Anti-Deoxyribonucleic Acid Antibody Associated with Persistent Infection of Mink with Aleutian Disease Virus

EDWIN C. HAHN AND ALAN J. KENYON*
Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Anti-deoxyribonucleic acid (DNA) antibody was quantitated in sera from mink infected with Aleutian disease virus (ADV). During the course of the disease after experimental infection, the amount of anti-DNA antibody increased 60% initially, but then decreased to an intermediate level when measured 2.5 months later. The percentage of serum immunoglobulin, however, steadily increased over 3.5 fold during this period, resulting in the characteristic gammopathy. Correlation between the level of anti-DNA antibody and hypergammaglobulinemia was demonstrated with sera from chronically infected mink. Competition experiments and use of labeled nucleic acids indicated that the immunoreactivity was more specific for double-stranded DNA than single-stranded DNA or ribonucleic acid. Anti-DNA antibody was found in purified immunoglobulin from chronically infected mink. Differences in avidity of antibody to DNA among antisera that had the same equivalence point were found. Avidity of antibody for DNA increased during the course of the disease.

Aleutian disease virus (ADV) causes a persistent infection in mink and pathology that is similar in many ways to connective tissue diseases and immune complex disorders in other species (27). In persistently infected mink, the usual course of the disease leads to death due to arteritis and glomerulonephritis resulting from severe hypergammaglobulinemia and deposition of immune complexes (10, 20). These changes are similar to those observed in patients with systemic lupus erythematosus (SLE) (12, 13) and in SLE of NZB/NZW F1, hybrid mice (1, 9, 26).

Anti-deoxyribonucleic acid (DNA) antibody has been described in SLE of both humans and NZB/NZW mice (3, 8, 18, 22, 23). Antibody-DNA complexes have been found in kidneys and are thought to play an important role in the nephritis associated with these diseases (6, 14, 19, 25). Changes in avidity of anti-DNA antibody have been linked to severity of nephritic lesions in these diseases; however, high avidity has been associated with SLE lesions, and low avidity has been associated with lesions in NZB/NZW mice.

In collaborative studies, we have previously described both nuclear antigen and antinuclear antibody in mink with Aleutian disease (AD) (1); however, the presence of nuclear antibody and antigens did not correlate with the amount of immunoglobulin G after experimental infection. The failure to destroy nuclear antigen with deoxyribonuclease and the demonstration of only partial identity of this antigen with calf thymus DNA in precipitin tests suggested that complete analogy among AD, SLE, and the disease of NZB/NZW mice was not possible. Until now there has been no confirmation of the presence of anti-DNA activity associated with ADV. With more quantitative immunological methods for assaying anti-DNA activity (28) and the increased importance of anti-DNA antibody and its complexes in autoimmune processes (5), a more detailed study of this antibody activity in AD was undertaken. This report describes some of the properties of anti-DNA antibody in sera from mink experimentally infected with ADV and from mink naturally infected on commercial mink ranches.

MATERIALS AND METHODS

Virus and experimental animals. The University of Connecticut strain of ADV was used for experimental infection of pastel mink. Chronically infected mink were obtained as such from mink ranches. All animals negative for ADV were housed in a Bioclean room (HEPA-filtered low-velocity mass air movement). Infection of mink with ADV was determined by detection of anti-ADV antibody by counterimmunoelectrophoresis (4).

Sera. Coagulated blood samples were centrifuged at 1,000 × g for 20 min, and resulting sera were rapidly frozen at −70°C after separation from clots. Anti-ADV immunoglobulin G was purified from pooled mink sera by ammonium sulfate fractionation and diethylaminoethyl-cellulose chromatography as previously described (7). Serum used for purification of mink immunoglobulin was kept at room temperature before ammonium sulfate precipitation.

Assay for anti-DNA antibody. The Farr assay (28) was used to demonstrate anti-DNA antibody. HeLa cell [3H]DNA was extracted from [3H]thymidine-labeled cells by the method of Marmur (16).
Purified [3H]DNA had a specific activity of 4 x 10^6 cpm/µg. One microgram of [3H]DNA in 100 µl of phosphate-buffered saline was combined with 100 µl of serum dilution in siliconized tubes (10 by 75 mm).

Mixtures of [3H]DNA and serum samples were held at 5°C for 16 to 20 h. Antibody-DNA complexes were precipitated by addition of 5 volumes of cold 60% saturated ammonium sulfate (the ammonium sulfate was adjusted to neutral pH with NaOH). After 30 min at 5°C, samples were centrifuged for 10 min at 1,000 x g. The supernatant was decanted. The precipitate was resuspended with 1 ml of 50% saturated ammonium sulfate and centrifuged again. The pellet from the second wash was dissolved in 1 ml of water and transferred to a scintillation vial for counting.

Analysis of serum proteins. The concentration of immunoglobulin G relative to total serum protein was determined by electrophoresis on cellulose acetate membranes (Microzone system, Beckman Instruments, Inc., Fullerton, Calif.). The percentage of immunoglobulin was based on densitometric integration of stained electrophoretic patterns (Phoroscope electrophoresis densitometer, Millipore Corp., Bedford, Mass.).

RESULTS

Anti-DNA antibodies. To establish serum levels of anti-DNA antibodies in infected and control mink, 10 infected and 10 control sera were analyzed. The results in Table 1 indicate a wide range of values for the various sera from chronically infected mink. The infected animals had, on the average, over four times higher levels of anti-DNA antibodies than control mink. The differences in the means of the two groups were determined to be highly significant by a two-tailed Student t test (P = 0.001). Three of the sera from infected mink gave values which appeared close to the control values. Control values averaged 383 cpm: in all experiments to date, the range of control values was 157 to 750 cpm with similar input of [3H]DNA.

Increases in anti-DNA antibody occurring after infection with ADV were studied and compared with the development of gammopathy. At the time of infection, levels of immunoglobulin were close to normal (Table 2), yet the amount of anti-DNA antibody detected at this time was above what had been shown in previous experiments (Table 1) to be uninfected control values. During the course of the study, the percentage of immunoglobulin of total serum protein rose from an average of 9.2% at the time of infection to 32.8% at 86 days after infection. In a one-tailed test, differences in the percentage of immunoglobulin were significant at both 35 and 86 days (P = 0.0008 and 0.0005, respectively). The percentage of anti-DNA antibodies rose initially for the group (P = 0.05), but then dropped to an intermediate level not significantly different from that at the time of infection (P = 0.19). Comparison of the relative amounts of anti-DNA antibody with the percentage of immunoglobulin indicated that with time after infection anti-DNA antibody represented an increasingly smaller proportion of the total immunoglobulin.

Correlation between anti-DNA antibody and hypergammaglobulinemia was not observed after relatively short-term infection of mink (Table 2), but was observed with samples from long-term, chronically infected mink (Fig. 1). In the latter study, random samples of serum from a ranch where all animals were chronically infected from the time of birth were tested for both percentage of immunoglobulin and anti-DNA antibody. The correlation most closely fits an exponential relationship between anti-DNA antibody and total immunoglobulin (P = 0.001).

Anti-DNA equivalence. Various sera, positive for anti-DNA antibody, were tested at different dilutions in the Farr assay (Fig. 2). Four sera studied here showed reduced counts in the precipitate at both antigen excess and antibody excess. The peak of maximal activity was sharp. This equivalence point occurred between serum dilutions of 1/16 and 1/32. With these and subsequently other sera, it was found that there was a correspondence between high activity in terms of amount of binding and equivalence points at higher serum dilutions. This was shown when sera taken at different times after infection were compared. With increasing time after infection, the equivalence point moved toward more dilute serum concentrations as the fraction of DNA precipitated increased.

Reaction of [3H]DNA with purified mink immunoglobulin. Proof that the precipitate in the Farr assay is truly the product of an antibody-antigen reaction was obtained by reacting various dilutions of purified mink anti-ADV im-

<table>
<thead>
<tr>
<th>Table 1. Anti-DNA antibody in ADV-infected mink sera*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADV infected</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>2,354</td>
</tr>
<tr>
<td>2,680</td>
</tr>
<tr>
<td>447</td>
</tr>
<tr>
<td>2,029</td>
</tr>
<tr>
<td>2,501</td>
</tr>
<tr>
<td>457</td>
</tr>
<tr>
<td>663</td>
</tr>
<tr>
<td>1,379</td>
</tr>
<tr>
<td>1,585</td>
</tr>
<tr>
<td>2,208</td>
</tr>
</tbody>
</table>

* Sera from ADV-infected and ADV-free ranches were tested at a final dilution of 1/16. In the absence of serum, 230 cpm, or 4.6% of input counts, was precipitated. In a t test for significance, t = 4.026, indicating P = 0.001 for 9 df.  
* Mean ± standard deviation of the mean.
munoglobulin G with [³H]DNA. The purified mink immunoglobulin reacted as did mink serum (Fig. 3). The equivalence point for purified mink immunoglobulin corresponded to a concentration of 300 μg of immunoglobulin per ml.

There appeared to be an antibody component detectable at an eightfold antibody excess that reacted with the DNA only when a small amount of control serum was added to each reaction tube. In plots of activity against concentration, this component, apparent as only a shoulder in the samples without added control serum, was increased in activity to higher levels in the presence of dilute control serum. The possibility that the added control serum was adding some complement which was able to add to complexes formed by nonprecipitating antibody-antigen complexes was tested in an independent experiment. When guinea pig complement was included in samples of purified immunoglobulin, the fraction of precipitated DNA was increased.

Anti-DNA positive serum that had been heat inactivated (56°C for 30 min) reacted with DNA to the same extent as serum that was not inactivated. This would suggest that complement was not involved; however, complement components not sensitive to heat or other serum components could still bind to the immune complexes. Heated control serum reacted strongly with DNA. Centrifugation of heated control serum (100,000 × g for 1 h) reduced the DNA reactivity, indicating that the enhanced reactiv-

**Table 2. Changes in anti-DNA antibody after ADV infection**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Days after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (preinoculation)</td>
</tr>
<tr>
<td></td>
<td>% DNA*</td>
</tr>
<tr>
<td>99</td>
<td>44</td>
</tr>
<tr>
<td>94</td>
<td>21</td>
</tr>
<tr>
<td>82</td>
<td>25</td>
</tr>
<tr>
<td>43</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>48</td>
</tr>
</tbody>
</table>

\[ \bar{x} \pm sdm^c = 31.6 \pm 13.4 \quad 9.2 \pm 2.8 \quad 50.2 \pm 17.8 \quad 27.8 \pm 8.6 \quad 40.2 \pm 15.8 \quad 32.8 \pm 10.3 \]

* Percentage of input counts precipitated in the Farr assay.

b Determined by densitometric integration of stained membranes after electrophoresis of sera.

\[ \bar{x} = \text{mean} \]

\[ s = \text{standard deviation} \]

\[ n = \text{number of observations} \]

\[ d = \text{deviation} \]

\[ s_d = \text{standard deviation} \]

\[ \bar{x} = \frac{\sum x_i}{n} \]

\[ s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}} \]

\[ s_d = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n}} \]

\[ r = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum (x_i - \bar{x})^2 \sum (y_i - \bar{y})^2}} \]

\[ \beta = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sum (x_i - \bar{x})^2} \]

\[ \epsilon = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s_1^2/n_1 + s_2^2/n_2}} \]

\[ F = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ G = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ H = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ I = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ J = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ K = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ L = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ M = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ N = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ O = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ P = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ Q = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ R = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ S = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ T = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ U = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ V = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ W = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ X = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ Y = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ Z = \frac{\sum (x_i - \bar{x})^2}{\sum (y_i - \bar{y})^2} \]

\[ a = \text{an amount} \]

\[ b = \text{another amount} \]

\[ c = \text{a constant} \]

\[ d = \text{a degree} \]

\[ e = \text{an element} \]

\[ f = \text{a factor} \]

\[ g = \text{a group} \]

\[ h = \text{a height} \]

\[ i = \text{an index} \]

\[ j = \text{a job} \]

\[ k = \text{a key} \]

\[ l = \text{a letter} \]

\[ m = \text{a mass} \]

\[ n = \text{a number} \]

\[ o = \text{an object} \]

\[ p = \text{a point} \]

\[ q = \text{a question} \]

\[ r = \text{a rate} \]

\[ s = \text{a symbol} \]

\[ t = \text{a term} \]

\[ u = \text{a unit} \]

\[ v = \text{a variable} \]

\[ w = \text{a weight} \]

\[ x = \text{a number} \]

\[ y = \text{a number} \]

\[ z = \text{a number} \]
ity of heated control serum was due to protein aggregation.

**Antibody specificity.** The results in Tables 3 and 4 show the effects of competition by components of DNA and synthetic polynucleotides for antibody binding sites. Addition of unlabeled DNA to the reaction reduced the amount of labeled DNA precipitated in the assay (Table 3). Thymidine and adenosine did not inhibit in the assay; in fact, both compounds increased the amount of [3H]DNA that was precipitated. Polyribonucleotides had little effect on the amount of DNA precipitated (Table 4), whereas the single-stranded deoxyribohomopolymer increased the precipitated counts and the copolymer, polydeoxyadenylate-polydeoxycytidylate, competed with [3H]DNA. Other experiments with radioactively labeled transfer ribonucleic acid (tRNA) and ribosomal RNA indicated that the antibody in mink sera has a lower affinity for RNA than for DNA.

To determine the specificity of the reaction of antibody with DNA, various amounts of unlabeled DNA were added to the reaction. The results (Fig. 4) indicated for the two concentra-

![Graph](image)

**TABLE 3. Competition by DNA and nucleosides**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Serum</th>
<th>cpm precipitated ± standard deviation of the mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Mink</td>
<td>1,471 ± 138</td>
</tr>
<tr>
<td>100 µl of DNA</td>
<td>Mink</td>
<td>62 ± 11 (P = 0.001)</td>
</tr>
<tr>
<td>(1 mg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µl of DNA</td>
<td>Mink</td>
<td>288 ± 29 (P = 0.002)</td>
</tr>
<tr>
<td>100 µl of thymi-</td>
<td>Mink</td>
<td>2,132 ± 60 (P = 0.01)</td>
</tr>
<tr>
<td>dine (1 mg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µl of thymi-</td>
<td>Mink</td>
<td>1,991 ± 634 (P = 0.1)</td>
</tr>
<tr>
<td>dine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µl of aden-</td>
<td>Mink</td>
<td>2,541 ± 348 (P = 0.01)</td>
</tr>
<tr>
<td>osine (1.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µl of aden-</td>
<td>Mink</td>
<td>2,154 ± 354 (P = 0.07)</td>
</tr>
<tr>
<td>osine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>189 ± 26</td>
</tr>
</tbody>
</table>

* Each assay in triplicate contained 100 µl of serum at a dilution of 1/10, 10 µl of [3H]DNA, and the additions noted. Final assay volume was 0.3 ml in every case. The significance of values compared with the control values was determined by Student's t test.

![Graph](image)

**TABLE 4. Competition by polynucleotides**

<table>
<thead>
<tr>
<th>Amt precipitated</th>
<th>Addition</th>
<th>cpm</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>1,335 ± 55</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Polyadenylate</td>
<td>1,752 ± 28</td>
<td>131 (P = 0.01)</td>
</tr>
<tr>
<td></td>
<td>Polycytidylate</td>
<td>1,425 ± 63</td>
<td>107 (P = 0.7)</td>
</tr>
<tr>
<td></td>
<td>Polyadenylate-polycytidylate</td>
<td>1,287 ± 222</td>
<td>96 (P = 0.4)</td>
</tr>
<tr>
<td></td>
<td>Polydeoxyadenylate</td>
<td>2,077 ± 36</td>
<td>156 (P = 0.01)</td>
</tr>
<tr>
<td></td>
<td>Polydeoxyadenylate-polydeoxycytidylate</td>
<td>147 ± 2</td>
<td>11 (P = 0.003)</td>
</tr>
</tbody>
</table>

* All polynucleotides were added to give a final concentration of 10 µg/ml. They were present throughout the incubation period. See legend to Table 3.
tions of serum tested that unlabeled DNA competed almost completely with the [3H]DNA. As expected, equal amounts of unlabeled and labeled DNA reduced the amount of labeled DNA precipitated by about half.

**Antibody avidity.** The avidity of mink antibody for DNA was examined by following the breakdown of preformed antibody-[3H]DNA complexes after addition of unlabeled DNA. The results of a typical experiment performed with two sera are shown in Fig. 5. Within the first 10 min after addition of excess unlabeled DNA, the relative amount of precipitated counts was reduced to 30 to 50% of the initial value. Although the two sera tested had similar equivalence points (see Fig. 1), they had differing avidities.

In another experiment, sera were taken at different times before and after infection and tested for avidity. The results of this experiment (Fig. 6), and experiments with sera from several other mink, showed that after infection there was a marked increase in the avidity of the antibody for DNA. Antibody obtained at later times after infection was not disassociated by addition of unlabeled DNA.

**Native versus denatured DNA.** To compare the antibody specificity for native and denatured DNA, freshly prepared DNA was heat denatured and compared with portions of native DNA. The percentage of counts precipitated as complexes at various concentrations of serum is shown in Fig. 7. The equivalence point for the two samples of DNA was the same. A higher percentage of native DNA reacted at the higher concentrations of mink serum. At antigen excess, the differences between native DNA and denatured DNA were less apparent.

**DISCUSSION**

By using the Farr assay, anti-DNA antibody was consistently demonstrated in sera from ADV-infected mink. The anti-DNA antibody level early after infection did not correlate with the level of gammopathy as has been reported for “nuclear” antigens and antinuclear antibodies (1). In chronically infected populations, however, there was an exponential relation between anti-DNA antibody and gammopathy. Highest anti-DNA antibody levels were observed only in sera that displayed gammopathy.

AD gammopathy has been considered to be mainly anti-ADV antibody (2, 4, 17, 21); however, there is information suggesting that not all of the drastically elevated antibody is specific for viral antigens. First, anti-ADV levels plateau after infection, whereas levels of total immunoglobulin continue to increase (2); second, anti-ADV titers, but not gammopathy, are reduced in mink treated with levamisole (A. J. Kenyon, R. Kassel, G. Notani, and E. C. Hahn, Fed. Proc. 35:529, 1976); and third, ADV-infected mink are
capable of producing antibody to other antigens (11, 15). These results showing anti-DNA antibody associated with ADV support the concept that not all the AD antibody is antiviral; however, the fact that the relative level of anti-DNA antibody decreased from day 35 to 86 (Table 2) indicates that anti-DNA antibody does not play a major role in the continuing hypergammaglobulinemia, but could be important during the later pathogenic stages of the disease (Fig. 1).

Competition experiments indicated that the serum was forming precipitable complexes with DNA. The ability of purified mink anti-ADV immunoglobulin G to react in the Farr assay proves that true antibody-DNA complexes are being formed. Since in that experiment (Fig. 3) dilute control serum could increase complex formation by purified immunoglobulin at intermediate serum concentrations, it would appear that there are at least two types of antibody in the pooled immunoglobulin sample. It is not known whether the component that can bind with control serum is binding complement or some other component of whole serum. Increased binding of immunoglobulin in the presence of control serum is not related merely to the situation occurring at antigen excess because the increase is seen only at intermediate immunoglobulin concentrations. At antigen excess (dilutions 1/64 through 1/4,096), addition of dilute control serum did not increase complexing of the [3H]-DNA.

Mink anti-DNA antibody had more affinity for double-stranded DNA than for single-stranded DNA (Fig. 7). The preferred specificity of antibody for double-stranded DNA has been seen with sera from SLE patients showing renal involvement (14). Single-stranded specificity, not associated with the disease process, is caused by nonspecific reaction of complement components with single-stranded DNA. Since heating mink sera did not change the quantity of anti-DNA antibodies detected, binding of complement is not a major constituent of the immune complexes formed in the assay.

The avidity experiment (Fig. 6) showed that individual serum samples had variable antibody avidities. Results of others have indicated that antibody avidity for DNA decreases in the case of NZB mice as the mice develop nephritic lesions (24, 25). The opposite relation was, however, found for avidity in human SLE; high-avidity antibody to DNA was correlated with increased glomerulonephritis (6, 14). Our results with AD indicate that anti-DNA antibody avidity increased with time after infection. If Steward's proposal (24) is correct, that low-avidity antibody is pathogenic, then it would follow that the pathology of AD is not due to the anti-DNA antibody. In AD, the late lesions may be caused more by antiviral antigens and not anti-DNA antibody. Failure to detect later increases in specific antibody to ADV may be caused by increases in ADV antigen, which could block the free anti-ADV and contribute to the precipitation of immune complexes. Although AD may have some similarities to other autoimmune diseases, the mechanisms that cause the various diseases may be different.

ACKNOWLEDGMENTS

The able assistance of E. W. Shores is appreciated. We thank Edwin Michaels for valuable discussion of the statistics of this study.

This investigation was supported by Public Health Service grant CA-15604 awarded by the National Cancer Institute and in part by core grant CA-08748 from the National Cancer Institute.

LITERATURE CITED