A DNA-Binding Protein in the Serum of Certain Mammalian Species
(Dog/mink/acidic serum protein/protein purification)

R. THOBURN, A. I. HURVITZ, AND H. G. KUNKEL

Rockefeller University and *The Animal Medical Center, New York, N.Y. 10021

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ABSTRACT Various mammalian species contain an anionic serum protein that reacts specifically with native DNA. It is considerably less reactive with single-strand DNA and does not react with monodeoxyribonucleotides, homopolyribonucleotides, or duplexes of homopolyribonucleotides. Synthetic dA·dT was an effective inhibitor of the reaction with native DNA, while Micrococcus luteus DNA and dG·dC were not inhibitory.

This protein was encountered in the course of studies on DNA antibodies. Although it reacted with red cells coated with DNA and gave agar precipitation bands, it was clearly distinct from DNA antibodies. It was found in the serum of all animals of a given species, migrated as an α-β globulin, and did not cross-react with gammaglobulins. It reacted with DNA in solution to give precipitation curves that were strongly influenced by changes in ionic strength. The protein was isolated from canine serum by precipitation with DNA and purified to homogeneity, as judged by immunoelectrophoretic and electrophoretic criteria.

A similar protein was found in mink, equine, and other sera, but not in human sera. Previous studies on DNA antibodies in certain experimental animals may have given false positive results due to this protein.

A wide variety of proteins that interact with DNA have been described in both mammalian and nonmammalian systems. The vast majority have been isolated from cells and are the subject of considerable current interest (1–9). In serum, however, only a limited number of proteins have been described that react with DNA under physiologic conditions of pH and ionic strength. Antibodies to single-stranded DNA have been produced in experimental animals after the DNA has been complexed with a protein carrier (4) and they, as well as antibodies to native DNA, occur spontaneously in patients with systemic lupus erythematosus (5–7), and in New Zealand mouse disease (8–10). Their presence has been suggested in Aleutian disease of mink (11) and in hamsters with schistosomiasis (12). The very basic complement component, C1q, reacts with DNA and mimics antibodies to DNA under certain conditions (13). During studies on DNA antibodies in various animal species, we encountered an acidic serum protein that showed special reactivity with native DNA. This report describes some of the properties of this DNA-binding protein, which has been isolated and characterized.

MATERIALS AND METHODS

Sera. All sera were separated from clots promptly after bleeding; hemolyzed samples were not used. Sera from animals were obtained from various sources, but the majority of the samples were obtained from the Rockefeller University animal quarters. Mink sera were collected primarily from animals maintained at the University by A.H.; a few were collected on a local mink ranch. Both Aleutian and non-Aleutian genotypes were studied; these included mink with naturally-acquired and experimentally-induced Aleutian disease. Sera from mink with experimentally-induced disease were a gift of A. J. Kenyon. All sera were inactivated at 56° for 30 min.

Polynucleotides. Native calf-thymus DNA, Escherichia coli DNA, Clostridium perfringens DNA, and salmon-sperm DNA were obtained from Worthington Biochemical Corp. M. luteus DNA and synthetic polynucleotides were purchased from Miles Laboratories Inc. The dA·dT was a gift from Dr. Alexander Tomasz. Single-stranded DNA was prepared by heating native calf-thymus DNA at 100° for 10 min, followed by immediate immersion in an ice bath. Nuceloprotein was prepared by the method of MIRSKY and POLLISTER (14).

Procedures. Hemagglutination tests were performed as described (15). The test used formalinized Rh-positive erythrocytes (type O), which were tanned and incubated with DNA. Equal volumes of antigen in McIlvaine's buffer and a 1% suspension of tanned erythrocytes were used. Calf-thymus native DNA (50 μg/ml) was incubated with cells for 60 min at 37° in McIlvaine's buffer (pH 4.9). Single-stranded DNA was labeled with [3H]actinomycin for its stabilizing effect on the hemagglutination pattern and incubated with the erythrocytes in McIlvaine's buffer pH 4.7 at 37° for 15 min. The optimal concentration of single-stranded DNA was 10 μg/ml.

50 μl of serum was diluted serially with 1% normal rabbit serum in phosphate-buffered saline (pH 7.2) and 50 μl of this buffer was added to each well of microtiter plates. One drop (50 μl) of a 1% suspension of coated cells was added to each well. The settling pattern was read in 2 hr. The specificity of the hemagglutination reaction was determined by inhibition of the hemagglutination reaction with appropriate antigen. Binding to single-stranded DNA was assayed after prior absorption of sera with native DNA, so that all reactivity with this molecule was eliminated.

Quantitative inhibition studies of red cells coated with native DNA were performed with an endpoint dilution of antisera added to a serial dilution of antigen. The last dilution of antigen giving maximal inhibition was used to determine the inhibition by the antigen. Comparative inhibitory activity of various polynucleotides on the reaction of canine sera with DNA were determined.

0.6% Agarose gel was prepared with Seakem agarose (Maine Colloid, Inc., Springfield, N.J.) in 0.05 M phosphate buffer (pH 7.2)–0.001% sodium azide (16).

Quantitative precipitation curves were performed on canine serum inactivated at 56° for 30 min and dialyzed against

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0.05 M phosphate buffer (pH 7.2), overnight. Different amounts of DNA were added to 0.1 ml of dialyzed serum and the volume was adjusted to 0.2 ml with the phosphate buffer. The tubes were incubated at 37° for 60 min and at 4° overnight, spun, decanted, and washed three times with cold phosphate buffer. Protein in the precipitates was quantitated by the Folin–Ciocalteau method. Similar studies were done in buffers of higher ionic strength.

A linear 10–40% sucrose gradient in pH 7.2 phosphate buffer–saline was used to determine the sedimentation characteristics of the protein. A 1:2 dilution of 0.1 ml of serum was applied to the gradient and spun at 35,000 rpm for 16 hr at 4°. The locations of IgG and IgM proteins (75 and 198 markers, respectively) were determined immunologically with antisera developed to canine myeloma proteins. The effect of reduction and alkylation on the isolated, relatively purified protein was also studied by this technique. The protein was incubated for 1 hr at 37° in 0.2 M 2-mercaptoethanol, and alkylated with a 10% excess of iodoacetamide.

Isoelectric point determinations on the relatively purified protein were performed (17) on Whatman No. 3 electrophoresis paper.

**Purification of Protein.** 50 ml of canine serum was dialyzed against phosphate buffer overnight at 4°, then 12.5 mg of calf-thymus native DNA (equivalence) was added. The mixture was incubated at 37° for 1 hr and at 4° for 2 hr. The precipitate was centrifuged at 670 × g and washed four times with cold phosphate buffer. The washed precipitate was suspended in 5 ml of 0.05 M phosphate–0.05 M NaCl buffer (pH 6.9). 0.25 mg of DNase and cobalt chloride (final concentration 0.01 M) were added. The suspension was stirred at room temperature for 3 hr and dialyzed against the suspension buffer overnight until most of the precipitate had dissolved. The suspension was cleared by centrifugation at 20,000 rpm for 30 min. The supernatant (4.5 ml) was applied to a 3 × 86-cm column containing packed Biogel equilibrated with 0.3 M phosphate buffer (pH 5.3). The flow rate was adjusted to 15 ml/hr and 4.5-ml fractions were collected.

The active pool obtained from the exclusion volume of the column (about 26 ml) was concentrated by precipitation with ammonium sulfate, 35 g per 100 ml, at 4°, and the supernatant was removed by siphon. The wet precipitate was dialyzed and applied to a block of Pevikon C-870 in barbital buffer (pH 8.6), ionic strength 0.05. Electrophoresis was for 16 hr, at a potential gradient of 8 V/cm. The block was cut into 0.25-cm wide segments; each segment containing the active protein was eluted twice with 5 ml of barbital buffer. The active fractions were pooled and concentrated by ammonium sulfate precipitation. The precipitate was suspended in 0.05 M phosphate buffer (pH 7.2) and dialyzed overnight. Final purification of the material obtained from the block was by sucrose density gradient centrifugation.

Antiserum to the DNA-binding protein was produced in rabbits by injection of 1 mg of the DNA–protein precipitate in complete Freund’s adjuvant into multiple sites over a 4-week period. 1 Week after the immunization was completed, the animals were bled. Purity of the isolated protein was tested by Agarose double-diffusion, by immunoelectrophoresis against antiserum prepared to the DNA–protein precipitate, and by polyacrylamide gel disc electrophoresis. A 37% polyacrylamide gel, as modified from Davis, was used for electrophoretic studies (18). Phosphorus was determined on the isolated protein by S. Theodore Bell (19).

**RESULTS**

**Measurement of the DNA-binding protein**

The acidic protein was first detected in dog serum by the agglutination of red cells coated with native DNA, a standard method for the determination of DNA antibodies. All of 16 normal dog sera agglutinated these coated red cells, to titers up to 1/512. Zone electrophoresis experiments indicated that the serum protein involved migrated in the α-β region, and not in the area where immunoglobulins are ordinarily found. After isolation the protein failed to react with anti-immunoglobulin sera, indicating that it was not an immunoglobulin.

The reaction of canine serum with native DNA was also demonstrated in an Agarose double-diffusion system (Fig. 1). Serum from a patient with antibodies to native DNA and two normal human sera are included for comparison. The reaction of DNA with the human serum containing DNA antibodies and the canine serum produced precipitin lines that fused. In addition, sera from several other species also formed precipitin lines with DNA, but some of these were less reactive than canine serum. The reaction between canine serum and DNA could be abolished by DNase treatment of DNA fractions.

**TABLE 1. Reaction of sera from various species with native DNA and single-strand DNA, demonstrated by a hemagglutination technique**

<table>
<thead>
<tr>
<th>Species</th>
<th>Number positive*</th>
<th>Native DNA</th>
<th>Single-stranded DNA</th>
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<tbody>
<tr>
<td>Dog</td>
<td>16 (16)</td>
<td>512</td>
<td>32</td>
</tr>
<tr>
<td>Mink</td>
<td>53 (62)</td>
<td>512</td>
<td>32</td>
</tr>
<tr>
<td>Horse</td>
<td>5 (6)</td>
<td>128</td>
<td>32</td>
</tr>
<tr>
<td>Sheep</td>
<td>6 (6)</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>Cow</td>
<td>9 (10)</td>
<td>128</td>
<td>64</td>
</tr>
<tr>
<td>Cat</td>
<td>4 (4)</td>
<td>512</td>
<td>64</td>
</tr>
<tr>
<td>Mouse†</td>
<td>0 (30)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0 (10)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Monkey</td>
<td>0 (10)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Man</td>
<td>0 (25)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The total number tested in given in parentheses.
† Included SWR, RF/Un, C57 BR/6J, C57 BL/6J strains.
DNA, but not by RNase or trypsin. Trypsin treatment of the isolated protein abolished the binding reaction with DNA.

Although normal human sera did not react with native DNA by hemagglutination and precipitation methods, sera from various mammalian species reacted with native DNA in the hemagglutination assay (Table 1). Special attention was paid to mink sera because of previous reports of DNA antibodies in minks with and without Aleutian disease. The vast majority of mink sera from both Aleutian and non-Aleutian genotypes reacted with DNA. There appeared to be no correlation between DNA binding and genotype of the mink or the presence or absence of Aleutian disease, nor was there a change in titer after inoculation of uninfected mink with Aleutian disease virus. In addition, sera from certain other species, including cat, horse, and cow, showed a similar non-antibody binding reaction. Hamster and guinea-pig sera, although not shown in the table, also gave binding reactions with DNA. Sera from man, monkey, rabbit, and mouse failed to show the reaction.

Additional information on the nature of the reaction was secured from precipitation curves. The largest amount of precipitate was obtained with 0.05 M phosphate buffer (pH 7.2). When the ionic strength of the buffer was increased, the amount of precipitate was reduced at each point on the curve, to minimal values with a 0.25 M buffer. These curves demonstrated zones of excess DNA-binding protein, equivalence, and excess DNA. Supernatants from the zone containing excess binding protein agglutinated DNA-coated cells, while supernatants from zones of equivalence and excess DNA were unreactive.

Specificity of the acidic protein for DNA

The hemagglutination inhibition assay was a useful tool for studying the specificity of binding reactions with various proteins. In these experiments, a hemagglutination-inhibition assay with calf-thymus native DNA as the coat was used. The inhibitory capacity of several nucleotides and soluble nucleoprotein were compared. Less than 1 µg/ml of calf-thymus native DNA gave complete inhibition, while 30-60 times more single-stranded DNA was needed in contrast. Deoxymononucleotides and soluble nucleoprotein failed to inhibit the reaction with native DNA. Synthetic polyribonucleotides, including double- and single-stranded RNA, were also not inhibitory. In addition, organic anions such as sulfobromophthalein (5 mg/ml), probenecid (1 mg/ml), and bilirubin failed to inhibit the binding reaction. These inhibition studies illustrate the specificity of this protein for native DNA.

To determine whether this protein reacted more specifically with certain types of DNA, native DNA from several different sources were compared in the hemagglutination-inhibition assay. The DNA-binding protein was selective in its reaction with DNA. The synthetic double-stranded DNA, dA·dT, was the most effective inhibitor (Table 2), giving complete inhibition at a concentration of 0.07 µg/ml. DNA isolated from C. perfringens was inhibitory at an average concentration of 0.4 µg/ml. It was a slightly better inhibitor than DNA from salmon sperm, calf thymus, or E. coli. DNA isolated from M. luteus, known to have a high guanine-cytosine content, and dG·dC, a synthetic double-stranded DNA, were not inhibitory at a concentration of 125 µg/ml. The interaction of actinomycin with guanine in calf-thymus native DNA did not alter the inhibitory activity compared with unlabeled calf-thymus native DNA. This finding supports the lack of inhibition by dG·dC and the considerable specificity of this reaction.

Physical-chemical characteristics

The protein could be purified readily by precipitation with native DNA. It was then solubilized by DNase treatment, and further purified by standard techniques of column chromatography, preparative zone electrophoresis, and sucrose density gradient centrifugation. Fig. 2 shows the banding of the isolated protein in a 3% polyacrylamide gel. It failed to penetrate the gel at higher gel concentrations.

The protein migrated as a distinct band in the β region on cellulose acetate. Similarly, when proteins of whole canine serum were separated by zonal electrophoresis the reactive material could only be eluted from a restricted area in the α2-β region of the block. These studies reveal the anionic properties of this protein and differentiate it from various basic proteins, such as histone, C1q, and gammaglobulins. Mobility was determined at various pH values with the purified protein; a solution of hydrolyzed dextran was used as a reference substance to determine the extent of electro-osmotic flow. The concentration of the DNA-binding protein was kept constant. These experiments showed an isoelectric point

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**Table 2.** Hemagglutination-inhibition studies with calf-thymus native DNA as the coat and native DNA from various sources as the inhibitor

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>G + C mol %</th>
<th>µg/ml needed for inhibition</th>
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<tbody>
<tr>
<td>dA·dT</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>C. perfringens</td>
<td>26.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Salmon sperm</td>
<td>44.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>45.0</td>
<td>1.1</td>
</tr>
<tr>
<td>E. coli</td>
<td>51.0</td>
<td>1.9</td>
</tr>
<tr>
<td>M. luteus</td>
<td>75.0</td>
<td>&gt;125</td>
</tr>
<tr>
<td>dG·dC</td>
<td>100.0</td>
<td>&gt;125</td>
</tr>
</tbody>
</table>

of pH 5.4. Zonal electrophoresis of serum showed the DNA-binding protein of mink was also an acidic molecule that migrated in a location similar to the canine protein.

Information on the size of the protein was obtained by sucrose density gradient centrifugation, as well as by column chromatography. The DNA-binding protein was localized by the hemagglutination assay to the region between the 19S and 7S markers. Reduction and alkylation of the isolated protein resulted in dispersion of the DNA-binding protein throughout the gradient. Most of the binding protein was in the lower one-third of the gradient, but reactive material was localized in the upper one-third of the gradient as well. It appeared that reduction and alkylation split some of the protein into subunits and resulted in the aggregation of another portion. The protein appeared in the void volume of a Sephadex G-200 column, indicating a molecular weight above 400,000.

Immuno-electrophoretic analysis of the isolated DNA-binding protein showed a single line with a mildly anodal migration when developed by an antiserum prepared to the DNA-protein precipitate. Immunochemical examination of this protein by Ouchterlony's double-diffusion technique also gave a single precipitin band. The concentration of the protein in canine serum was about 100 μg/ml. The protein did not contain phosphorus.

**DISCUSSION**

The protein described in this study appears to be of special interest because it binds native DNA at physiologic pH and ionic strength and shows considerable specificity for certain types of DNA. Although most of the studies were done on dog serum, evidence was obtained that the protein in the other species was similar. The DNA-binding protein was readily differentiated from DNA antibodies in that it was found in the serum of all animals of a given species, migrated as an α-β globulin, and did not crossreact with immunoglobulins. Previous studies have suggested the presence of DNA antibodies in mink sera, especially those with Aleutian disease (11) and in hamster sera with schistosomiasis (12). The presence of the DNA-binding protein in these sera may well have lead to confusion in these interpretations.

The affinity of the DNA-binding protein for DNA is considerably reduced by an increase in the ionic strength from 0.05 to 0.25 M, suggesting the importance of electrostatic forces [although increasing ionic strength is also known to affect the secondary structure of the nucleic acid (20)]. The failure of soluble nucleoprotein to inhibit the binding reaction suggests that the condensed state of this form of DNA conceals the reactive sites, or that histone and nonhistone proteins are blocking the determinants on DNA. Like many intracellular DNA-binding proteins described in lower forms this protein has a net negative charge at physiological pH and may share a positively charged groove with the DNA chain (21).

The specificity of the canine protein for DNA is closely restricted to the native DNA structure. It is less reactive with single-stranded DNA and does not react with monodeoxyribonucleotides, homopolyribonucleotides, or duplexes of homopolyribonucleotides. Since electrostatic interactions are generally dependent on the conformation of the macromolecules involved and since native DNA has higher affinity constants for monovalent and divalent ions than single-stranded DNA, it may be on this basis that the protein is more reactive with the native DNA molecule. The fact that binding was considerably increased with DNA containing high molar ratios of adenine and thymine suggests that it also may result from specific interactions with several nucleic acid bases.

The biologic significance of this protein has not been established, but several possibilities might be considered. It might relate in some fashion to the acidic proteins associated with DNA in the interphase chromosomes of higher organisms (22, 3). The protein could also resemble one of the polymerases. Finally, it might well serve a carrier function for DNA, much as serum protein carries carriers with hormones.

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