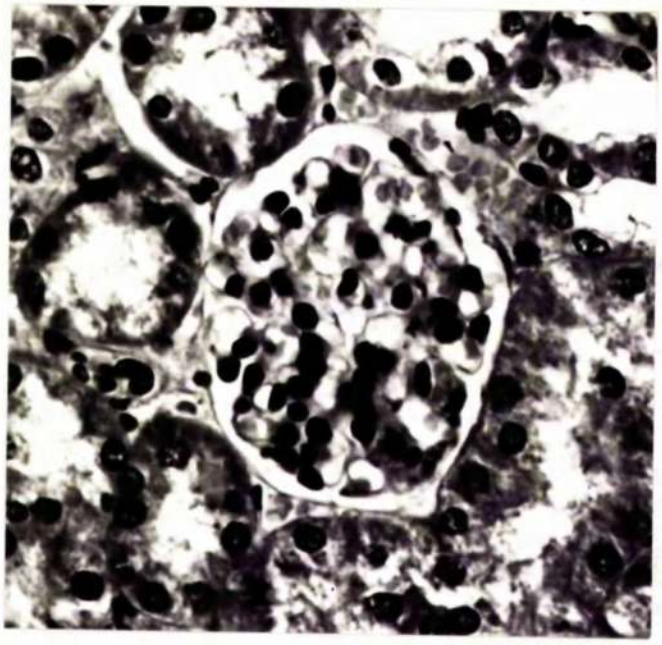
Various parameters graded + to ++++ according to severity

Fig. 76:- Glomerulus from a control mouse, showing normal cellularity and patent capillary loops.

(HE X 400)

Fig. 77:- Proliferative glomerulonephritis in a mouse which received 7 daily inoculations of CAV immune complexes. There is swelling of the tuft, increased cellularity with expansion of mesangial areas, and occlusion of capillary loops.

(HE X 400)



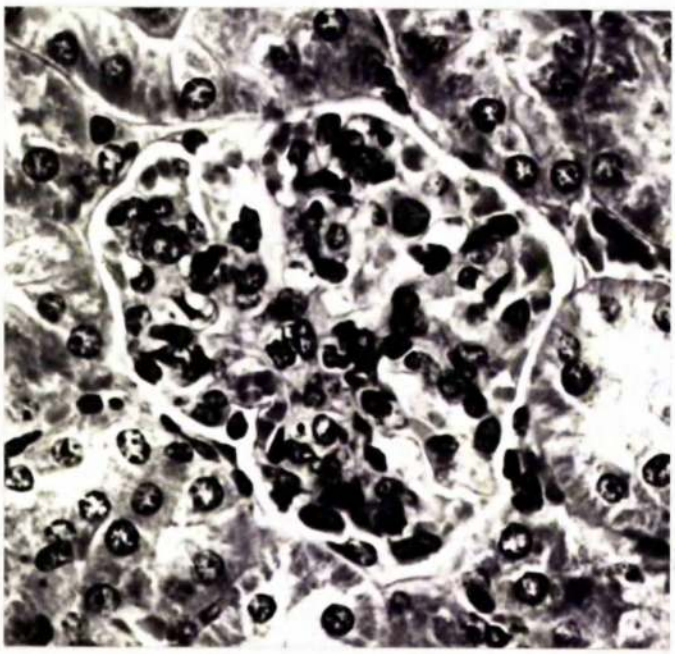


Fig. 78:- Glomerulus of mouse which received 5 daily inoculations of CAV immune complexes, showing granular deposits of canine IgG in the mesangial regions.

(Immunofluorescence X 400)

Fig 79:- Glomerulus of mouse which received 7 daily inoculations of CAV immune complexes, showing granular deposits of CAV antigen in mesangial regions.

(Immunofluorescence X 400)

Fig. 80:- Glomerulus of mouse which received 7 daily inoculations of CAV immune complexes, showing granular deposits of mouse complement (C3) in a similar distribution to antibody and antigen.

(Immunofluorescence X 400)

Fig. 81:- Section of glomerulus from mouse which received 4 daily inoculations of CAV immune complexes: Small electron-dense deposits (small arrows) are present in the mesangial matrix and there is extension of mesangial cytoplasm (large arrow) into the axial region of a capillary loop, where it has partially displaced the endothelium. M = Mesangial cell; E = Endothelial cell; Ep = Epithelial cell.

(Electron microscopy X 15,000)

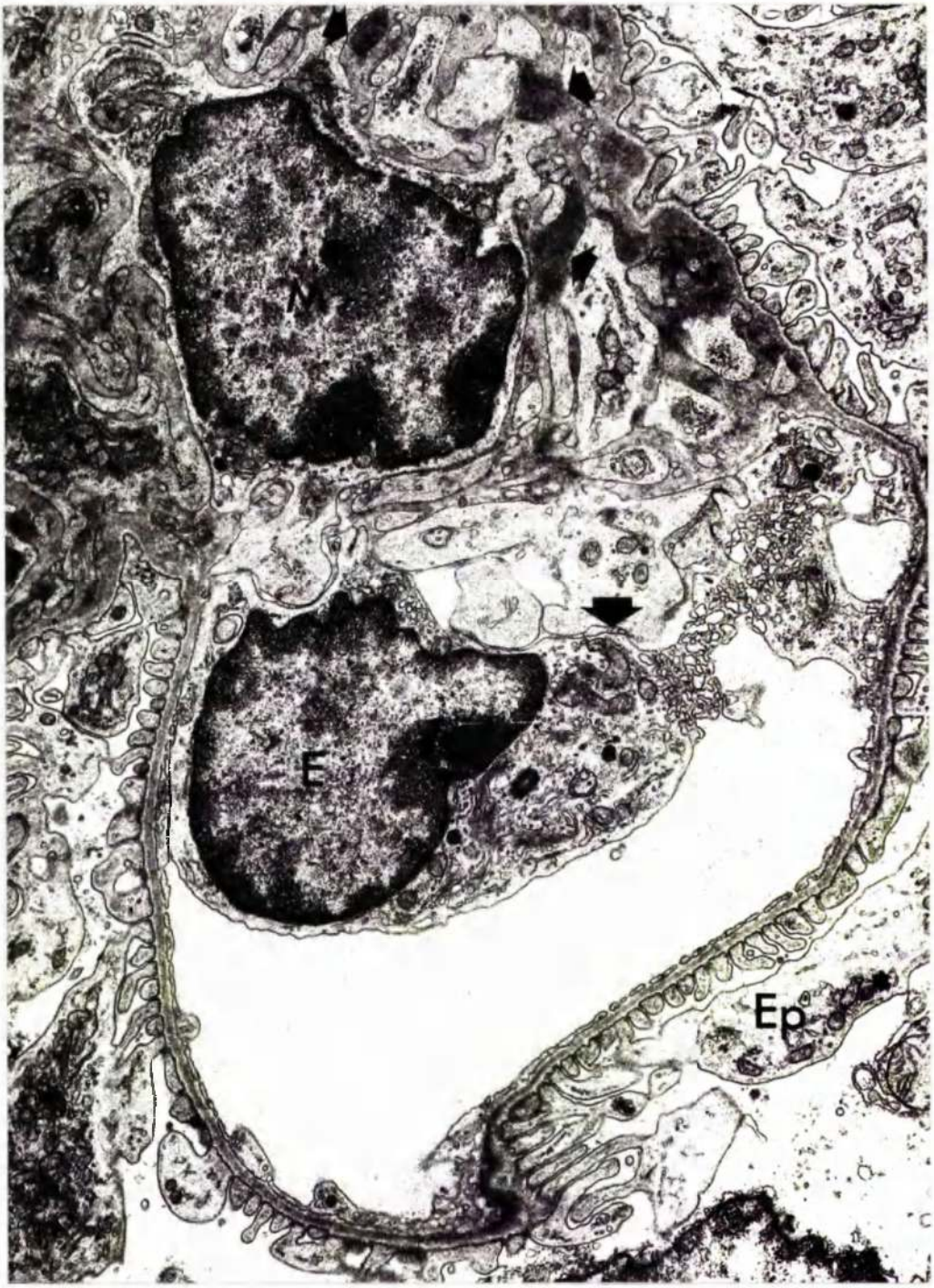

N
E
Ep

Fig. 82 (a):- Section of glomerulus from a mouse which received 5 daily inoculations of CAV immune complexes: There is marked expansion of mesangium (M) and part of the cytoplasm of a cell, located in the mesangium, can be seen extending into a capillary loop (arrows). There is also fusion of epithelial cell (Ep) foot processes.

(Electron microscopy X 14,000)

(b) (insert):- An electron dense deposit can be seen in a sub-endothelial location (Arrow). E = Endothelial cell.

(Electron microscopy X 6,000)

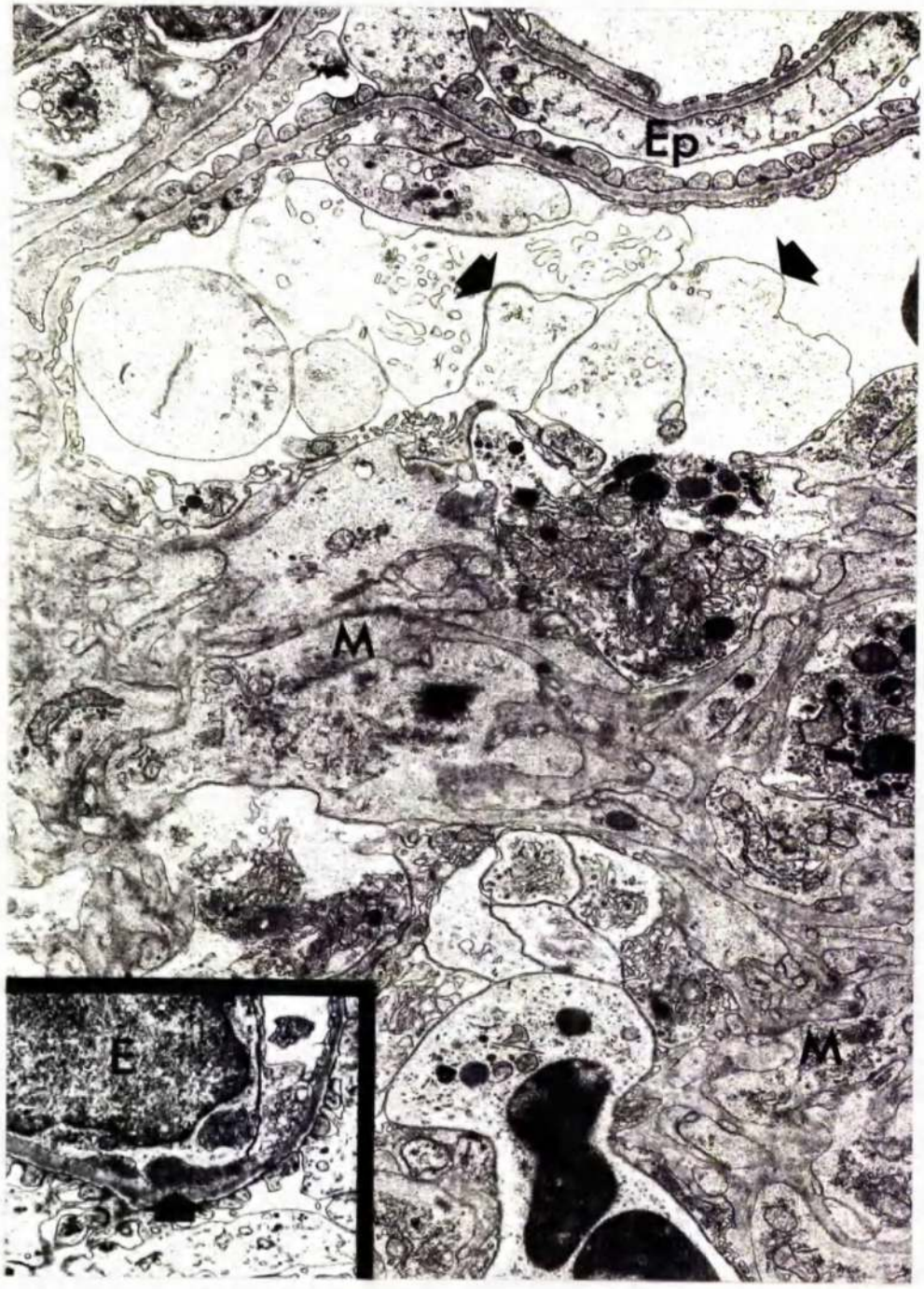

Ep
M
M
E

Fig. 83:- Section of glomerulus from a mouse which received 7 daily inoculations of CAV immune complexes: A large monocyte (Mon) can be seen in a capillary loop; there is also marked expansion of the adjacent mesangium.
M = Mesangial cell.

(Electron microscopy X 10,000)

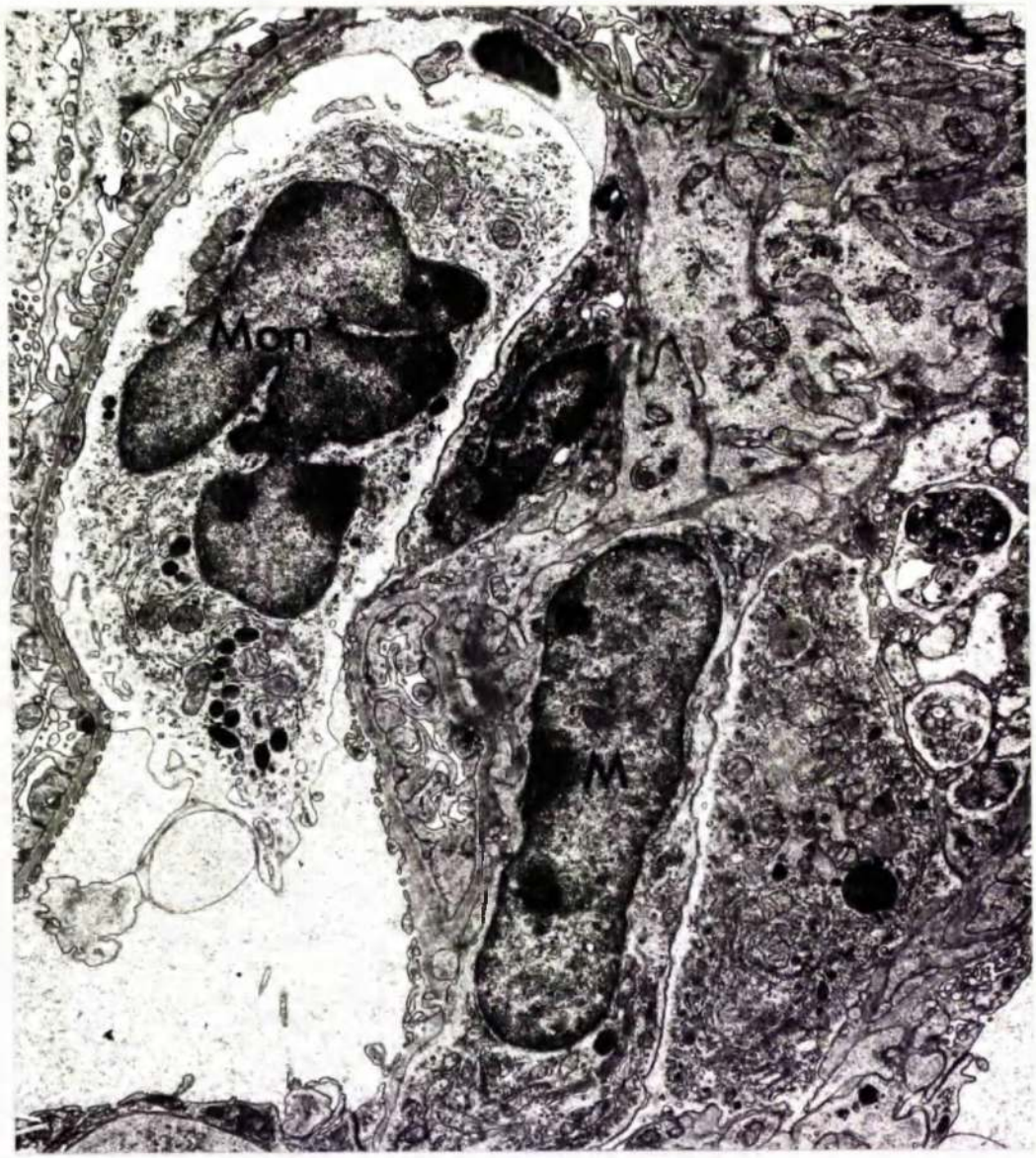
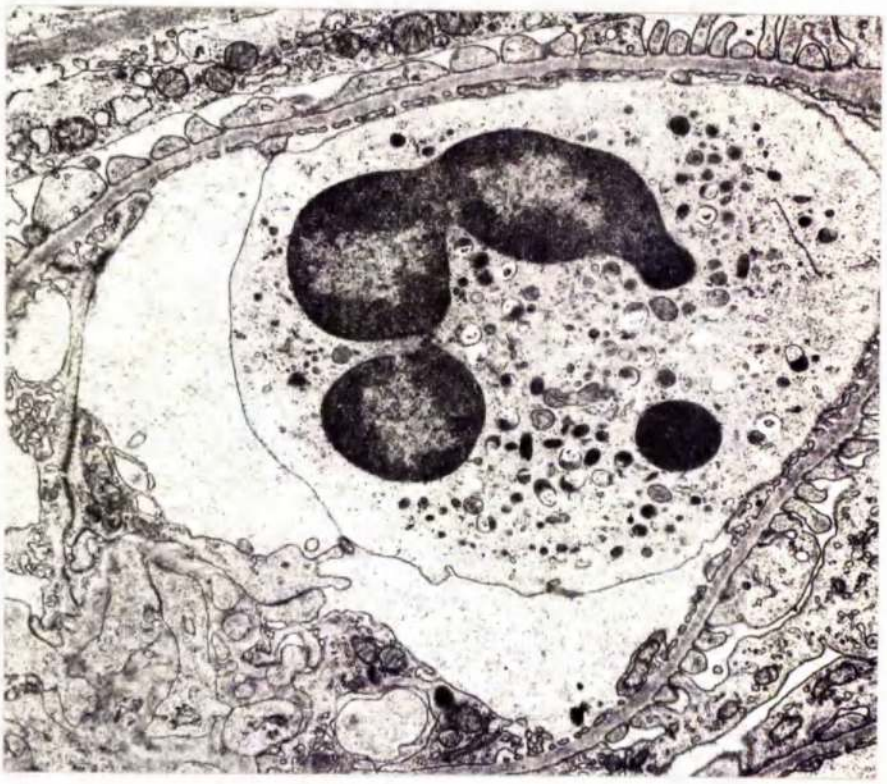

Mon
M

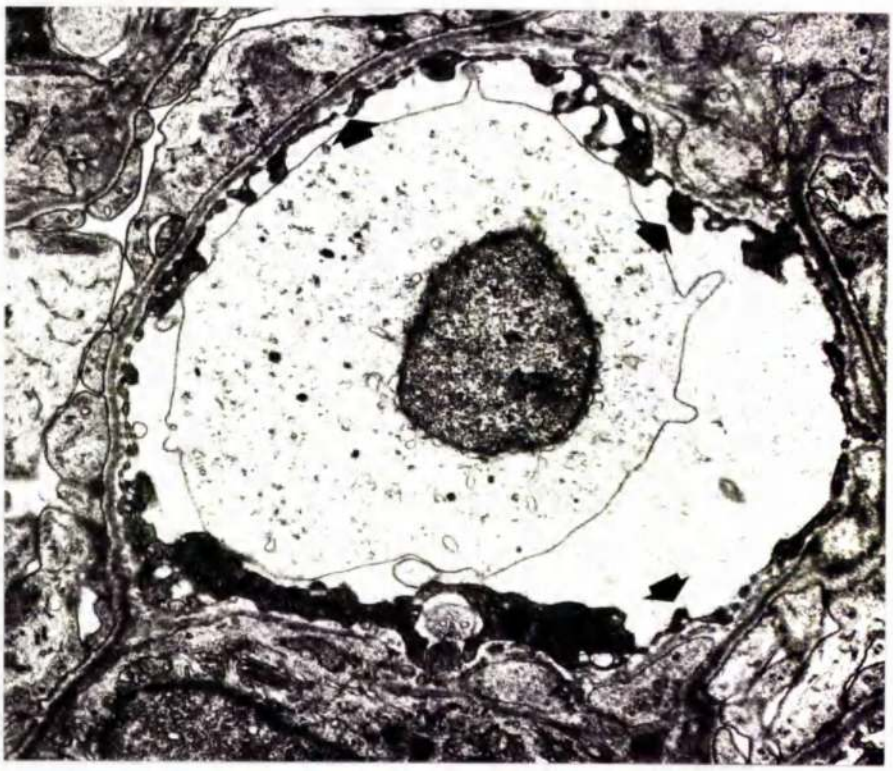
Fig. 84:- Section of glomerulus from a mouse which received 5 daily inoculations of CAV immune complexes, showing a polymorphonuclear leukocyte lodged in a capillary loop.

(Electron microscopy X 12,000)

Fig. 85:- Section of glomerulus from a mouse which received 7 daily inoculations of CAV immune complexes: There is necrosis of the glomerular endothelium (arrows) which appears electron-dense and, in places, is partially detached from the GBM.

(Electron microscopy X 12,000)





(b) Dogs

Clinical and Macroscopic Findings

Throughout the course of the experiment, neither inoculated nor control dogs showed any clinical abnormalities. At necropsy, all inoculated and control dogs showed a normal macroscopic appearance.

Histological Findings

Mild histological changes were found in the glomeruli of all dogs receiving 4 or more doses of complexes. Affected glomeruli showed expansion of the mesangial regions accompanied by segmental mesangial hypercellularity (Fig. 86). In 3 dogs (Nos. 96, 99 and 102), the lesions were diffuse, affecting all of the glomeruli, whereas in the remaining animals they were focal in nature, as not all glomeruli were affected. The remaining inoculated and control dogs appeared histologically normal.

Immunofluorescence Findings

The results of immunofluorescence studies are summarised in Table 15. Diffuse granular deposits of IgG were detected in the mesangial regions of the glomeruli in all dogs receiving 3 or more doses of virus antigen-antibody complexes (Fig. 87). The intensity of fluorescence varied from one animal to another, although, in general, heavier deposits were found in those dogs receiving 5 or more doses of complexes. Similar granular deposits of complement were found in the glomeruli of 6 dogs, all of which received 4 or more doses of complexes. Viral antigen, however, was not detected in the kidneys of any of the inoculated or control dogs.

Ultrastructural Findings

All dogs receiving 3 or more doses of virus antigen-antibody complexes showed glomerular ultrastructural alterations. In general, these changes were mild, but were most marked in those dogs showing the most severe histological lesions. There was an increase in mesangial matrix and mesangial cells often showed marked cytoplasmic activity in the form of increased amounts of endoplasmic reticulum and mitochondria. Mesangial cytoplasmic processes extended into the axial regions of the capillary loops and sometimes between the endothelium and the GBM; these changes resulted in displacement of the endothelium and a reduction in diameter of many capillary loops. Furthermore, in the most severely affected glomeruli, electron-dense dead or dying endothelial cells were found partially detached from the underlying GBM (Fig. 88). In most of the dogs, poorly electron-dense granular deposits were found in subendothelial locations (Fig. 90); occasional electron dense deposits were also found in the mesangial matrix (Fig. 89).

The glomeruli of dogs examined after 2 doses of complexes and control dogs showed no ultrastructural abnormalities.

Table 15:- Immunofluorescence findings in dogs receiving preformed CAV immune complexes.

Dog No.	Number of daily doses of complexes	Glomeruli		
		IgG	C3	CAV
91	2	-	-	-
92	2	-	-	-
93	3	+	-	-
94	3	+	-	-
95	4	+	-	-
96	4	+ +	+	-
97	5	+ +	+	-
98	5	+	+	-
99	6	+ + +	+	-
100	6	+ +	+	-
101	7	+	-	-
102	7	+ + +	+ +	-
	Virus protein			
103	3	-	-	-
104	5	-	-	-
105	7	-	-	-
106	Uninoculated Control	-	-	-
107	" "	-	-	-
108	" "	-	-	-

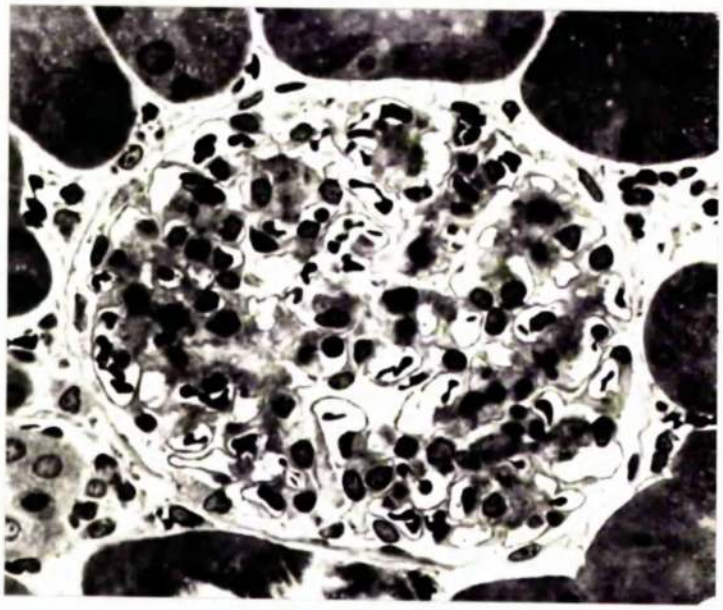
Degree of fluorescence graded + to + + + +

Fig. 86:- Glomerulus from a dog which received 6 daily inoculations of CAV immune complexes, showing expansion and segmental hypercellularity of the mesangial regions.

(Mallory's borax methylene blue, 1μ X 400)

Fig. 87:- IgG in the glomerulus of a dog which received 6 daily inoculatior of CAV immune complexes: Diffuse granular fluorescence can be seen in the mesangium.

(Immunofluorescence X 300)



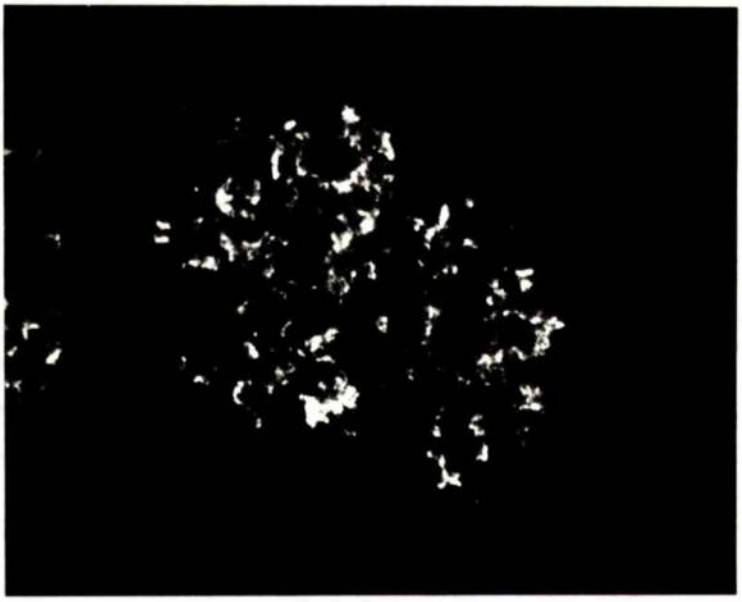


Fig. 88:- Section of glomerulus from a dog which received 7 daily inoculations of CAV immune complexes: There is marked mesangial expansion; extension of mesangial cytoplasmic processes (arrows) beneath capillary endothelium has resulted in displacement of the latter . The endothelial nuclei are pyknotic and their cytoplasm appears electron-dense. An increase in mesangial cytoplasmic organelles can also be seen. M = Mesangial cell; E = Endothelial cell; Ep = Epithelial cell.

(Electron microscopy X 10,000)

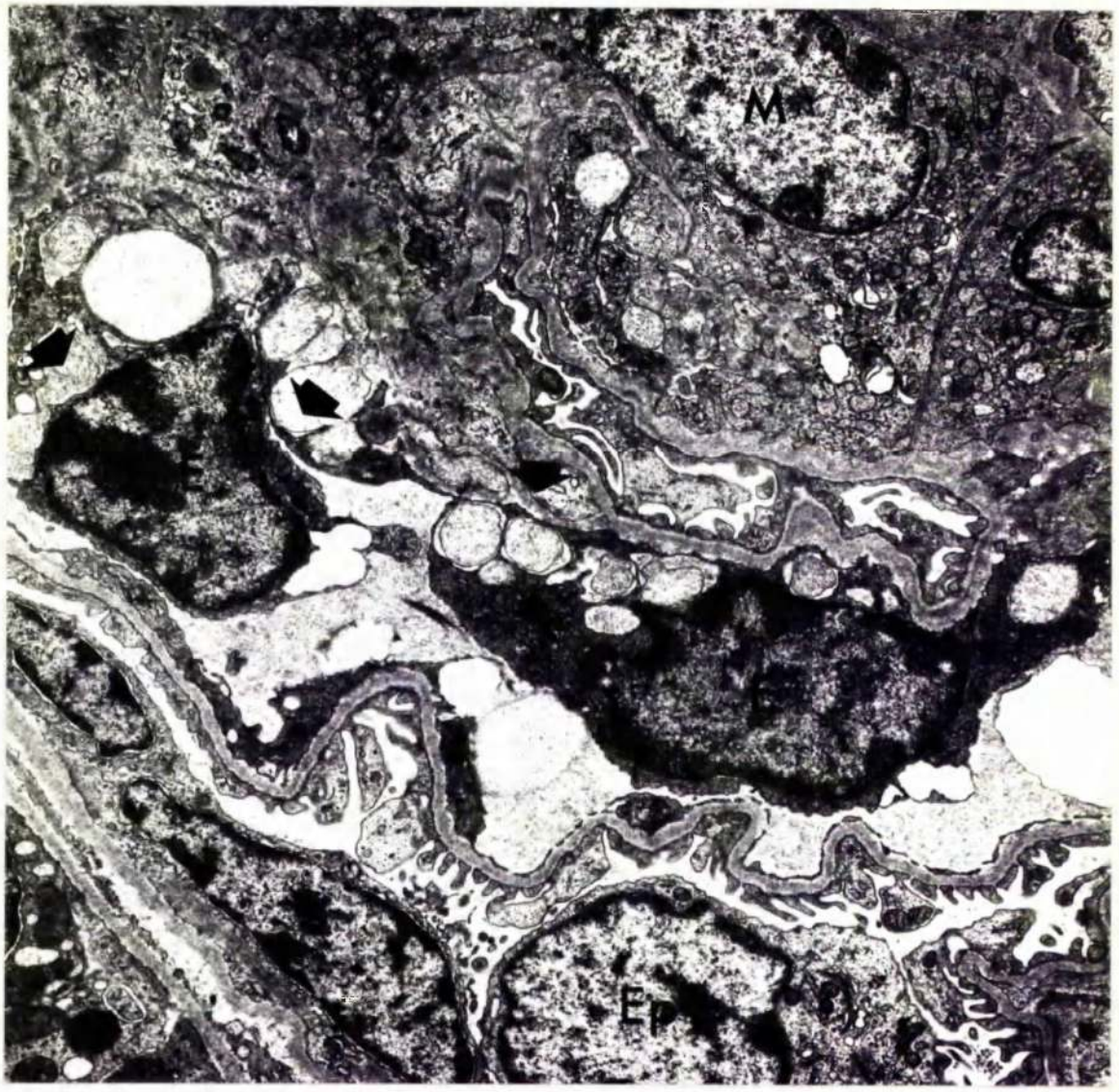
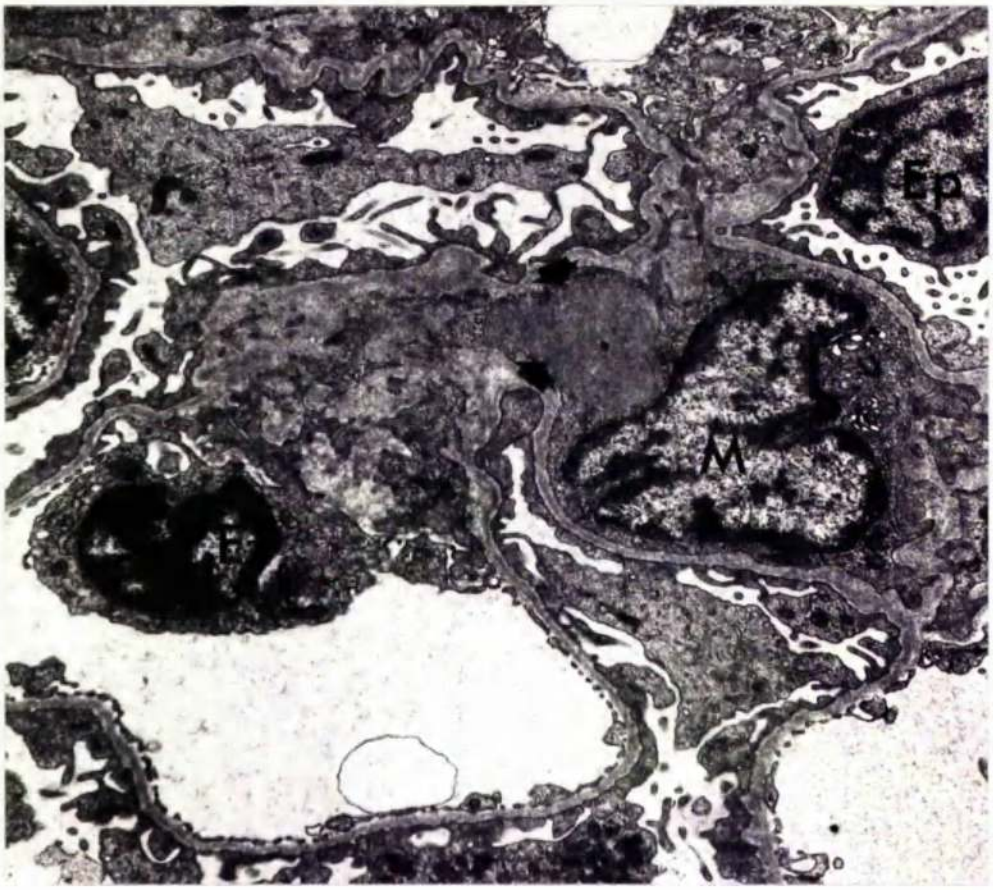

M
Ep

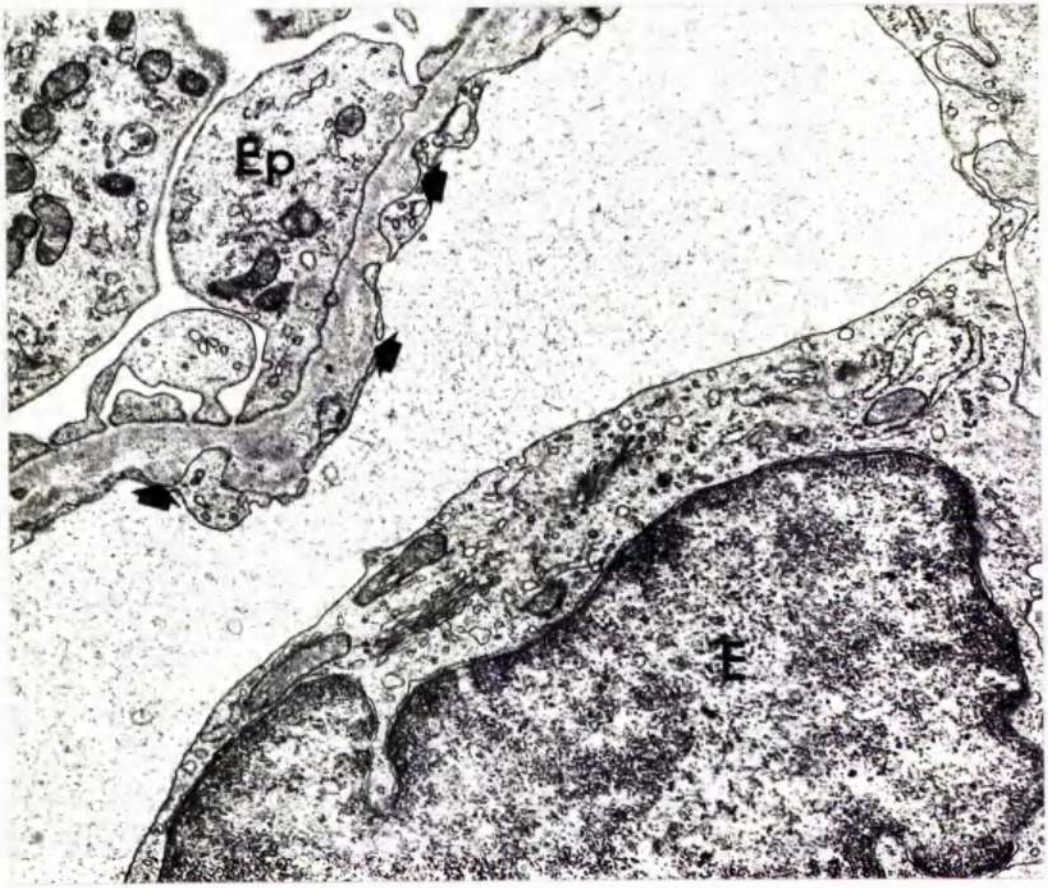
Fig. 89:- Section of glomerulus from a dog which received 7 daily inoculations of CAV immune complexes: An electron dense deposit (arrows) can be seen in the mesangium. M = Mesangial cell; E = Endothelial cell; Ep = Epithelial cell.

(Electron microscopy X 10,000)

Fig. 90:- Section of glomerulus from a dog which received 6 daily inoculations of CAV immune complexes: Small poorly electron dense deposits (arrows) can be seen in a sub-epithelial location. E = Endothelial cell; Ep = Epithelial cell.

(Electron microscopy X 16,000)


Ep
M
Ep


Ep
E

DISCUSSION

In the present study, repeated inoculation of preformed virus antigen-antibody complexes resulted in proliferative glomerulonephritis in 37 per cent of inoculated mice. The glomerular changes were similar, but much less severe than those described in the previous section of this thesis associated with experimentally induced CAV infection in dogs.

By means of immunofluorescence, deposits of canine IgG were demonstrated in the glomeruli of all mice receiving 3 or more daily doses of complexes. As was the case with CAV infected dogs, the immune deposits were mainly confined to the mesangial regions of the glomeruli, a distribution which is considered characteristic of the deposition of relatively large poorly soluble complexes (Germuth and Rodriguez, 1973). Deposits of viral antigen and complement were also detected from the 3rd day onwards but were present in smaller amounts and were not found in all mice. Failure to detect viral antigen in some mice in which canine IgG was demonstrated, is probably related to variation between individual mice in the amounts of complexes deposited in the glomeruli. Because many of the antigenic sites in the deposited complexes are covered by antibody, it is likely that antigen will not be detectable when only small amounts of complexes are deposited in the glomeruli. This would account for the finding that those mice which showed the most severe glomerular histological changes had the heaviest deposits of viral antigen, as assessed by immunofluorescence.

Under the electron microscope, numerous small electron-dense deposits were found in the mesangial regions of the glomeruli and these were accompanied by expansion of matrix and an increase in number of mesangial cells. In the most severely affected mice, polymorphonuclear and mono-

nuclear leukocytes were also found lodged in the capillary loops and less frequently in the mesangium. Similar glomerular lesions were described by Okumura et al., (1971) in mice receiving preformed bovine serum albumen immune complexes, although, in the latter experiment, inoculated mice showed more severe glomerular changes with heavier infiltrates of polymorphonuclear and mononuclear leukocytes.

As heterologous antigens (i.e. CAV protein and canine derived anti-CAV antibody) were used in the inoculation of the present series of mice, it might have been expected that the host would respond by producing an immunological response against these antigens. Immunofluorescence studies, however, failed to detect mouse IgG in the glomeruli of any of the inoculated animals.

Dogs inoculated with preformed virus antigen-antibody complexes developed much less severe glomerular changes than were observed in mice. Although all dogs receiving 3 or more daily doses of complexes consistently showed diffuse mesangial deposits of IgG in the glomeruli, it was not possible to demonstrate viral antigen. Diffuse histological changes consisting of mild expansion of mesangial matrix and segmental hypercellularity, were found in only 3 dogs. By means of electron microscopy, isolated electron dense deposits were found in the mesangium and poorly electron-dense deposits were sometimes found in subendothelial sites. Failure to produce severe glomerular changes in dogs was almost certainly due to insufficient glomerular deposition of complexes, which would also account for the inability to demonstrate viral antigen by immunofluorescence. This might have been expected, since, on a volume per body weight basis, the doses of complexes which the dogs received were much smaller than those administered to mice. However, other factors, such as the degree of antigen excess and thus the solubility of the complexes and the time intervals between consecutive doses, probably also influenced the quantities of complexes deposited in the glomeruli.

SECTION V : IMMUNE COMPLEX GLOMERULONEPHRITIS IN DOGS NATURALLY INFECTED WITH CAV

INTRODUCTION

MATERIALS AND METHODS

RESULTS

Tables 16-18 ; Figures 90-113.

DISCUSSION

INTRODUCTION

The experimental studies described in the previous sections of this thesis have demonstrated that CAV may induce renal damage in a number of different ways. During acute systemic infection, glomerular lesions are attributable to both direct lytic and immune complex mediated mechanisms, while recovered animals may develop an immunologically mediated focal interstitial nephritis as a sequel to persistence of virus in the renal tubules.

Infection of glomerular cells during naturally occurring acute systemic CAV infection has been recognised since Rubarth's initial description of the disease in 1947; similarly, it is well established that dogs recovering from infection may develop focal interstitial nephritis (Hartley, 1958; Wright, 1967b). However, immune complex mediated glomerular lesions have not been recorded in association with naturally occurring CAV infection. Over the course of the last year of this study, a small survey of natural cases of CAV infection was carried out, in order to compare the renal lesions occurring in naturally infected dogs with those which developed during experimental infection. In this section, an account is given of the histological, immunofluorescence and ultrastructural findings in the kidneys of 10 dogs which either died or were in various stages of recovery from acute CAV infection.

MATERIALS AND METHODS

Animals

The 10 dogs were all referred for clinical examination to the Department of Veterinary Medicine, University of Glasgow and subsequently died or were destroyed by intravenous injection of pentabarbitone sodium. Eight of these dogs were not vaccinated against CAV, while the vaccination history of the remaining 2 animals (Nos. 1 and 4) was unknown. The age, breed and sex of the dogs are given in Table 16.

Renal Biopsy Procedure

A renal biopsy was performed on Case Nos. 7 and 8, 2 days after the onset of clinical illness. The procedure was carried out according to a method described by Osborne et al., (1967). Under general anaesthesia induced by sodium thiopentone ("Intraval", May and Baker Ltd., Dagenham), a small area of skin on the upper left flank was shaved and prepared for surgery. While the left kidney was immobilised by abdominal palpation, a small incision was made in the skin and a disposable renal biopsy needle (2N-27-02, Travenol Laboratories Inc., Deerfield, Illinois) was inserted through the abdominal skin and into the kidney. The piece of renal tissue thus obtained was divided into 2 portions and examined histologically and by immunofluorescence in the same manner as described for necropsy material.

Histological Immunofluorescence and Ultrastructural Procedures

All dogs were subjected to a comprehensive macroscopic and histopathological examination as soon as possible after death. With the the exception of 3 dogs which died, pieces of kidney were subjected to ultrastructural examination as described in the section on "materials and methods". Kidney tissue from all dogs was examined by immunofluorescence for the presence of IgG, B1C globulin (C3) and CAV antigen.

In addition, the kidneys of 2 dogs (Nos. 7 and 10), in which there was extensive interstitial infiltrates containing plasma cells, were examined by the indirect "sandwich" fluorescence test in order to determine whether or not the plasma cells contained anti-CAV antibody.

Biochemistry

Where possible, blood and urine samples were obtained at necropsy. Urine was examined for the presence of protein by the turbidometric method using standard sulphosalicylic acid and blood urea nitrogen levels were measured by the Standard Technicon AAII-1 method.

Elutian Procedures

Kidney tissue from 6 dogs was subjected to elution procedures as described in the section on "materials and methods".

Serology

Anti-CAV antibody levels in serum samples and renal eluates were estimated using the indirect immunofluorescence technique.

RESULTS

Clinical History and Macroscopic Findings

Case No. 1: This was a 3 year old Cairn terrier which died 36 hours after developing an illness characterised by progressive depression, anorexia, shivering and eventually recumbancy. A more detailed examination showed pallor and mild jaundice of the mucous membranes, petechiation of the gums and moderate enlargement of the superficial lymph nodes.

At necropsy, the carcase was jaundiced and there were petechial haemorrhages on the gums and ventral surface of the tongue. Other findings were characteristic of acute systemic CAV infection i.e. the presence of an enlarged pale mottled liver, oedema of the wall of the

Table 16: Age, breed and sex of naturally occurring cases of CAV infection.

Dog No.	Age	Breed	Sex
1	3 years	Cairn terrier	F
2	3 months	Collie cross	F
3	8 months	Alsatian	M
4	5 months	Papillon	M
5	6 months	Foxhound	F
6	"	"	M
7	"	"	M
8	"	"	M
9	"	"	M
10	5 months	Shetland collie	M

gall bladder, widespread enlarged haemorrhagic lymph nodes and petechial haemorrhages in the thymus. In addition, however, there were multiple small haemorrhages on the surfaces of both kidneys (Fig. 91); these haemorrhages were present throughout the kidney substance.

Case No. 2: This was a 3 month old Collie cross dog which was presented with depression, fever (105°F) and anorexia. Over the next 4 days, the dog became progressively more depressed, dehydrated and reluctant to move. There was enlargement of all superficial lymph nodes and, on abdominal palpation, the animal showed considerable discomfort. Mild jaundice of the mucous membranes was apparent on the 4th day, at which time, since the dog had become recumbent, euthanasia was carried out.

Case No. 3: This was an 8 month Alsatian which developed clinical illness characterised by depression, anorexia and moderate enlargement of superficial lymph nodes. By the 4th day, there was little change except that jaundice had developed; however, 2 days later the animal was slightly brighter and its appetite was returning, although jaundice was still apparent. On the 8th day following the onset of clinical illness the dog was presented for euthanasia.

At necropsy, there was moderately severe jaundice, although the liver appeared relatively normal. The only other notable feature was the presence of several focal haemorrhages in both kidneys.

Case No. 4: This was a 5 month old Papillon which developed anorexia, depression and mild jaundice. After 3 days, the dog showed some improvement and corneal opacity developed in the right eye. Subsequently, however, the animal's condition deteriorated and it began to show vague signs of an alimentary disturbance, namely anorexia, marked discomfort on abdominal palpation and occasional vomiting. Death occurred 12 days after the onset of clinical signs.

At necropsy, a segment of distal ileum, 5cm in length, was found to be infarcted due to the presence of a thrombus in the adjacent mesenteric vessel. Petechial haemorrhages were found in the kidneys, though they were not so numerous as in Case No. 1. The only additional finding was diffuse corneal opacity of the right eye.

Case Nos. 5-9: This was a litter of 6 month old unvaccinated Foxhound puppies. Case No. 5 died following a severe acute illness lasting 36 hours. During this period, the animal showed progressive dullness and inappetance and jaundice became apparent in the terminal stages. There was enlargement of superficial lymph nodes, petechiation of lips, gums and vulva and purplish discoloration of the abdominal skin. Terminally, the animal became recumbant, the heart rate was markedly increased and the pulse became progressively weaker.

At necropsy, the carcase was jaundiced; there was purplish discoloration of the abdominal skin and petechial haemorrhages were found on the lips and gums and in the vulval and vaginal mucosae. Haemorrhages, similar to those seen in Case No. 1, were present in both kidneys. Other findings were characteristic of acute systemic CAV infection (vida supra).

Subsequently, the remaining 4 dogs in the litter developed a mild illness characterised by lethargy, pyrexia (103-104°C) and varying degrees of superficial lymph node enlargement. Submandibular and brisket oedema was present in Case No. 6. Two days later, all 4 animals were showing marked clinical improvement; at this stage, unilateral corneal opacity developed in 2 dogs (Nos. 6 and 7). Needle beopsy specimens were obtained from the kidney of 2 of these animals, 2 days after the onset of clinical illness. The 4 dogs were then killed at varying intervals following clinical recovery.

At the necropsy of Case No. 6 occasional small white foci of

interstitial nephritis were found in the renal cortices. In addition, there was diffuse corneal opacity of the right eye. Case No. 7 showed more severe focal interstitial nephritis with multiple small white foci 1-2mm in diameter scattered throughout the cortex and medulla of both kidneys; on cut section, these foci were often seen as narrow white streaks extending across the cortical width. In addition, the left eye showed diffuse corneal opacity. Case Nos. 8 and 9 showed no macroscopic abnormalities.

Case No. 10: This was a 5 month old Shetland collie which developed a mild transient illness characterised by anorexia and depression. After 3 days, the dog showed marked clinical improvement and at this stage, unilateral corneal opacity developed. Seventeen days after the onset of clinical illness, the dog was presented for euthanasia. At the time of death, the only apparent clinical abnormality was slight corneal opacity of the right eye.

At necropsy, apart from slight unilateral corneal opacity, macroscopic abnormalities were cofined to the kidneys which showed a focal interstitial nephritis. The surfaces of both kidneys were studded with numerous small white foci 1-3mm in diameter (Fig 92). On cut section, many of these foci appeared as pale streaks extending into the deep cortex; similar small foci of interstitial nephritis were found in the medulla.

Histological Findings

In 3 dogs, (Nos. 1, 2 and 5) the histological picture was characteristic of acute CAV infection. There was focal hepatic necrosis, haemorrhagic lymphadenitis and, in the kidneys, numerous inclusions were found in glomerular endothelial and mesangial cells. The glomerular cells were also swollen, there was mesangial hypercellularity, and many

glomeruli showed an increased content of polymorphonuclear leukocytes (Fig. 93). In addition, 2 of these dogs (Nos. 1 and 5) showed occasional haemorrhage into the filtration spaces and scattered foci of tubular and interstitial haemorrhage.

Apart from unilateral anterior uveitis in 4 animals and necrosis of a segment of bowel in Case No. 4, histological abnormalities were confined to the kidneys in the remaining 7 dogs. In 4 of these animals,(Nos 3, 4, 6 and 9) there was mesangial expansion and segmental hypercellularity affecting some, but not all glomeruli (Figs. 94 and 95). In addition, occasional inclusions were found in mesangial cells in Case No. 3; this animal also showed a few scattered foci of tubular haemorrhage.

Foci of interstitial nephritis were found in 5 dogs. In 3 of these animals (Nos. 1, 4 and 6), only a few small focal accumulations of cells consisting of lymphocytes, macrophages and plasma cells were found. However, in the remaining 2 dogs (Nos. 7 and 10), numerous large foci of interstitial cellular infiltration were found in both cortex and medulla. In both animals, inclusions were frequently encountered in tubular epithelial cells and sometimes in cells lying free in tubular lumina within the cortical and medullary foci (Fig. 96). The cortical cellular infiltrates, which showed a high rate of mitotic activity, consisted mainly of lymphocytes, large mononuclear cells and plasma cells. Necrotic tubules were found within the lesions; these sometimes contained inclusions and were often surrounded by accumulations of macrophages and polymorphonuclear leukocytes. Other intact tubules showed cytoplasmic swelling and vacuolation and often contained necrotic cellular debris, among which lymphocytes and polymorphonuclear leukocytes were sometimes found (Fig. 98). In comparison with lesions found in the cortex, those in the medulla contained many more necrotic tubules and these were characteristically surrounded by polymorphonuclear

leukocytes and large numbers of macrophages, with only a few lymphocytes and plasma cells (Fig. 97). A notable feature of some of the cortical and medullary foci in both of these dogs, was the presence of multinucleated giant cells which were particularly numerous in Case No. 10 (Fig. 99). Evidence of early interstitial fibrosis was present in Case No. 10, particularly around the margins of the larger cellular foci where an increase in the number of interstitial fibroblasts was apparent.

Immunofluorescence Findings

The results of the immunofluorescence studies are summarised in Table 17. Granular deposits of IgG were detected in the mesangial regions of the glomeruli in 7 dogs (Nos. 1-7). In 6 of these animals, the deposits were found diffusely in all the glomeruli whereas, in one dog (No. 7), many contained only segmental deposits (Figs. 100 and 101). The glomeruli of the remaining 3 dogs, which were examined at 17, 35 and 49 days, contained no specific deposits of IgG. Elsewhere in the kidney, groups of fluorescing plasma cells were found in 5 dogs (Fig. 105). In 2 of these animals (Nos. 1 and 4), they were restricted to a few isolated small foci, found mainly in the medulla; the remaining 3 dogs (Nos. 6, 7 and 10) showed larger accumulations of specific IgG fluorescing plasma cells mainly in the cortex.

Complement (C3) was detected as diffuse fine granular mesangial staining in the glomeruli of 4 dogs (Nos. 1-4) (Fig. 102).

When stained for the presence of CAV antigen, discrete fluorescing virus-infected cells were detected in the majority of glomeruli and in the endothelium of interstitial capillaries in the kidneys of 3 dogs (Nos. 1, 2 and 5). A third animal (No. 3) showed only occasional fluorescing glomerular cells. In addition, however, in Case Nos. 1-5, fine granular deposits of CAV antigen were found in the mesangial regions

Table 17: Naturally occurring CAV infection: Immunofluorescence patterns in the liver and kidneys.

Dog No.	Time examined after clinical onset	Liver	Immunofluorescence					
			Glomeruli				Tubules	Plasma cells
		CAV	CAV		IgG	C3	CAV	IgG
			Discrete cellular	Fine granular				
1	36 hours (D)	+	+ +	+ +	+ + +	+	+	+
2	4 days	+	+ +	+ +	+ + +	+ +	-	-
3	8 days	-	+	+	+ +	+	-	-
4	12 days (D)	-	-	+	+ +	+	+	+
5	36 hours (D)	+	+ +	+	+ +	-	-	-
6	7 days	-	-	-	+	-	+ +	+
7	2 days*		-	-	+	-	-	-
	21 days	-	-	-	+	-	+ + +	+ +
8	2 days*		-	-	+	-	-	-
	35 days	-	-	-	-	-	-	-
9	49 days	-	-	-	-	-	-	-
10	17 days	-	-	-	-	-	+ + +	+ + +

Degree of fluorescence graded + to + + + +

D = Died

* = Renal biopsy

of all glomeruli, in a similar pattern to that observed for IgG (Fig. 103). Virus antigen was not detected in the glomeruli of the remaining 5 dogs. Foci of fluorescing tubular epithelial cells were detected in both cortex and medulla in 5 dogs (Nos. 1, 4, 6, 7 and 10) and these were particularly numerous in Cases Nos. 7 and 10 (Fig. 104). CAV antigen was also found in large amounts in the liver of Case Nos. 1, 2 and 4.

Renal biopsy material from Case Nos. 7 and 8 examined by immunofluorescence, each contained 2 glomeruli. In both animals, the glomeruli contained diffuse granular deposits of IgG but were negative for the presence of CAV antigen and complement.

Kidney sections from Case Nos. 7 and 10, which were stained by the indirect immunofluorescence test, contained foci of fluorescing plasma cells indicating the presence of anti-CAV antibody (Fig. 106).

Ultrastructural Findings

Of the 7 dogs whose kidneys were examined by electron microscopy, 2 (Nos. 2 and 3) showed diffuse glomerular ultrastructural changes. In both animals numerous electron dense deposits were found scattered throughout the mesangial regions of the glomeruli. This, together with an increase in matrix and segmental hypercellularity, resulted in expansion of mesangial regions which impinged on endothelial cells and caused partial collapse of many of the capillary loops. Many mesangial cells showed phagocytic activity and their cytoplasm often contained electron dense granules and increased amounts of endoplasmic reticulum (Fig. 107). Glomerular endothelial and epithelial cells were swollen and vacuolated and, in the case of epithelial cells, this often resulted in areas of fusion of the foot processes. In Case No. 2, which showed the most severe ultrastructural changes, moderate numbers of polymorphonuclear leukocytes and large monocytes were also found lodged in

the glomerular capillary loops. In this animal, virus particles were found in the nuclei of mesangial cells (Fig. 108) which were sometimes disrupted with release of virus into the surrounding mesangial matrix. Small clumps of virus particles were also occasionally observed in membrane-bound vesicles in the cytoplasm of otherwise uninfected mesangial cells.

Less severe changes were found in the glomeruli of a further 2 dogs (Nos. 6 and 7). In these animals, there was mild expansion of mesangial matrix, occasionally accompanied by granular electron dense deposits (Fig. 109). In Case No. 6, this was also, in some areas, associated with an increase in mesangial cells. In both animals, electron-dense, vacuolated, dying endothelial cells were found (Fig. 110); these cells were sometimes partially detached from the GBM and were being replaced by adjacent healthy endothelial cells.

Glomeruli examined from the remaining 3 dogs (Nos. 8, 9 and 10) showed a normal ultrastructural appearance.

Foci of interstitial nephritis were located in both cortex and medulla in 2 dogs (Nos. 7 and 10). In these animals, cortical lesions were composed of lymphocytes, plasma cells, macrophages and large monocytes and many mitotic configurations were observed (Fig. 111). Medullary infiltrates contained large numbers of macrophages, accompanied by focal accumulations of polymorphonuclear leukocytes and only a few lymphocytes and plasma cells (Fig. 112). Necrotic tubules were found more frequently in the medulla and were usually surrounded by a heavy infiltrate of macrophages and polymorphonuclear leukocytes. In both cortex and medulla, intact tubules found within the infiltrates showed evidence of degenerative changes consisting of cytoplasmic swelling and vacuolation and marked mitochondrial swelling. Occasionally, virus infected tubular epithelial cells were observed in these tubules; however,

virus particles were more often present in tubular lumina where they were found lying among degenerating infected cells and necrotic cellular debris (Fig. 113). In such tubules, lymphocytes and polymorphonuclear leukocytes were often observed migrating through the epithelium and lying among the necrotic cellular debris in the lumen. Virus particles were also occasionally found within intact or degenerating cells lying in the interstitium. Early fibroblast activity was noted, particularly around the margins of the interstitial foci, where increased numbers of fibroblasts and early collagen deposits were found.

Biochemical Findings

The results of urine protein and blood urea nitrogen estimations are presented in Table 17. The 5 dogs, in which diffuse granular deposits of IgG and viral antigen were detected in the glomeruli by immunofluorescence, all showed significantly elevated levels of protein in the urine (i.e. >50mg/100ml), ranging from 77 to 276mg/100ml. Only one dog (No. 5) showed a significantly elevated level of blood urea nitrogen.

Serological Findings

The results of anti-CAV antibody estimations on serum and renal eluates are summarised in Table 17. Of the 5 dogs from which serum was obtained, 2 showed rising titres of antibody to CAV in paired serum samples and all 5 animals had titres of 256 or more when they were killed; these were all dogs which had shown clinical recovery. Low levels of anti-CAV antibody were detected in all 5 kidney eluates. In one of these dogs, deposits of immunoglobulin were not found in the glomeruli and the eluted antibody was therefore, presumably, derived from interstitial plasma cells.

Table 18:- Naturally occurring CAV infection: Biochemical and Serological findings.

Dog No.	Time examined after clinical onset	Anti-CAV Antibody		Urine Protein mg/100ml	Blood Urea Nitrogen mg/100ml
		Serum	Eluate		
1	30 hours (D)	ND	2	77	ND
2	4 days	-	-	94	18
3	8 days	ND	2	162	33
4	12 days (D)	ND	2	102	ND
5	36 hours (D)	ND	ND	276	83
6	7 days	256	ND	0	41
7	2 days*	4			
	21 days	512	1	10	28
8	2 days*	16			
	35 days	512	ND	7	21
9	49 days	512	ND	9	18
10	17 days	512	8	32	37

D = Died

* = Renal biopsy

ND = Not done

Fig. 91:- Kidney from Case No. 1, showing multiple petechial haemorrhages.

Fig. 92:- Kidney from Case No. 10, showing multiple small white foci of interstitial nephritis.

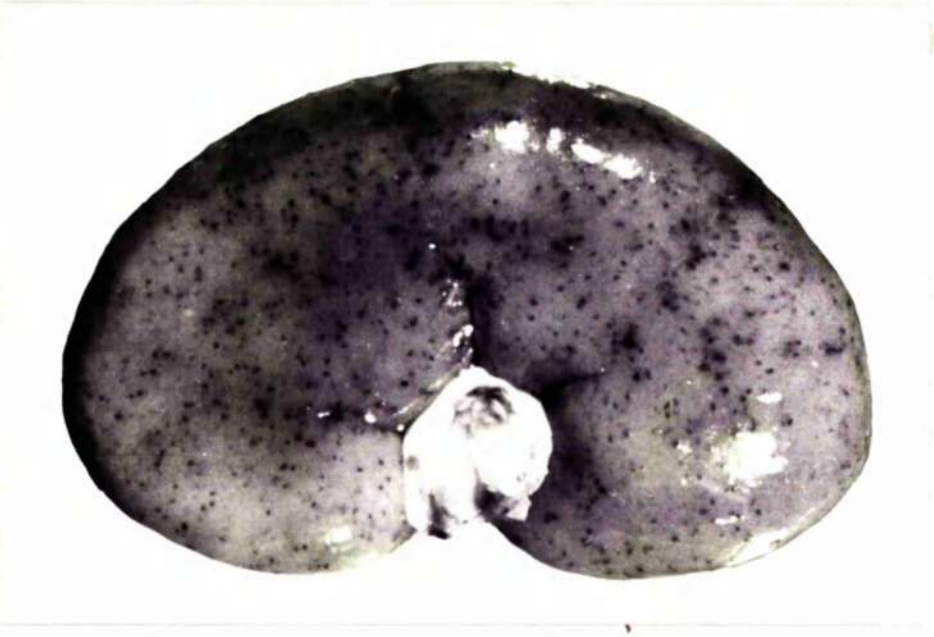


Fig. 93:- Glomerulus from Case No 1: The glomerular cells are swollen and there is an increase in mesangial cellularity resulting in occlusion of capillary loops. Several intranuclear inclusion bodies can also be seen.

(HE X 400)

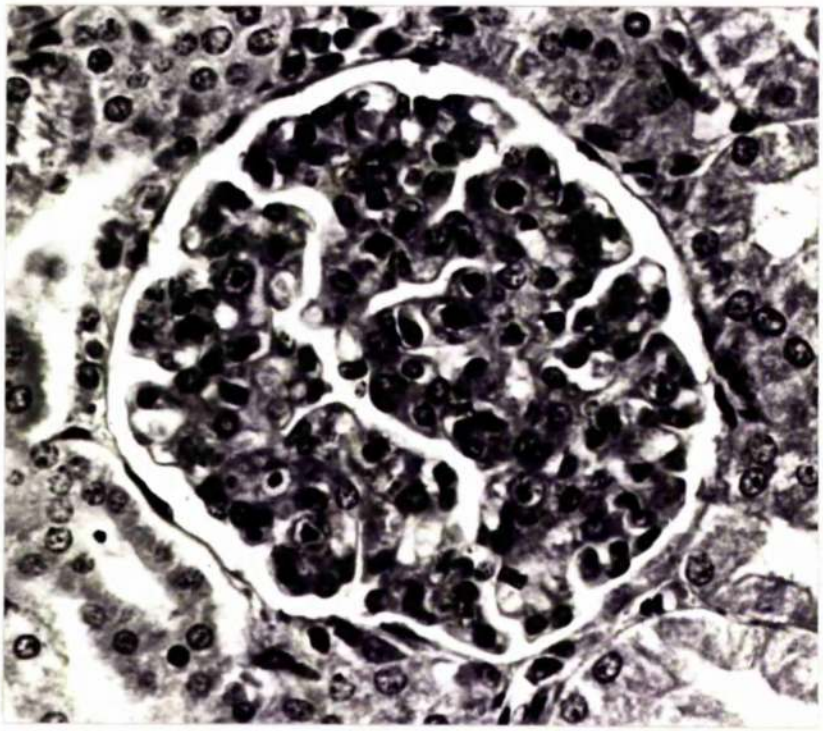
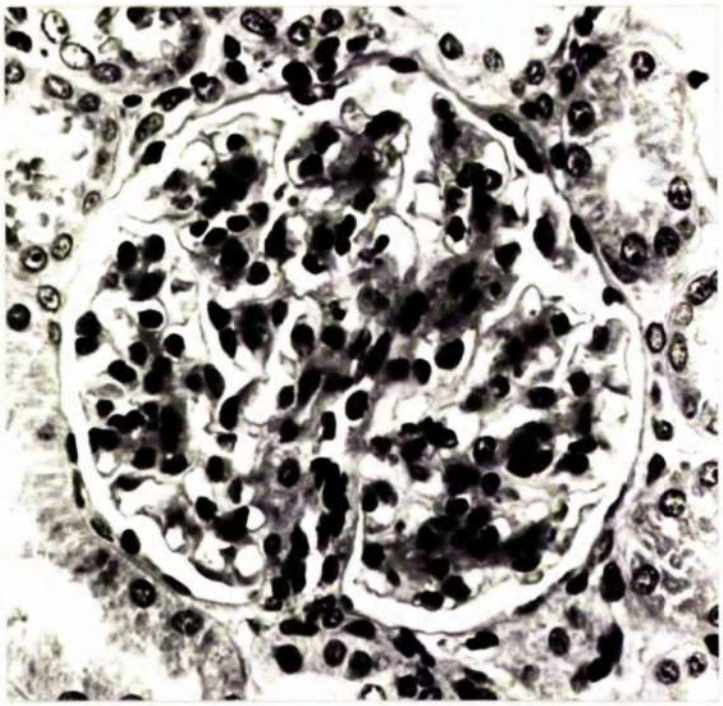


Fig. 94:- Glomerulus from Case No. 3, showing expansion and segmental hypercellularity of mesangial regions.

(HE X 400)

Fig. 95:- Another glomerulus from Case No. 3 shows a normal histological appearance.

(HE X 400)



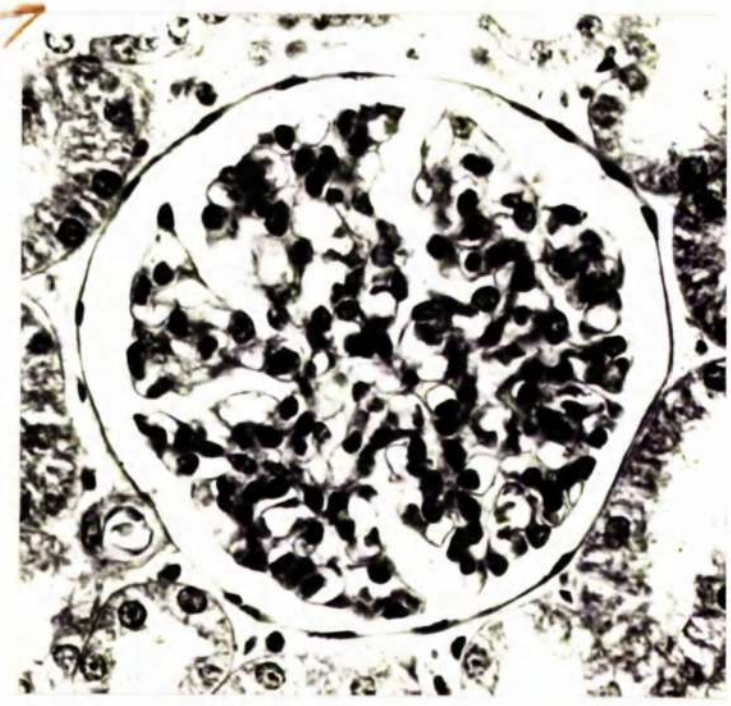
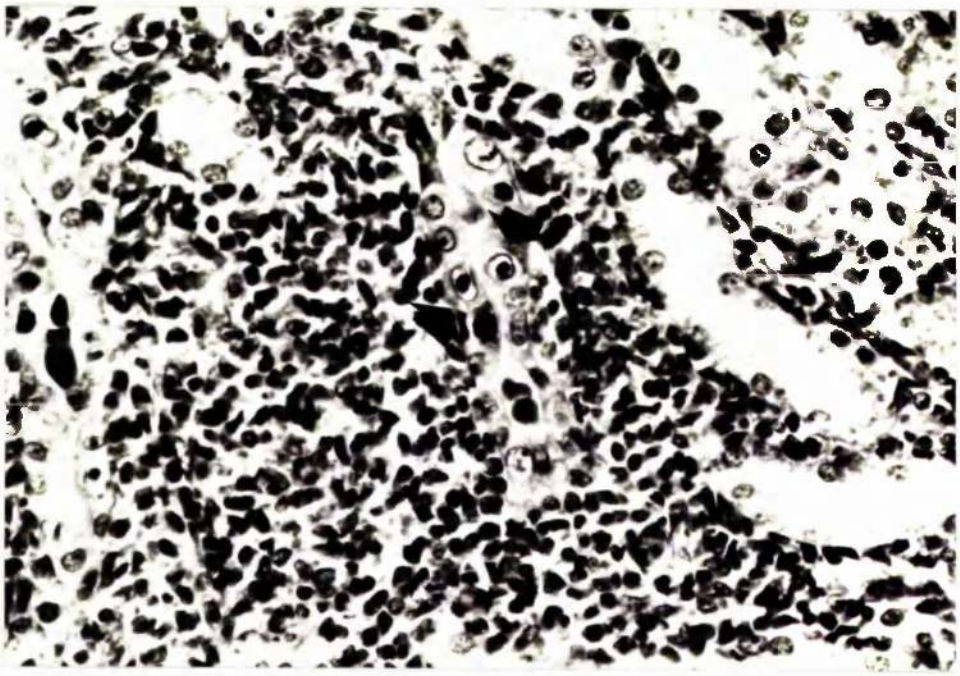


Fig. 96:- CAV interstitial nephritis in Case No. 7: Within this cortical focus, intranuclear inclusion bodies (arrows) can be seen in tubular epithelial cells.

(HE X 300)

Fig. 97:- CAV interstitial nephritis in Case No. 10: A transverse section of medullary tubules shows the remains of a necrotic tubule surrounded by large numbers of macrophages and a few polymorphonuclear leukocytes.

(HE X 450)



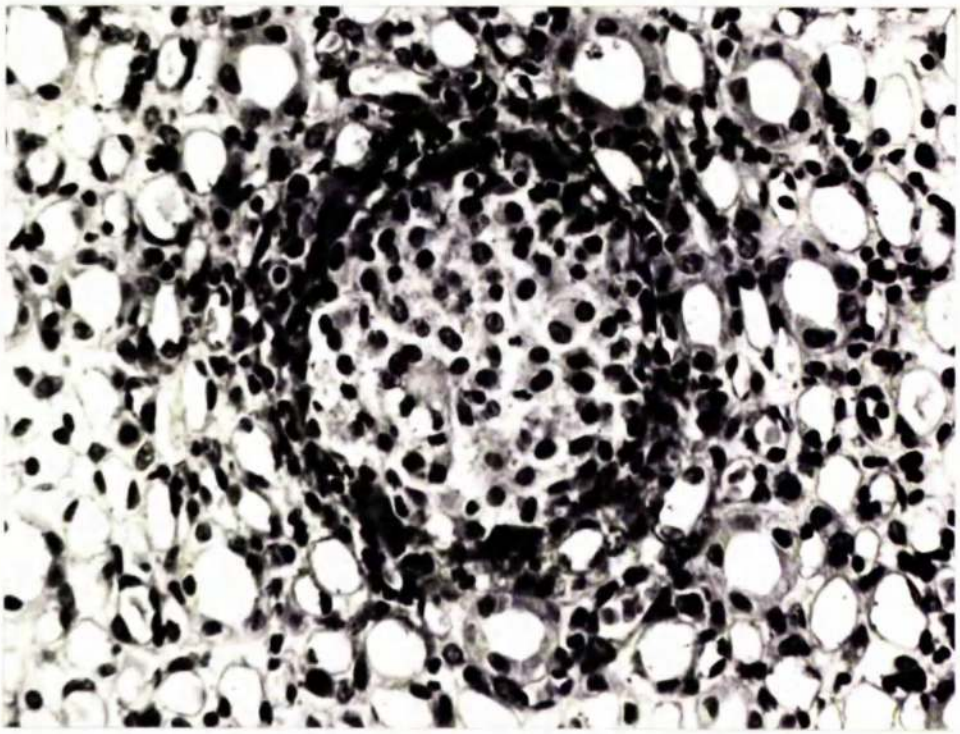
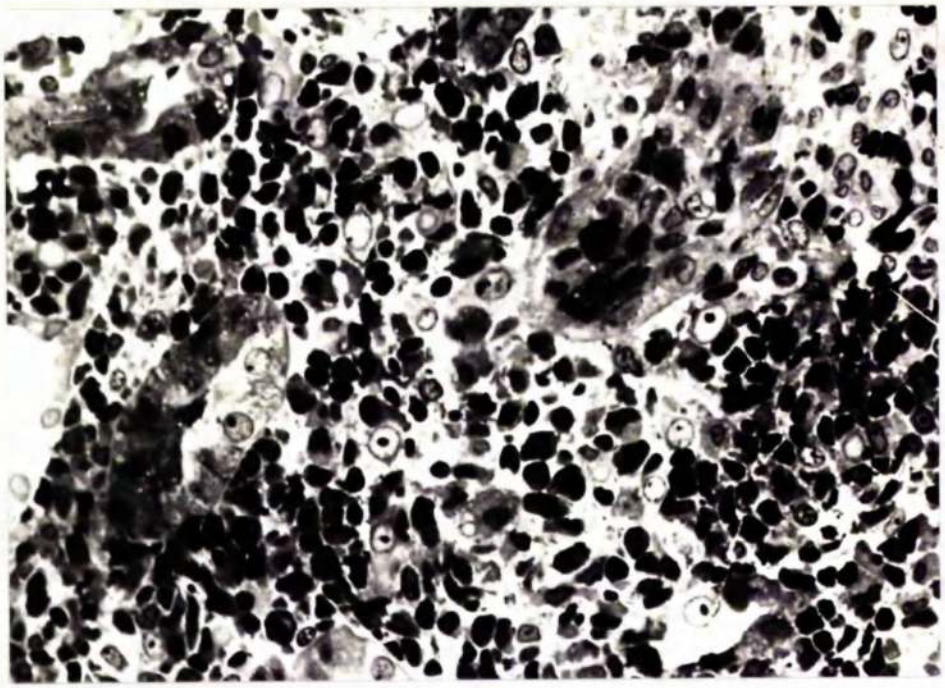


Fig. 98:- CAV interstitial nephritis in Case No 10: The cellular infiltrate is composed of a mixture of lymphocytes, plasma cells and macrophages.

(Mallory's borax methylene blue, 1μ section X 400)

Fig. 99:- CAV interstitial nephritis in Case No 10: This cellular focus contains numerous multinucleated giant cells.

(HE X 300)



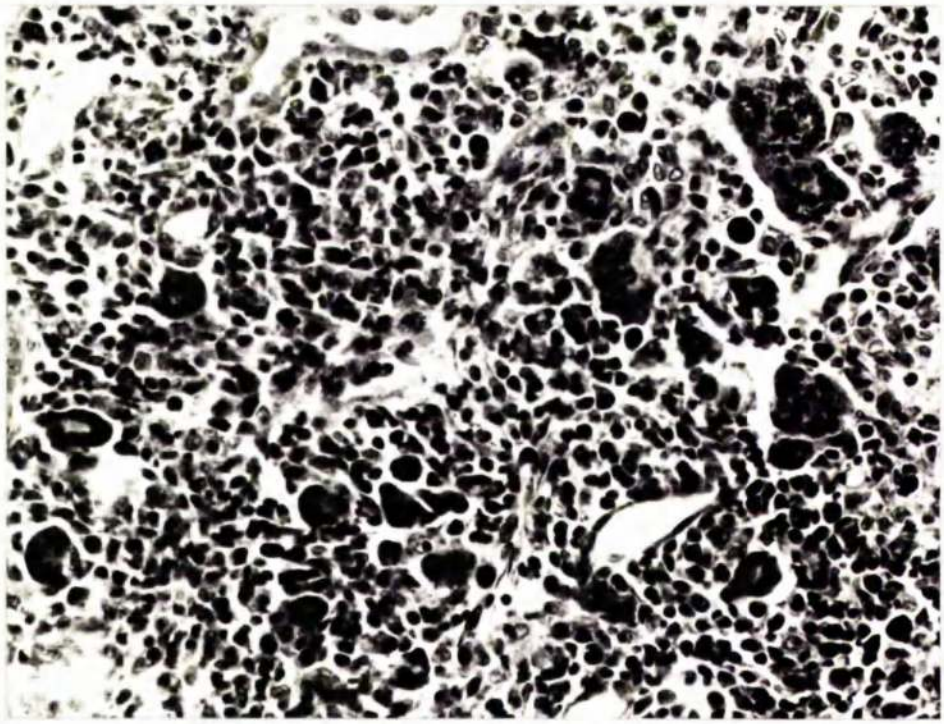


Fig. 100:- IgG in a glomerulus from Case No. 1: Diffuse granular fluorescence can be seen in the mesangial regions.

(Immunofluorescence X 400)

Fig. 101:- IgG in a glomerulus of Case No. 7: Coarse granular fluorescence is confined to segments of the glomerulus.

(Immunofluorescence X 400)

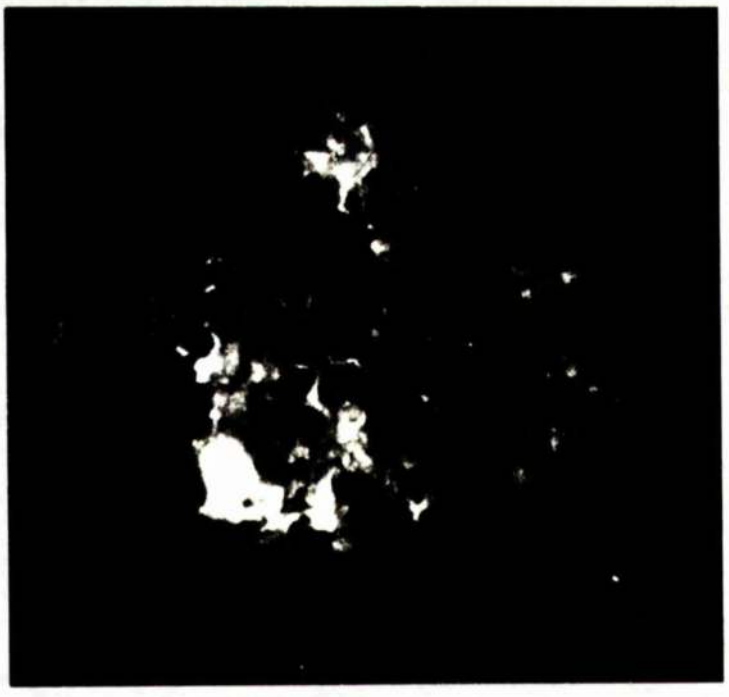


Fig. 102:- Complement in a glomerulus from Case No. 2: Diffuse granular fluorescence can be seen in the mesangial regions.

(Immunofluorescence X 400)

Fig. 103:- CAV antigen in a glomerulus from Case No. 2: Fine granular fluorescence is present in the mesangial regions; a discrete fluorescing cell can also be seen.

(Immunofluorescence X 400)

Fig. 104:- Section of kidney from Case No. 7 stained for CAV antigen: Specific antigen fluorescence can be seen in tubular epithelial cells.

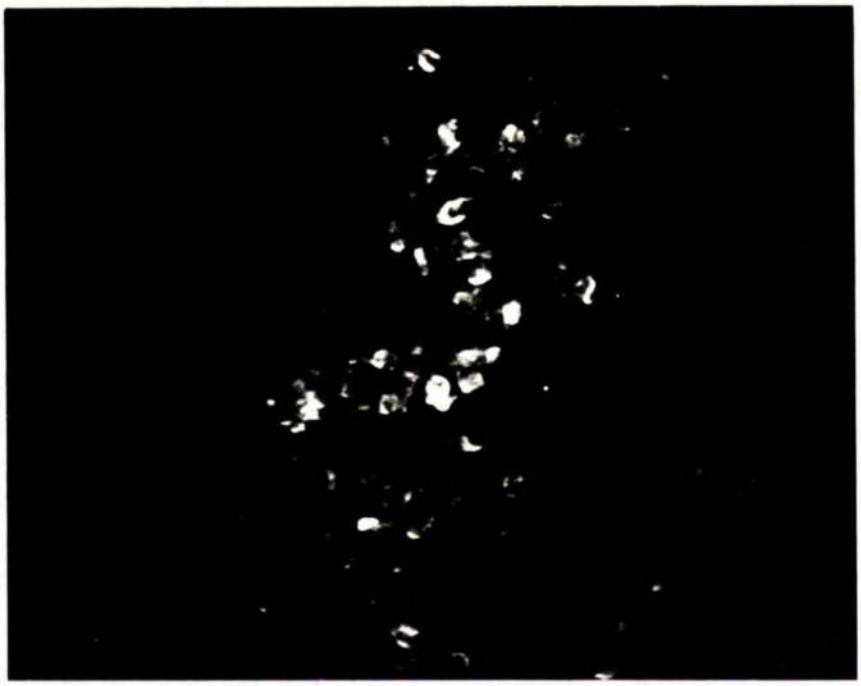
(Immunofluorescence X 400)

Fig. 105:- CAV interstitial nephritis, Case No. 10: The cellular infiltrate contains numerous IgG staining plasma cells.

(Immunofluorescence X 250)

Fig. 106:- CAV interstitial nephritis, case No. 10: A section of kidney stained by the indirect immunofluorescence test for anti-CAV antibody shows a small group of fluorescing plasma cells.

(Immunofluorescence X 400)



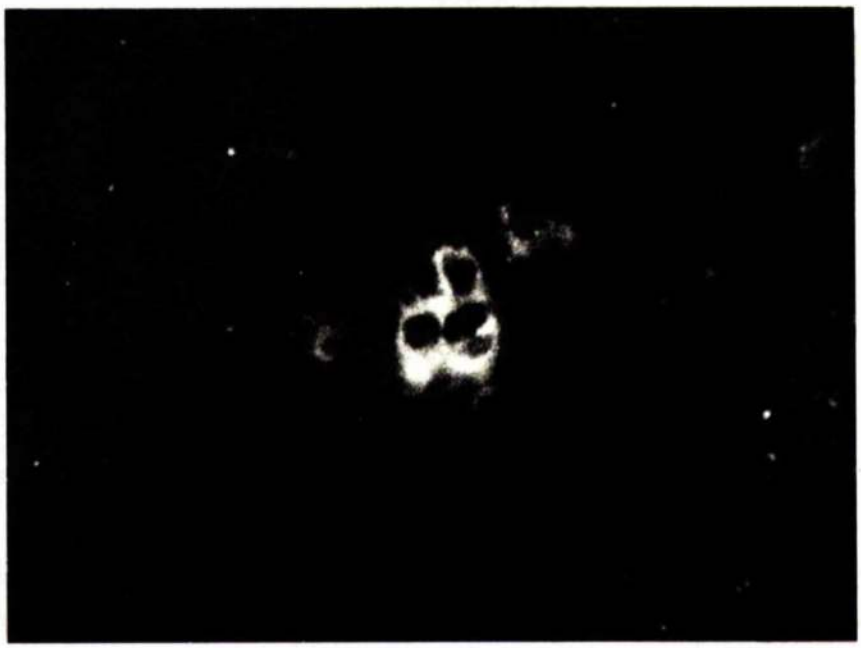
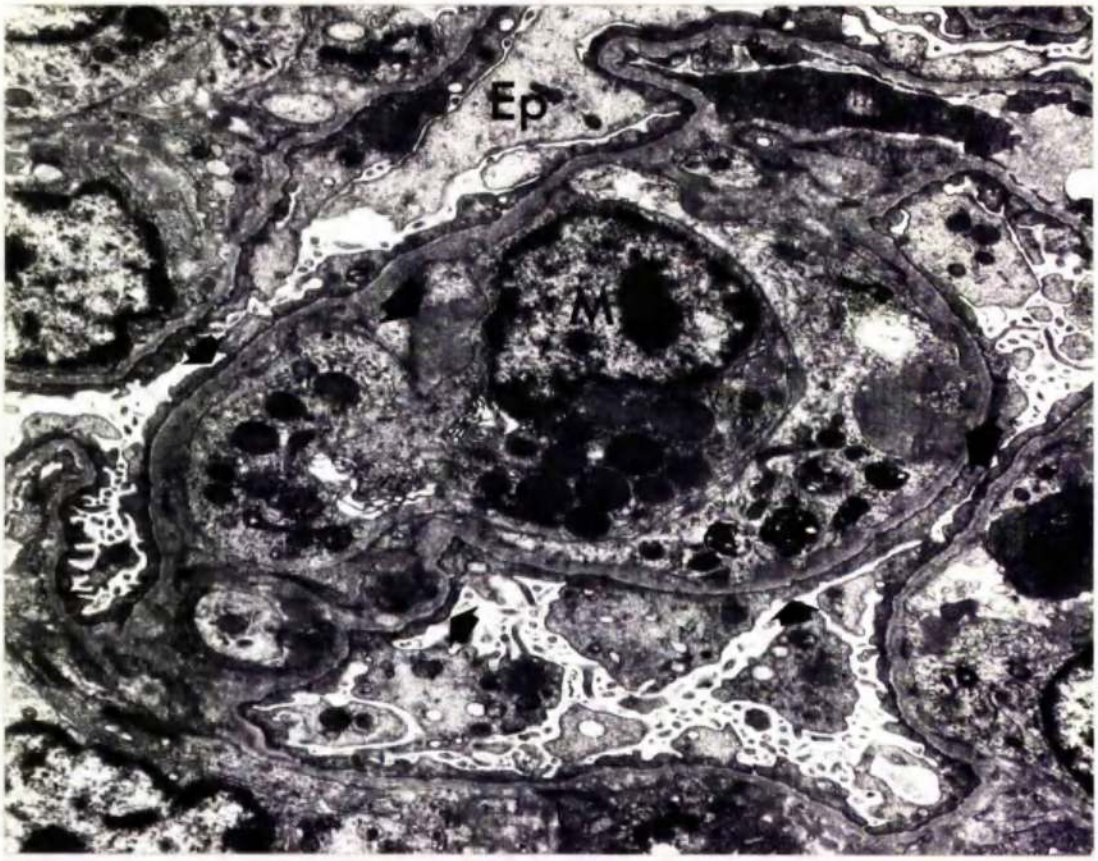


Fig. 107:- Section of glomerulus from Case No. 2: Irregular electron-dense deposits (large arrows) are present in the mesangium; a mesangial cell (M) can be seen containing numerous large electron-dense granules. There is also extensive fusion (small arrows) of the epithelial cell (Ep) foot processes.

(Electron microscopy X 10,000)

Fig. 108:- Section of glomerulus from Case No. 2: Virus particles can be seen in the nucleus of a mesangial cell (M); small electron-dense deposits (arrows) are also present in the surrounding mesangial matrix. Ep = Epithelial cell.

(Electron microscopy X 15,000)


Ep
M

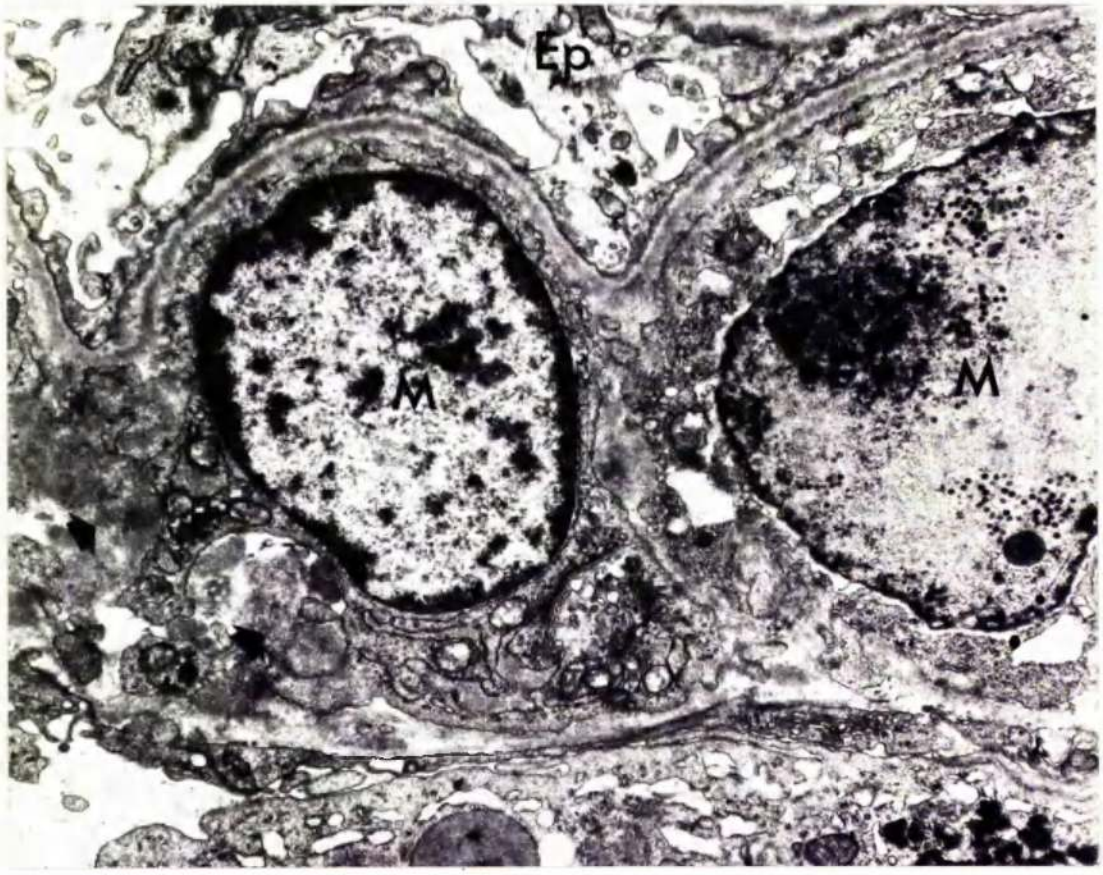

Ep
M
M

Fig. 109:- Section of glomerulus from Case No. 6: Electron-dense deposits (*) can be seen in the mesangial matrix.
M = Mesangial cell.

(Electron microscopy X 15,000)

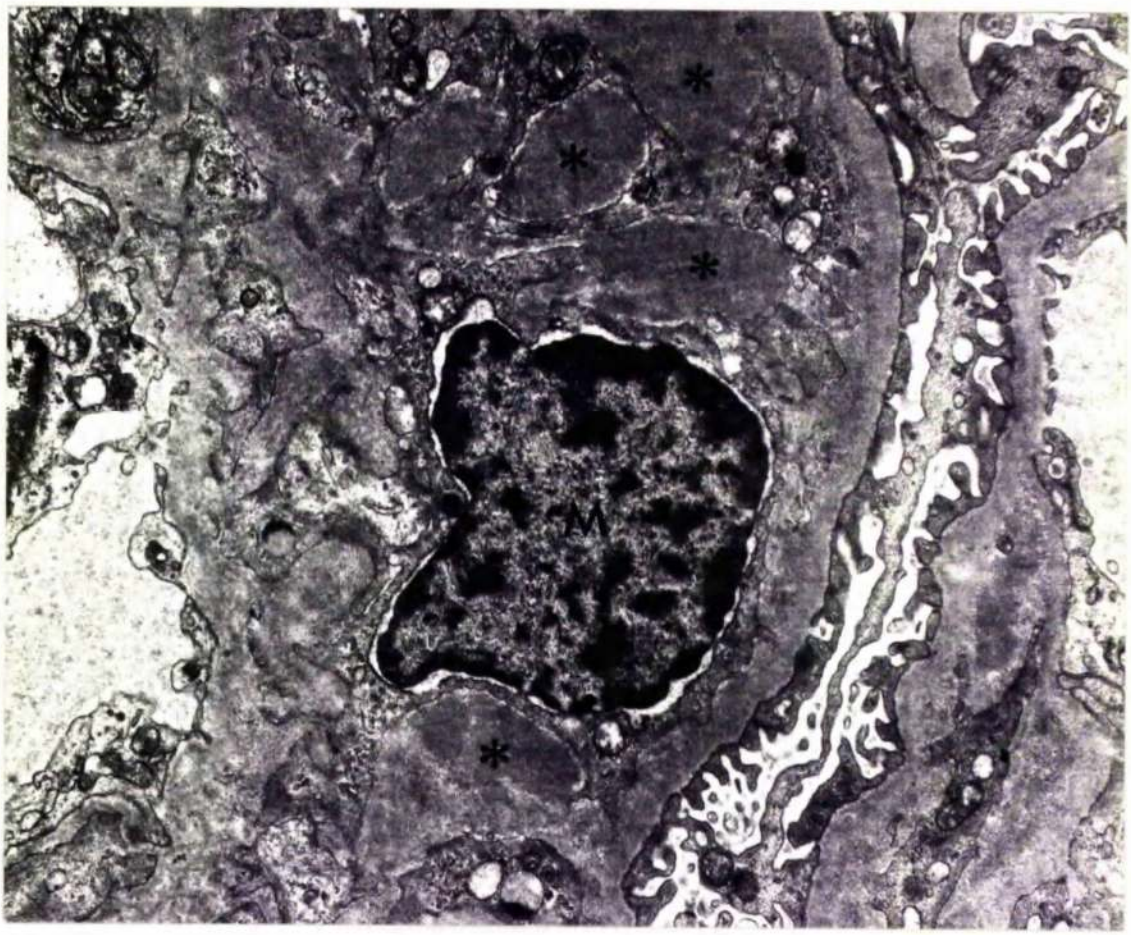

M

Fig. 110:- Section of glomerulus from Case No. 6: The glomerular endothelium (arrows) appears shrunken, electron-dense and vacuolated. M = Mesangial cell.

(Electron microscopy X 10,000)

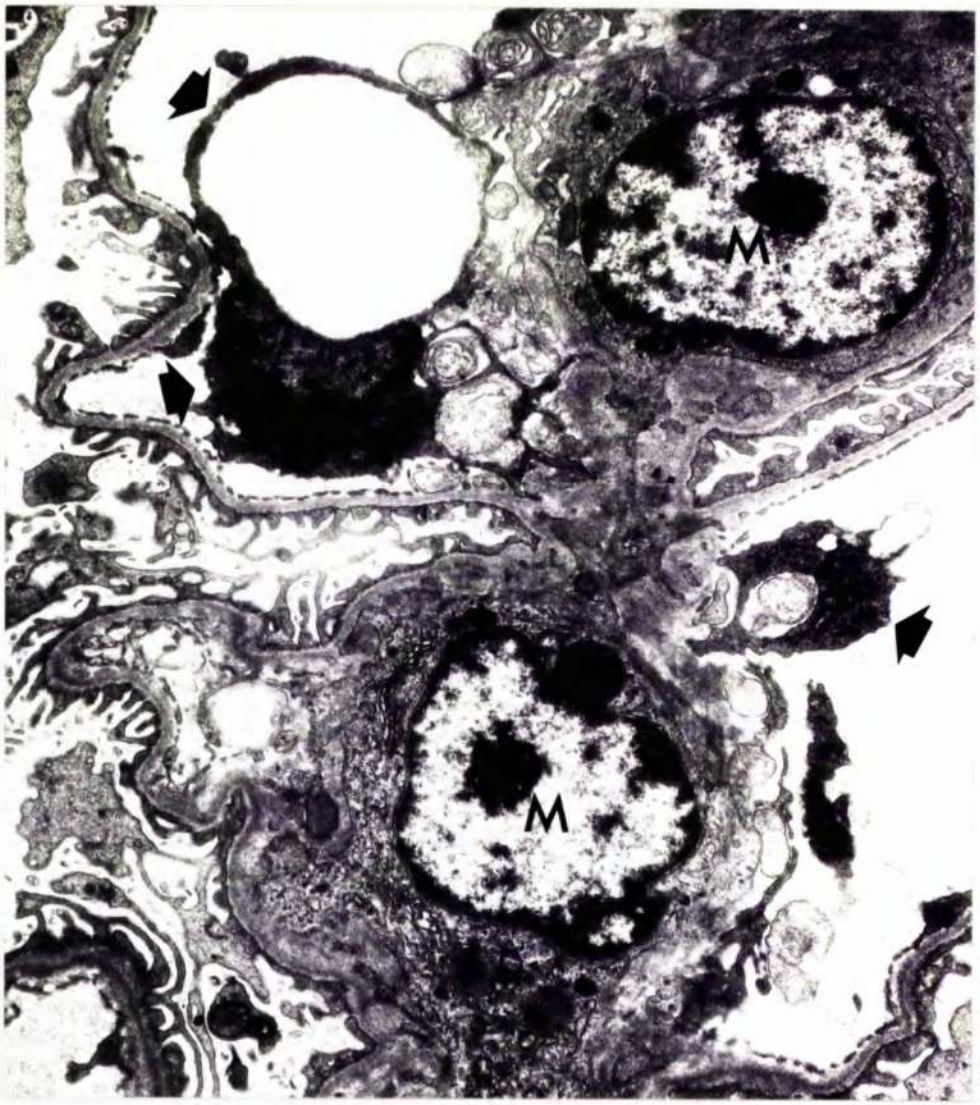

M
M

Fig. 111 (a):- CAV interstitial nephritis, Case No. 10: A section of cortical cellular infiltrate shows a mixture of lymphocytes (L) and plasmablast (P).

(Electron microscopy X 6,000)

(b) (Inset):- A lymphocyte from the same lesion can be seen undergoing mitotis.

(Electron microscopy X 6,000)

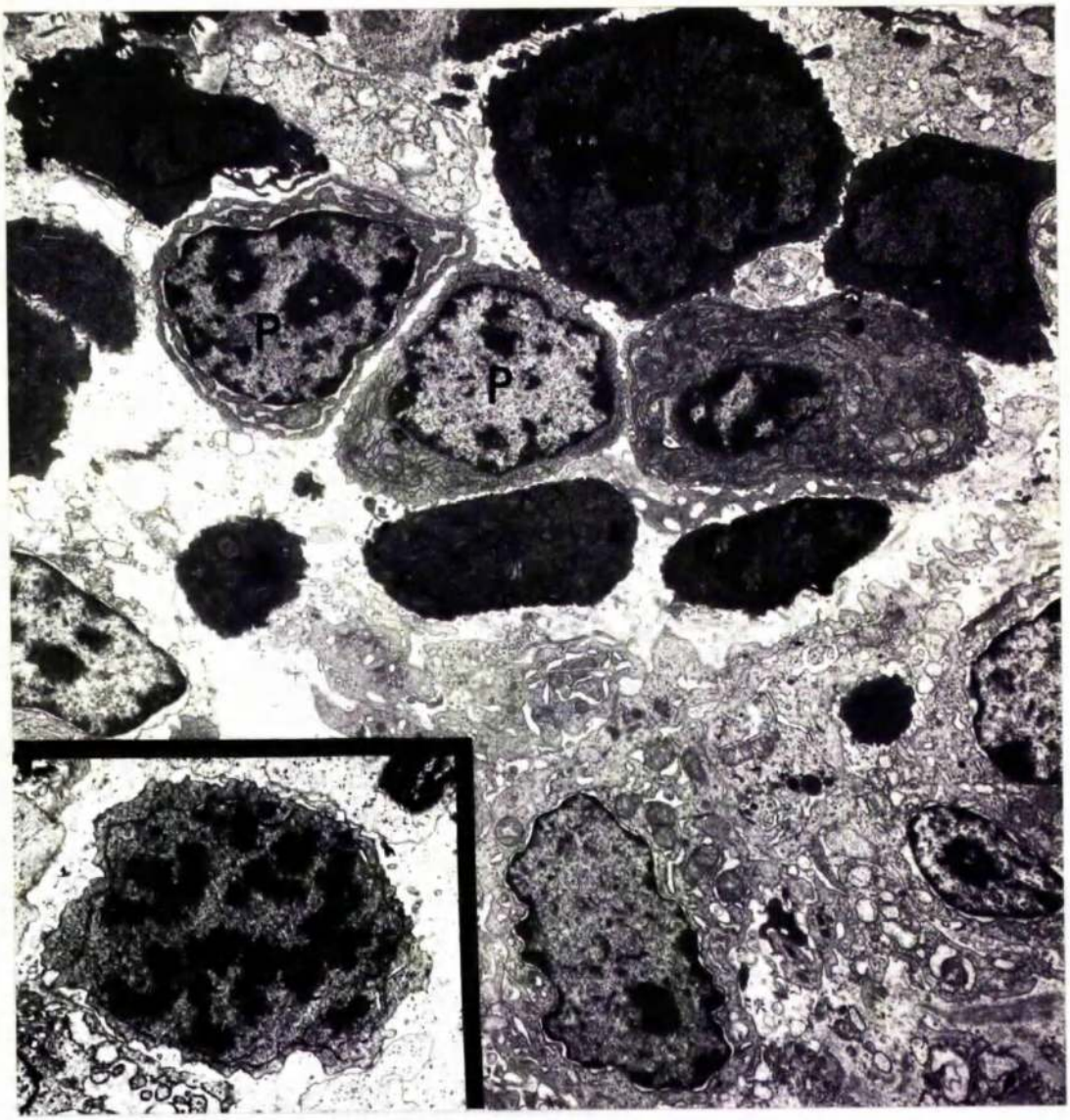

P
P

Fig. 112:- CAV interstitial nephrits, Case No. 10: Section of medullary cellular infiltrate, showing numerous active macropahges (Ma). A polymorphonuclear leukocyte (Pm) can also be seen.

(Electron microscopy)

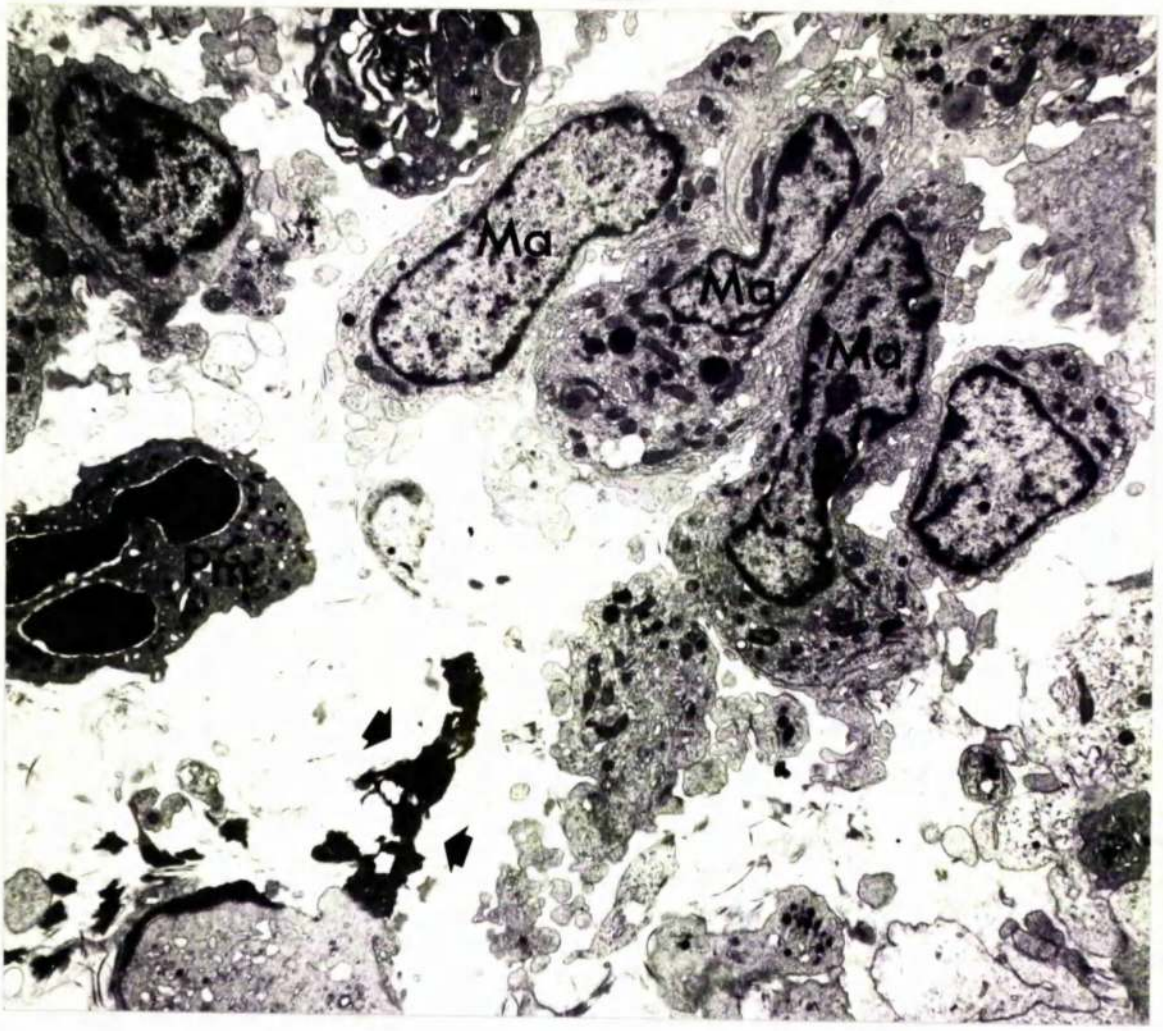
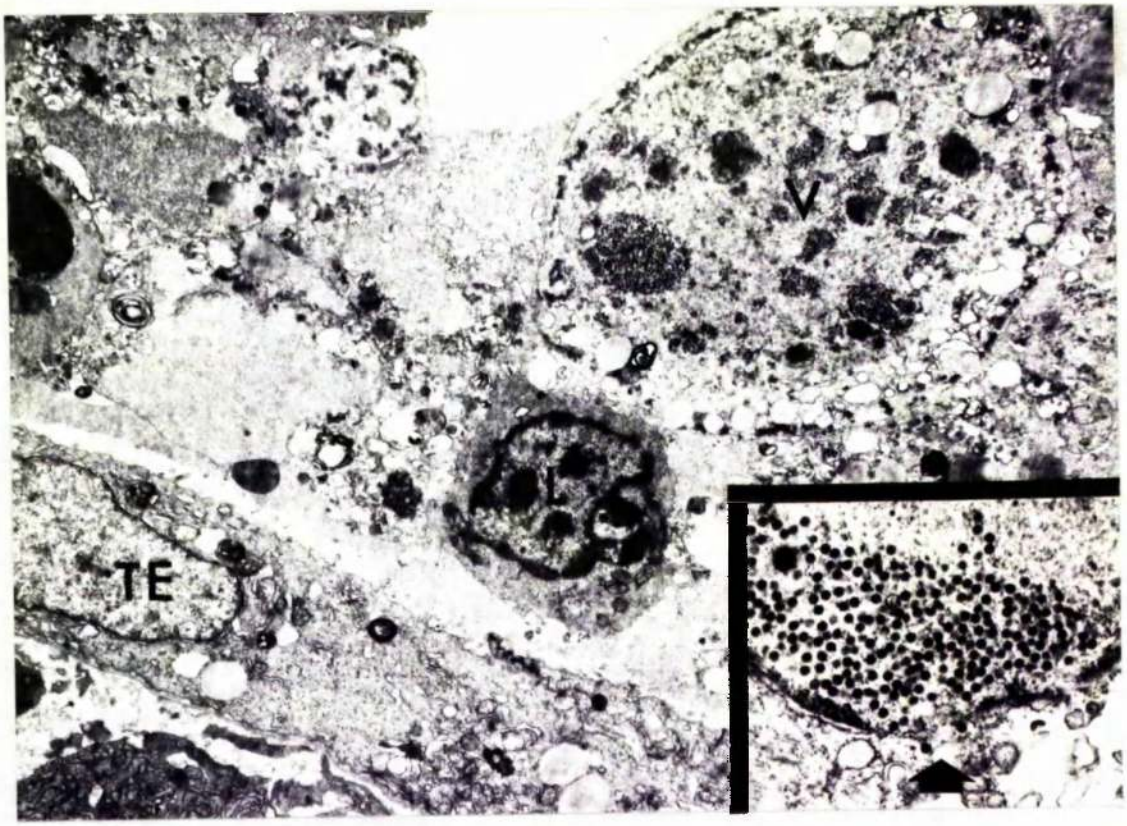

Ma
Ma
Ma
Pm

Fig. 113 (a):- CAV interstitial nephritis, Case No. 10: Section of medullary tubule containing a lymphocyte (L) and a degenerating virus-infected cell (V) lying among necrotic cellular debris. TE = Tubular epithelium.

(Electron microscopy X 6,000)

(b) (Insert) Part of the nucleus of an infected cell found in the lumen of the same tubule, showing virus particles escaping through the nuclear membrane (arrow).

(Electron microscopy X 16,000)


V
TE

The range of renal lesions found in the present series of dogs closely resembled that observed in dogs experimentally infected with CAV, as described in sections I and III. In those animals which died during the systemic phase, direct infection of glomerular cells resulted in lytic cellular damage. Widespread foci of interstitial nephritis were also found in 2 of the clinically recovered dogs. In these animals, the cellular infiltrates, particularly in the cortex, contained large numbers of lymphocytes and plasma cells and, by means of an indirect immunofluorescence test, local anti-CAV antibody production was demonstrated in the plasma cells. In other sites, however, the finding of large populations of macrophages and relatively few plasma cells surrounding infected tubules, was suggestive of a cell mediated hypersensitivity reaction. In addition to the above lesions, a number of dogs showed evidence of immune complex mediated glomerular disease. Granular deposits of immunoglobulin, viral antigen and complement were found in the mesangial regions of the glomeruli and this was associated with varying degrees of mesangial expansion and cellular proliferation and elevated levels of proteinuria. These findings,along with the detection of anti-CAV antibody in renal eluates, are in agreement with the results obtained in experimental studies (see Section III) and establish that the glomerular changes are mediated by the deposition of virus antigen-antibody complexes. CAV is, therefore, the first viral antigen, indeed antigen of any kind, which has been shown to be involved in immune complex mediated glomerular disease in the dog. Moreover, the finding of less severe histological changes and a gradual decrease in the glomerular deposits of IgG in those animals examined at 7, 21 and 35 days after clinical illness, suggests that the glomerular lesion is transient and unlikely to lead to progressive renal failure.

SUMMARY AND CONCLUSIONS

The results of the present study indicate that there are 3 major mechanisms by which CAV may inflict renal injury (see Table 19):-

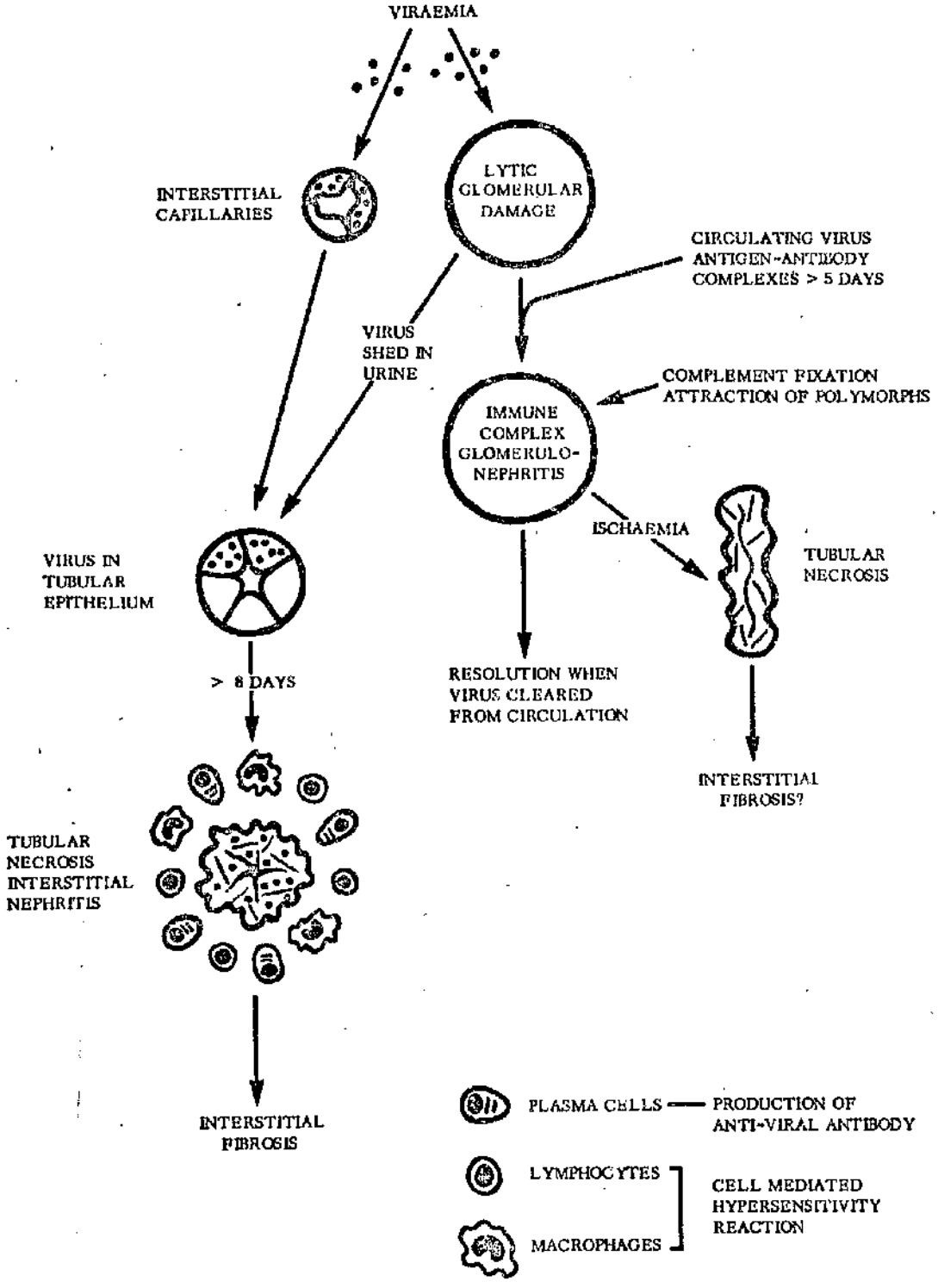
1. During systemic infection, the virus replicates in glomerular endothelial and mesangial cells resulting in cellular desruption and release of GIM antigens into the urine.

2. Infected dogs, which survive until the stage when antibody production commences, also develop a proliferative glomerulonephritis. Evidence was provided that this developed as a result of deposition of circulating virus antigen-antibody complexes. In response to these deposits, there was proliferation of mesangial cells and expansion of mesangial matrix resulting in marked swelling of the glomerular tufts and occlusion of capillary loops. These changes were exacerbated by infiltration of polymorphonculear leukocytes into the tufts, an event which was probably related to fixation of complement by deposited complexes. At this stage, scattered foci of proximal tubular necrosis were also found; these did not appear to be associated with direct virus infection but were considered to have developed secondary to glomerular ischaémia.

Following recovery from systemic infection, the glomerular lesions appear to resolve. Since virus antigen is rapidly cleared from the circulation, immune complexes are no longer formed and the immune deposits which are already present in the glomeruli are gradually removed by mesangial cells. Thus, it would appear that, although CAV is capable of inducing a relatively severe glomerulonephritis accompanied by proteinuria and haematuria, because of the transient nature of the lesion, it is unlikely to give rise to progressive renal failure. It is, however, probable that, in some cases, focal interstitial fibrosis will result from resolution of necrotic tubular foci.

3. In addition to the glomerular lesions described above, a percentage of dogs which recover from systemic infection develop focal interstitial nephritis as a sequel to persistence of virus in tubular epithelium. In addition to tubular necrosis produced by direct lysis of infected cells, tissue damage also undoubtedly results from the infiltration of cells into the surrounding interstitium. That the presence of these cells appears to represent an immunological response to the virus, was supported by the demonstration of anti-viral antibody production by plasma cells within the lesions. However, other morphologic features of the lesions suggest that cell mediated hypersensitivity reactions may also be involved; this is one aspect of the disease which requires further investigation. Once virus is successfully eliminated from the kidney, the interstitial lesions resolve by fibrosis. Although, in most cases, this is of no functional significance, it may be that in a few individuals the resultant scarring is sufficiently severe to result in renal failure; this, however, has yet to be confirmed.

Table 19 :— THE RELATIONSHIP BETWEEN CAV INFECTION AND RENAL DISEASE



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CANINE ADENOVIRUS NEPHROPATHY

by

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SUMMARY

The purpose of the present investigation was to carry out a detailed histological, immunofluorescence and ultrastructural study of the renal lesions which occur during canine adenovirus (CAV) infection. An attempt was made to define the mechanisms by which the virus inflicts renal injury and in particular, to determine the role of the immune response in the development of renal lesions.

It was shown that 3 main mechanisms of renal damage occurred in both experimentally induced and naturally occurring CAV infection:

1. During acute systemic infection, replication of virus in glomerular endothial and mesangial cells was shown to result in lytic cellular damage and release of glomerular basement membrane antigens into the urine

2. In dogs which survived to the stage when anti-viral antibody first appeared, there was also deposition of immune complexes in the glomeruli which resulted in a proliferative glomerulonephritis. Results of immunofluorescence and elution studies indicated that the deposited complexes were composed of viral antigen and anti-viral antibody. Furthermore, repeated inoculation of mice and dogs with virus antigen-antibody complexes prepared in vitro, resulted in similar but less severe glomerular lesions. In dogs recovering from systemic infection, there was a gradual reduction in severity of glomerulonephritis. At this stage, due to elimination of virus antigen, immune complexes were no longer present in the circulation and there was removal by mesangial cells of immune deposits already present in the glomeruli.

3. However, although the glomerular lesions appeared to resolve, a percentage of dogs recovering from systemic infection developed focal interstitial

nephritis in response to persistence of virus in tubular epithelium. As evidence that the interstitial cellular infiltrate represented an immunological response to the virus, anti-viral antibody production was demonstrated in plasma cells found within the lesions. However, the morphological appearance of the lesions, which often contained large numbers of active macrophages surrounding virus-infected tubules, suggested that cell mediated hypersensitivity reactions may also be involved.