Aleutian Disease of Mink

Prevention of Lesions by Immunosuppression

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Mink that were homozygous recessive for the Aleutian gene (*aa*) were inoculated with Aleutian disease virus (ADV) and simultaneously treated with cyclophosphamide (Cy). Control mink were inoculated with ADV. All mink were injected with bovine serum albumin (BSA) and their anti-BSA antibody response was measured to monitor the influence of drug therapy on the humoral antibody response. Formation of anti-BSA antibody was markedly suppressed and the hypergammaglobulinemia and development of AD lesions was inhibited in the Cy-treated mink. The non-Cy-treated control mink developed characteristic signs and lesions including glomerulonephritis and arteritis. The nontreated ADV-infected mink, but not the Cy-treated ADV-infected mink, had glomerular deposition of C3 and gamma globulin. Both groups had high titers of virus in their blood. These results indicate that the development of ADV lesions can be prevented by immunosuppressive treatment and further implicate host immune mechanisms in the pathogenesis of Aleutian disease (Am J Pathol 66:543–556, 1972).

ALEUTIAN DISEASE (AD) is a persistent viral infection of mink characterized by hypergammaglobulinemia (HGG), hepatitis, arteritis, glomerulonephritis and widespread proliferation of plasma cells.[1–5] Studies on the pathogenesis of the lesions have suggested that they are immunologically mediated.[5–7] Antiviral antibody in high titers [8,9] and virus-antibody complexes have been demonstrated in the serum of infected mink.[6] Glomerular deposition of gamma globulin (IgG) and complement (C3) have been reported.[5] Ultrastructural observations of the glomerular lesions have revealed a proliferative glomerulitis with subendothelial and mesangial deposition of electron-dense granular material and increases in the numbers of mesangial cells and amounts of mesangial matrix.[10,11] Staining of affected arteries has shown deposits of viral antigen, C3 and gamma globulin.[12] Furthermore, the plasmacytosis characteristic of this disease may be the result of chronic antigenic stimulation.[8]

In the current study, cyclophosphamide (Cy) was employed to

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further clarify the pathogenesis of the lesions in AD-affected mink. Experiments were conducted to determine whether treatment with Cy could inhibit the development of lesions and the glomerular deposition of IgG and C3 in experimentally infected mink. The drug prevented the development of AD lesions, HGG and the glomerular deposition of IgG and C3, even though virus titers of 10^4 to 10^5 minimum infective $dose_{50}$ (MID_{50}) were present in the blood. These findings further indicate a role for the immune processes in the development of lesions in AD.

Materials and Methods

Animals

Healthy *aa* mink of both sexes, 8–12 months of age, were used throughout this study. All mink were screened for AD by serum levels of gamma globulin (GG) determined by electrophoresis on cellulose acetate. Any mink whose serum level of GG was 17% or higher was excluded. All mink were given 10 mg of oxytetracycline and 10 mg of neomycin in their drinking water daily for the prevention of intercurrent bacterial infections.

Treatment Groups

Two levels of Cy were used to treat ADV-inoculated mink.

One group (A), composed of 20 mink, received 10^4 MID_{50} of AD virus intraperitoneally, Cy at a dosage of 10 mg/kg three times a week for 13 weeks and bovine serum albumin (BSA) at a dose of 5 mg at 2-week intervals for three injections. The initial injection of BSA was incorporated with Freund's complete adjuvant and given subcutaneously while the other two injections were administered intraperitoneally without adjuvant.

The second group (B), composed of 10 mink, received 10 mg/kg of Cy intraperitoneally twice weekly for 8 weeks and AD virus and BSA as group A. After 8 weeks, Cy treatment was terminated in 3 mink in Group B while Cy therapy was continued for an additional 8 weeks in 3 others.

The third group (C or control group), composed of 16 mink, was given AD virus and BSA, but not Cy. Additional controls included 4 mink given Cy only and 4 mink given BSA only.

Pretreatment Renal Biopsies

Kidney biopsies were obtained from the mink in groups A and C 2 weeks before treatment and used as control tissues in the immunofluorescence and histologic studies. Small pieces of renal cortex were taken surgically and divided into two parts. One was frozen in a mixture of dry ice and isopentane and the other was fixed in 10% neutral formalin for light microscopy.

Antibody Response to BSA

All mink in group A and half in group C (8 mink) were bled at 0, 2, 5, 9 and 13 weeks for counts of peripheral leukocytes, titrations of anti-BSA antibody and determinations of serum gamma globulin. The mink in group B and the remainder in group C were bled every 2 weeks for similar determinations. Peripheral leukocyte counts were made with a model B Coulter counter.

A micropassive hemagglutination technic similar to that described by Sever [13] and Campbell *et al* [14] was used for anti-BSA titration. The plates were read after 8–12 hours of incubation.

Collection and Evaluation of Tissue

Blood was collected from all surviving mink at the end of the trials. Mink dying during the experiments and those killed at the end of the experiments were necropsied. Kidneys were snap-frozen for immunofluorescent examination and pieces of liver, spleen, kidney, stomach, intestine, urinary bladder, lung, heart, lymph nodes, skin and brain were fixed in 10% buffered neutral formalin and in Carnoy's fixative for histopathologic examination. Tissue sections were stained with hematoxylin and eosin (H&E) and periodic acid–Schiff procedures. AD lesions in liver, kidney and blood vessels were graded as negative (−), mild (+), moderate (2+), and severe (3+) according to Henson *et al.*[4]

Immunofluorescence

Direct immunofluorescence was employed to determine the deposition of mink IgG and complement (C3) in the renal glomeruli, as previously described.[5] Kidney stained with fluorescein-labeled rabbit antisera against mink IgG, C3 and albumin was observed for the number of glomeruli stained and the intensity, amount and distribution of staining within individual glomeruli.

Virus Titration

Samples of whole blood collected from 3 Cy-treated mink in group A and 3 non-Cy-treated mink in group C were assayed for AD virus by mink titration after 13 weeks of infection. One milliliter of 10^{-2} through 10^{-5} dilutions of whole blood from each mink was inoculated intraperitoneally into each of 2 AD-free Aleutian-type mink. Blood samples from the inoculated mink were collected by toenail bleeding before inoculation and at 2-week intervals after inoculation and the serum levels of gamma globulin determined by electrophoresis. Gamma-globulin levels greater than 20% of the total serum proteins were considered to be a positive indication of AD. Final determinations were made 3 months after inoculation.

Results

Serologic Studies

Cy treatment influenced the antibody responses to BSA and the gamma-globulin levels. Anti-BSA antibody was not detected in 33, 72, 100 and 100% of the Cy-treated, ADV-inoculated mink in group A at 2, 5, 9 and 13 weeks of treatment, respectively. At a dosage level of 10 mg/kg. twice a week (group B), anti-BSA antibody was not detected in 22, 30, 43 and 50% of the mink at 2, 4, 6 and 8 weeks after inoculation, respectively. In contrast, antibody was detected in all sera obtained from non-Cy-treated ADV-inoculated mink in group C and in the 4 animals given BSA only.

The primary response in those mink that formed antibody in groups

A and B and in all the mink in group C was similar, but an anamnestic response, which was prominent in non-Cy-treated mink, did not occur in the suppressed group. No anti-BSA antibody appeared in the 3 mink in which Cy treatment was terminated after 8 weeks.

The gamma-globulin level of the untreated mink (group C) rose progressively from an initial mean of 4% to 37% by the thirteenth week. Meanwhile, the mean level of group A given 10 mg/kg three times a week remained at 5%. The gamma-globulin levels of the Cy-treated mink (group B) given drug twice a week remained at approximately 9% for the 8-week trial period.

A progressive rise in the serum IgG occurred in 3 mink in group B when Cy treatment was stopped after 8 weeks. At the time therapy was withheld, the mean IgG level in these mink was 9%, but it increased to 13% within 2 weeks after cessation of treatment and to 34% by 8 weeks. The serum levels of gamma globulin in the 3 mink with continued Cy treatment after the initial 8 weeks was 13%; after 8 more weeks it was 11%.

Leukopenia was present in all Cy-treated mink. The degree of depression varied somewhat, but was more pronounced in the mink given the higher dosages.

Lesions

All ADV-inoculated mink not treated with Cy developed characteristic gross and histologic lesions of AD. The lymph nodes, livers, spleens and kidneys were grossly enlarged, and the livers and kidney were mottled with small, white foci. Microscopically, extensive proliferation and infiltration of mononuclear cells (predominantly plasma cells) were found in the lymph nodes, spleens, livers and kidneys of non-Cy-treated mink.

Lesions of AD were not observed in any of the Cy-treated, ADV-inoculated mink given the higher dosage of drug. Two of the mink given the lower dosage, however, had mild AD lesions. These changes were a few mononuclear cells in the livers and kidneys and a mild glomerulonephritis. No other lesions were seen in any of the other Cy-treated mink.

The livers from drug-treated mink did not have any infiltrations of mononuclear cells, but they had centrolobular and periportal hepatocellular degeneration attibutable to Cy toxicity. Mononuclear cells formed large irregular collections in the hepatic portal areas of the non-Cy-treated infected mink. Small foci of these cells were present in the hepatic sinusoids also. Marked intrahepatic proliferation of bile duct

occurred in 4 animals. Fig 1A and 1B compare liver sections from treated and nontreated mink. Marked necrosis with lymphoid depletion occurred in the lymph nodes and spleens of treated mink. Severe fibrinoid necrosis and subacute or chronic inflammatory changes were observed in the medium-size arteries of 2 non-Cy-treated, AD virus–infected mink (Fig 2). No vascular lesions were observed in any of the Cy-treated, AD virus–inoculated mink.

Infiltrations of mononuclear cells occurred in the kidneys of non-Cy-treated mink and were mainly located in the cortex. No renal cellular infiltration was observed in any treated mink. Tubular atrophy and hyperchromasia of the epithelial cells attributable to Cy toxicity were observed in the renal cortices of treated mink. Fig 3A and 3B compare kidney sections from Cy-treated and non-Cy-treated mink.

The non-Cy-treated, AD-affected mink had glomerulonephritis with hypercellularity. Finely granular, amorphous, eosinophilic material was deposited in the capillary walls and mesangial areas of the affected glomeruli. A small number of polymorphonuclear leukocytes were present in some glomeruli. Glomeruli in the Cy-treated mink kidneys and in the pretreatment biopsies were not affected. Fig 4A and 4B compare the glomeruli from the Cy-treated and non-Cy-treated mink.

The mink in which Cy treatment was stopped after 8 weeks developed AD lesions similar to those found in the non-Cy, AD virus–inoculated group. The 3 mink in which Cy therapy was continued did not develop AD lesions when killed 8 weeks later.

Immunofluorescence

Bright fluorescence was observed in all the glomeruli of non-Cy-treated mink upon staining with fluorescein-tagged anti–mink IgG and anti–mink C3, but not with anti–mink albumin. A granular pattern of fluorescence was observed in the capillary walls and in the mesangial areas (Fig 5). No staining with either anti-IgG, anti-C3 or anti-albumin was seen in the glomeruli of Cy-treated mink except in 2 animals given the smaller amounts of drug. The pretreatment renal biopsies all lacked fluorescence.

Viral Titration

The *in vivo* titration of whole blood from 3 nontreated and 3 treated mink demonstrated AD virus in all 6. Blood from all 3 Cy-treated mink titered 10^4 MID_{50}/ml. The end point was reached in only 1 of the 3 non-Cy-treated animals evaluated. The titer in this animal was 10^5 MID_{50}/ml. The titer in the other 2 mink was 10^5 or higher.

Discussion

The humoral immune response monitored by the formation of anti-BSA antibody was suppressed by Cy and appeared to be directly related to the level of drug administered. Thus, the percentage of Cy-treated mink with completely suppressed formation of anti-BSA antibody increased in group A from 32% after the second week to 100% 9 weeks after treatment was initiated. Less suppression of the anti-BSA response was noted in the group of mink given less drug (group B). Complete suppression of the primary immune response by Cy treatment has been reported several times.[15–18] Less suppression during the primary response may be attributable to either an inadequate drug dose or to the use of adjuvant with the test antigen. Maguire and Steers [19] observed inhibition of precipitating antibody formation in Cy-treated guinea pigs injected with ovalbumin, but the drug did not prevent antibody formation when ovalbumin was injected with adjuvant.

Cy treatment exerted a pronounced influence on the development of AD lesions (Table 1). There was no rise in serum gamma globulin, no gross or microscopic lesions and no deposition of IgG and C3 in the glomeruli of AD-infected, Cy-treated mink given the drug more frequently and of 8 out of 10 mink given the lower dosage. Mink given AD virus had large amounts of virus in their blood 13 weeks after inoculation, regardless of Cy therapy.

The lack of lesions with high levels of virus in the Cy-treated mink suggests that direct viral propagation is not responsible for the development of the lesions in AD. This is in contrast to the findings by Weiner *et al* [20] of increased susceptibility of mice to several acute virus infections and depression of antibody responses induced by Cy therapy. A strong cytotoxic and lymphocytic action of Cy was indicated in the present study by the depletion of lymphoid cells in the spleens and lymph nodes of drug-treated mink. The cumulative effect of prolonged Cy treatment observed in this study suggests that suppression of anti-BSA antibody formation and inhibition of HGG resulted from destruction of immunocompetent cells.

Deposition of immune complexes is thought to cause the arteritis and glomerulonephritis in AD.[5,6,12] Recent investigations in this laboratory [12] and by Porter et al [9] suggest that part, if not all, of the gammaglobulin increase in AD-infected mink is antibody directed against AD viral antigens. Since Cy treatment prevented the development of HGG, it seems likely that the drug prevented anti-AD antibody response and could have prevented the formation of antigen-antibody complexes and the initiation of lesions by their tissue deposition.

Table 1—Summary of the Effect of Cyclophosphamide Therapy on the Development of Aleutian Disease (AD)

Parameters	Results in mink inoculated with Aleutian disease virus	
	Cy-treated*	Non-Cy-treated
Anti-BSA antibody formation	+	3+
Hypergammaglobulinemia	—	3+
Gross lesions of AD	—	3+
Microscopic lesions of AD	— to +	3+
Gamma globulin and complement (C3) deposits in glomeruli	— to ±	2+ to 3+
Virus titer of whole blood	10^4 MID_{50} ml	10^5 MID_{50} ml†

* Cy indicates cyclophosphamide.
† Highest dilution of whole blood used.

Other workers have evaluated the influence of Cy on immunologically induced diseases. Sharon and Pollard [20] reported that lesions of lymphocytic choriomeningitis virus infection in adult mice were completely suppressed by Cy therapy although the mice remained viremic. Similarly, Russell and co-workers [22,23] and Casey [24] reported that antinuclear antibody formation was decreased, lifespan significantly prolonged and the incidence of glomerular lesions markedly reduced in Cy-treated NZB × NZW mice.

Induction of tolerance to serum proteins and cellular antigens by Cy has been reported.[16,17] In the present study, mink treated with Cy and injected with AD virus and BSA failed to develop anti-BSA antibodies for 8 weeks after cessation of Cy therapy. However, HGG and AD lesions developed in these same animals beginning 2–3 weeks after Cy therapy was stopped. Thus, tolerance to AD virus apparently did not occur.

Cy was not well tolerated by the mink at the dosages used. Depression, anorexia, cyanosis, and leukopenia were frequent. Hepatic degeneration, necrosis and depletion of lymphoid tissue, renal tubular epithelial necrosis, and necrotic or hemorrhagic cystitis have been described in dogs and rats poisoned with Cy.[25] Most of these changes were observed in the mink in this study.

Previous work from this and other laboratories has strongly suggested that AD is an immunologically mediated disease. The studies reported here have shown that the use of an immunosuppressive drug (Cy) will prevent lesions of AD, without preventing viral replication. These

findings further incriminate immunologic processes in the pathogenesis of AD.

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[*Illustrations follow*]

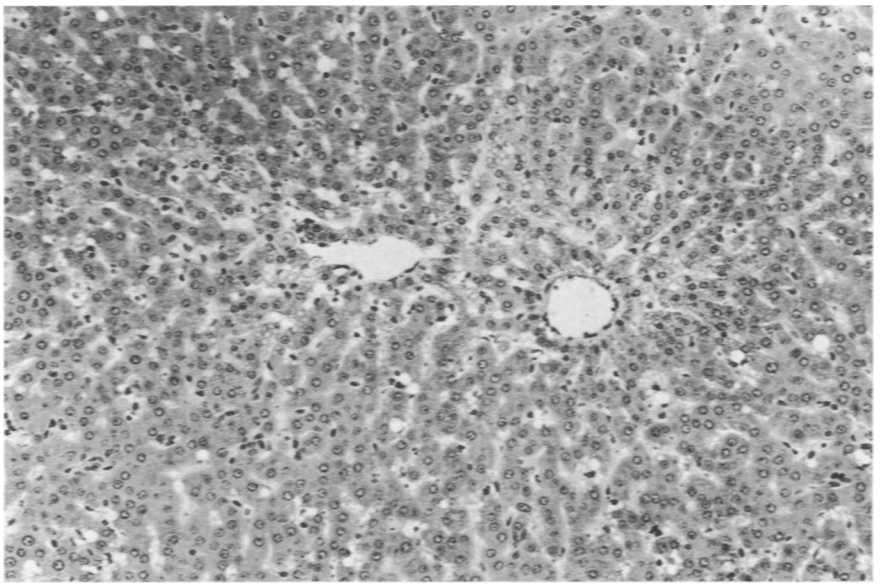

Fig 1A—Liver from a AD virus–inoculated, Cy-treated mink (H&E, × 140).

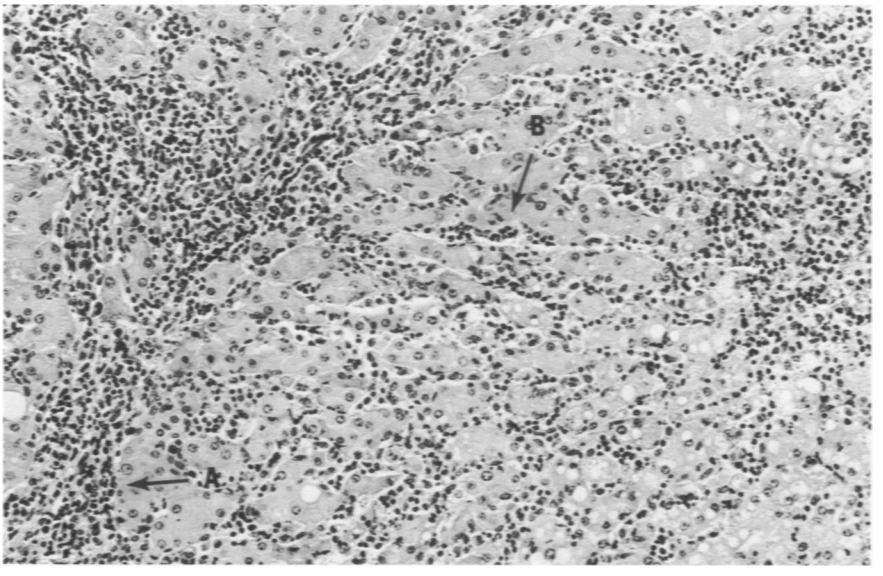

Fig 1B—Liver from an AD virus–inoculated, non-Cy–treated mink. Many mononuclear cells are in the portal (*A*) and in the perisinusoidal areas (*B*) (H&E, × 140).

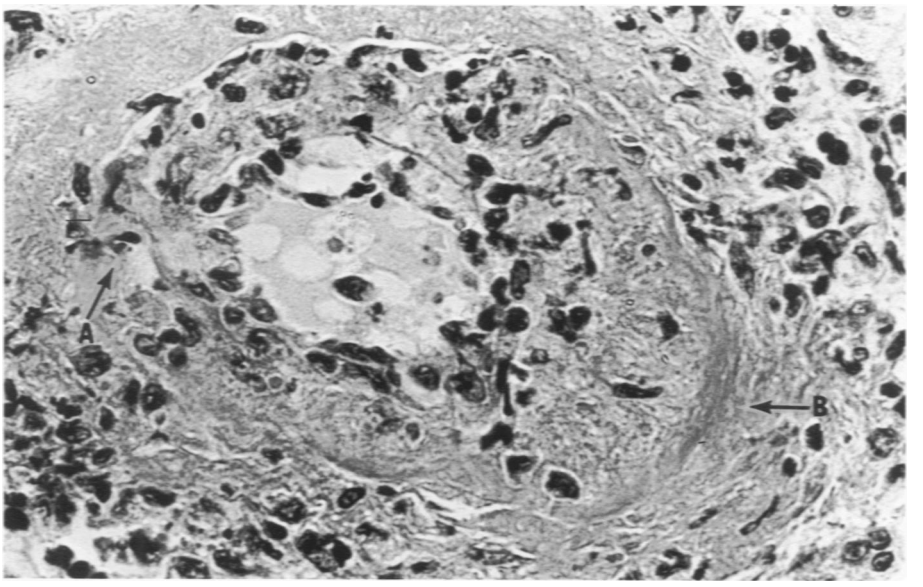

Fig 2—Small muscular artery in an AD virus–inoculated, non-Cy mink. There is myolysis, neutrophilic infiltrate (*A*), and fibrinoid formation (*B*) (H&E, × 275).

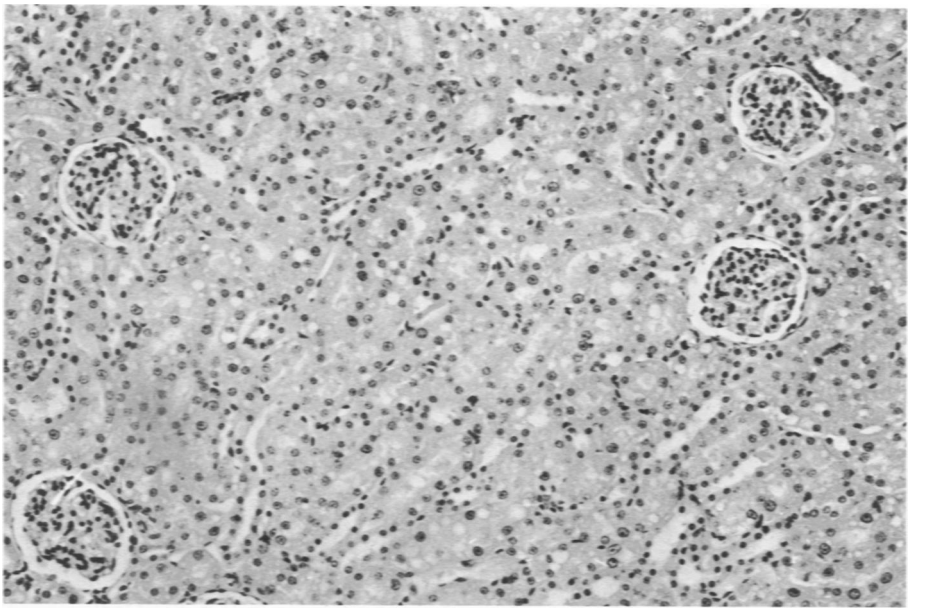

Fig 3A—Renal cortex from an AD virus–inoculated, Cy-treated mink (H&E, × 150).

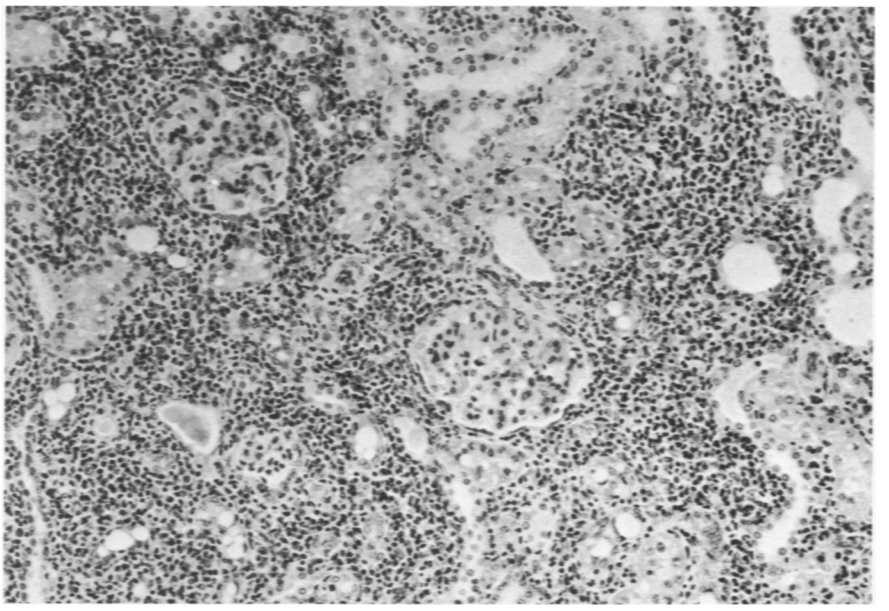

Fig 3B—Renal cortex from an AD virus–inoculated, non-Cy–treated mink. There is an intense interstitial infiltration of mononuclear cells, mainly plasma cells, glomerular thickening and eosenophilic casts. (H&E, × 150).

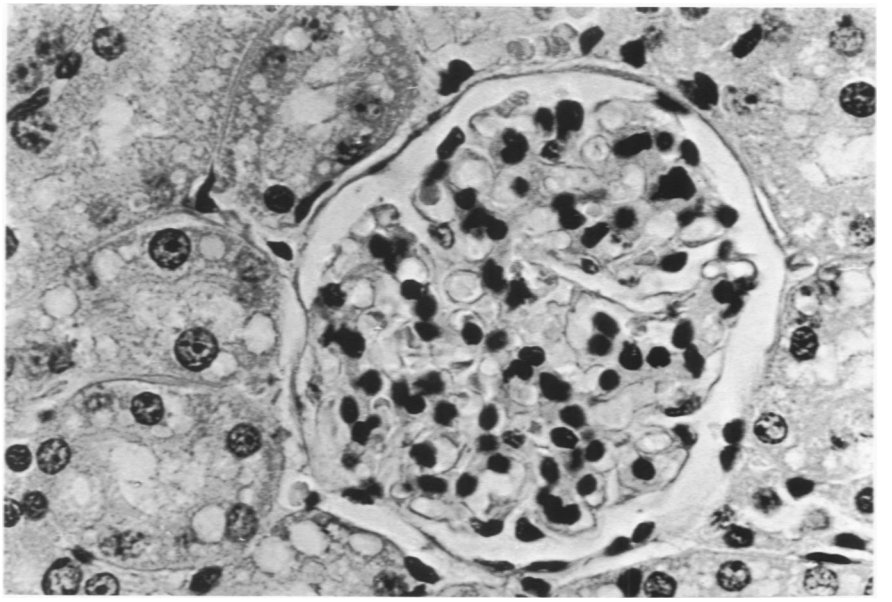

Fig 4A—Normal glomerulus from a Cy-treated, AD virus–inoculated mink (H&E, × 400).

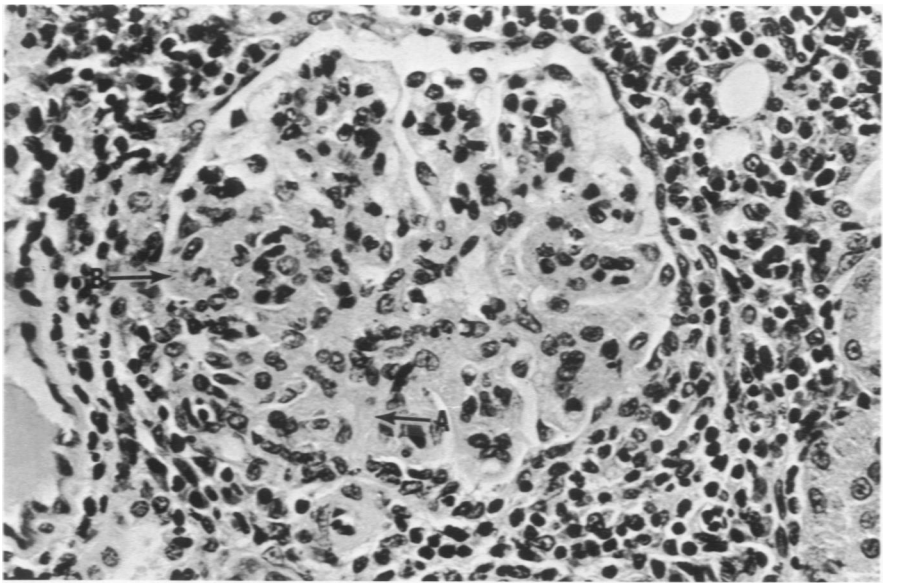

Fig 4B—Glomerulus from a non-Cy–treated, AD virus–inoculated mink. The glomerulus contains granular eosinophilic material (*A*) and a few neutrophils (*B*). Lack of patent capillaries and the presence of periglomerular cellular infiltrate are also evident (H&E, × 400).

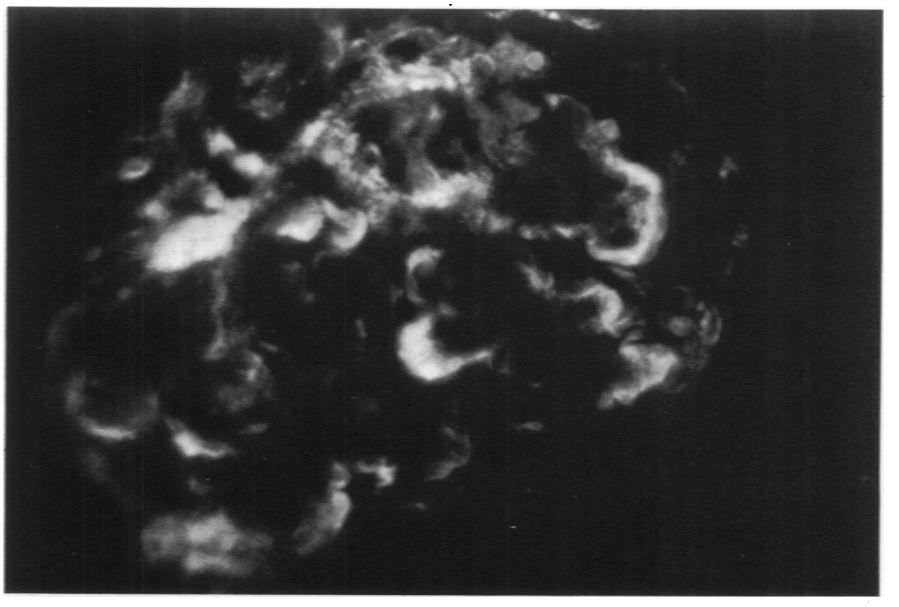

Fig 5—Glomerulus from a AD virus–inoculated, non-Cy–treated mink stained with anti-mink C3. C3 is present in the walls of the capillaries and in the mesangial areas (Cy, × 400).