

Pathogenesis of Aleutian Disease of Mink: Nature of the Antiglobulin Reaction and Elution of Antibody from Erythrocytes and Glomeruli of Infected Mink

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Erythrocytes from mink chronically infected with Aleutian disease virus (ADV) gave positive antiglobulin reactions with rabbit anti-mink immunoglobulin (Ig)G, anti-mink C3, and anti-mink serum, but did not react with anti-mink IgM. The strongest reaction was observed with anti-mink C3. Immunoelectrophoresis demonstrated that serum from rabbits injected with erythrocytes from ADV-infected mink gave a precipitin line with normal mink serum in the beta globulin region corresponding to C3. When normal mink erythrocytes were exposed to serum from ADV-infected mink, they were not sensitized, demonstrating that the antibodies in these mink sera were not directed against erythrocyte antigens. Glycine-hydrochloride buffer treatment of erythrocyte stromata and isolated glomeruli from ADV-infected mink yielded eluates containing serum proteins in the gamma globulin region which appeared to be IgG, and in the beta and alpha globulin regions which are probably complement components. In both erythrocyte and glomerular eluates, anti-ADV antibody was demonstrated. These findings suggested that the positive direct antiglobulin test and glomerulonephritis in Aleutian disease is due to the persistence of ADV and formation and deposition of ADV antigen-antibody-complement complexes on the erythrocyte surfaces and in glomerular capillaries.

Aleutian disease (AD) of mink is an immunological disease caused by a persistent virus infection. The disease is slowly progressive and invariably fatal (9). AD was so named because it was first recognized in the Aleutian coat-colored mink; however, it is now known that the disease occurs in all color phases of mink (4, 7). The disease is characterized by a systemic proliferation of lymphoid cells and generalized plasmacytosis, hypergammaglobulinemia, glomerulonephritis, arteritis, and hepatitis (7, 9, 15, 16). It has been demonstrated that serum from AD virus (ADV)-infected mink have infectious virus-antibody complexes (17), and anti-ADV antibody titers were extremely high in hypergammaglobulinemic serum (2, 3, 13, 18). Glomerulonephritis in this disease was characterized by granular deposition of host immunoglobulin and complement along the glomerular capillary walls and mesangia (7, 15, 18). Very recently (in 1973), ADV was isolated from

ADV-infected mink tissues (10; Cho and Ingram, *Nature*, in press).

In 1966, Saison and co-workers reported that erythrocytes from ADV-infected mink agglutinated when reacted with rabbit anti-mink globulins (19). They suggested that the positive direct antiglobulin test (Coombs test) was due to antibody, and that the antibody was either (i) autoantibody to normal erythrocyte membrane antigens, (ii) autoantibody to erythrocyte membrane components made autoantigenic through the direct or indirect effects of the viral infection, or (iii) antibody to an antigen (perhaps viral) attached or adherent to the erythrocyte membrane (20).

The present investigation was undertaken to identify and characterize the serum proteins responsible for the positive antiglobulin reaction in AD. In a second set of experiments, glomeruli were isolated from the kidneys of ADV-infected mink. Eluates of the glomeruli

were prepared and the specificity of the antibody in these eluates was determined.

MATERIALS AND METHODS

Experimental animals. Aleutian (aa genotype), standard dark (AA genotype), and pastel (AA or aA genotype) mink, 3 months to 1 year of age, were obtained from the AD-free herd of the Field Station of the Ontario Veterinary College. All of the mink were tested by immunoelectrosmoporesis (IEOP) (2; Cho and Ingram, Can. J. Comp. Med., in press) and none had antibody against ADV antigen. Rabbits weighing 2 to 3 kg were purchased commercially and used for the preparation of antisera against mink serum proteins.

Virus. Source of ADV and preparation of inoculum were described elsewhere (Cho and Ingram, Can. J. Comp. Med., in press).

Preparation of rabbit antisera against mink serum proteins. Rabbits were immunized with mink antigens in Freund incomplete adjuvant. The following antisera were produced: anti-C3 (RAC3); anti-complement (RAC); anti-immunoglobulin (Ig)G (RAIgG), anti-IgM (RAIgM), and anti-mink serum (RAS).

Mink antisera against *Brucella abortus* and *Salmonella typhimurium* "O" antigens were used to prepare antigen-antibody complexes. These antisera collected at 1 week and 10 weeks after immunization with *Salmonella* and *Brucella* antigens, respectively, were used as the source of mink IgM and IgG.

Antigen-antibody complexes were prepared by incubating the antigens with the appropriate antisera in the presence of 0.01 M ethylenediaminetetraacetic acid (EDTA) at 37 C for 2 h. The complexes were washed four times with 0.85% NaCl containing 0.01 M EDTA and twice with 0.85% saline. The sediments were suspended in saline, emulsified in Freund incomplete adjuvant, and inoculated subcutaneously into rabbits three times at 12-day intervals.

Zymosan-mink-complement complexes were prepared by the methods of Mardiney and Müller-Eberhard (14) and used as the source of mink antigens for the production of RAC3. *Salmonella typhimurium* "H" antigen and specific rabbit antiserum were used to prepare immune complexes. These complexes were added to fresh normal mink serum and incubated at 5 C for 18 h and then at 37 C for 30 min. The antigen-antibody-complement complexes were washed six times with cold saline and used to immunize rabbits to produce rabbit anti-mink complement (RAC).

For the preparation of RAIgG, the rabbit antisera were absorbed repeatedly with antigen-antibody complexes composed of *Salmonella typhimurium* "O" antigen and mink antiserum collected 1 week after immunization. RAIgM sera were absorbed with mink IgG fractions eluted with 0.0175 M phosphate buffer solution (pH 6.3) by diethylaminoethyl-cellulose chromatography.

RAC and RAC3 were each absorbed four times with immune complexes composed of *Salmonella typhimurium* "O" antigen-mink antibody and *Brucella abortus* antigen-mink antibody.

The specificity of the reagents was tested by immunoelectrophoretic analysis. After absorption, only precipitin lines for IgG, IgM, and β 1c were observed with appropriate reagents and normal mink serum (Fig. 1a, 1b, 2, 3, 4).

Each rabbit antiserum used in this investigation was absorbed six times with washed normal red blood cells pooled from Aleutian, pastel, pearl, and standard dark mink.

Preparation of rabbit anti-ADV-infected mink erythrocytes serum. For the preparation of rabbit anti-ADV-infected mink erythrocytes, 6 ml of 50% mink erythrocyte suspension collected from six infected mink which gave positive antiglobulin reactions were injected intravenously into two rabbits twice at an interval of 7 days, and 14 days after the second injection serum was collected and absorbed with normal mink red blood cells as described above.

Direct antiglobulin test. Mink blood was collected by cardiac puncture into equal volumes of Alsever solution. The cells were washed at least five times with 0.85% saline and made up to a 2% suspension in 0.85% saline. One drop of each cell suspension was added to one drop of each antiglobulin reagent. Cells and serum were thoroughly mixed and incubated at 37 C for 1 h. At the end of the reaction, cells from each tube were carefully removed with a Pasteur pipette and spread on a slide for microscope examination.

Preparation of erythrocyte stromata from ADV-infected mink. The method of Kochwa and Rosenfield (11) was followed with some minor modifications. For the preparation of erythrocyte stromata of ADV-infected mink, 50 ml of blood was collected from four infected mink showing strong direct antiglobulin reactions and thoroughly washed as described above. The packed cells were pooled and were made up to a 10% suspension in saline. For the preparation of the red cell stromata, the red cells were lysed by adding 2.5 ml of a 0.5% solution of digitonin for each 50 ml of the 10% red cell suspension and stirred for 1 min. This material was centrifuged at $52,800 \times g$ for 30 min, and the supernatant fluid was discarded. Erythrocyte stromata were washed six times with large volumes of cold isotonic saline. The pellets were reacted with phosphate-buffered saline (PBS, 0.01 M in 0.14 M NaCl, pH 7.4) for 1 h and after centrifugation at $52,800 \times g$ for 30 min, the supernatant fluid was collected. The erythrocyte stromata were utilized for the preparation of eluates.

Preparation of acid eluates from the erythrocyte stromata. Glycine-hydrochloride buffer, half volume of whole blood (0.1 M, pH 3.0), was added to the sedimented erythrocyte stromata and mixed thoroughly. The stromata were incubated at room temperature for 20 min and centrifuged at $52,800 \times g$ for 30 min. The supernatant fluids were collected and neutralized with 1 N NaOH to pH 7.0. The eluates were concentrated with polyethylene glycol. Anti-ADV antibody was assayed by IEOP, and immunoelectrophoresis was carried out to examine the serum proteins contained in the eluates. Erythrocytes from normal mink were treated similarly and served as a control.

Isolation of glomeruli and preparation of glomerular eluates from normal and ADV-infected

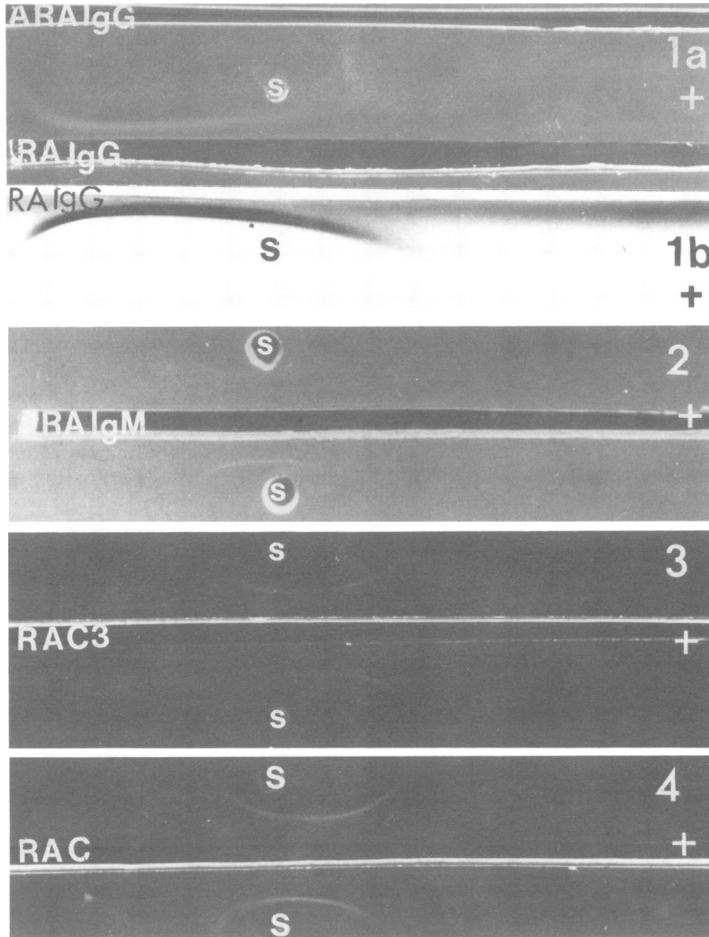


FIG. 1-4. Immunoelectrophoretic patterns formed by rabbit antibodies to mink serum proteins. Abbreviations: S, normal mink serum; RAIgG, rabbit anti-mink IgG (1a, b); ARAIgG, rabbit anti-mink IgG absorbed with mink IgG (1a); RAIgM, rabbit anti-mink IgM (2); RAC3, rabbit anti-mink C3 (3); RAC, rabbit anti-mink complement (4).

mink kidney. Kidney cortex collected from 10 ADV-infected mink 4 months or later after infection was cut into small pieces and washed five times with 0.85% saline. For the isolation of glomeruli, the method of Greenspon and Krakower (6) was followed with minor modifications. Fragments of the cortex were forced through a stainless steel sieve of 60 mesh (0.25 mm). The paste passed through the mesh, was collected and washed with 0.85% saline five times by centrifuging at $1,100 \times g$ for 15 min. The sediment was mixed with an 82% sucrose solution. It was then centrifuged at $39,000 \times g$ for 3 min. The intact isolated glomeruli were thus brought to the top. This top layer was collected and washed with 0.85% saline five times, with centrifugation at $70 \times g$ for 10 min and finally at $2,500 \times g$ for 15 min. Glomeruli of more than 90% purity (Fig. 5) were suspended in 0.85% saline and stored at 5 C for 18 h to remove soluble proteins. The glomerular suspension was frozen and thawed twice and washed twice with 0.85% saline at $2,500 \times g$ for 15 min. The pellet was reacted with PBS for 1 h, and after centrifugation at

$2,500 \times g$ for 15 min the supernatant fluid was collected as the PBS eluate. Glycine-hydrochloride buffer (50 ml/g of pellet) (0.1 M, pH 3.0) was added to the pellet and incubated for 1 h at room temperature. After centrifugation at $2,500 \times g$ for 15 min, the supernatant fluid was collected and neutralized with 1 N NaOH to form the acid eluate.

Both PBS and acid eluates were concentrated with polyethylene glycol. Antibody activity was assayed by IEOP, and immunoelectrophoretic analysis was carried out to examine the serum proteins eluted from the isolated glomeruli. Eluates from glomeruli collected from normal mink kidney served as a control.

Immunofluorescent test for detection of immune complexes in kidneys. RAIgG and RAC3 were conjugated with fluorescein isothiocyanate (FITC). Frozen kidney blocks were cut with a cryostat. To remove soluble proteins, the sections were washed in PBS for 30 min at room temperature and were then stained with FITC-conjugated RAIgG or RAC3 for 40 min at room temperature. After staining, all the specimens

were washed in PBS for 30 min with several changes of the buffer, rinsed with distilled water, mounted in buffered glycerine, and examined.

RESULTS

Antiglobulin test with red blood cells from ADV-infected mink. Direct antiglobulin tests were run on the red blood cells from five Aleutian and two standard dark mink infected with ADV. Red blood cells were collected at 5 months after infection, and tests were conducted to determine whether these cells were coated with IgM, IgG, C, or C3. Erythrocytes from ADV-infected mink gave positive reactions with RAC3 and RAC. RAIgM gave negative reactions, whereas RAIgG gave positive reactions in four and negative reactions in three infected mink (Table 1). At 5 months after ADV infection, anti-ADV antibody titers in the serum ranged from 10,000 to 40,000 (Table 1). One drop of normal mink serum mixed with one drop of antiglobulin serum and incubated for 1 h at 37 C failed to cause a direct antiglobulin reaction with erythrocytes from ADV-infected mink, which showed a positive reaction with the antiglobulin reagents before absorption with normal mink serum.

As a control, four Aleutian mink were injected with spleen suspension from a normal Aleutian

mink. Five weeks after immunization, direct antiglobulin tests were run on red blood cells with RAIgG, RAIgM, RAC, RAC3, and RAS. All cells gave negative results (Table 1).

Indirect antiglobulin test with serum from ADV-infected mink. To determine if serum from ADV-infected mink contains antibodies against normal mink erythrocytes, 1 vol of a 10% suspension of normal mink red blood cells was treated with 4 vol of undiluted ADV-infected mink serum for 1 h at 37 C. After thorough washing, RAS was added. No agglutination of red blood cells was observed. This experiment showed that infected mink serum did not sensitize normal mink erythrocytes.

Production of antibody against serum proteins on the surface of erythrocytes of ADV-infected mink. Two rabbits were injected with ADV-infected mink erythrocytes which showed positive direct antiglobulin reactions, and 14 days after the second injection sera were collected and tested for antibody activity against normal mink serum. Immunoelectrophoretic analysis, using these antisera, showed a very faint precipitin line with normal mink serum in the beta globulin region corresponding to C3. The position of the precipitin line was similar to Fig. 3.

Demonstration of anti-ADV antibody in

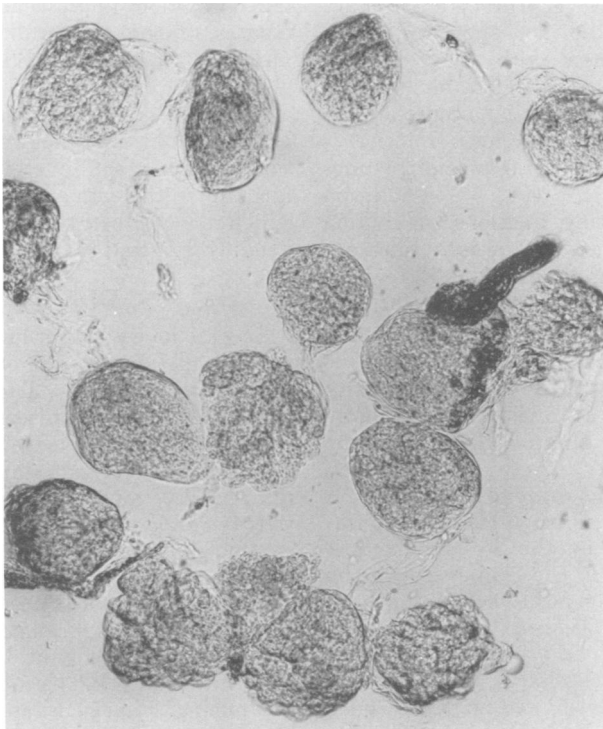


FIG. 5. Isolated glomeruli from Aleutian disease virus-infected mink kidney prepared for antibody elution.

TABLE 1. Direct antiglobulin reactions of red blood cells from ADV-infected and normal mink

Group	Mink no.	Serum anti-ADV antibody titers ^a	Types of antiglobulin				
			RAIgG	RAIgM	RAC	RAC3	RAS
ADV-infected	A2	20,000	++ ^b	-	+	+++	320 ^c
	A4	10,000	++	-	-	+++	80
	A12	40,000	-	-	-	+	320
	A13	20,000	+	-	++	++	160
	A18	10,000	-	-	++	++	40
	S10	40,000	+	-	+	++	320
	S12	10,000	-	-	++	+	160
	Normal	A21	0	-	-	-	-
A22		0	-	-	-	-	-
S1		0	-	-	-	-	-
S2		0	-	-	-	-	-
			-	-	-	-	-

^a Anti-ADV antibody was assayed by immunoelectroosmophoresis.

^b Degree of agglutination of erythrocytes. Undiluted antiglobulins were used.

^c Reciprocals of the highest dilutions of rabbit anti-mink serum which caused the agglutination of erythrocytes.

the acid eluates from ADV-infected mink erythrocyte stromata. By the treatment with digitonin, red blood cells were lysed. The resultant stromata were intact and were free of hemoglobin after several washings. Anti-ADV antibody was eluted by glycine-hydrochloride buffer treatment of the red cell stromata from ADV-infected mink (Fig. 6). PBS treatment of the same erythrocyte stromata failed to yield any antibody activity. Erythrocyte stromata from normal mink did not show anti-ADV antibody after treatment with either glycine-hydrochloride buffer or PBS (Table 2). Immunoelectrophoretic analysis showed that the acid eluate of erythrocyte stromata from ADV-infected mink yielded several precipitin lines when developed with RAS (Fig. 7). In addition to the slower migrating fractions of gamma globulins which appeared to be IgG, lines appeared in the alpha and beta globulin regions, which were consistent with the suggestion that the acid eluates contained complement components.

Elution of proteins in glomerular deposits and demonstration of anti-ADV antibody from the glomerular eluates. Sections of the kidneys from 10 ADV-infected mink, which were collected 4 months or more after infection and used as the sources of glomeruli preparations, were examined for the presence of host IgG and complement in the glomeruli. All the sections showed strong positive staining with FITC-conjugated RAIgG and RAC3. Fluorescence showed lumpy and granular patterns along the glomerular capillary walls (Fig. 10). Normal kidney sections showed no fluorescence with FITC-conjugated RAIgG and RAC3.

The acid eluates of the isolated glomeruli from ADV-infected mink kidney showed antibody activity against ADV antigen but no reaction with control antigen or with normal kidney suspension (Table 2, Fig. 8). When normal kidney sections were incubated with the acid eluates from AD glomeruli at room temperature for 40 min, then washed and stained with FITC-conjugated RAIgG, the kidney sections failed to show fluorescence. Neither the PBS eluates of the glomeruli from ADV-infected mink nor the acid nor PBS eluates from normal glomeruli had anti-ADV antibody. Immunoelectrophoresis of the glomerular eluate from ADV-infected mink showed precipitin lines in the gamma globulin region corresponding to IgG, and in the beta and alpha globulin regions, which may represent mink complement components (Fig. 7 and 9).

DISCUSSION

Several investigators have reported that AD in mink has some of the characteristic features of autoimmune states. The hypothesis that AD is an autoimmune disease is based on the reports of a direct positive Coombs test (19, 20), demonstration of anti-gamma globulin factor (21), and circulating deoxyribonucleic acid (DNA) and anti-DNA antibody (1). Anti-gamma globulin factors, which were generally of low titer, were found in 68% of 32 normal mink sera, compared with 72% of 42 mink infected with AD (21). A high concentration of DNA was detected in sera of mink both before and after ADV infection (1). The incidence and quantity of "nuclear" antigens and anti-nuclear antibody detected by immunofluorescence were

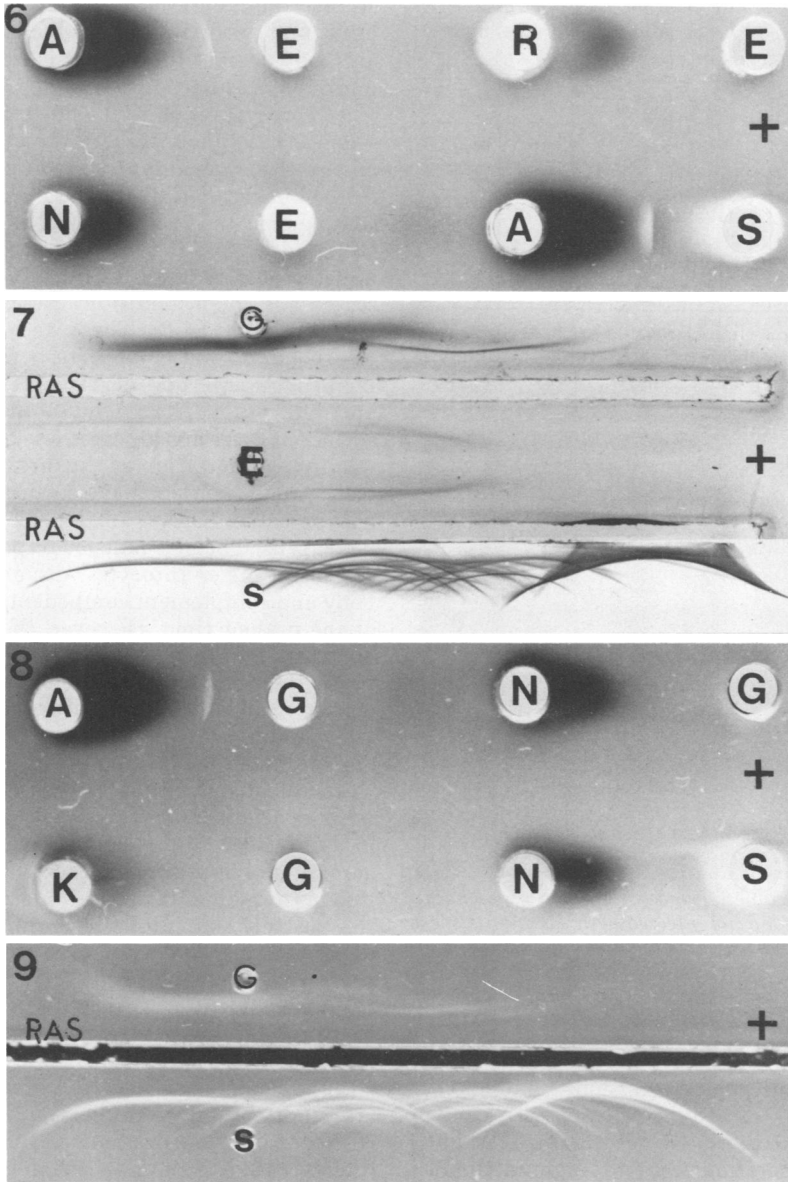


FIG. 6. Immunoelectrosmophoresis of acid eluate from erythrocytes of AD-infected mink.

FIG. 7. Immunoelectrophoresis of acid eluate from erythrocytes and from glomeruli of AD-infected mink.

FIG. 8. Immunoelectrosmophoresis of acid eluate from glomeruli of AD-infected mink.

FIG. 9. Immunoelectrophoresis of acid eluate from glomeruli of AD-infected mink. Abbreviations: A, ADV antigen prepared from AD-infected mink tissue; N, negative (control) antigen prepared from normal mink tissues; S, mink anti-ADV serum; E, acid eluate from erythrocyte of AD-infected mink; R, normal mink erythrocyte stromata; G, acid eluate from glomeruli of AD infected mink; K, normal mink kidney suspension; RAS, rabbit anti-mink serum.

greater in sera from mink after ADV infection than in sera taken from mink before ADV infection. A pre-AD serum pool showed nuclear fluorescence with human white blood cell nuclei at dilutions up to 1:16, whereas the titer of the post-AD serum pool was 1:64. However, the

presence of nuclear antigens and anti-nuclear antibodies did not correlate with the degree of hypergammaglobulinemia. The significance of anti-gamma globulin factors and anti-DNA-antibody as pathogenic factors in AD is unknown at present.

TABLE 2. *Antibody to Aleutian disease virus antigen in eluates from erythrocytes and glomeruli of normal and Aleutian disease-infected mink^a*

Disease status	Eluate from:	Eluted at:	
		pH 3	pH 7.4
Normal	Erythrocytes	-	-
	Glomeruli	-	-
Infected	Erythrocytes	+	-
	Glomeruli	+	-

^a None of the eluates reacted with antigens prepared from normal mink tissues, normal mink erythrocytes, or normal mink kidney.

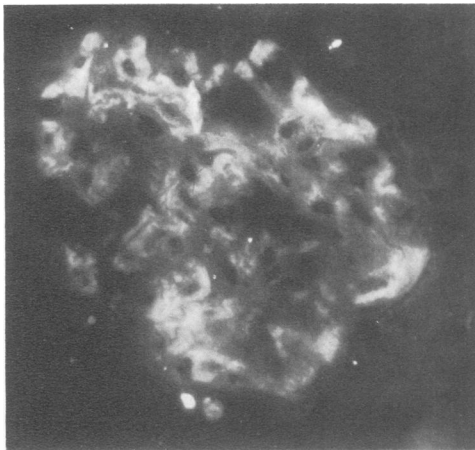


FIG. 10. *Immunofluorescent reaction of glomeruli from kidney of a mink infected with Aleutian disease virus; unfixed frozen section stained with FITC-conjugated rabbit anti-mink IgG. IgG is present in the capillary walls and mesangium and forms a granular pattern.*

Fluorescein-labeled normal- and ADV-infected mink sera did not react with cells of normal mink tissues (15, 18). Anti-ADV antibody titers, determined by the immunofluorescence test, were extremely high, and at 60 days after ADV infection the geometric mean antibody titer reached 100,000 (18). The antibody titers were correlated with the degree of hypergammaglobulinemia (13, 18; Cho and Ingram, *Proc. Can. Fed. Biol. Soc.* 15:299, 1972; Cho and Ingram, *Can. J. Comp. Med.*, in press). Porter et al. (16) failed to demonstrate the systemic lupus erythematosus (SLE) cell phenomenon or anti-nuclear factor in ADV infections of mink.

In the present investigation, erythrocytes from ADV-infected mink were shown to have IgG and complement components on their surfaces (Table 1, Fig. 7). The immunoglobulins

eluted from the erythrocytes of ADV-infected mink had anti-ADV antibody activity, but no antibody against the surface antigen of normal mink red blood cells.

Erythrocytes from ADV-infected mink gave a stronger reaction with RAC3 than with RAIgG, and erythrocytes from some mink reacted with RAC3 but not RAIgG (Table 1). By means of a complement-fixing antibody consumption test, Gilliland et al. (5) reported that previously undetectable red cell-bound IgG could be detected and quantified from the erythrocytes of patients with SLE. These red cells showed positive direct antiglobulin tests with anti-complement serum but negative reactions using anti-IgG serum. The amount of IgG fixed on the surface of these erythrocytes was below the level required for detection by the direct antiglobulin test. These workers have been able to elute anti-erythrocytic IgG from the reactive red blood cells. The mechanisms responsible for the coating of the erythrocytes with anti-ADV antibody and complement components are not clear at the present time. However, the erythrocytes apparently are coated with a complex of the virus, the antibody and complement and acid elution results in the separation of the antibody and complement. The virus antigen was not demonstrated in the eluates.

The renal glomerular lesions in ADV-infected mink resemble those in man with SLE (12) and in NZB mice (8). It has been reported that gamma globulin and complement were deposited in the glomeruli of ADV-infected mink, and the possible source of the immune complexes deposited in glomeruli were ADV-antibody-complement complexes (7, 15) or circulating DNA-anti-DNA antibodies (7) as was reported in SLE (12). However, the acid eluate from glomeruli of ADV-infected mink did not react with the nuclei of normal mink kidney cells (15). Porter and co-workers (18) eluted anti-ADV antibody, as determined by the immunofluorescence test, from ADV-infected mink kidney and concluded that the pathogenesis of the glomerulonephritis of AD is related to the formation of viral antigen-antibody-complement complexes which lodge in glomerular capillaries. In the present investigation, serum proteins eluted from isolated glomeruli of ADV-infected mink kidney contained antibody against the ADV antigen as assayed by IEOP. These results supported the earlier finding of Porter and co-workers (18).

The present investigations strongly suggested that the positive direct Coombs reaction and glomerulonephritis in AD were due to the persistence of the virus and formation and deposi-

tion of ADV antigen-antibody-complement complexes on the surfaces of erythrocytes and in glomerular capillaries.

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