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MESANGIAL LOCALIZATION OF IMMUNE COMPLEXES IN EXPERIMENTAL CANINE ADENOVIRUS GLOMERULONEPHRITIS

N. G. WRIGHT, W. I. MORRISON, H. THOMPSON AND H. J. C. CORNWELL

From the Department of Veterinary Pathology, University of Glasgow Veterinary School, Bearsden Road, Glasgow, Scotland

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Summary.—Each of a group of 14 dogs was infected experimentally by an intravenous dose of canine adenovirus calculated to allow survival until the initial stages of antibody production; the kidneys of infected dogs were examined during the period of 4–14 days after administration of virus. Proliferative glomerulonephritis with localization of IgG, C3 and viral antigen in mesangial regions was demonstrated. With the electron microscope, electron dense deposits were found scattered throughout the mesangium. There was proliferation of mesangial cells, infiltration into the glomerular tuft of polymorphonuclear leucocytes and, in some cases, focal glomerular necrosis with intracapsular and tubular haemorrhage. By means of an indirect immunofluorescence test, anti-viral antibody was detected in kidney eluates; anti-kidney antibody was not present.

GLOMERULONEPHRITIS is a feature of a number of viral infections of animals. Thus, equine infectious anaemia virus (Banks, Henson and Maguire, 1972), lymphocytic choriomeningitis virus in mice (Kajima and Pollard, 1970), Aleutian disease virus in mink (Henson *et al.*, 1969), swine fever virus (Cheville, Mengeling and Zinober, 1970), coxsackie B4 virus in mice (Sun *et al.*, 1967) and leukaemia viruses in mice (Mellors *et al.*, 1971) have all been shown to be involved in the development of immunologically mediated glomerular disease.

In the dog, a range of morphological types of glomerulonephritis similar to those occurring in the human has recently been described (Murray and Wright, 1974). The search for aetiological factors in canine glomerulonephritis has included investigation of the role of the common canine viruses. The purpose of the present paper is to describe the development of glomerulonephritis in dogs infected experimentally with canine adenovirus.

MATERIALS AND METHODS

Virology.—The strain of canine adenovirus (CAV) employed in this study was originally isolated by means of dog kidney tissue culture from the kidney of a dog suffering from adenoviral interstitial nephritis. Preliminary results showed that 1 ml of virus suspension containing 10^7 tissue culture infectious doses, 50% ($TCID_{50}$) was lethal in 4 days when inoculated intravenously into antibody-free dogs. The aim of the present experiment was to create a situation where infected dogs survived until and during the initial period of antibody production. This was established by diluting virus pools to 10^3 $TCID_{50}$ per ml, thus prolonging the survival time.

Experimental animals.—Fourteen, 16-week old antibody-free dogs weighing approximately 6 kg were given 1 ml of virus suspension containing 10^3 $TCID_{50}$ intravenously. The times of

examination of these animals are given in Table I. Eight control animals were given 1 ml of uninfected tissue culture suspension intravenously and killed in pairs on Days 4, 6, 8 and 10 after inoculation. These animals were used for comparison of kidney histology, ultrastructure and immunofluorescence.

Urine was aspirated from the bladder at necropsy and examined for the presence of protein by the method of Lowry *et al.* (1951).

Serology.—Anti-CAV antibody was prepared in dogs and conjugated with fluorescein isothiocyanate (FITC) as previously described (Wright *et al.*, 1973*a*). Serum samples from both test and control animals were examined before commencement of the experiment and at necropsy by an indirect immunofluorescence test. Briefly, doubling dilutions of sera were exposed to known positive infected liver sections (from a case of systemic adenovirus infection) for 30 min and, after thorough washing in phosphate buffered saline (PBS, pH 7·1) the sections were then stained for 30 min with rabbit anti-dog globulin (diluted 1 : 20) also conjugated with FITC. After washing, the sections were examined for the presence of fluorescing hepatic cells by means of a Leitz Orthoplan fluorescence microscope equipped for incident light fluorescence.

The kidneys of 4 control and all infected dogs were minced, washed repeatedly in PBS and eluted with 0·02 mol/l citrate buffer (pH 3·2) according to the method of Lambert and Dixon (1968). The eluates were concentrated 20-fold and tested by an indirect immunofluorescence test (as described above) for the presence of anti-CAV antibody. Sections of normal dog kidney were also employed for the detection of antibody in the eluates directed against kidney tissue.

Histological, immunofluorescence and ultrastructural procedures.—Small portions of kidney were fixed in mercuric chloride–formol, embedded in paraffin wax and sectioned at 6 μm. Sections were stained with Mayer's haemalum and eosin, McManus's periodic acid-Schiff, chromotrobe-methenamine silver and martius scarlet blue.

For immunofluorescence studies, washed frozen sections of kidney were fixed in acetone for 10 min and stained with either rabbit anti-dog IgG, goat anti-dog C3 or anti-CAV antibody all conjugated with FITC. The sections were washed and mounted in PBS before examination with the fluorescence microscope. Suitable control sections were employed with all the conjugates.

Electron microscopy was carried out on 6 animals. Under general anaesthesia, the kidneys were removed and portions sliced into small blocks less than 1 mm thick. The latter were immersed in cold paraformaldehyde–glutaraldehyde, subsequently post-fixed in 1% osmium tetroxide and embedded in Araldite. Ultrathin sections were mounted on copper grids, stained with uranyl acetate and lead citrate and examined with an AEI 6B electron microscope.

RESULTS

Clinical findings

The inoculated animals were clinically normal until the 3rd day after administration of virus, when they all developed pyrexia in the region of 104°–106°F. On the 4th day they were depressed, anorexic and, on palpation, showed signs of anterior abdominal pain. There was congestion of mucosae and enlargement of superficial lymph nodes. Deaths occurred from the 4th day onwards (see Table I) and in those animals surviving to 6 days after infection jaundice could be detected. The 2 animals which survived longer than 9 days showed marked clinical improvement, with return of appetite and gradual disappearance of jaundice.

Macroscopic findings

The 12 dogs which died or were killed up to and including the 9th day after inoculation of virus showed macroscopic changes characteristic of acute systemic adenovirus infection (infectious canine hepatitis); hepatomegaly, serofibrinous peritonitis and haemorrhagic lymphadenitis were the main findings. Jaundice was also evident in all animals examined from the 6th to the 10th day after infection. In addition, however, 7 dogs showed multiple small petechial renal

TABLE I.—*Canine Adenovirus Glomerulonephritis: Histological Features*

Dog no.	Day examined	Glomerulo-nephritis	Inclusions: Glomeruli	Inclusions: Capillaries	Interstitial infiltration	Tubular haemor-rhage	Tubular necrosis	Urine protein mg/100 ml
1	4 (D)	++	+	+	—	—	—	ND
2	5 (D)	++	+	+	—	—	—	ND
3	5 (D)	++	++	+	—	+	+	107
4	6	+	+	+	—	—	—	ND
5	6	++	+	+	—	—	—	ND
6	6	+++	+++	+	—	—	—	71
7	7 (D)	++	+	+	+	—	+	150
8	7 (D)	++	++	+	+	++	++	61
9	8 (D)	+++	++	+	++	+	++	ND
10	8 (D)	++++	++	+	+	++	++	220
11	9 (D)	++++	—	—	+	++	+	ND
12	9	++++	+	+	+	++	—	500
13	10	+++	—	—	++	++	++	15
14	14	++	—	—	++	+++	+++	2

+ to ++++ = Indices of severity of lesions.
— = No lesion.
D = Died.
ND = Not done (bladder empty at necropsy).

haemorrhages, present mainly in cortical regions; these lesions were first recorded at 7 days after administration of virus and were present in all animals examined thereafter (Fig. 1).

Histological findings

The main histological changes are summarized in Table I. In all animals, diffuse cytological changes were found in the glomeruli; all the glomeruli were involved to approximately the same extent. 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 with occlusion of capillary

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EXPLANATION OF PLATES

FIG. 1.—Kidney from an infected dog, 8 days after administration of virus; many small haemorrhagic foci can be seen.

FIG. 2.—Glomerulus from a control dog showing normal cellularity and patent capillary loops. H. & E. × 160.

FIG. 3.—Glomerulus from an infected dog, 5 days after administration of virus. The tuft is normal in size and cellularity but there is distinct expansion of mesangial areas and occlusion of capillary loops. A single intranuclear occlusion body can be seen (arrow). H. & E. × 160.

FIG. 4.—Glomerulus from an infected dog, 9 days after administration of virus. The tuft is swollen and the capillary loops are collapsed; the hypercellularity is due to infiltration by polymorphonuclear leucocytes and a moderate increase in the number of mesangial cells. H. & E. × 160.

FIG. 5.—Fibrinous exudation (small arrows) and haemorrhage (large arrow) into Bowman's capsule, 9 days after administration of virus. H. & E. × 160.

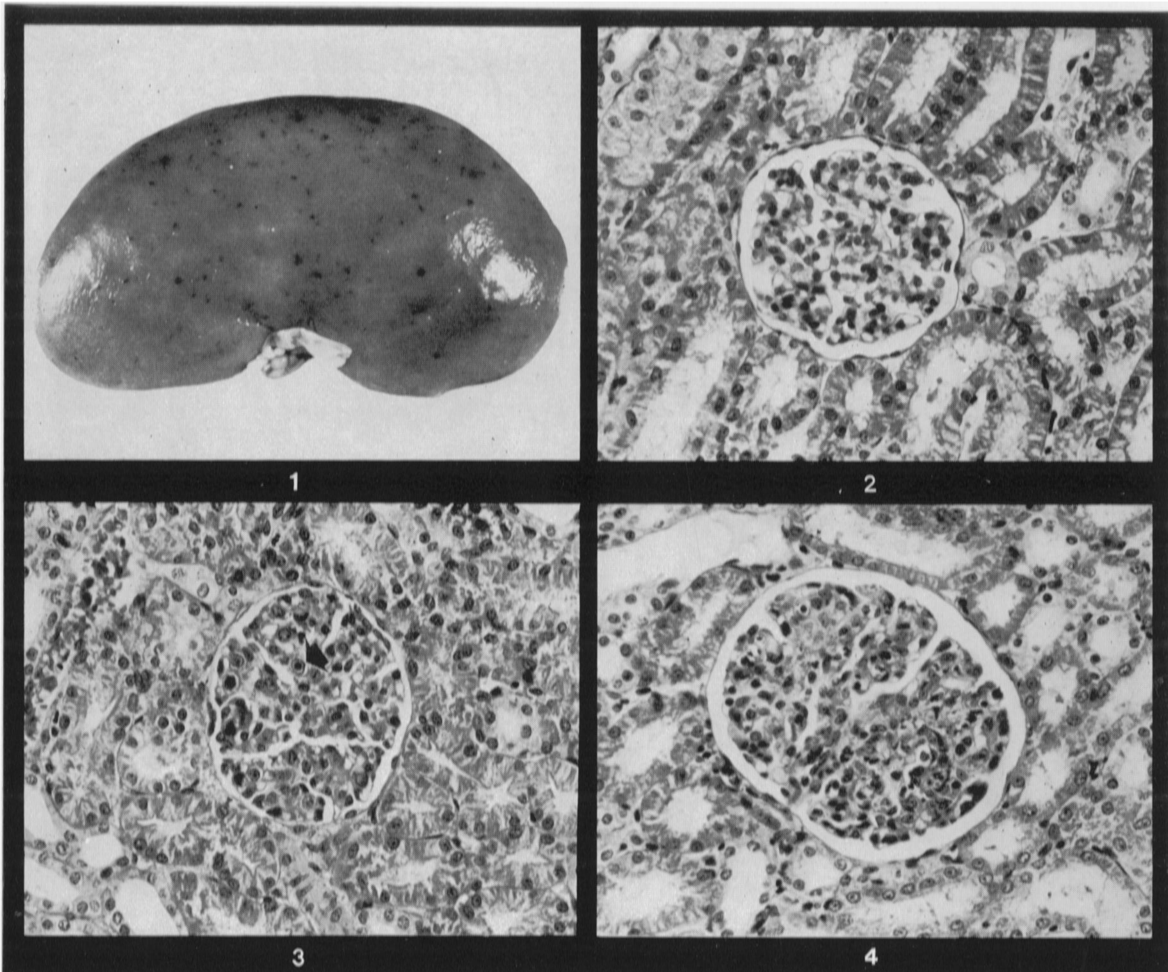
FIG. 6.—CAV antigen occurs as fine streaks of fluorescence in mesangial areas; 2 larger cellular deposits can also be seen; 7 days after administration of virus. Immunofluorescence × 160.

FIG. 7.—Lumpy mesangial deposits of IgG, 9 days after administration of virus. Immunofluorescence × 160.

FIG. 8.—Glomerulus from the same dog stained for C3. Immunofluorescence × 160.

FIG. 9.—Necrotic mesangial cell, 9 days after inoculation of virus. Numerous CAV virions can be seen scattered among cellular debris. Electron microscopy × 10,300.

FIG. 10.—Electron dense deposits (arrows) in mesangial areas, 9 days after administration of virus. Electron microscopy × 8,575. M = Mesangial cell; E = Epithelial cell.



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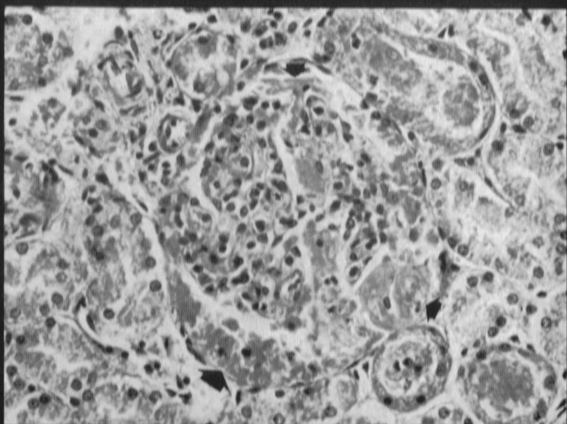

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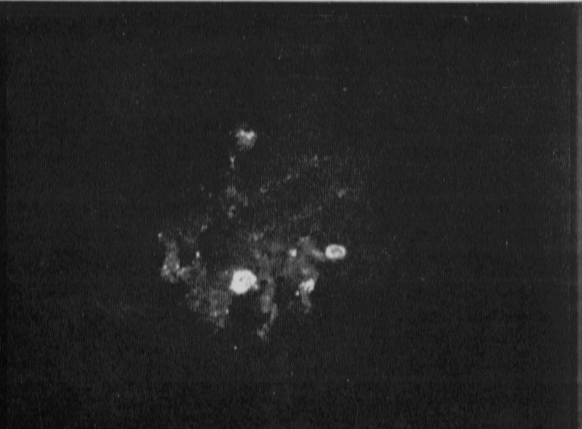

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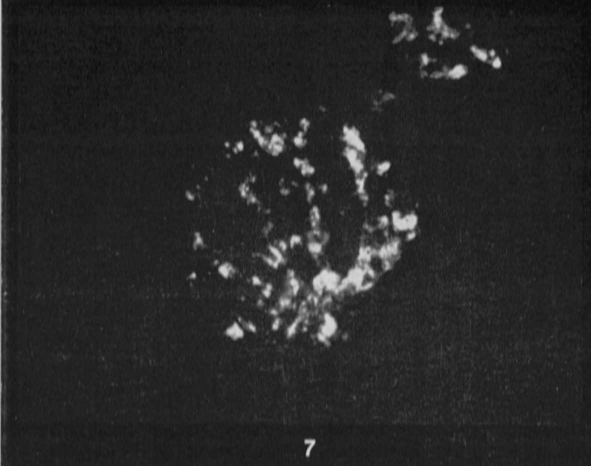

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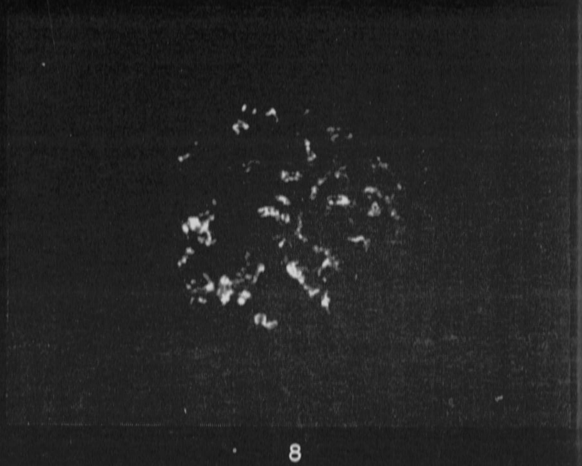

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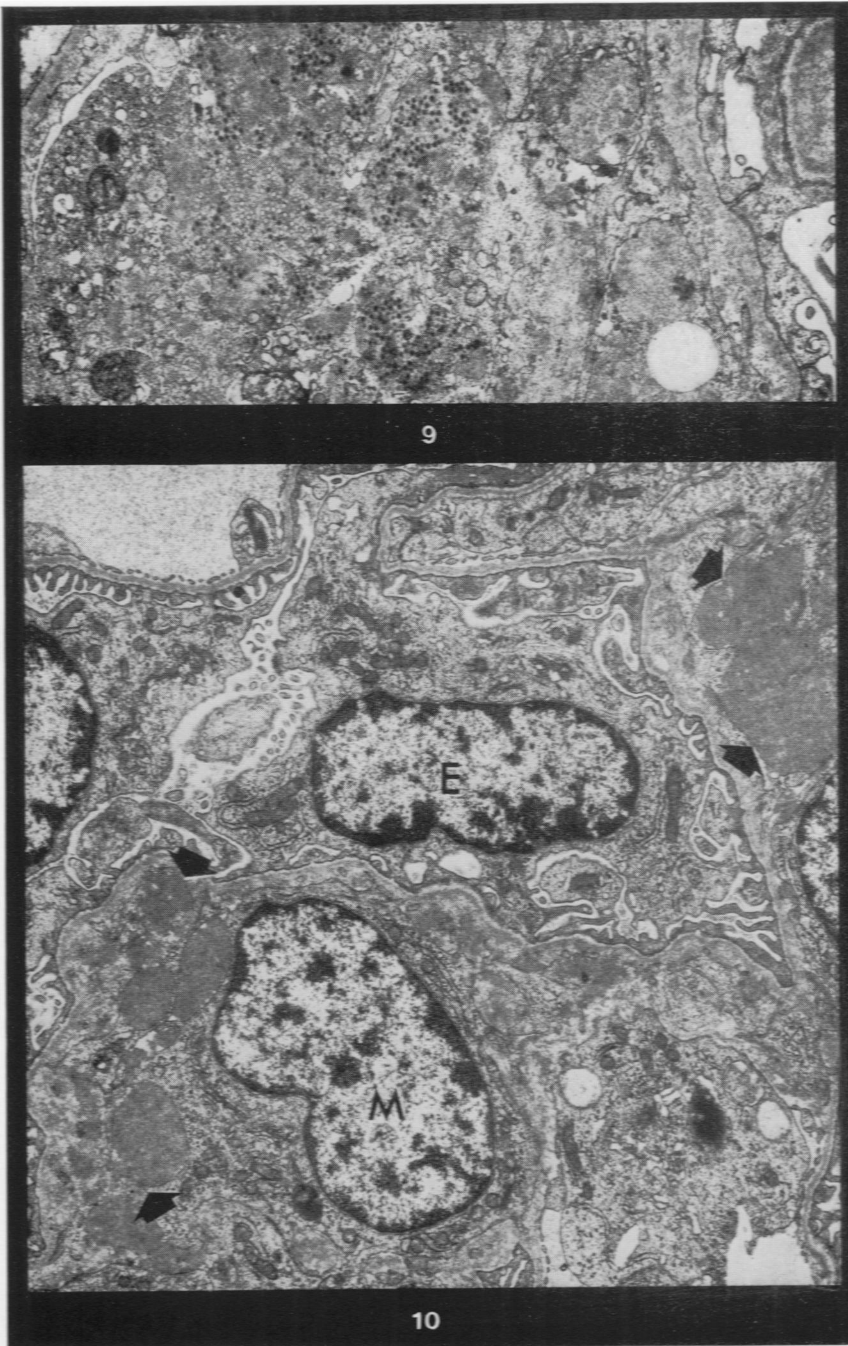

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loops (Fig. 3). Occasional polymorphonuclear leucocytes were found lodged in the loops and granular debris was noted in Bowman's spaces. From 6 days onwards more severe cytological changes developed. Proliferation of mesangial cells became evident at this time and was responsible for marked expansion of mesangial areas with accentuated lobulation of the tufts. The resultant glomerular hypercellularity was further augmented by large scale infiltration of polymorphonuclear leucocytes into the tufts (Fig. 4); at the height of the disease on Days 8 and 9 these cells had penetrated the mesangium and urinary spaces. In addition to these proliferative changes, some glomeruli showed capillary thrombosis, leading to segmental areas of tuft necrosis with liberation of red cells and fibrin into the urinary spaces. Occasionally there was extensive capsular with subsequent tubular, haemorrhage (Fig. 5). The latter lesions, together with foci of interstitial haemorrhage, were the basis of the haemorrhagic lesions seen at necropsy. Characteristic adenovirus intranuclear inclusion bodies were found in all animals up to and including Day 9 after infection. Inclusions varied in number from glomerulus to glomerulus but never exceeded 6 per glomerulus (rated +++ in Table I); they were present in endothelial and mesangial cells. Elsewhere in the kidney, inclusions were found in small numbers in vascular endothelium of interstitial capillaries and larger renal blood vessels and, in one case killed on Day 10, in proximal epithelial cells.

Focal areas of tubular epithelial necrosis were detected as early as 5 days after infection. These lesions were confined to the cortex, particularly proximal epithelium and in only one case (dog 13) were they associated with the presence of inclusion bodies. From 7 days until the end of the experiment, focal interstitial infiltrates of macrophages, large lymphoid cells and plasma cells were found. These were, in the main, confined to the cortex although in many dogs a few small foci occurred around collecting tubules in the medulla.

Immunofluorescence findings

The immunofluorescence patterns are summarized in Table II. When kidneys from infected dogs were stained for the presence of CAV antigen, fluorescing glomerular cells were detected in varying numbers up to 9 days after administration of virus; likewise, a small number of fluorescing cells were noted in extraglomerular vascular endothelium. Thus, the pattern of antigen fluorescence at this stage showed a close correlation with the distribution of inclusion bodies although, in general terms, the number of fluorescing cells was greater than the number of inclusion bodies seen on light microscopy. In addition, however, on Days 7, 8, 9 and 10 fine granules of antigen fluorescence were also observed in mesangial areas (Fig. 6). In only one case (dog 13) was specific antigen fluorescence present in a few tubular epithelial cells. The animal killed on Day 14 did not show any specific staining for antigen in the glomeruli, vascular endothelium or tubules.

IgG was not detected in the kidneys of the dog killed on Day 4. On Day 5, however, fine mesangial granules of fluorescence were observed. This pattern of fluorescence persisted until the end of the experiment on Day 14. As the disease progressed larger lumpy deposits of IgG were also detected, again in mesangial areas (Fig. 7). These larger fluorescing deposits persisted until Day 9. A few interstitial plasma cells were also stained from the 6th day onwards. C3 was not

TABLE II.—*Canine Adenovirus Glomerulonephritis: Immunofluorescence Patterns*

		Viral antigen		IgG			IF titres	
Dog no.	Day examined	Glomeruli	IE cells	Glomeruli	Plasma cells	C3	Serum	Eluate
1	4 (D)	++ Cellular	+	—	—	—	—	—
2	5 (D)	++ Cellular	+	+ Fine mes. granules	—	—	—	—
3	5 (D)	+++ Cellular	+	+ Fine mes. granules	—	—	—	—
4	6	++ Cellular	+	++ Lumpy Fine mes. granules	—	—	—	—
5	6	+++ Cellular	+	+++ Lumpy Fine mes. granules	—	—	—	—
6	6	+++ Cellular	+	++ Fine mes. granules	+	—	—	—
7	7 (D)	+ Cellular Fine mes. granules	—	+++ Lumpy Fine mes. granules	+	—	1 : 4	—
8	7 (D)	++ Cellular Fine mes. granules	+	+++ Lumpy Fine mes. granules	+	+ Lumpy	1 : 2	1 : 4
9	8 (D)	+ Cellular Fine mes. granules	+	+++ Lumpy Fine mes. granules	+	++ Lumpy	1 : 64	1 : 2
10	8 (D)	++ Fine mes. granules	+	++++ Lumpy Fine mes. granules	+	+ Lumpy	1 : 8	1 : 4
11	9 (D)	+ Fine mes. granules	—	+++ Lumpy Fine mes. granules	+	+ Lumpy	1 : 2	1 : 16
12	9	++ Cellular Fine mes. granules	+	++++ Lumpy Fine mes. granules	+	+ Lumpy	1 : 16	1 : 32
13	10	+ Fine mes. granules	—	++ Fine mes. granules	+	—	>1 : 512	1 : 32
14	14	—	—	+ Fine mes. granules	+	—	>1 : 512	1 : 128

D = Died.
IE = Interstitial endothelial.
mes. = Mesangial.
Lumpy = Coarse granules of fluorescence in mesangial areas.
+ to ++++ = Indices of degree of fluorescence.
— = No fluorescence.
IF = Immunofluorescence.

detected in glomeruli until the 7th day and persisted until the 9th day; the deposits of C3 were lumpy and, again, confined to the mesangial region (Fig. 8).

Ultrastructural findings

Only the dogs sacrificed on Days 6 (3), 9 (1), 10 (1), 14 (1) were examined with the electron microscope; the remaining animals died and their tissues were thus unsuitable for ultrastructural studies. The dog killed on Day 6 showed marked swelling and vacuolation of all the glomerular cell types, with subsequent partial or complete occlusion of the capillary lumina. The thin endothelial lining of capillary loops was frayed and often had detached from the glomerular basement membrane (GBM). A few capillary loops contained polymorphonuclear leucocytes 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 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 the GBM appeared normal at this stage. Elsewhere in the kidneys there were no remarkable alterations. Adenovirus virions were observed in the nuclei of swollen endothelial and mesangial cells. Virus particles were observed in greatest numbers beneath the nuclear membrane which in some necrotic cells was disrupted, with subsequent release of virus into the cytoplasm (Fig. 9).

On Days 9 and 10, the ultrastructural changes had increased in intensity with, in many instances, necrosis of endothelial and mesangial cells and with the appearance of red blood cells and strands of fibrin in the urinary spaces. Numerous polymorphonuclear leucocytes were found lodged in the capillary loops and occasionally had penetrated the mesangial matrix. The latter was distinctly expanded and the nuclei of mesangial cells showed accentuated lobulation. The main feature of affected glomeruli at this stage was the presence of large irregular electron dense deposits scattered throughout the mesangium (Fig. 10). These deposits extended into the axial region of the capillary loops and smaller deposits were occasionally observed beneath swollen endothelium at these sites. However, deposits were never seen in the peripheral portions of the loops. Only on rare occasions were free virions located in the deposits.

Biochemical and serological findings

Proteinuria with levels up to 500 mg/100 ml was observed in 6 of the 8 dogs examined. Although IgG was deposited in the glomeruli as early as 5 days post infection, circulating antibody was not detected until 7 days; by Days 10 and 14, however, titres had risen to $> 1 : 512$. Anti-viral antibody was also detected in kidney eluates from the 7th day onwards (Table II); anti-kidney antibody was not present in these eluates.

Control animals

None of the control dogs showed clinical or macroscopic abnormalities, nor did they have glomerular lesions as judged by histological, ultrastructural and immunofluorescence examinations; proteinuria was not detected in any animal.

DISCUSSION

A previous study has shown that during the systemic phase of CAV infection and before development of circulating antibody, virus localizes in endothelial and mesangial cells in the glomeruli and in addition to lytic changes in these cells, GBM antigens are released into the urine (Wright *et al.*, 1973*a*). The present work has extended these studies and has established that CAV infected dogs develop an immune complex glomerulonephritis during the initial period of antibody production and clearance of virus from the circulation. The localization of these viral immune complexes was mainly mesangial and only rarely were deposits found in subendothelial regions. The mesangial pattern of complex deposition, as seen by immunofluorescence and electron microscopic examination, is in accordance with that seen in conditions where there is formation of relatively large and poorly soluble complexes in the circulation (class II complexes, Germuth and Rodriguez, 1973). The general appearance of the glomerular lesions was similar to those recently described in mice inoculated with preformed CAV antibody complexes (Wright *et al.*, 1973*b*), but in the present study the mesangial proliferative changes were more extensive and necrosis of the tuft with subsequent fibrinous exudation were also observed.

CAV antigen, IgG and C3 were found simultaneously in damaged glomeruli in a granular or lumpy distribution. Moreover, antibody directed against CAV but not against kidney tissue was detected in kidney eluates from the 7th day onwards. Early in the course of the experiment, CAV antigen occurred only in cellular foci but from the 7th day onwards (when circulating antibody was first detected) it was also seen as fine granules in the mesangium. This latter pattern of fluorescence suggested deposition of antigen/antibody complexes from the circulation, rather than simply deposition of antibody on to cell associated antigen implanted in the glomerulus. Some preliminary evidence for the presence of circulating complexes has recently been obtained; mesangial localization of canine IgG was found in mice which had received repeated intravenous doses of serum from affected dogs (Wright *et al.*, unpublished work). This passive transfer occurred only with serum taken at the height of the disease, when antibody titres were still low and when deposition of complexes in the kidneys of donor dogs was maximal. Transfer was not obtained early in the disease (no circulating antibody and minimal deposition of complexes) nor late in the disease (high circulating antibody and minimal deposition of complexes).

Proteinuria (in excess of 50 mg/100 ml) was detected in 6 of the 8 dogs examined. The dogs killed on the 10th and 14th days had normal levels of 15 mg and 2 mg respectively. 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.

Focal interstitial nephritis with intranuclear inclusions in necrotic tubular epithelium, particularly in the collecting tubules, is a common occurrence in dogs recovering from the acute systemic phase of CAV infection (Wright, Cornwell and Thompson, 1971); these lesions are of maximal intensity 15–25 days after inoculation of virus and are associated with a viruria. In the present study, where experimentally infected animals were examined up to 14 days after infection, virus, with the exception of one animal, was not detected in tubular epithelial cells at any

level of the nephron. Thus the necrotizing lesions of the proximal epithelium were presumably not due to direct lytic action of virus. A more plausible explanation might be local ischaemia due to occlusion of glomerular capillaries.

When the experiment was terminated at 14 days, the severity of the glomerular lesions appeared to be abating and, although mesangial deposits of IgG could still be seen, antigen and C3 were no longer detectable. Whether CAV induced glomerulonephritis occurs only as a transient lesion during the early period of recovery from systemic infection or also leads in some cases to the establishment of progressive glomerular disease is currently being explored.

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