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Immunoglobulin Classes of Aleutian Disease Virus Antibody

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Aleutian disease virus (ADV) persistently infects mink and causes marked hypergammaglobulinemia. Immunoglobulin class-specific antisera were used to define the total immunoglobulin of each class by radial immunodiffusion and the immunoglobulin class of ADV-specific antibody by immunofluorescence in experimentally and naturally infected mink. Electrophoretic gamma globulin closely reflects the immunoglobulin G (IgG) level in mink, and the majority of the increased immunoglobulin and ADV antibody in infected mink is IgG. IgM becomes elevated within 6 days after infection, reaches peak levels by 15 to 18 days, and returns to normal by 60 days after infection. The first ADV antibody demonstrable is IgM, and most mink have virus-specific IgM antibody for at least 85 days postinfection. Serum IgA levels in normal mink are not normally distributed, and ADV infection causes a marked elevation of IgA. Low levels of ADV-specific IgA antibody can be shown throughout the course of infection. Failure of large amounts of virus-specific IgG antibody to inhibit the reaction of virus-specific IgM and IgA antibodies suggests that the various classes of antibodies are directed against spatially different antigenic determinants. The IgM and IgA were shown not to be rheumatoid factors.

Aleutian disease (AD) is a common persistent viral infection of mink and represents a major economic problem to the mink industry. Aleutian disease virus (ADV) is an autonomous parvovirus (3, 29). Mink with AD develop a systemic plasmacytosis with markedly increased serum gamma globulin, and glomerulonephritis and arteritis are caused by tissue deposition of immune complexes (reviewed in references 21 and 31). The striking elevation of gamma globulin in AD has been shown to be caused by overproduction of immunoglobulin G (IgG) (28). Specific ADV antibody levels are very high in persistently infected mink (2, 5, 10, 25, 30).

The present study was undertaken to ascertain whether elevations of serum IgA and IgM occur after ADV infection and to follow the time course of development of ADV antibody of each immunoglobulin class.

MATERIALS AND METHODS

Animals. Ranch-raised mink (*Mustela vison*) were obtained from and maintained at the Fur Breeders' Agricultural Cooperative, Midvale, Utah. The uninfected mink were 4 to 7 months of age when tested and included 25 black, 7 pastel, and 20 Aleutian animals. Mink infected for timed intervals included 28 pastel and 48 Aleutian animals of a similar age. These mink did not have demonstrable ADV antibody before experimental infection. Naturally infected mink were sacrificed at 7 months of age and included 10 black, 10 pastel, and 13 Aleutian animals, each of which had histological lesions of AD.

Virus. Mink were infected intraperitoneally with 10^5 pastel mink 50% infectious doses of in vivo passage 3 of the Utah-1 strain of ADV (30).

Antiserum production and characterization. Mink IgG was prepared by three precipitations of serum at half saturation with $(NH_4)_2SO_4$, dialysis of the precipitate against 0.0175 M sodium phosphate buffer (pH 7.0), and application and elution of the protein on a column of DE-52 cellulose (Whatman) using the same buffer. Rabbits were immunized with 1 mg of this material three times at 3-week intervals using complete Freund adjuvant. The rabbits produced antibody only to mink IgG, as shown by immunoelectrophoresis and immunodiffusion tests. Three grams of IgG prepared as described above was reduced and alkylated by the method of Fleischman and co-workers (13) as modified by Coe (6), and L chains were isolated. The L chains were copolymerized with bovine serum albumin by the method of Avrameas and Ternynck (1) and used to absorb a rabbit antiserum prepared against mink IgG to render it specific for gamma chain.

Rabbit antiserum to mink alpha chain was made by the method of Coe and Hadlow (7) using mink intestinal washings as the source of IgA and unabsorbed rabbit antibody to mink IgG as the precipitating agent. This antiserum was rendered IgA specific by absorption with a copolymer of mink IgG and bovine serum albumin.

We were unable to obtain monospecific antiserum to mink mu chain by the method of Coe and Hadlow (7); therefore, we used a monospecific rabbit antiserum to human mu chain to precipitate mink IgM from heated (56°C for 30 min) mink serum containing 0.01 M EDTA. Rabbits were immunized with the washed precipitate, and the antiserum was rendered mink mu chain specific by absorption with mink IgG-bovine serum albumin copolymer.

Each absorbed antiserum was shown to react with a single different mink serum protein in immunoelectrophoresis and double diffusion in agar, and the antisera to mink IgG and IgM were shown to cross-react with the corresponding purified human immunoglobulins. Completeness of absorption was tested by using double diffusion in agar. As an additional test for completeness of absorption, purified ADV antibody of each immunoglobulin class was tested by indirect immunofluorescence.

Measurements of immunoglobulin. Serum protein electrophoresis was performed and controlled with cellulose acetate strips as described previously (30). Serum protein concentration was measured with a refractometer. Radial immunodiffusion was used at equilibrium for measurement

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TABLE 1. Serum protein and Aleutian disease viral antibody levels in normal and infected mink

Mink group	No. studied	Serum protein (g/dl)	Electrophoretic gamma globulin (mg/dl)	IgG (mg/dl)	IgM (mg/dl)	Reciprocal of geometric mean titer in antibody-positive mink (% with antibody)		
						IgG	IgM	IgA
Normal	52	6.91 ± 0.81	633 ± 252	480 ± 214	23 ± 12	0 (0)	0 (0)	0 (0)
Infected								
1–6 days	16	6.61 ± 0.26	424 ± 166	319 ± 121	36 ± 20	0 (0)	10 (6)	0 (0)
8–12 days	17	7.05 ± 1.27	633 ± 250	491 ± 185	43 ± 27	10 (6)	15 (71)	10 (6)
15–18 days	8	7.41 ± 1.01	783 ± 156	712 ± 194	74 ± 30	34 (100)	18 (75)	20 (25)
30 days	14	8.04 ± 0.68	2,076 ± 494	1,356 ± 535	51 ± 44	848 (100)	28 (71)	14 (29)
60 days	14	8.33 ± 0.65	2,847 ± 620	1,891 ± 618	22 ± 13	6,106 (100)	31 (79)	27 (64)
85 days	7	9.09 ± 1.08	5,125 ± 1,109	4,296 ± 734	16 ± 2	10,000 (100)	40 (71)	25 (86)
Naturally infected	33	8.22 ± 0.93	3,179 ± 1,288	2,468 ± 867	22 ± 10	5,722 (100)	27 (73)	30 (67)

of immunoglobulin concentration (22), and a secondary mink serum standard was included with all tests. Primary standardization was done by using mink IgG prepared as described above and mink IgA and IgM prepared from heated (56°C for 30 min) 0.01 M EDTA-treated mink serum applied to an affinity column containing the IgG fraction of rabbit antiserum to mink alpha or mu chain. The antibody was coupled to Sepharose 4B beads by the cyanogen bromide method of March and co-workers (23) at pH 6.5, and absorbed antigen was eluted from the column with 4 M urea (pH 7.0). The eluates were concentrated and dialyzed against phosphate-buffered saline and shown to contain only the desired immunoglobulin by using double diffusion in agar. Protein concentrations were measured at 280 nm and assumed an $E_{280}^{1\%}$ of 13. Spectrophotometric accuracy was controlled by glass optical density standards, and amplifier gain was adjusted to a tolerance of ±1%.

Assay for ADV antibody. Acetone-fixed cover slips of ADV-infected CRFK cells were used as antigen-containing targets in indirect immunofluorescence assays using the immunoglobulin class-specific antisera described above (10).

Sucrose density gradients. Diluted mink serum samples were separated on 10 to 26% sucrose gradients by the method of Forghani and co-workers (14), except that the gradients were centrifuged for 4.4 h at 65,000 rpm in an SW65 rotor.

Absorption of possible rheumatoid factors. Selected mink sera were absorbed with 9 mg of insoluble heat-aggregated (69°C for 15 min) mink IgG per ml for 0.5 h, and the sera were tested for ADV antibody of each immunoglobulin class before and after absorption.

Data analysis. Data were analyzed by using the BMDP statistical programs of the Health Sciences Computing Facility, University of California at Los Angeles (11). Both parametric and nonparametric analyses were performed.

RESULTS

Specificity and reproduciblity of the assays. The area of the radial immunodiffusion rings was linear with concentration for mink IgG, IgA, and IgM, indicating that equilibrium was reached for each test. Eighty-three to 105 individual assays of the secondary standards showed standard deviations of ±2.8% for IgG, ±2.3% for IgA, and ±2.7% for IgM. No cross-reactivity of the three antisera could be shown by double diffusion in agar or by immunofluorescence with purified ADV antibody of each immunoglobulin class. The reproducibility of the ADV antibody titers was approximately the same as previously reported (10) or ± one dilution. It should be emphasized that the IgG antibody titers presented in this study are not comparable with those of our previous studies which used anti-IgG rather than gamma chain-specific antisera. A direct comparison using 53 pairs of determinations showed that the present test for IgG antibody had 1/11 of the sensitivity of our previous tests.

Immunoglobulin and antibody results. All parameters studied in control and infected mink except for IgA levels were shown to be log-normally distributed (Table 1). Parametric statistical analysis of the results for all mink at all times is shown in Table 2. The total serum protein, electrophoretic gamma globulin, and IgG were significantly increased in the experimentally infected mink, and this increase first became evident 15 to 18 days after infection. The same parameters were also markedly increased in naturally infected mink. Although no effect of coat color on total serum protein was observed, infected Aleutian mink had a significantly higher electrophoretic gamma globulin and IgG level than did infected pastel or black mink. Serum IgG measured by radial immunodiffusion averaged 81% of the electrophoretic gamma globulin. IgG antibody to ADV was found in one mink infected for 12 days and in all mink infected for 15 days or more. The IgG anti-ADV continued to increase through 85 days after experimental infection and reached very high titers for this type of test.

Although IgM levels for the total group of mink did not increase progressively with time, there was a significant increase in IgM within 6 days after infection ($P < 0.02$), and the IgM was threefold the level of uninfected mink 15 to 18 days after infection ($P < 0.001$). IgM was still significantly increased 30 days after infection but was at the level of uninfected mink thereafter. IgM anti-ADV was first found in one mink 6 days after infection and was present in approxi-

TABLE 2. Results of parametric statistical analyses of the data shown in Table 1

Finding	Probability values	
	Days after infection	Mink coat color
Log total serum protein	0.004[a]	0.424
Log electrophoretic gamma globulin	<0.001[a]	0.016[a]
Log IgG	<0.001[a]	0.003[a]
Log IgM	0.073	0.316

[a] Significant at the 0.050 level.

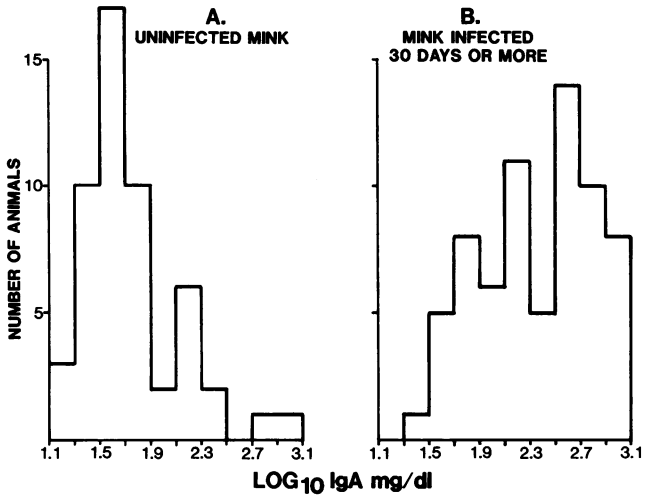

FIG. 1. (A) The wide range of serum IgA concentrations in 52 uninfected mink. Mink infected with Aleutian disease virus for 1 to 18 days had IgA concentrations similar to those of the uninfected mink (not shown). (B) Sixty-eight mink infected for 30 days or more with Aleutian disease virus had a marked increase in serum IgA concentration.

mately three-quarters of mink tested at later times after infection. IgM antibody titers remained quite low.

Serum IgA was not normally distributed in uninfected mink and ranged from 15 to 1,003 mg/dl. The distribution of IgA levels in normal mink is shown in Fig. 1A. The serum IgA distribution of mink infected for 1 to 18 days was nearly identical to that of uninfected mink (data not shown). The serum IgA in mink infected for 30 or more days was markedly and significantly increased ($P < 0.001$), and the distribution is shown in Fig. 1B. The effect of coat color on IgA levels did not quite reach statistical significance ($P = 0.060$). IgA anti-ADV was first found in one mink 12 days after infection and was present in less than one-third of mink infected for 30 days or less. A majority of mink infected for 60 days or more had IgA anti-ADV, and the titers were relatively low.

No statistically significant interaction between time after infection and coat color was observed for any measurement. Nonparametric statistical analyses did not show any additional relationships.

Other results. Nine sera from mink infected for 30 days or more were separated on sucrose gradients and analyzed for antibody of each immunoglobulin class. Two mink which did not have IgM antibody to ADV demonstrable in whole serum had such antibody in the lowest part of the gradients, and one mink without demonstrable IgA antibody in whole serum had antibody in the intermediate portion of the gradient. The percentage of mink with IgM and IgA antivirus antibody shown in Table 1 thus is probably underestimated.

Affinity-purified IgA or IgM with ADV antibody activity was mixed with equal amounts or a 10-fold excess of IgG with ADV antibody activity and tested by immunofluorescence. The intensity of the reaction of IgM or IgA ADV antibody was somewhat reduced but not abolished by an excess of IgG ADV antibody, suggesting that the IgM and IgA antibodies may react with spatially different ADV determinants than the IgG anti-ADV.

Eight sera with strong IgM and IgA anti-ADV reactions were absorbed with 9 mg of heat-aggregated mink IgG per ml. The absorptions did not remove the IgM or IgA reactivity with ADV antigen, indicating that the IgM and IgA were not rheumatoid factors.

DISCUSSION

This study, using immunological identification of mink immunoglobulin classes, confirms earlier physicochemical studies which showed that IgG is the principal immunoglobulin that is increased in Aleutian disease (20, 28). The time course of the immune response to ADV is similar to that found for other viruses (36), although ADV infection does not induce the formation of neutralizing antibody (29, 30). The vast majority of ADV antibody demonstrable 30 days or more after infection is of the IgG class. Gamma globulin as measured by serum electrophoresis is mostly IgG.

ADV infection of mink causes a larger increase in serum immunoglobulin than other persistent viral infections cause in other species. However, lactic dehydrogenase virus infection of mice causes a significant increase in IgG2a and occasionally causes an increase in IgG1 (26, 27). Horses infected with equine infectious anemia virus develop about double the normal level of IgGa within 2 months, and this immunoglobulin remains elevated for at least a year. The infected horses have a concomitant decrease in serum IgG(T) (24).

A transient but significant increase in serum IgM levels was found in early AD, and the first ADV antibody demonstrated was of this class. Similar observations have been made in horses infected with equine infectious anemia virus (24). The majority of mink with late AD retain specific antiviral antibody of the IgM class. Some workers consider a prolonged IgM response to be indicative of persistent infection with such viruses as measles or rubella (9, 15). However, a relatively large dose of a nonreplicating virus (poliovirus in rabbits) may produce an IgM antibody response for at least 25 weeks (36). The IgM and IgA anti-ADV shown in the present study apparently represents specific antivirus antibody, since antibody activity could be shown with the purified immunoglobulin and was not removed with heat-aggregated IgG. Rheumatoid factors are known to cause false-positive IgM antibody tests in the presence of IgG antivirus antibody (34, 37). The lack of inhibition of IgM and IgA ADV antibody by an excess of IgG antibody suggests that the antibodies of various classes may be directed against spatially different viral determinants (33).

The enormous range of IgA levels found in mink was surprising. Human IgG, IgA, and IgM are distributed in a log-normal fashion in large samples (4, 35), and the observations in the present study are also distributed in a log-normal fashion with the exception of IgA. Human IgD concentrations are distributed in a similar nonnormal fashion (12, 38). ADV infection of mink caused a large increase in IgA 30 or more days after infection, although specific antibody titers of this class were quite low.

The glomerulonephritis of AD is characterized by proliferation of mesangial and endothelial cells, an increase in mesangial cell cytoplasm, and a marked increase in mesangial matrix material. Electron microscopy has shown deposits compatible with immune complexes both in a subendothelial location in capillary loops and in the cytoplasm of mesangial cells (16, 17). Immunofluorescence studies have shown a preferential deposition of IgA (32) or IgM (19) in both locations. The glomerulonephritis of AD thus has both morphological and immunological similarities to human mesangial proliferative glomerulonephritis (8), in which there may be selective deposits of IgA or IgM, often following a respiratory viral infection. A similar mesangial proliferative

glomerulonephritis with selective deposition of IgA and IgM has been produced in mice by immunization with anionic dextran sulfate (18). The finding of markedly increased IgA levels in mink with AD in the present study, together with the similarities of the glomerulonephritis of AD to human IgA nephropathy, suggests that a thorough immunological evaluation of AD glomerulonephritis should be undertaken. Such a study should also attempt to assess whether ADV antigen in glomeruli has any unusual electrical charge properties similar to those of the anionic dextran sulfate which caused IgA nephropathy in mice (18).

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