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Immunology

Evidence for a naturally occurring anti-spermine antibody in normal rabbit serum

(polyamines/anti-polyamine antibody/polyamine-binding protein/naturally occurring antibody)

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ABSTRACT **Normal rabbit serum contains an IgG-like component(s) that binds spermine as well as other polyamines. This molecule(s) has the same physicochemical properties as anti-spermine antibody obtained from rabbits immunized with spermine-thyroglobulin conjugates. However, the polyamine-binding IgG-like molecule(s), unlike anti-spermine antibody, is incapable either of precipitating a spermine-bovine serum albumin complex or of binding to a spermine-Sepharose column. These results suggest the existence of a naturally occurring anti-polyamine antibody that may have defective antibody function.**

The polyamines spermidine and spermine are aliphatic cations synthesized by all eukaryotic cells, and numerous studies have indicated that polyamines play an important role in the regulation of cell growth and function (for reviews, see refs. 1–6). Further, clinical studies suggest that alterations in polyamines in some tissues and body fluids can be utilized in the diagnosis and prognostic evaluation of certain diseases (7–11).

Specific antibodies against polyamines would be highly desirable in various areas of polyamine research. Antibodies can be used to develop simple and sensitive radioimmunoassays for polyamines and to provide a powerful tool to dissect biological function of polyamines at the cellular and molecular levels. Recently, several investigators have reported the development of anti-polyamine antibodies and their application to immunological analysis of polyamines (12–15). These antibodies, however, have been shown to exhibit substantial crossreactivity (12, 13). In an attempt to obtain a more specific anti-polyamine antibody, we have undertaken the purification and characterization of an anti-spermine antibody from rabbit serum, using normal serum as a reference. In this report, we present evidence indicating that normal serum contains a naturally occurring anti-spermine antibody, which appears to have defective antibody function.

MATERIALS AND METHODS

Materials. Spermine, spermidine, putrescine, bovine thyroglobulin, bovine gamma globulin, and horseradish peroxidase were purchased from Sigma. Sepharose-4B, staphylococcal protein A-Sepharose, dextran T 70, and Norit SX-II charcoal were obtained from Pharmacia. Crystalline bovine serum albumin (hereafter referred to as "albumin") and Freund's complete adjuvant were obtained from Calbiochem. [^{3}H]-Spermine (44 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was purchased from New England Nuclear. DEAE-cellulose was obtained from Serva (Heidelberg, West Germany). Ultrogel AcA 44 was purchased from LKB Laboratories (Bromma, Sweden). Goat anti-rabbit whole serum and anti-rabbit IgG were purchased from Miles Yeda (Rehovot, Israel).

Immunization of Rabbits and Preparation of Sera. The macrophage harvesting method described by Johnston (16) was used with a slight modification. Male New Zealand White rabbits (3.5–4 kg body weight) were injected intraperitoneally with 300 μg of spermine-thyroglobulin prepared by the method of Skowsky and Fisher (17) and dissolved in 1 ml of isotonic saline containing 4 mg of untreated charcoal. Four hours later, peritoneal exudate was collected with 100 ml of normal saline and centrifuged at $1000 \times g$ for 10 min. The cells were resuspended in 2 ml of saline, half of which was given back to the animal intravenously and the other half of which was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously at the back neck area and the thigh at seven or eight spots. Boosters were given biweekly by subcutaneous injection into the back neck of 1 mg of spermine-thyroglobulin suspended in Freund's adjuvant. At least 14 weeks after the first immunization, about 50–80 ml of the blood was collected from each rabbit and the serum was separated and pooled. Nonimmune serum (40–50 ml from each rabbit) was obtained from untreated rabbits. Seven rabbits were used for obtaining either immune or nonimmune serum.

Precipitin Assay of Nonimmune and Immune Sera with Spermine-Albumin. The precipitin reaction was detected by the Ouchterlony double-diffusion test (18) in a 1% agarose gel plate prepared with saline. Diffusion was allowed to proceed for 18 hr at room temperature.

[^{3}H]Spermine Binding Assay. The reaction mixture consisted of 0.8 ml of 0.05 M borate buffer (pH 8.0) containing 0.3 mM KCl, 0.2 mM $CaCl_2$, and 0.1 mM $MgCl_2$, 0.1 ml of various amounts of [^{3}H]spermine, and 0.1 ml of the fractionated serum samples (A_{280} = 1.0) as indicated in *Results*. [^{3}H]Spermine was purified by high-voltage electrophoresis at 30 V/cm on Whatman 3 MM paper with 0.1 M sodium citrate buffer, pH 4.3, prior to use. At the end of 2 hr of incubation at 37°C, 0.1 ml of charcoal/dextran suspension (5 g of Norit SX-II and 0.1 g of dextran T 70 in 100 ml of borate buffer) was added to each reaction mixture and mixed thoroughly. Following centrifugation at $1000 \times g$ for 20 min, 0.8 ml of the supernatant was collected and the radioactivity was measured in 10 ml of toluene-base liquid scintillation cocktail.

Spermine-Sepharose Binding Test of Serum Samples. Spermine coupled to Sepharose was prepared as described by Cuatrecasas *et al.* (19). Briefly, 20 ml of Sepharose 4B was dispersed in 20 ml of distilled water, 2 g of CNBr was added, and the pH of the suspension was maintained at 11.0–11.3 with NaOH. After activation, the Sepharose beads were washed thoroughly with cold 0.1 M bicarbonate buffer (pH 9.0), 120 mg of spermine dissolved in 20 ml of the same buffer were added, and the mixture was incubated overnight at 4°C. The

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Abbreviation: P_i/NaCl, phosphate-buffered saline.

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spermine-Sepharose conjugates contained approximately 1–2 μmol of spermine per 1 ml of gel. The spermine-Sepharose column was prepared by using 10 ml of gel per column (2 × 5 cm), which was washed successively with P_i/NaCl (0.01 M sodium phosphate-buffered 0.14 M saline, pH 7.4, free of Mg^{2+} and Ca^{2+}), 0.1 M ethanolamine (pH 10.8), P_i/NaCl (pH 7.0), 0.17 M glycine·HCl buffer (pH 2.3), and P_i/NaCl. Test protein samples were applied to separate spermine-Sepharose columns and washed thoroughly with P_i/NaCl. The amount of protein applied in a 1-ml solution varied between 5 and 100 mg. For elution of the adsorbed fraction, 0.17 M glycine·HCl buffer (pH 2.3) was used, and the absorbance at 280 nm was monitored.

Immunoelectrophoresis. Immunoelectrophoresis was performed in sodium barbital buffer, (pH 8.6), ionic strength 0.05, as described (20), and a 1:4 dilution of the barbital buffer was used for the preparation of the agarose gel. Electrophoresis was carried out at 2 mA/cm for 75 min at 10°C. After electrophoresis, precipitation arcs were developed with goat anti-rabbit IgG or goat anti-rabbit whole serum as described (20).

Complement Fixation Test. The method described by Mayer *et al.* (21) was followed. The reaction mixture consisted of 1 ml of spermine-albumin (5 μg/ml), 1 ml of guinea pig serum [45 CH_{50} units (see below) per ml, preadsorbed with sheep erythrocytes], and 1 ml of whole nonimmune or immune serum or fractions from nonimmune and immune rabbit sera, each diluted 1:20 in P_i/NaCl. The reaction mixture was incubated at 37°C for 1 hr. Portions of the reaction mixture (0.15–0.35 ml) were then mixed with 1 ml of sheep erythrocytes (5×10^8 cells per ml) that had been sensitized with anti-sheep erythrocytes as described below. The reaction mixture was incubated at 37°C for 1 hr, and centrifuged at 1000 × *g* for 10 min. The resulting supernatant was measured for remaining complement activity by the absorbance at 541 nm. Less than 5% consumption of the total input complement activity was judged as negative. The sheep erythrocytes were sensitized by incubating 1×10^9 cells per ml with 1:400-diluted rabbit anti-sheep erythrocytes at 37°C for 30 min in a total volume adjusted to 7.5 ml with barbital buffer containing 0.1% gelatin and 100 mM $MgCl_2$ as described (21). One unit of CH_{50} is defined as the amount of complement required for causing 50% lysis of sensitized sheep erythrocytes (21). The spermine-albumin conjugates were prepared according to the procedure of Skowsky and Fisher (17) and contained at least 10 mol of spermine per mol of albumin.

DEAE-cellulose chromatography was performed on a 2.8 × 30 cm column with 10 mM and 50 mM sodium phosphate buffer (pH 7.5). Gel filtration was carried out with a 1.5 × 85 cm column of Ultrogel, using P_i/NaCl. Samples were applied in a volume of 4 ml of P_i/NaCl that contained approximately 80 mg of protein. The column was calibrated with blue dextran (average 2,000,000 daltons), bovine gamma globulin (160,000 daltons), and horseradish peroxidase (40,000 daltons) as reference substances.

RESULTS

Immune serum obtained from rabbits immunized with spermine-thyroglobulin conjugates showed hapten-specific antibody activity as evidenced by precipitin formation between immune serum and spermine-albumin in the Ouchterlony double diffusion test (Fig. 1). No precipitin was formed between nonimmunized rabbit serum and spermine-albumin. Both sera were fractionated by addition of solid ammonium sulfate, and the proteins precipitated at 50% saturation were collected by centrifugation. The precipitates contained essentially all of the serum immunoglobulin. The precipitates were dissolved in 10 mM phosphate buffer (pH 7.5), dialyzed against 500 vol of the same buffer overnight, and subjected to DEAE-cellulose chromatography. Each column was washed with 10 mM phosphate buffer (pH 7.5) and then eluted with 50 mM phosphate buffer (pH 7.5). Fig. 2 shows the elution profile of protein during DEAE-cellulose chromatography of the two serum samples. The two profiles were similar: the first peak (fraction I), containing the majority of immunoglobulin, emerged during the washing; the second peak was eluted by 50 mM phosphate buffer (fraction II). The amount of protein in both fractions was larger in the immune serum sample than in the nonimmune serum sample, probably because the immune serum contained a higher amount of immunoglobulin. When fractions I and II were examined for spermine-albumin precipitating activity by the Ouchterlony double diffusion test, only fraction II from the immune serum gave a positive reaction (data not shown).

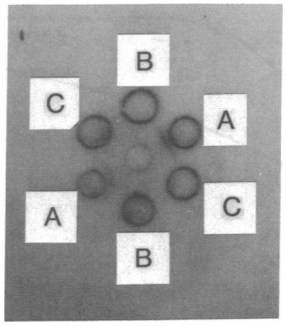

FIG. 1. Ouchterlony double diffusion test of nonimmune and immune serum for hapten-specific antibody activity. Center well, spermine-albumin; wells A, immune serum; wells B, nonimmune serum; wells C, saline.

Fractions I and II derived from both nonimmune and immune serum were tested for [^{3}H]spermine-binding activity (Fig. 3). Fraction I from either serum lacked the binding activity, whereas fraction II from either serum was capable of binding [^{3}H]spermine. The observed slight difference in the binding capacity of the two fractions II is largely due to experimental variations.

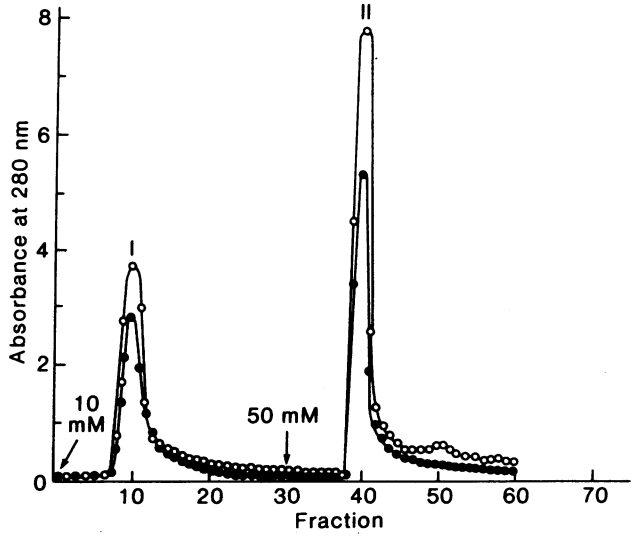

FIG. 2. Profile of partially purified nonimmune (●) and immune (○) sera on DEAE-cellulose chromatography. The nonimmune and immune serum precipitates obtained at 50% saturation with ammonium sulfate were collected by centrifugation and washed with 50% saturated ammonium sulfate. Final precipitates were resuspended in and dialyzed against 10 mM phosphate buffer (pH 7.5) and applied to a DEAE-cellulose column after removal of undissolved materials by centrifugation.

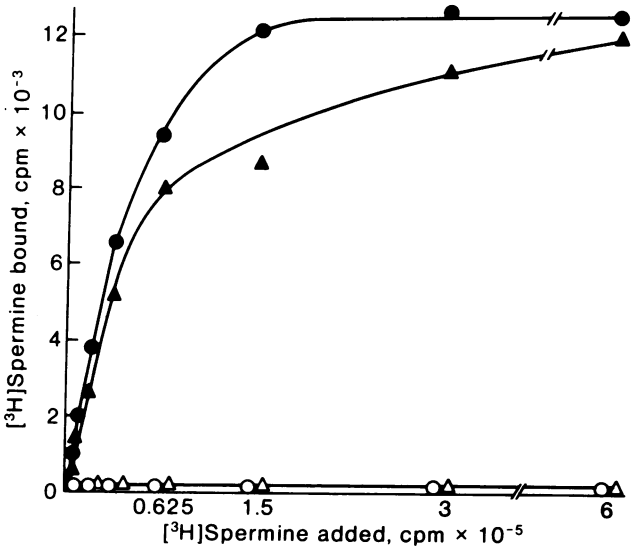

FIG. 3. [^{3}H]Spermine binding assay of fractions I and II from nonimmune and immune sera. From nonimmune serum: ○, fraction I; ●, fraction II. From immune serum: △, fraction I; ▲, fraction II.

The above results indicate that the fractions II derived from immune and from nonimmune serum contain [^{3}H]spermine-binding protein(s), but they differ from each other in terms of their ability to react with carrier (albumin)-bound spermine. In order to examine the structural properties of [^{3}H]spermine-binding protein(s) in these fractions, immunoelectrophoresis employing anti-rabbit IgG was carried out. As shown in Fig. 4, fraction II from normal serum formed precipitins with anti-rabbit IgG in the fast-moving-globulin region. Identical results were obtained with fraction II from immune serum (data not shown). These results strongly suggest that the two fraction II preparations similarly contain IgG that has higher negative charge than the other rabbit IgG. This is consistent with the data obtained with DEAE-cellulose chromatography.

Fig. 5 shows elution profiles of fractions I and II derived from nonimmune serum during molecular seive chromatography. Both fractions contained a major protein peak that eluted at the position of bovine gamma globulin (160,000 daltons). The small peak observed with fraction II was identified as α_2-macroglobulin by further immunoelectrophoretic analysis. Gel filtration of fractions I and II derived from immune serum gave essentially identical profiles (data not shown). These results thus indicate that fractions I and II from both sera contain a major protein species similar to gamma globulin in molecular weight.

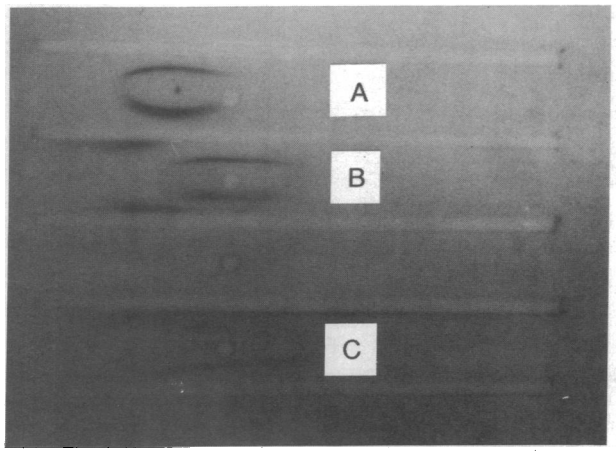

FIG. 4. Immunoelectrophoresis of fractions I and II from nonimmune serum. After electrophoresis, precipitin arcs were developed with goat anti-rabbit IgG. Well A, fraction I; well B, fraction II; well C, nonimmune whole serum.

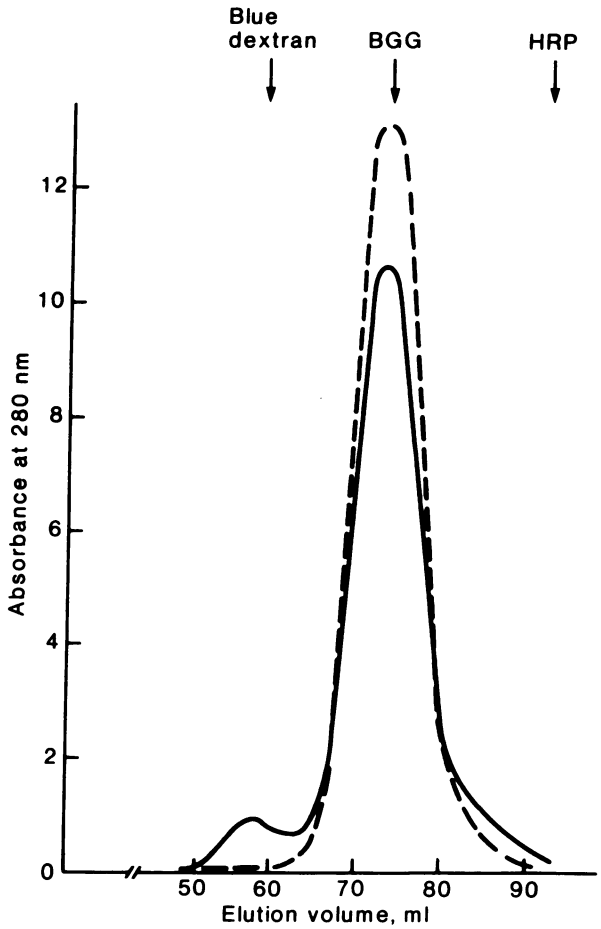

FIG. 5. Gel filtration pattern of fractions I (- - -) and II (——) from normal serum on Ultrogel AcA 44. Normal serum was collected and precipitated with 50% saturated ammonium sulfate. After dialysis against P_i/NaCl (pH 7.5), an 80-mg protein sample in 4 ml of P_i/NaCl was applied onto a column (1.5 × 85 cm) preequilibrated with P_i/NaCl. The column was calibrated with blue dextran, bovine gamma-globulin (BGG), and horseradish peroxidase (HRP) as reference substances.

Because the IgG-like substance in fraction II of nonimmune serum was indistinguishable from that in immune serum in terms of physicochemical properties, its functional properties as antibody were examined (Table I). As described above, this material failed to form precipitin with spermine-albumin. Similarly, it was incapable of binding to spermine-Sepharose, whereas the corresponding fraction derived from immune serum bound to the beads. The addition of nonimmune serum did not interfere with the ability of immune serum to precipitate spermine-albumin (data not shown). The results of an antigen-specific complement fixation test indicated that the fraction derived from immune serum efficiently removed the complement activity from guinea pig serum, whereas that from nonimmune serum failed to do so. Fraction II from both nonimmune and immune serum bound [^{3}H]spermine, but treatment of each fraction with either anti-rabbit IgG (goat) or protein A coupled to Sepharose resulted in loss of the binding activity. On the other hand, fractions I from both nonimmune and immune sera gave negative results when they were examined by the above tests for anti-spermine antibody activity.

Table 1. Functional properties of normal and immune serum antibodies

Property	Normal serum			Immune serum		
	Whole serum	Fraction I	Fraction II	Whole serum	Fraction I	Fraction II
Precipitin formation with spermine-albumin	−	−	−	+	−	+
Binding to spermine-Sepharose	−	−	−	+	−	+
Complement fixation with spermine-albumin	−	−	−	+	−	+
Binding of [^{3}H]spermine						
No pretreatment (control)	+	−	+	+	−	+
Anti-rabbit IgG pretreatment	−	−	−	−	−	−
Protein A-Sepharose pretreatment	−	−	−	−	−	−

It was conceivable that the Ouchterlony test using spermine-albumin complex or the complement fixation test of fraction II from nonimmune serum might give negative results because of a low concentration of anti-spermine IgG-like substance in the original serum. Therefore, in an attempt to isolate and concentrate this material, spermine-Sepharose affinity chromatography was performed by applying as much as 20–25 times more protein of fraction II from nonimmune serum (about 100 mg). However, essentially all of the protein passed through the column, and the concentrated eluate contained no detectable amount of protein and gave negative results in terms of [^{3}H]spermine binding, the most sensitive test for the functional properties of anti-spermine antibody. These results thus indicate that the IgG-like substance in nonimmune serum is a functionally deficient antibody.

Fig. 6 shows that both fractions II were capable of binding spermidine as indicated by competition experiments. However, spermidine caused a greater inhibition of [^{3}H]spermine binding by fraction II from immune serum than by fraction II from nonimmune serum. This difference may be due to the presence of anti-spermine antibody in immune serum that cross-reacts with spermidine or may reflect the difference in the affinity of the two fractions II for spermidine. Similar results were obtained with competition experiments with putrescine (data not shown).

DISCUSSION

In this paper, we have presented the evidence indicating the presence of a naturally occurring polyamine-binding IgG-like substance in normal rabbit serum. This material has the same physicochemical properties as anti-spermine antibody but appears to be functionally defective as complete antibody. Whether this deficiency involves alteration in molecular constitution of immunoglobulin or is merely the result of interference by other substances in normal serum remains to be elucidated. The latter possibility, however, is unlikely because the mixing of nonimmune serum with immune serum did not interfere with the ability of immune serum to precipitate spermine-albumin. The observation that the naturally occurring IgG-like substance is unable to form precipitin with spermine-albumin or bind to spermine-Sepharose suggests that it does not have a sufficiently high valency for antigen binding. The possibility that the negative results of the Ouchterlony test or the complement fixation test of fraction II from nonimmune serum is due to the minuteness in amount of the IgG-like spermidine-binding substance in the original serum appears unlikely because such material(s) was not detectable in the concentrated eluate from the spermine-Sepharose affinity chromatography using a large amount of fraction II. It is of interest that both the polyamine-binding substance in normal serum and anti-spermine antibody in immune serum were recovered in fraction II, which contains more negatively charged IgG, rather than in fraction I, which contains the majority of IgG species.

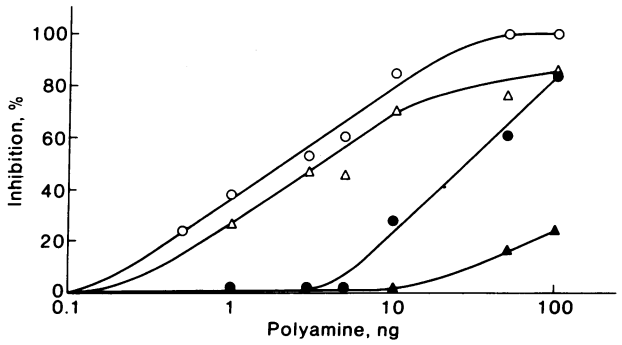

FIG. 6. Spermidine and spermine inhibition of [^{3}H]spermine binding by nonimmune and immune sera. The [^{3}H]spermine binding assay was carried out in the presence of increasing amounts of spermidine (●, ▲) or spermine (○, △). The data are expressed as percentage of inhibition by the indicated amount of unlabeled spermine or spermidine. △ and ▲, Nonimmune serum; ○ and ●, immune serum.

The present findings raise an important question as to the origin and function of the naturally occurring IgG-like material. Recently it was shown (22, 23) that sera and other body fluids contain polyamine-peptide conjugates. Earlier studies showed (24–26) that polyamines can be incorporated into various proteins by transglutaminases. In addition, polyamines are known to be of widespread biological occurrence and to form tight complexes with nucleic acids, proteins, and phospholipids (1–6). Spermine and spermidine are also present as a component of antibiotics (27). It is possible that these polyamine-containing macromolecules could act as antigen to elicit the production of anti-polyamine antibody. Whether such a naturally occurring anti-polyamine antibody is found in other mammalian species is not yet known. Recently a naturally occurring anti-putrescine antibody in human sera was reported by Roch *et al.* (28).

At present, the physiological function of this naturally occurring IgG-like substance is not known. The present data indicate that it does not function physiologically as an antibody. One possibility is that this material, by virtue of its ability to bind polyamines, serves to modulate the serum level of free polyamines, which is known to increase markedly in various physiological and disease states (7–11, 29). Alternatively, this IgG-like species may represent a biologically inactive, altered form of native anti-polyamine antibody produced sometime in the earlier life history of the animal. The existence of naturally occurring antibodies against various homologous antigens has been demonstrated in several autoimmune diseases (30–33).

The present study points out several difficulties in obtaining specific antibodies to polyamines. Our anti-spermine antibody preparation showed cross-reactivity with spermidine and

putrescine. Such low specificity has also been reported by others (12, 13). Further, the presence of a naturally occurring polyamine-binding IgG-like protein in serum could obscure the specificity and sensitivity of immunological assay procedures for polyamines. In addition, preliminary studies indicate that other serum proteins such as α_2-macroglobulin and albumins can also bind polyamines with significant degrees of affinity. Our present finding, that anti-polyamine antibody binds to spermine-Sepharose, whereas the naturally occurring IgG-like polyamine-binding protein does not do so, suggests that spermine-Sepharose affinity chromatography in combination with conventional purification methods may facilitate isolation of pure anti-spermine antibody.

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