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Autoimmunity in Aleutian Disease: Contribution of Antiviral and Anti-DNA Antibody to Hypergammaglobulinemia

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The contributions to Aleutian disease gammopathy of specific antiviral antibody and an autoimmune component, anti-DNA antibody, were studied with pastel ranch mink naturally infected with Aleutian disease virus. Specific antibody activities were determined by countercurrent immunoelectrophoresis and radioimmune assay, respectively. Gamma globulin levels (percent γ) were determined by serum electrophoresis. Within an infected mink population, it was possible to predict the level of gammopathy from measurement of the two antibody levels. For the mink serum samples used, there was better correlation between anti-DNA antibody levels and total serum immunoglobulin than between anti-Aleutian disease virus antibody titers and percent γ. With serum samples taken over a 2-week interval, significant increases were measured in anti-DNA antibody and percent γ. Increases in anti-Aleutian disease virus titers during this period were not significant. The results suggest that the continuing increases in serum immunoglobulin in Aleutian disease virus-infected mink are due to both a specific antiviral response and an autoimmune response, as reflected in generation of anti-DNA antibody.

Aleutian disease (AD) is an immunopathological disorder of mink induced by persistent infection with AD virus (ADV) (16). The main lesions of the disease are caused by overproduction of immunoglobulin and formation and deposition of immune complexes (10, 15, 19). Antibody to viral antigens can be detected as early as 7 days (17) after infection. Titers of specific antibody to the virus increase during the months after infection but usually reach a plateau (4). Total circulating immunoglobulin continues to increase throughout the course of the disease, however, even though titers of specific antiviral antibodies have reached a maximum. The resultant gammopathy does not appear to be a general polyclonal increase in all immunoglobulins, since ADV infection does not cause an increase in antibody titer to unrelated antigens (13, 17). A characteristic of AD that may contribute to the persistence of the virus is that antiviral antibody is nonneutralizing (18). This phenomenon appears to be a property of the virus and not the host, since antiviral antibody from other infected species also fails to neutralize (9).

The progressive increase in gamma globulin associated with AD has led many investigators to consider that factors other than viral stimulation may contribute to gammopathy. Consideration of a plasmacytoma as the cause of the disease has not been substantiated (see reference 19 for review); however, the possibility of an autoimmune component in AD is suggested by several pieces of evidence. Positive direct Coombs' reactions in ADV-infected mink have been reported (7, 22), associated with anti-ADV antibody on the erythrocyte cell surface and anemia (14, 22). Recently, high titers of antisperm antibodies have been associated with male infertility in uninfected black mink (26). Treatment of infected mink with the immunomodulator levamisole reduces the level of anti-ADV titer but not gammopathy (A. J. Kenyon, R. Kassel, G. Notani, and E. C. Hahn, Fed. Proc. 35:529, 1976). Antinuclear antibody and nuclear antigen have been described in mink sera from animals infected with ADV (3). Anti-DNA antibody has been demonstrated after infection of mink with ADV (8, 21, 23). The importance of anti-DNA antibody and its immune complexes have been demonstrated in several autoimmune processes, including systematic lupus erythematosus (12) and the autoimmune disease of NZB-NZW F1 hybrid mice (25). These studies suggest the likelihood that the hypergammaglobulinemia of chronic AD is partially the result of an autoimmune component.

The results reported here compare anti-ADV, anti-DNA, and total gamma globulin levels of various sera obtained from naturally infected

TABLE 1. Antibody levels in mink sera

Sample	Anti-ADV titer[a]	Anti-DNA (cpm)[b]	Percent γ[c]
1	0	441	10.5
2	0	10	7.0
3	0	93	9.0
4	0	10	8.0
5	0	388	9.0
6	0	585	7.0
7	40	2,040	10.0
8	80	16,323	27.0
9	1	412	10.5
10	10	4,357	12.5
11	10	5,672	15.0
12	640	7,255	20.0
13	2	109	13.5
14	4	13	12.0
15	320	8,826	27.0
16	8	77	8.0
17	10	10	7.1
18	20	10	12.0
19	20	10	9.1
20	20	3,735	12.5

[a] Reciprocal of the highest serum dilution that reacted in countercurrent immunoelectrophoresis.

[b] Ammonium sulfate precipitable counts of [^{3}H]DNA at a 1/32 dilution of serum.

[c] Expressed as percentage of total serum protein.

mink. The relative contribution of specific viral and anti-DNA components to the gammopathy of Aleutian disease is analyzed.

MATERIALS AND METHODS

Sera. Blood samples were obtained by cardiac puncture of randomly selected ranch mink from several ranches in northern Illinois. All mink were 6 months old, of the pastel color phase, and heterozogous for the Aleutian gene. AD was endemic in the herds, with little change in frequency of positives, so that mink could be assumed to be infected at or near the time of birth. Sera were obtained from clotted blood, centrifuged at 1,000 × g for 20 min, and frozen once until used.

Measurement of immunoglobulin. Sera were electrophoresed on cellulose acetate membranes (Microzone system; Beckman Instruments, Inc., Fullerton, Calif.) and the percentage of gamma globulin (percent γ) was determined by densitometric integration of stained electrophoretic patterns. The percent γ is expressed relative to the total applied serum protein. Several samples assayed in triplicate yielded coefficients of variation in the range of 2.6 to 12% for means in the range of 7 to 50 percent γ.

Assay for anti-ADV. Titers of specific antiviral antibody were obtained by assaying twofold dilutions of mink sera by counterimmunoelectrophoresis (6) with ADV antigen prepared from mink tissue (United Animal Science, Middleton, Wis.). The titers are expressed as the reciprocal of the last dilution showing a positive precipitin reaction.

Assay for anti-DNA antibody. Anti-DNA antibody was assayed by radioimmune assay by the method of Farr (27) as previously described (8). Levels of anti-DNA antibody were determined with the same batch of [^{3}H]DNA, so to this extent, all the results have been standardized. Titers are expressed as the counts per minute of activity precipitated with 50% saturated ammonium sulfate in the assay.

Statistical analysis. Differences in means were compared by the Wilcoxon-Mann-Whitney rank sum test. Since this test is not as stringent as Student's t test, the level of significance was accepted at $P \leq 0.01$. Correlation among the variables was shown by multiple regression analysis. Partial correlation between two variables under conditions where there is no contribution from the third variable were determined from correlation coefficients (r) by the formula:

$$r_{12\cdot3} = \frac{(r_{12} - r_{13}\, r_{23})}{\sqrt{(1 - r_{13}^2)\,(1 - r_{23}^2)}}$$

(see reference 24).

RESULTS

Components of gammopathy. Sera from randomly selected pastel mink from a single ranch were analyzed for anti-ADV antibody, anti-DNA antibody, and total percent γ. The results (Table 1) indicated a large variation in the levels of the three parameters tested. In general, control mink lacking antibody to ADV (samples 1 through 6) had low levels of both total gamma globulin and anti-DNA antibody. Statistical analysis of these data is shown in Table 2. Correlation between the independent variables, anti-ADV and anti-DNA, were very low (r = 0.488). Better correlation was shown between anti-ADV titer and percent γ (r = 0.609), and a still better correlation was shown between anti-DNA and percent γ (r = 0.903). A multiple correlation of anti-ADV and anti-DNA as a predictor of percent γ yielded a multiple correlation coefficient (R = 0.9234), indicating that the two independent variables accounted for 85.27% of the variation shown in the dependent variable, percent γ. A value of r this large or larger could occur by chance alone, with $P = 8.5 \times 10^{-8}$. The relations among the variables can be expressed by the multiple regression equation I = 0.00849D + 0.00111V + 9.028, where I is percent γ, D is the counts per minute of anti-

TABLE 2. Multiple linear regression of data in Table 1[a]

Variable	Mean ± SD
A: anti-ADV (1/titer)	59.3 ± 154.0
D: anti-DNA (cpm)	2,517.8 ± 4,232.0
I: percent γ	12.3 ± 5.9

[a] Correlations: r_{AD} = 0.488; r_{AI} = 0.609; r_{DI} = 0.903. Multiple correlation: R = 0.9234. Variance-ratio test: F(2, 17) = 49.21; $P = 8.5 \times 10^{-8}$. Regression analysis: I′ = 0.00849A + 0.00111D + 9.03. Standard error, 2.27.

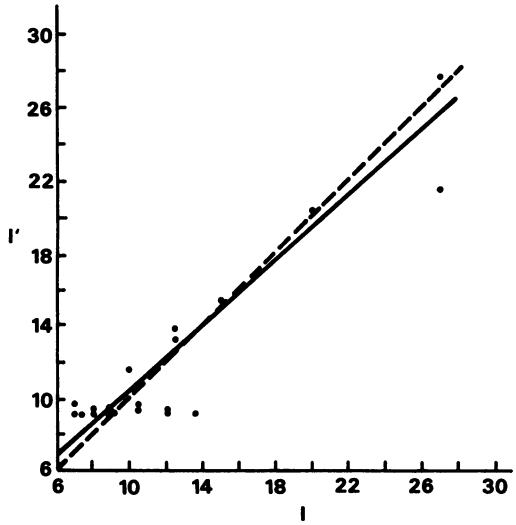

FIG. 1. Comparison of actual and predicted values of percent γ. Values of percent γ (I') predicted by the equation in Table 2 are plotted for each actual value (I). -------, Theoretical ideal relation; ——, regression of data.

DNA antibody, and V is the reciprocal of the anti-ADV titer. The constant in the equation represents the level of percent γ that would exist in the absence of any contribution from anti-ADV or anti-DNA antibody. This calculated value is within 0.5 standard deviation of the actual mean for the uninfected mink sera in this experiment (8.42 ± 1.36 percent γ). To examine the validity of this equation, data for anti-DNA and anti-ADV levels (Table 1) were used to calculate predicted I' values for each of the serum samples. These predicted values of I' were compared graphically with the actual values of percent γ for the sera (Fig. 1). The regression of I' on I is very close to the expected relation between the predicted and actual values for percent γ. The largest disparity occurred in uninfected mink samples containing the smallest amounts of gamma globulin. This would indicate that the level of gamma globulin in samples close to the normal range are more dependent on variables other than levels of anti-ADV or anti-DNA antibody. Two other independent sets of sera that were analyzed resulted in similar equations with slightly different coefficients for D and V.

A contour plot of the multiple regression equation is shown in Fig. 2. The major contribution to percent γ is from the autoimmune component, with very little contribution of specific antiviral antibody in the absence of a contribution from anti-DNA antibody.

Change in antibody components with time. In a separate experiment, sera were obtained from

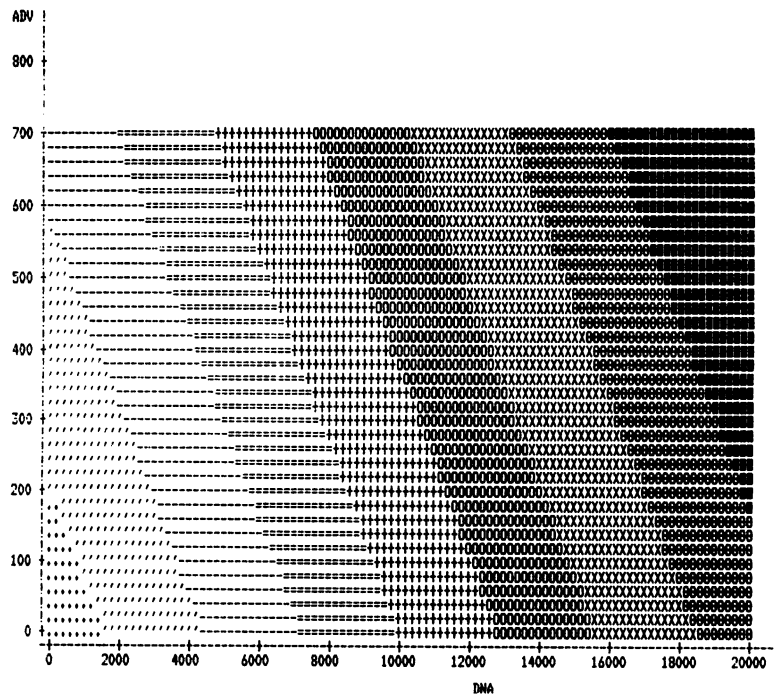

FIG. 2. Contour plot of the regression equation. Computer-generated values for the variables are plotted with the dependent variable, percent γ, represented by the contour shade.

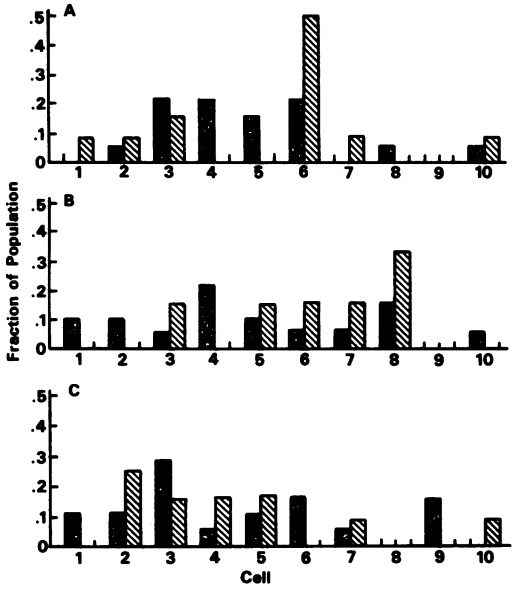

FIG. 3. Histogram of antibody levels at different times. A, Anti-ADV titer (range, 1 to 10,000); B, anti-DNA counts per minute (range, 14 to 17,287); C, percent γ (range, 3 to 50.75). Values are expressed as the log of the data and divided into 10 equal cells. Stippled, early bleeding; hatched, 2 weeks later. Analysis of this data is shown in Table 3.

infected mink taken randomly from a second closed herd at two time periods, 2 weeks apart. Distributions of anti-ADV antibody, anti-DNA antibody, and percent γ at both time points are shown in Fig. 3. In this representation, the largest increases were seen in anti-DNA antibody and total percent γ. The results of statistical analysis of these data are shown in Table 3. The data have similarly shaped distributions but are not distributed normally. Because of this deviation from a normal distribution, statistical analysis utilized the nonparametric, Wilcoxon-Mann-Whitney rank sum test to compare means. A comparison of the means for the three variables indicated that all increased during the 2-week period. The increase in anti-ADV titer was not significant ($P = 0.118$). However, there was a significant increase in the total gamma globulin ($P = 0.00228$) and a significant increase in the levels of anti-DNA antibody ($P = 0.00175$), despite a large variation in individual values. These data suggest better correlation between the increases in anti-DNA antibody and total gamma globulin than between anti-ADV antibody and gamma globulin.

Uninfected mink (a total of 20 sera) exhibited an increase in percent γ from 6.5 ± 1.4 to 8.8 ± 2.9 and a decrease in counts per minute in the Farr assay from 261 ± 120 to 224 ± 367 over the 2-week period. Neither of these changes were significant ($P = 0.0239$ and $P = 0.0495$, respectively) at the $P \leq 0.01$ level.

Partial correlation among the variables. It is mathematically possible to evaluate correlations between two variables in the absence of any contribution from other variables. This analysis was performed with the data shown in Table 1 and for the two sets of data shown in Fig. 2. The partial correlation coefficient for anti-ADV antibody and anti-DNA antibody showed a high degree of significant correlation only at the early period (Table 4) of the second experiment. Excellent correlation was indicated between anti-DNA antibody and total immunoglobulin levels for all three sets of data. A correlation between anti-ADV levels and percent γ levels was indicated in the second but not the first experiment.

Independence of anti-ADV and anti-DNA. In the preceding sections, little correlation between anti-ADV and anti-DNA was noted except in one case. To directly test for cross-reactivity between anti-ADV and anti-DNA antibody activities, a competition experiment was performed by counterimmunoelectrophoresis assay with a serum possessing an anti-ADV titer of 1/320. Titers of anti-ADV activity were unaffect-

TABLE 3. Increase in antibody with time

Function	Percent γ	1/Anti-ADV titer	Anti-DNA (cpm)
Sample 1 ($x \pm$ SD)[a]	11.6 ± 8.4	692 ± 2,410	1,613 ± 4,019
Sample 2 ($x \pm$ SD)[a]	19.8 ± 10.4	922 ± 2,860	6,146 ± 5,693
Δx	8.2	230	4,533
% Increase	71	33	281
z_x[b]	2.836	1.185	2.921
Q (2)[c]	0.00228	0.118	0.00175
Significance ($P \leq 0.01$)	Yes	No	Yes

[a] Independent serum samples (Fig. 3) were obtained from different AD-positive mink at two times, 2 weeks apart. $n_1 = 18$; $n_2 = 12$.

[b] Normal deviate for x.

[c] Probability function, Pr ($Z \leq z$).

TABLE 4. Correlation among variables

Statistic	Data set[a]		
	1	2	3
df (n = 3)	17	29	14
Correlation coefficient			
r_{AD}	0.4880	0.9750	0.5429
r_{AI}	0.6090	0.6497	0.7237
r_{DI}	0.9030	0.7310	0.7895
Significance levels			
r at 5%	≥0.456	≥0.355	≥0.497
r at 1%	≥0.575	≥0.456	≥0.623
Partial correlation coefficient[b]			
$r_{AD.I}$	−0.1818 (NS)	0.9640 (0.01)	−0.0672 (NS)
$r_{DI.A}$	0.8750 (0.01)	0.5775 (0.01)	0.6843 (0.01)
$r_{AI.D}$	0.4489 (NS)	0.4157 (0.05)	0.5725 (0.05)

[a] Data set 1 is from Table 1; data sets 2 and 3 are samples 1 and 2 in Table 3 with AD-negative samples added.
[b] Partial correlation coefficients are calculated from the three correlation coefficients by the equation shown in Materials and Methods and reflect the correlation of two variables, x_1 and x_2, under conditions of constant x_3. The significance of each (in parentheses) is determined from the significance levels at the particular number of degrees of freedom.

ed by addition of up to 1 mg of salmon sperm DNA to the antibody wells, whereas addition of 5 μl of ADV antigen to the antibody wells reduced the titer of anti-ADV activity by 80-fold.

DISCUSSION

In this study, a comparison was made between two components of an immune response—the specific response to the virus that causes the disease and an autoimmune response. Although it is clear that these two independent variables are not the only immunoglobulins contributing to the gammopathy, the fact that it is possible to predict total gamma globulin levels on the basis of these two variables indicates that whatever the specificities of the additional variables are, they are probably related to one of the measured variables. The coefficients of the measured variables in the regression equation above probably reflect these related activities (Table 2).

The general low levels of gamma globulin and the increases in antibody activities that occurred over the 2-week period (Table 3) might be interpreted to indicate that either some of the mink were not infected for the full 6 months or that the virus strain was of relatively low virulence. Normally, higher gamma globulin levels are observed in mink infected with ADV for over 6 months (17). A contribution of low temperature to the formation of DNA–anti-DNA immune complexes is suspected from recent data. Under laboratory conditions, temperatures below 37°C favor the formation of DNA–anti-DNA immune complexes in mink serum (unpublished data). Since sera were collected at pelting in late fall, the contribution of ambient temperatures as well as virulence of the virus strain must be also considered as a source of sample variation.

The data in Tables 3 and 4 indicate that better correlation is shown between anti-DNA levels and total gamma globulin, particularly at a later time after infection. This confirms earlier results which indicated an inconsistent relationship between anti-DNA antibody and the gammopathy of AD early after experimental infection (8). It appears that a major contribution to the late gammopathy associated with AD in pastel mink is in the form of an autoimmune response. This autoimmune contribution seen in pastel mink may not be as readily seen in homozygous Aleutian mink, in which a relatively rapid and continuous increase in antiviral antibody occurs (4).

The particular association of anti-DNA antibody with many autoimmune diseases (12, 25) and the reasons that these particular antibody-antigen complexes are deleterious to the host give little indication of how the activity has arisen. The changes in levels of anti-DNA antibody with time in infected mink sera were not seen in the controls (Table 3), indicating that the autoimmune component is not a general feature of mink superimposed on AD, although a tendency toward autoreactivity is suggested by several studies (7, 22, 26). Rather, it is likely that the autoimmune component is triggered by the viral infection. Previous results obtained with experimental AD infection (8) showed a transi-

tory increase in anti-DNA antibody occurring early after infection. There did not, however, appear to be coupling of anti-DNA antibody levels to the gammopathy during the first months after infection. The initial consistent contribution to increased immunoglobulin G is probably antiviral, as has been indicated by the work of others (17). In the present studies with sera from mink infected up to 6 months, both activities contribute, with anti-DNA predominant.

Both general and specific mechanisms can be proposed to account for the increases in anti-DNA antibody that have been noted. In a general way, later triggering of self-perpetuating anti-DNA antibody could be the result of the constant buildup of toxic immune complexes (15, 19) causing release of host debris, including cellular DNA. Recent results indicating absence of suppressor T cell function under conditions of treatment with anti-DNA–DNA complexes supports the idea that this immune complex may have unique properties (2). Results in other systems have suggested that increased anti-DNA antibody levels are a consequence of polyclonal B cell stimulation (11). This possibility is not likely in the case of AD, since a decrease in antibody titer to nonviral antigens presented before infection has been noted after ADV infection (13). Increased anti-DNA antibody might also arise through general reduction in immune complex clearance brought about by direct or indirect effects of the virus on the reticuloendothelial system.

Alternatively, anti-DNA activity might arise through a more specific consequence of the persistent viral infection. It is not clear why anti-DNA antibody is such a consistent feature of late AD gammopathy when other autoimmune activities have not been consistently observed (16). The most likely explanation for the uniqueness of this response is that DNA becomes particularly immunogenic. Such immunogenicity could occur if there is binding of viral proteins to host DNA or, by analogy, if the protein bound to the guanine-cytosine-rich, double-stranded DNA hairpin of the parvovirus H-1 (20) turns out to be a nonvirion protein such as that described for ADV (5) and other parvoviruses (16). The capsid proteins of ADV (1) and H-1 parvoviruses (S. Rhode, personal communication) have high affinity for DNA and chromatin. Such high binding of viral proteins with host DNA could produce a strong haptenic stimulus.

A correlation between anti-DNA antibody levels and anti-ADV titers was observed at the first but not the second time period in the experiment shown in Table 3. If anti-DNA antibody were a result of specific immunogenic association of viral proteins with viral or host DNA, a more consistent correlation would be seen between anti-DNA and anti-ADV levels. Should the recent demonstration of DNA in association with purified AD antigen (1) prove not to arise as an artifact of virus isolation, then the specific mechanism of the genesis of anti-DNA antibody by ADV would be supported.

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