

METABOLISM AND FUNCTION OF GAMMA GLOBULIN IN ALEUTIAN DISEASE OF MINK*, †

BY DAVID D. PORTER, M.D., FRANK J. DIXON, M.D.,
AND AUSTIN E. LARSEN, D.V.M.

(From the Division of Experimental Pathology, Scripps Clinic and Research Foundation,
La Jolla, California, and the Utah Mink Growers Cooperative, Midvale)

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Aleutian disease (AD) of mink is characterized by a systemic proliferation of plasma cells. Secondary to this plasma cell proliferation there is marked hypergammaglobulinemia, necrotizing vasculitis, proliferation of fibrillary polysaccharide containing material in the mesangial areas of the glomeruli, and some thickening of the glomerular capillary basement membranes (1-4). The extent of the tissue lesions is closely correlated with the degree of hypergammaglobulinemia in AD-affected mink (5). AD is readily transmitted to normal mink by cell-free ultrafiltrates, and the agent is sedimentable by ultracentrifugation, strongly suggesting that it is a virus (6, 7).

The increased gamma globulin is initially heterogeneous electrophoretically, and in 10 to 20 per cent of mink with AD followed for a year, the gamma globulin becomes more homogeneous, resembling the globulin in human or mouse myeloma. Mink developing the homogeneous globulin show Bence Jones proteinuria, but no myeloma-like bone lesions (8).

This infectious, apparently viral, disease combines many of the serologic, cellular, and tissue changes in myeloma on one hand and in immunologically induced vascular and renal diseases on the other. The present studies were undertaken in an attempt to define the pathogenetic mechanisms involved in AD, particularly the function, if any, of the gamma globulins and to compare the physical and metabolic characteristics of normal and AD gamma globulins.

Materials and Methods

Animals.—Ranch-raised mink were obtained from the Utah Mink Growers Cooperative, and were judged to be normal or to have AD on the basis of serum protein electrophoresis (5). Mink with a serum gamma globulin of 20 per cent or more of the total serum protein were considered to have AD. The presence or absence of AD was confirmed by gross and histologic examination of the tissues at the time of sacrifice of the mink.

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Preparation of Proteins.—Normal mink serum was partially precipitated by the addition of ammonium sulfate to 50 per cent saturation. Albumin was prepared from the fraction soluble in 50 per cent ammonium sulfate by a continuous molarity gradient elution from carboxymethylcellulose starting with sodium phosphate buffer 0.01 M pH 6.0. Gamma globulin was prepared from the material insoluble in 50 per cent ammonium sulfate by dialysis against 0.0175 M sodium phosphate buffer pH 7.0 and elution from diethylaminoethylcellulose using the same buffer. Bovine gamma globulin (Armour Laboratories, Chicago, lot W30512) and egg albumin (Pentex, Inc., Kankakee, Illinois, lot 4) were obtained commercially, *Megathura crenulata* hemocyanin was purified from the hemolymph by preparative ultracentrifugation. Proteins were trace-labeled with I^{131} (9).

Electrophoresis and Ultracentrifugation.—Paper strip electrophoresis employed a Beckman model R system and model RB analytrol scanner (Beckman Instruments, Inc., Palo Alto California). Immunoelectrophoresis was done by the micromethod of Scheidegger (10).

Analytical ultracentrifugation was performed in a Beckman model E machine (Beckman Instruments, Inc.) operated at 59,780 RPM using Schlieren optics. Photographic plates were measured with a microcomparator and sedimentation coefficients corrected to water at 20°C.

Preparation of Antiserums.—Antiserums to whole, normal, and AD-affected mink serums, gamma globulin, albumin, and fibrin were prepared in rabbits by 3 monthly injections of the material in Freund's complete adjuvant, followed by 12 weekly injections of the soluble protein. Antiserums were checked for specificity by immunoelectrophoresis. Rabbits and a horse were injected with homogenates of AD-affected mink tissue in complete Freund's adjuvant and the serums were subsequently absorbed with normal mink serum and tissues.

Fluorescent Antibody Technique.—The fluorescent antibody technique employed methods previously described (11). Serums were labeled with fluorescein isothiocyanate and absorbed with liver powder or eluted from diethylaminoethylcellulose with 0.05 M sodium phosphate buffer (12). Frozen sections and cell smears from normal and AD-affected mink were fixed in alcohol-ether, acetone, or formalin. Ten AD-affected mink serums and 5 normal mink serums, rabbit anti-mink gamma globulin, albumin, and fibrin, and rabbit and horse anti-AD-affected mink tissue were used as fluorescent stains.

Antibody Determinations.—The antigen precipitating capacity of antiserums for hemocyanin, bovine gamma globulin, and egg albumin was measured by the technique of Talmage and Maurer (13) and antibody to *Shigella flexneri* was measured by the agglutination of a 0.025 per cent suspension of whole organisms.

Complement Fixation.—Quantitative serum complement assays on mink serums were done by the method of Osler, Strauss, and Mayer (14). Semiquantitative complement fixation employing normal and AD-affected mink serums and normal and AD-affected tissue extracts as antigen were done by the LBCF method (15).

Rheumatoid Factor Assay.—Assays for rheumatoid factor were done by Dr. Morris Ziff and Dr. H. H. Fudenberg employing sheep cells sensitized with normal mink gamma globulin which were reacted with normal and AD-affected mink serums.

L. E. Cell Preparation.—Lupus erythematosus (L.E.) cell preparations were done by the method of Hargraves (16) using 10 normal and 15 AD-affected mink blood samples.

Serologic Test for Syphilis.—VDRL and Mazzini tests were performed on 4 normal and 6 AD-affected mink serums.

Serum Protein Half-Life.—Normal and AD-affected mink were given potassium iodide in their drinking water throughout the experiment. Each animal received 2.0 μ c of I^{131} mink albumin or gamma globulin intraperitoneally, the injection contained 1 mg protein in 1 ml. The mink were counted in a whole body scintillation counter for 5 biologic half-lives of the protein. The protein half-life was determined by the slope of the semilogarithmic plot of disappearance of I^{131} from the animal, corrected for isotopic decay. Urinary protein loss was

determined by sulfosalicylic acid turbidity measurements of 24 hour urine specimens. Urine was examined for trichloroacetic acid-precipitable radioactivity.

The Effect of AD on Unrelated Antibody Formation.—Fourteen normal and 10 AD-affected mink were given one dose of complete Freund's adjuvant containing 10 mg each of hemocyanin, bovine gamma globulin, and egg albumin. The mink were bled 30 days after receiving the adjuvant.

Fifty-four normal mink were given 4 doses of incomplete Freund's adjuvant containing 10 mg each of hemocyanin, bovine gamma globulin, and egg albumin, and 1 mg of *Shigella flexneri* whole killed organisms at 25 day intervals. At the time of the last injection of adjuvant 33 of the mink were given AD-infectious material intraperitoneally. Four months later the mink receiving the AD virus had developed hypergammaglobulinemia of 50 per cent or more of the total serum protein. The antibody levels of the AD-affected and normal mink were measured on the day of the virus inoculation and 4 months later to determine if AD and/or the development of hyperglobulinemia would affect preexisting and continuing unrelated antibody formation.

RESULTS

Serum Protein Half-Lives.—The results of the albumin and gamma globulin half-life studies in normal and AD-affected mink are shown in Table I. The mean half-life of albumin in 4 normal mink was 3.3 days, and in 4 AD-affected hypergammaglobulinemic mink it was 2.9 days; the mean values are not significantly different using the t test. The mean half-life of normal gamma globulin in 5 normal mink was 3.45 days and in 5 AD affected mink it was 2.2 days, the difference of the means is highly significant using the t test (P less than 0.001). There was no significant proteinuria in this group of mink, and there was no loss of trichloroacetic acid-precipitable radioactivity in the urine.

Ultracentrifugation of Mink Serums.—Mink gamma globulin from either normal or AD-affected mink prepared by DEAE-cellulose chromatography showed a single homogeneous peak in the analytical ultracentrifuge with $S_{20,w} = 6.4$ at infinite dilution. Whole diluted serums from 43 AD-affected mink and 10 normal mink were examined in the analytical ultracentrifuge. The AD-affected serums had an average of 5.6 gm/100 ml of gamma globulin as compared with 0.7 gm/100 ml for the normals. The serums from AD-affected mink showed a large increase in the $S_{20,w} = 6.4$ component, and in addition, two components not seen in normal mink serums. The abnormal components were often quite heterogeneous in their sedimentation behavior and ranged from 9 to 17 and 22 to 25 $S_{20,w}$, and these components represented up to 6 per cent of the total serum protein. The two abnormal components were markedly enriched in euglobulin preparations. In the presence of 6 M urea or pH 3.2 glycine buffer these components dissociated to form 6.4S and 18S units, together with a small amount of material lighter than 6S. Removal of urea from such a dissociated preparation resulted in reappearance of the two abnormal components, which, however, were more heterogeneous in their sedimentation behavior than before the urea treatment. These characteristics indicate that

the two abnormal components found in AD-affected mink serums are protein-protein complexes. Immunoelectrophoresis of partially purified complexes indicated that they were composed, at least in part, of gamma globulin. Text-fig. 1 shows 2 serums with complexes, their euglobulins demonstrating relative enrichment in the complexes, and the euglobulins in urea showing dissociation of the complexes. Text-fig. 2 shows a euglobulin with both types of complexes.

Of the 43 AD serums tested, 31 had 22S to 25S complexes, 21 had 9S to 17S complexes, and only 7 showed neither type of complex. The presence or absence of complexes did not correlate with the presence or absence of vascu-

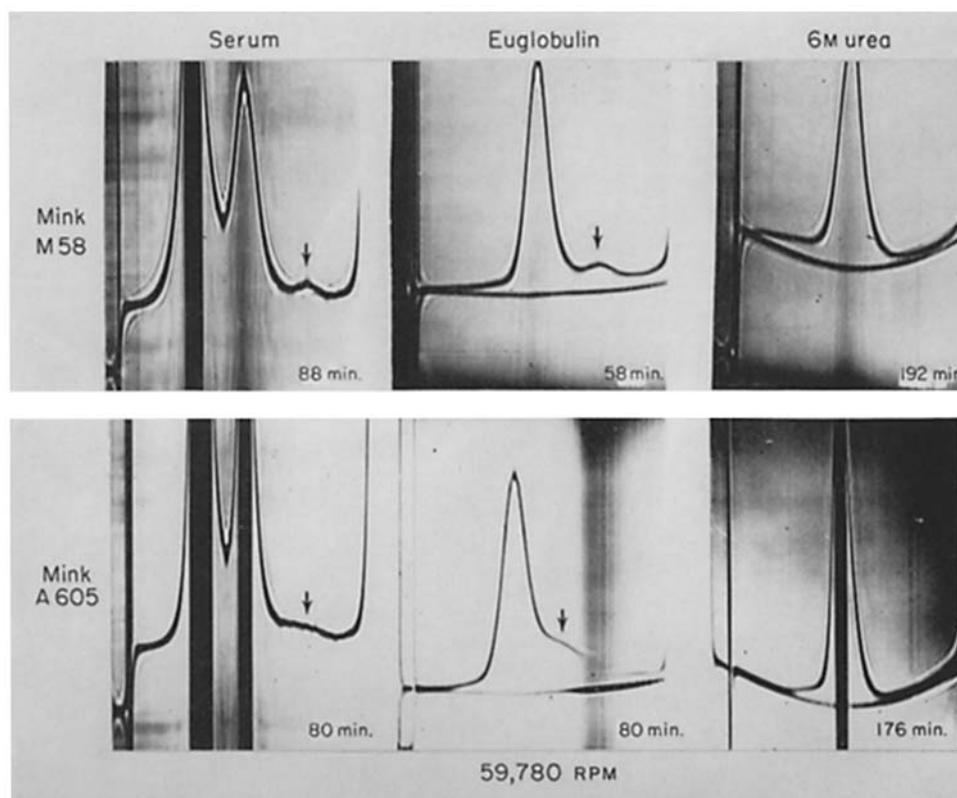
TABLE I
Half-Life of γ -Globulin and Albumin in Normal and Aleutian Disease-Affected Mink

Color/genotype	Total protein gm/100 ml	γ -Globulin		Albumin		Uri-nary protein loss mg/day	Half-life	
		per cent	gm/100 ml	per cent	gm/100 ml		γ -Glo-bulin days	Albumin days
Normal								
Black AA	7.4	11.7	0.86	58.2	4.31	7, 6	3.1	2.8
Pastel Aa	7.4	17.8	1.31	63.1	4.67	0, 1	3.8	3.3
Pastel Aa	7.3	14.4	1.05	68.6	5.01	4, 0	3.25	3.7
Pastel Aa	7.4	15.4	1.13	61.6	4.56	4, 2	3.5	3.45
Pastel Aa	7.3	14.5	1.06	66.5	4.85	4	3.6	—
Aleutian disease affected								
Pastel Aa	9.9	40.4	3.99	41.7	4.13	4, 10	2.3	3.1
Pastel Aa	9.4	45.4	4.26	32.8	3.08	3, 2	2.15	2.5
Pastel Aa	9.6	38.7	3.71	43.3	4.15	6, 3	2.1	2.8
Aleutian aa	9.2	44.5	4.09	38.7	3.56	2, 9	2.3	3.1
Pearl aa	15.2	74.0	11.23	14.9	2.26	17	2.1	—

litis in these mink. Since all animals in this group had advanced glomerular lesions the complexes did not appear to be directly related to the development of this lesion. Mink serums with either or both types of complexes had normal levels of hemolytic complement.

Fluorescent Antibody Results.—Arteritic lesions in AD-affected mink stained strongly with anti-gamma globulin, and weakly or not at all with antifibrin and antialbumin. An example of a fluorescent antibody stain of an arteritic lesion is shown in Figs. 1 *a* and 1 *b*, together with a hematoxylin and eosin stained section of a similar vessel. Glomeruli affected by AD stained weakly with all three antiserum proteins in a mesangial pattern characteristic of non-specific proteinuria (17). Ten AD and 5 normal fluorescent mink serums failed to stain any cells or tissues of AD-affected or normal mink, including autologous tissues. Rabbit and horse antisera to AD-infectious organ homoge-

nates failed to show any consistent pattern of staining of AD mink cells compatible with viral particles or materials. Results were identical with liver powder-absorbed or DEAE-eluted fluorescent antibody preparations, and likewise were identical using different methods of fixation.



TEXT-FIG. 1. Ultracentrifugal pattern of two Aleutian disease-affected mink serums showing the markedly elevated 6.4S peak and the presence of complexes which are indicated by the arrows. The complexes are 14S in the upper frames and heterogeneous in the lower. Euglobulin preparations show marked enrichment in the complexes, and the euglobulin in 6 M urea shows complete dissociation of the complexes to 6.4S units. Sedimentation is from left to right and the time on the photograph is from the attainment of full operating speed.

Complement Fixation.—Quantitative serum complement assays showed no difference between normal and AD-affected mink serums. The mean complement of 114 mink was 150 C'H50 units with a range of 53 to 250 C'H50 units.

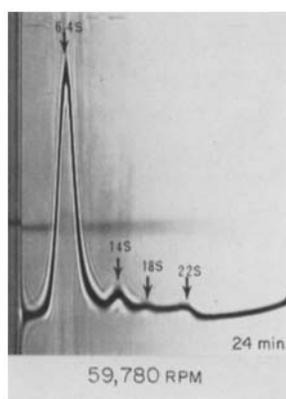
Semiquantitative complement fixation employing normal and AD-affected serums and infectious organ homogenates as antigen failed to show fixation of guinea pig complement.

Rheumatoid Factor Assay.—No rheumatoid factor activity was found using sheep cells sensitized with normal mink gamma globulin.

L.E. Cell Preparations.—No lupus erythematosus cells were found in preparations from normal or AD-affected mink.

Serologic Tests for Syphilis.—No reactivity of normal or AD-affected mink serums was found in VDRL or Mazzini tests.

Gel Diffusion.—Double diffusion in agar gel employing rabbit antisera to whole AD-affected mink serum which subsequently were absorbed with normal mink serum and as antigens either the immunizing AD serum or infectious tissue homogenate failed to show any reaction.



TEXT-FIG. 2. Ultracentrifugal pattern of Aleutian disease-affected mink euglobulin in 0.15 M NaCl showing complexes of 22S and 14S and normal components of 18S and 6.4S. Sedimentation is from left to right.

Effect of AD on Unrelated Antibody Formation.—Thirty days after a single injection of egg albumin, bovine gamma globulin (BGG), and hemocyanin in complete Freund's adjuvant, 14 normal mink had an average response of 816 μ g N antigen precipitated/ml antiserum to hemocyanin and 2.2 μ g N antigen precipitated/ml antiserum to BGG. Ten AD-affected mink had an average response of 224 μ g N antigen precipitated/ml antiserum to hemocyanin, and no response to BGG. Neither group made a detectable response to egg albumin. The mink antibody to hemocyanin was 6.4S gamma globulin as determined by immunoelectrophoresis, and showed a range of mobility as great as the same animal's gamma globulin reacted with rabbit anti-mink gamma globulin.

In the group of 54 mink given multiple antigens in adjuvant of which 33 mink were subsequently infected with the AD agent and became hypergamma-

globulinemic, the mink developing hypergammaglobulinemia did not show a corresponding increase in antibody titers to known antigens they were responding to before and during AD infection. The AD agent caused at least a 5-fold increase in the serum gamma globulin in this group of mink. The antibody titers to hemocyanin and *Shigella* fell comparably in both control mink and those becoming hypergammaglobulinemic due to the AD agent with the exception of 1 control mink which kept the same titer of antibody to each antigen. Six AD-affected mink and 2 controls showed rises in antibody levels to BGG of less than double the level of the time the mink were given the AD agent, while the remaining 46 showed a decrease in anti-BGG titers. As in the previous experiment, none of the mink made a detectable antibody response to egg albumin.

In all cases the normal mink receiving multiple antigens in adjuvant showed either no change in the amount of serum gamma globulin or only a transient rise in serum gamma globulin which promptly fell upon discontinuing immunization, in contrast to those either naturally or experimentally infected with AD agent which remained hypergammaglobulinemic for the remainder of their lives. Hyperimmunization of experimental animals usually does not produce hypergammaglobulinemia of the magnitude found in AD, but it is possible in at least some instances (18).

DISCUSSION

Aleutian disease of mink presents two problems; the mechanism of the hypergammaglobulinemia, and the pathogenesis of the glomerular and vascular lesions which morphologically resemble some lesions induced by immunologic injury. Although there is a good statistical correlation between the degree of hypergammaglobulinemia and the extent of glomerular lesions and the presence of arteritis in AD (5), for the purposes of discussion of pathogenesis it will be useful to separate these two facets of the disease.

Mink with AD have a significantly shortened gamma globulin half-life, indicating that the hypergammaglobulinemia is due to overproduction rather than to decreased catabolism of the protein. This result is similar to the observations in human myeloma and in hyperimmunized animals where in the presence of hypergammaglobulinemia the gamma globulin half-life is usually shortened (18, 19). The lack of effect of AD on albumin half-life is also in accord with studies on hypergammaglobulinemic rabbits (20), and is probably related to different degradatory mechanisms for these two serum proteins.

Analytical ultracentrifugation of AD affected mink serums showed that the increased γ -globulin was of the 6.4S type, and not macroglobulin, a result in accord with Kenyon and coworkers (21). The 6.4S mink γ -globulin was shown in this study to carry antibody activity to a known antigen. The finding of two classes of protein-protein complexes, 9S to 17S and 22S to 25S, which

consist at least in part of 6.4S γ -globulin units, in the serums of many mink with AD is quite similar to the demonstration of two classes of complexes in human rheumatoid arthritis serums (22, 23).

Gorham and coworkers (24) have shown that AD-affected mink do not have neutralizing antibody to the infectious agent, and that the hypergamma-globulinemic serum itself is infectious to a level of 10^5 ID₅₀/ml. Similarly in this study using fluorescent antibody and complement fixation methods, no antibody to the infectious agent has been detected in AD serums or in heterologous antisera prepared against infectious material.

The possibility has been raised that AD is an autoimmune disease (4, 6, 25-27). However, fluorescent AD mink serums have failed to show any reaction with normal or AD-affected mink tissues, including autologous tissues, suggesting that the increased gamma globulin does not represent auto- or isoantibody to any of the antigens present in the various organs tested. Complement fixation tests yielded negative results. Negative results were obtained with L.E. cell, rheumatoid factor, and syphilis serologic tests, which may be positive in some human connective tissue diseases. Therefore, within the technical limitations of the tests employed, no evidence for an autoimmune mechanism in AD has been found.

In an attempt to determine if the AD agent causes a proliferation of and increased gamma globulin production by all preexisting clones of plasma cells, mink previously immunized to a group of known antigens were given the AD agent in order to cause hypergammaglobulinemia. In no case did the antibody level to a known antigen rise in proportion to the rise in total gamma globulin level, instead, the antibody levels more often fell coincident with the rising gamma globulin. This indicates that the AD agent does not stimulate indiscriminantly the proliferation of preexisting clones of plasma cells but rather appears to have some specificity.

Since it has not been possible to attribute the hypergammaglobulinemia of AD to production of antibody against the infectious agent, the host's own tissues or to antigens to which the animal was responding prior to AD infection, it may be that it results from events unrelated to immunologic processes. The AD agent might either directly stimulate certain plasma cell precursors to multiply, or cover or delete a locus responsible for controlling the proliferative rate of the cells. It has been found that 10 to 20 per cent of mink affected by AD for a year show a transition from an electrophoretically heterogeneous hypergammaglobulinemia to an electrophoretically homogeneous, monoclonal type of hypergammaglobulinemia (8). The high rate of transformation into a monoclonal hypergammaglobulinemia together with the failure of AD to increase preexisting antibody formation is strongly in favor of the AD agent initially affecting a limited number of clones of plasma cells, one of which may become ascendant in time.

The vascular lesions of AD show some morphologic similarities to those experimentally produced by antigen-antibody complexes. In experimental animals it is known that amounts of antigen-antibody complexes too small to be detectable in the analytical ultracentrifuge are associated with the lesions of serum sickness and at least one type of experimental glomerulonephritis (28-30). In the AD-affected mink no direct evidence was found that the circulating protein-protein complexes were biologically harmful. Thus, the presence of complexes in AD is similar to the situation in rheumatoid arthritis, where most of the patients having complexes have clinically active disease and high rheumatoid factor levels, but no direct evidence for a harmful biological role for the complexes can be found (22, 23).

Consumption of complement often occurs in the course of *in vivo* antigen-antibody reactions associated with tissue injury. In serum sickness hemolytic complement often drops to less than half of the normal value coincident with the beginning of immune elimination of the antigen (29). However, the amount of complement utilized is not directly correlated with the development or severity of morphologic lesions. In nephrotoxic nephritis there is a sharp initial drop in complement level, and often a second transitory drop with the beginning of host response to the injected nephrotoxic serum, but normal complement levels are present after 7 to 10 days although an immunologic reaction persists for up to 3 weeks (17). Complement levels in chronic complex induced glomerulonephritis were found not to be consistently decreased (31). It appears that a reduced hemolytic complement level is associated with acute *in vivo* antigen-antibody reactions, but is not necessarily found in chronic *in vivo* antigen-antibody reactions. Thus, the finding of normal levels of hemolytic complement in AD-affected mink does not rule out the possibility of a chronic antigen-antibody reaction, which could conceivably cause tissue injury.

The arteritic lesions in AD show localization of serum gamma globulin, but not fibrin or albumin; this is similar to the arteritic lesions in human periarteritis nodosa which show selective localization of gamma globulin (32). The affected vessels in both mink and man show fibrinoid necrosis by light microscopy, but the cellular infiltration in AD is almost exclusively mononuclear, while in the human periarteritis it is predominantly polymorphonuclear leukocytes, more similar to the vasculitis in the Arthus reaction and serum sickness. The attraction of a different cell type in AD as compared with periarteritis nodosa suggests a difference in pathogenesis of the lesions.

The glomerular lesions of AD show only a mesangial localization of serum proteins, with no preferential localization of one protein. In contrast, complex induced nephritis and nephrotoxic serum nephritis show preferential deposition of gamma globulin and complement in the capillary walls (17, 30). AD also appears to differ from these other types of glomerular injury in that it features a marked proliferation of mesangial cells with only minor damage to the

remainder of the capillary wall, while experimental complex-induced, nephrotoxic serum-induced, or naturally occurring human glomerulonephritis primarily affects the peripheral glomerular capillary wall with variable damage to the mesangial areas. Again, this suggests that the glomerular lesions of AD may have a pathogenesis different from that of glomerular injury due to known or suspected immunologic insult. Fisher and coworkers (33) have described glomerular changes in multiple myeloma which have considerable resemblance to the glomerular damage in AD, and they believe the changes to be quantitatively related to the level of serum protein. The close association of serum protein levels and presence of tissue lesions in AD would make this idea attractive, but it fails to explain the presence of arteritis, which to our knowledge does not occur in multiple myeloma. An alternative explanation for the parallel of the serum protein changes and tissue lesions is that the virus may directly infect glomerular and arterial cells as well as cells of the plasma cell system, and that we see the result of similar degrees of infestation by the virus in different sites.

Aleutian disease of mink has been suggested as a good experimental model for human autoimmune or hypersensitivity diseases, primarily on the basis of some morphologic similarity of AD to the lesions of this group of diseases (4, 6, 25-27). The present studies have failed to show any immunologic role for the increased gamma globulin of AD in the production of the vascular and renal lesions which would argue against any usual immunopathologic process. While this may be the result of limitations in methodology, comparable methods have indicated participation of γ -globulin in human and experimental autoimmune and hypersensitivity diseases. Thus, until the pathogenetic mechanisms in AD become clearer, it would appear unwise to consider this naturally occurring infectious disease of mink as an experimental model for the study of human diseases of immunological origin. Further clarification of the nature of AD will require either *in vitro* cultivation of the infectious agent or an immunologic method of detecting the AD agent, neither of which has been achieved at this time.

SUMMARY

Aleutian disease-affected mink, which are markedly hypergammaglobulinemic, show a decreased half-life of the serum gamma globulin indicating that the hyperglobulinemia is due to increased production. No evidence that the gamma globulin was antibody to the infectious agent, to autologous or isologous tissues, or to antigens the animal was responding to prior to development of the disease was obtained. The increased gamma globulin was found to be of the 6.4S variety, and gamma globulin containing protein-protein complexes of 9S to 17S and 22S to 25S classes were observed in serums of affected mink. The findings are most consistent with the Aleutian disease virus acting as a direct and somewhat selective stimulus to plasma cell proliferation. There is no evidence that the arterial and glomerular lesions of Aleutian disease have

an immunologic pathogenesis. It seems possible that these vascular changes may be directly caused by the viral agent, or may be the result of the increased gamma globulin levels.

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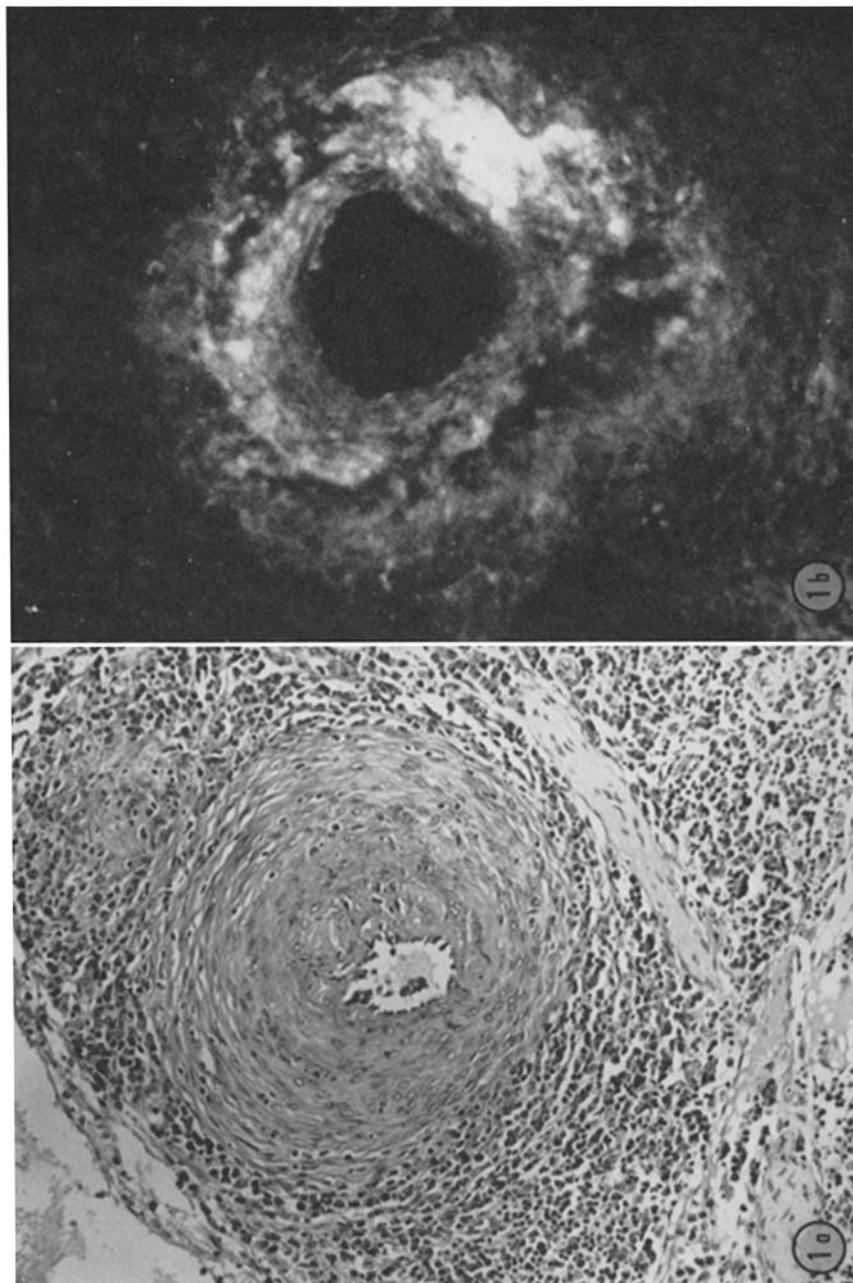
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EXPLANATION OF PLATE 72

FIGS. 1 *a* and 1 *b*. Renal arteries from an Aleutian disease-affected mink.

FIG. 1 *a*. The artery shows fibrinoid necrosis and a mononuclear cell infiltration when examined by conventional histologic technique. Hematoxylin and eosin stain, photomicrographed $\times 102$.

FIG. 1 *b*. Similar artery from the same mink stained with fluorescent anti-mink gamma globulin, with positive staining in the area of fibrinoid necrosis. Fluorescence micrograph, $\times 100$.



(Porter *et al.*: Gamma globulin in Aleutian disease of mink)