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EXPERIMENTAL ADENOVIRUS IMMUNE COMPLEX GLOMERULONEPHRITIS

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Summary.—A group of 40 mice were given daily intravenous inoculations of canine adenovirus–antibody complexes solubilized in antigen excess. Immunofluorescence tests showed deposits of these complexes in the glomeruli. Antigen, antibody and complement (C_3) were detected as streaks of fluorescence in mesangial areas; capillary loops were not stained. Although only 7 out of 40 (17%) inoculated mice developed proliferative glomerulonephritis, ultrastructural changes were found in the glomeruli of all animals from the third day onwards. In addition to proliferation of mesangial cells, deposits of electron dense material were detected in mesangial matrix and, less commonly, in subendothelial locations at the axial regions of the loops. Endothelial swelling and occasional necrosis, fusion of epithelial foot processes and extension of mesangial pseudopodia into the capillary loops were also detected.

THE LOCALIZATION of circulating immune complexes in the glomerulus and subsequent fixation of complement are important factors in the development of glomerulonephritis in man and animals (Dixon, 1968). Glomerulonephritis is now recognized to be a feature of many virus infections such as lymphocytic choriomeningitis (Oldstone and Dixon, 1967), coxsackie B4 infection (Sun *et al.*, 1967), equine infectious anaemia (Banks, Henson and McGuire, 1972) and leukaemia (Mellors *et al.*, 1971). Viral immune complexes have been demonstrated in these lesions by immunofluorescence and elution techniques, but the progression of glomerular lesions following inoculation of preformed viral–antibody complexes has never been studied.

The purpose of the present communication is to describe the sequential development of glomerular lesions in mice following repeated intravenous inoculation of soluble preformed adenovirus–antibody complexes.

MATERIALS AND METHODS

The virus used in this study 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). Infected cells were incubated at 37° until the cytopathic effects were complete, at which time the virus was harvested by repeated freezing and thawing. Virus suspensions were centrifuged at 2500 rev/min for 20 min at 4° to remove tissue culture debris and virus was subsequently sedimented by centrifugation at 25,000 rev/min for 45 min. The pelleted virus was resuspended in small volumes of phosphate buffered saline (PBS) pH 7·2 and the virus disrupted with release of viral protein by dialysis at 4° for 5 days against carbonate/bicarbonate buffer, pH 10·6 (Wilcox, Ginsberg and Anderson, 1963). This resulted in a solution of viral protein, the pH of which was adjusted

to neutrality by dialysis against PBS pH 7·2 for a further 2 days. Centrifugation was performed at 4000 rev/min for 15 min to remove any insoluble fragments and the viral protein solution was then stored at −25° until required. The protein concentration as measured by the method of Lowry *et al.* (1951) was 0·25 mg/ml.

Preparation of antiviral antibody.—Twelve-week old CAV antibody-free puppies were inoculated subcutaneously with 1 ml of CAV protein emulsified in Freund's complete adjuvant. Eight weeks later 1 ml of viral protein was administered intraperitoneally and after a further 10 days the animals were exsanguinated. The pooled serum was inactivated at 56° for 30 min and examined for the presence of neutralizing antibody to CAV; the titre of the serum was 1 : 10,000.

Preparation of complexes.—Soluble immune complexes were prepared according to minor modifications of the method of Okumura, Kondo and Tada (1971). A tube precipitation was employed using fixed volumes of antiviral antibody 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 antiviral antibody were mixed at equivalence and left at 4° for 48 hours. The opalescent complexes so formed were sedimented by centrifugation at 3000 rev/min for 20 min at 4°, washed twice in cold PBS and resuspended in 5 times excess viral protein. Solubilization of the complexes was obtained by lowering the pH to 2·4 by slow dropwise addition of 0·1 N HCl at 0° 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 3000 rev/min for 15 min.

Experimental animals.—Forty albino mice (Porton strain) aged 8–10 weeks received daily inoculations of 0·3 ml of soluble CAV immune complexes injected very slowly intravenously. Groups of at least 3 mice were killed daily at intervals of 1–12 days after initial inoculation, each mouse being sacrificed 24 hours after its last dose. As controls, 2 groups of 12 mice were given similar volumes of viral protein and anti-CAV antibody respectively and sacrificed at similar intervals. A further 10 mice were sacrificed as normal uninoculated controls for comparison of kidney histology, ultrastructure and immunofluorescence.

Histological, ultrastructural and immunofluorescence procedures.—Under general anaesthesia the kidneys were removed and portions of the cortices sliced into small blocks less than 1 mm thick. These were immersed in cold paraformaldehyde–glutaraldehyde, subsequently post-fixed in 2% 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.

Portions of each kidney were fixed in mercuric chloride–formol for 48 hours, embedded in paraffin wax and sectioned at 6 μm. Sections were stained with Mayer's haemalum and eosin, McManus's periodic acid-Schiff, methenamine silver and martius scarlet blue. For immunofluorescence studies, frozen sections of kidney were fixed in acetone for 10 min and stained with either rabbit anti-dog IgG, rabbit anti-mouse IgG, rabbit anti-mouse β1C (C_3) or anti-CAV antibody, all conjugated with fluorescein isothiocyanate. Stained sections were washed in PBS and examined by means of a Leitz "Orthoplan" fluorescence microscope. Suitable control sections were employed.

RESULTS

Histological findings

Proliferative glomerulonephritis was found in 7 out of 40 mice (17%) inoculated with viral immune complexes. This change was first detected after 4 daily doses of complexes (5th day) and consisted of proliferation of mesangial cells, resulting in focal areas of hypercellularity and expansion of the mesangium (Fig. 1 and 2). Many capillary loops were collapsed and devoid of red blood cells. Occasional polymorphonuclear leucocytes and mononuclear cells were found lodged in the capillary lumina. In these 7 mice all the glomeruli were affected to more or less the same extent and the tufts were distinctly enlarged. Proteinaceous casts were found in the tubules. In the remaining animals, the glomeruli were histologically normal.

Immunofluorescence findings

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. Viral antibody was detected in all mice as fine streaks of fluorescence in mesangial areas after the second dose of complexes (3rd day) (Fig. 3). There was a general variation of intensity of fluorescence from animal to animal but all experimental animals showed deposits of antibody up to the 12th day at the end of the experiment. Fluorescence staining for antigen (Fig. 4) and complement also appeared by the 3rd day, was weaker than that of antibody and was not detected in all animals. There was, however, a direct correlation between the degree of antigen fluorescence in the glomerulus and the development of proliferative glomerulonephritis. The 7 mice showing proliferative glomerulonephritis on light microscopy had the largest deposits of antigen. A further 26 animals showed weaker antigen fluorescence. Fluorescing deposits of C_3 were detected in 18 mice, including the 7 with proliferative glomerulonephritis. None of the experimental mice showed deposits of host (mouse) IgG in the glomeruli.

Ultrastructural findings

There was a wide range in ultrastructural abnormalities of the glomeruli whether or not proliferative changes were detected by light microscopy. The earliest change noted in the glomerulus was detected after 2 doses of complexes (3rd day) when evidence of mesangial activity was demonstrated in the form of extension of mesangial cytoplasmic pseudopodia into the axial region of the capillaries, often displacing swollen endothelial cytoplasm in these regions. At this time, small electron dense deposits were demonstrated in the mesangial matrix and, less commonly, trapped between endothelial cytoplasm and mesangial cytoplasmic projections. All these structural changes occurred to a varying degree in all mice inoculated with complexes. From the 5th day onwards further evidence of mesangial activity was detected, when an increase in the number of mesangial cells and in mesangial matrix was found (Fig. 5 and 6). This lesion was also present in inoculated mice although it was most prominent in the 7 mice which showed proliferative glomerulonephritis on light microscopy. Large macrophages with lobed nuclei and pale cytoplasm were frequently seen lodged within capillary loops and were occasionally observed pushing through capillary endo-

EXPLANATION OF PLATES

FIG. 1.—Glomerulus from a control mouse showing normal cellularity and patent capillary loops. H. and E. × 370.

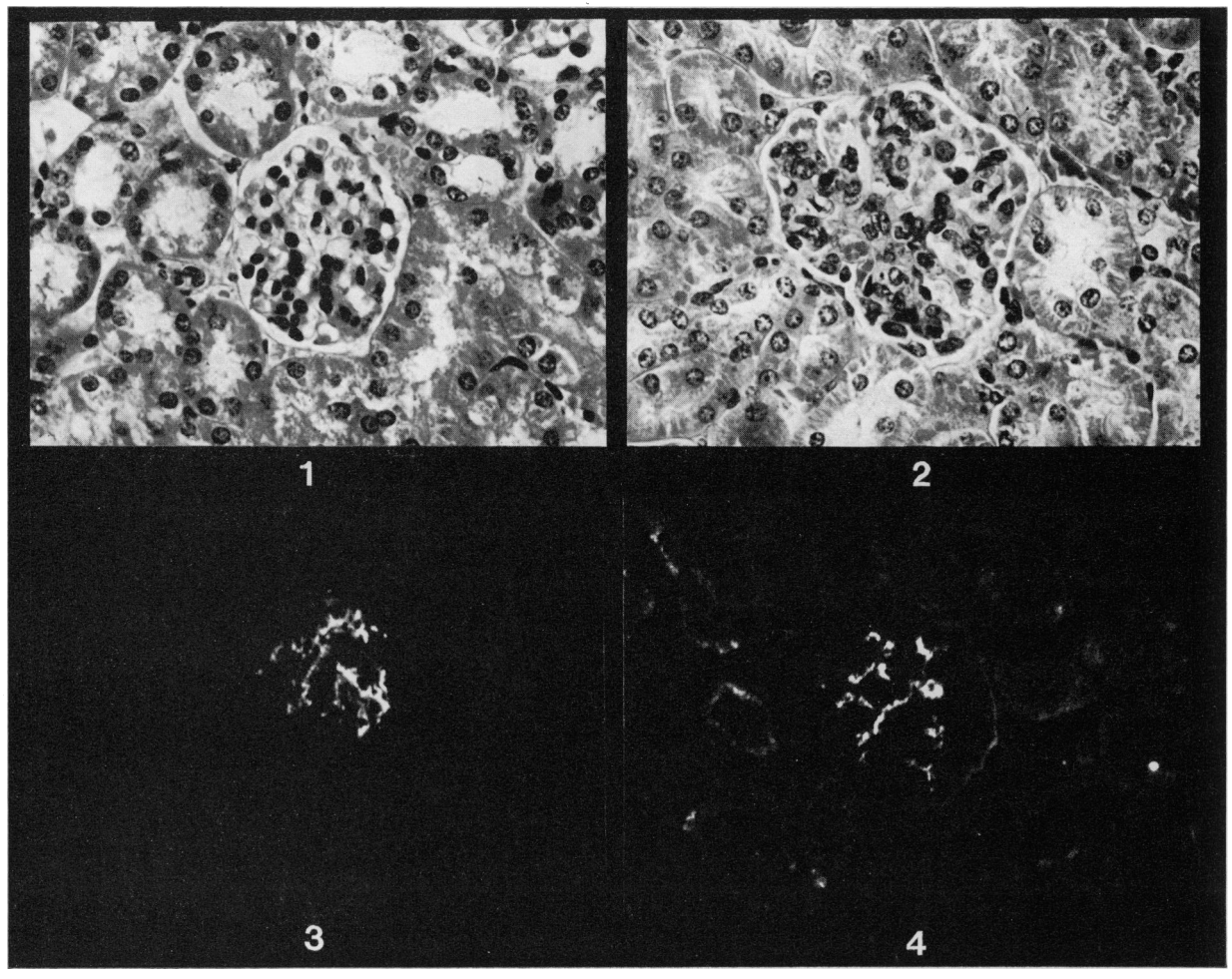
FIG. 2.—Proliferative glomerulonephritis in a mouse which received 7 daily inoculations of soluble complexes. There is swelling of the tuft, increased cellularity with expansion of mesangial areas and collapse of capillary loops. H. and E. × 370.

FIG. 3.—CAV antibody localized as streaks of fluorescence in mesangial areas. Capillary walls are unstained. This mouse received 5 daily inoculations of complexes. Immunofluorescence × 305.

FIG. 4.—CAV antigen deposited in similar locations as antibody. Seven daily injections of complexes. Immunofluorescence × 305.

FIG. 5.—Proliferative glomerulonephritis following 5 daily injections of complexes. Note the marked expansion of mesangium (M), mesangial cytoplasmic projections into a capillary loop (arrows) and fusion of epithelial foot processes (E). Electron microscopy × 10,000.

FIG. 6.—Electron dense deposits (arrows) beneath the endothelium (E). This mouse received 6 daily injections of complexes. Electron microscopy × 5000.



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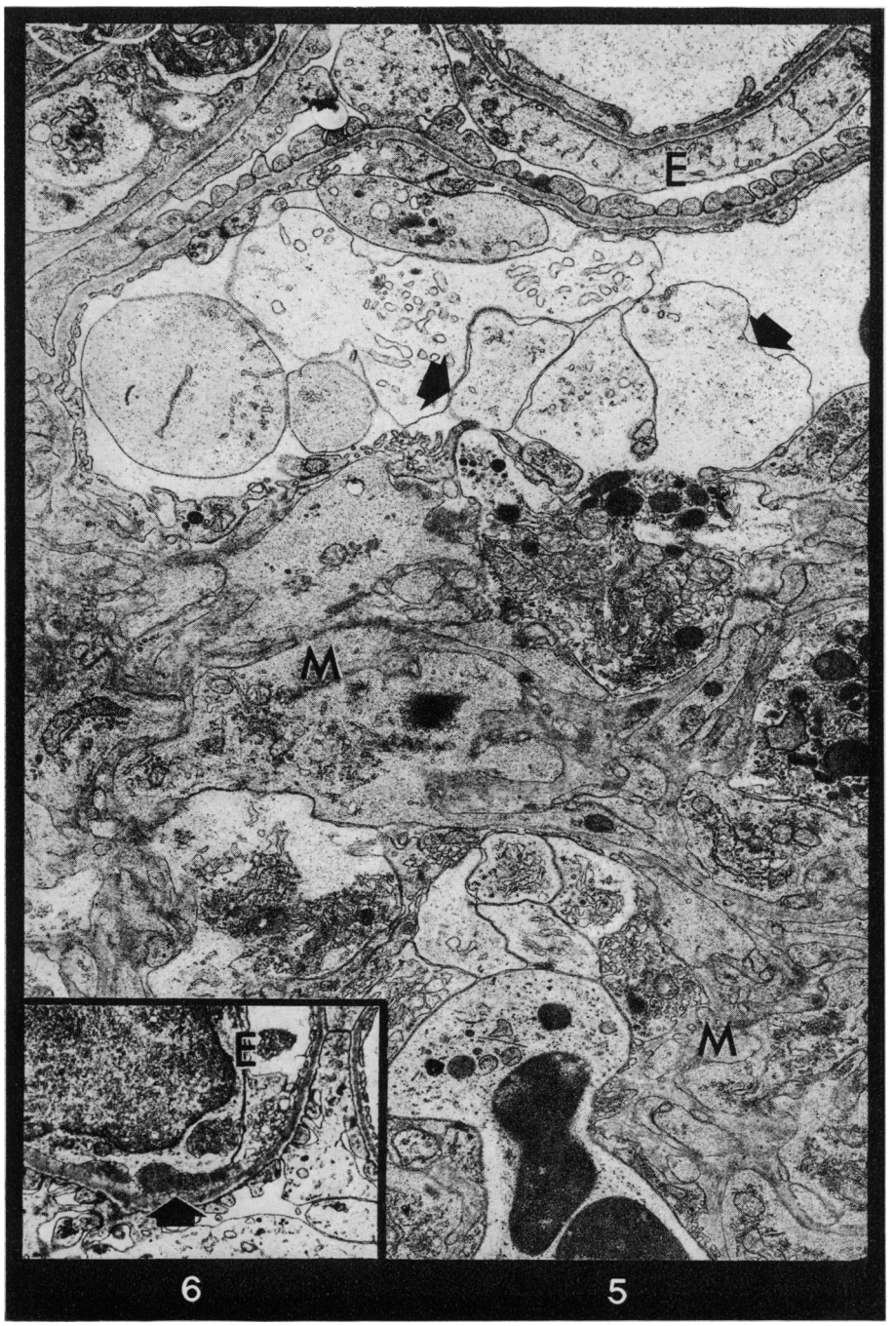

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thelium into the mesangium. Associated with those changes there was pronounced swelling of epithelial cells and focal areas of fusion of epithelial foot processes were also detected. A small number of polymorphonuclear leucocytes were also present within glomerular capillaries and a few electron-dense, dead or dying endothelial cells were found. All these ultrastructural abnormalities persisted to varying extent up to the close of the experiment on the 12th day.

Controls

Histological and ultrastructural abnormalities were not detected in any of the control animals. Likewise, immunofluorescence studies proved negative for adenovirus antigen, antibody, complement or host immunoglobulin (IgG).

DISCUSSION

Canine adenovirus is responsible for a range of conditions in dogs, particularly hepatitis (Rubarth, 1947), respiratory disease (Wright *et al.*, 1972) and renal disease (Wright, Cornwell and Thompson, 1971). During the systemic phase of the disease virus localizes in endothelial and mesangial cells in the glomerulus and, in addition to lytic changes in these cellular components, glomerular basement membrane antigens are released into the urine (Wright *et al.*, 1973). Animals recovering from the acute phase of the disease often develop focal interstitial nephritis associated with persistence of virus in the renal epithelium (Wright *et al.*, 1971) and occasionally mild focal mesangial proliferative changes are also present. Whether or not these latter changes are a result of the deposition of viral immune complexes during the normal immune elimination of virus from the circulation is not known. The purpose of the present experiment, as part of a series of studies into the interaction of CAV with the kidney, was to investigate the effect on the glomerulus of the arrival of circulating viral immune complexes. The mouse was chosen as the experimental model due to the difficulties in preparing sufficient quantities of viral protein to prepare the large volume of complexes necessary for inoculation into dogs.

In the present study, CAV antigen, CAV antibody and complement (C_3) were detected by immunofluorescence only in mesangial areas. With the electron microscope dense granular deposits were encountered in the mesangial matrix in association with proliferating mesangial cells, but only rarely were these deposits encountered in the capillary walls. Similar ultrastructural findings have been demonstrated in other experimental immune complex systems using bovine serum albumin as antigen (Okumura *et al.*, 1971). It would seem, therefore, that the glomerular mesangium is important in the clearance of circulating viral immune complexes from the circulation. The failure to demonstrate deposits in subepithelial sites following inoculation of preformed complexes may be a reflection of a number of factors, such as poor solubilization of complexes, the amount of complexes administered and the relatively short period of time of exposure of the glomerulus to these circulating complexes (12 days).

As heterologous antigens were used in the present immune complex system (*i.e.*, CAV protein and anti-CAV antibody) it might have been expected that the host (mouse) would respond by producing an immunological response against the viral antigens and antibody incorporated in the complexes implanted in the

glomerular mesangium. Immunofluorescence studies, however, failed to detect mouse IgG in the glomerulus of any of the inoculated animals.

The frequency of appearance and intensity of the immunofluorescence patterns varied from animal to animal but two main features emerged. Firstly, antigen, antibody and C_3 could not be detected until the 3rd day. Secondly, where large amounts of antigen could be detected in the glomerular mesangium, proliferative changes were most severe. The deposition of C_3 in mesangial regions was not associated with attraction of large numbers of polymorphonuclear leucocytes into the glomeruli.

The present study has shown that the arrival of circulating viral immune complexes in the glomerulus can induce a range of glomerular lesions culminating in proliferative glomerulonephritis. Parallel studies using the dog as the experimental model are in progress and preliminary results have, as in the mouse, confirmed that preformed CAV immune complexes inoculated intravenously localize in the mesangial areas of the glomerulus with, in a proportion of cases, subsequent mesangial proliferation. Further studies are in progress to ascertain the effects of prolonged administration of these complexes on the glomerulus.

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