ENZYME ACTIVATION BY ANTIBODY

I. Fractionation of Immune Sera in Search for an Enzyme Activating Antibody

T. SUZUKI, H. PELICHOVÁ AND B. CINADER

From the Departments of Medical Cell Biology, Medical Biophysics and Pathological Chemistry, Medical Sciences Building, University of Toronto

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Antisera to bovine pancreatic ribonuclease were fractionated on columns of DEAE Sephadex A-50 and the fractions were characterized by allotype content, electrophoretic mobility, the heterogeneity of heavy chains and the effect on enzyme activity.

Neutralizing antisera contained fractions which augmented ribonuclease activity in the presence of cyclic cytidylic or cyclic uridylic acid as substrate, but not in the presence of nucleic acid. Such antibodies were contained in a fraction P1 which could be separated as the first eluate from DEAE Sephadex A-50 and freed of small quantities of inhibiting antibody by rechromatography. The resulting fraction P1* was less heterogeneous than other fractions and its heavy chains had unique mobilities and showed a minimal tendency to polymerize.

The properties of these activating antibodies differed from those previously obtained by immunization with polyalanine-polytyrosine ribonuclease. Some other fractions, all more heterogeneous than P1, also showed neutralization properties which differed from those found with unfractionated antisera. This was particularly marked with fractions P3–P6, which gave an initial decrease in enzyme activity as a function of increasing levels of antibody, and a secondary increase in enzyme activity with yet higher antibody concentrations.

Most studies of antigen-antibody interaction have treated antigen and antibody as rigid molecules which were affected only in the immediate area of their interaction. However, there is evidence that even simple haptens show changes in their nuclear magnetic resonance spectra following combination with antibody (1), and that antibody itself may undergo a reduction in the number of possible conformations (2) or may undergo conformational alteration (3–6) upon combination with antigen.

The search for activating antibody is motivated by its potential usefulness in the analysis of antibody-imposed changes of antigen-conformation.

Antibodies were discovered as humoral factors which inhibited the biologic activity of bacterial toxins. Indeed, the first criteria for recognition and assay of antibody molecules were based on inhibitory activity. It has become apparent that inhibitory capacity is not a property of all antibodies elicited by biologically active molecules and that some antibodies may not affect biologic activity (7). This conclusion is consonant with the realization that well defined sites of macromolecules are responsible for biologic activity and that the inhibition of such activity depends on the effect of antibody on these selected sites, and hence on the specificity of antibody. More recently, it has been found that antibody molecules may even augment enzyme activity. This was reported for one particular fraction of antibody directed against amylase (8), for all antisera directed against penicillinase (9, 10) and for all ribonuclease-antisera from one particular animal (7, 11). The question arises whether this activat-
Activating function of antibody is a rare phenomenon, observed as a consequence of occasional genetic mutation of the antibody-forming mechanism, or whether activating antibodies are "normal" components of the immune response. If the latter were the case, this antibody fraction would have to be a minor constituent of a heterogenous family since it might otherwise constitute a serious biologic hazard to the antibody-forming animal. This paper summarizes our search for an activating-fraction in the serum of rabbits which were hyperimmunized with bovine pancreatic ribonuclease.

### Materials and Methods

**Ribonuclease A.** Bovine pancreatic ribonuclease was purchased from Sigma Chemical Co., St. Louis, Mo.

**Antiserum (goat) against normal rabbit γ globulin** (Operation No. 1390) was obtained from the Behringwerke, Marburg-Lahn, Germany.

**DEAE Sephadex A-50, Sephadex G-100, G-150 and G-200** were purchased from Pharmacia, Uppsala, Sweden.

**Barium salt of cyclic uridylic acid and sodium salt of cyclic cytidylic acid** were obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y.

**Yeast ribonucleic acid** (m. w., approximately 35,000) was obtained from Miles Laboratories, Elkhart, Ind.

**Immunization of rabbits with bovine pancreatic ribonuclease.** Animals were injected subcutaneously on days 0, 5, 10 and 15 with 2.5 mg of ribonuclease A, in complete Freund's adjuvant. On days 39, 41 and 43 the rabbits were injected intravenously with 1 mg of ribonuclease A in 0.15 M NaCl. Thereafter, the next intravenous injections were given on days 66, 68 and 70 (2 mg × 3), 101, 103 and 105 (4 mg × 3), 143, 145 and 147 (8 mg × 3). On days 206, 208, 210, 241, 243, 245, 303, 305, 307, 333, 335, 337, 360, 362, 364, 382, 384 and 386, the animals were given 16 mg/injection. On days 407, 409, 411, 477, 479, 481, 570, 572 and 574 the animals were injected intravenously with 32 mg ribonuclease/Injection. After the 303rd day, animals were desensitized with subcutaneous injections consisting of 0.1 ml of the solution used in the intravenous injections.

Animals were bled on days: 34, 48, 56, 75, 81, 110, 116, 152, 158, 215, 221, 249, 255, 312, 318, 343, 349, 369, 375, 391, 396, 416, 422, 456, 492, 549 and 555.

**Fractionation of antisera.** Normal rabbit sera and ribonuclease antisera were fractionated by chromatography on DEAE Sephadex A-50 using two consecutively applied gradients. Protein concentration was followed by determining absorbance at 280 nm. Immunoglobulin concentration was followed by assay with antiserum directed against allotypic specificity of light chain. Sera were first dialyzed against potassium phosphate buffer (pH 8.0, I = 0.05, 1 × 10⁻³ M Na₃) and were then centrifuged to remove any precipitated protein. Clear supernatants were applied to the column of DEAE Sephadex A-50 (3 by 45 cm) which had been equilibrated against potassium phosphate buffer (pH 8.0, I = 0.05). Elution with this buffer was continued until 400 ml had been collected in individual fractions of 10 ml. Thereafter, elution at pH 8.0 was continued by increasing the salt concentration, using a linear gradient. The required gradient was prepared in a mixing chamber which contained 1000 ml of potassium phosphate buffer (I = 0.05). Potassium phosphate buffer (I = 0.05, pH 8.0), containing NaCl, (I = 0.15), was kept in a chamber of the same cross-section as the mixing chamber and was allowed to flow into the mixing chamber. Approximately 80% of the immunoglobulins were eluted from the DEAE Sephadex A-50 column in at least nine distinguishable peaks (Fig. 1). After the completion of this gradient elution, a new gradient was applied, again at pH 8.0 with linearly increasing salt concentration. To this end, we mixed 500 ml potassium phosphate buffer (pH 8.0, I = 0.05) containing NaCl, (I = 0.15) with 500 ml of phosphate buffer (pH 8.0, I = 0.05) and containing NaCl (I = 1.0).

The position of fractions P1 to P12 is indicated in Figure 1. The basic fractions (P1 to P8) thus obtained were immunoglobulins of IgG type. Fraction P9 contained transferrin and was further purified by gel filtration on Sephadex G-150. The acidic fractions (P10 to P12) consisted of immunoglobulins of IgG and IgM types. These fractions were passed through Sephadex G-200 in order to obtain IgG free of IgM. All fractions (P1 to P12) were tested by double diffusion in agarose against bovine pancreatic ribonuclease A and were found to give a single precipitating zone.

**Specific isolation of ribonuclease-antibody.** Ribonuclease attached to p-amino-phenacetyl ether of
cellulose (PAP-cellulose) was used to remove antibody from antisera. The purified antibody and the antibody-free supernatant were then fractionated on DEAE Sephadex A-50. The immunoadsorbent, PAP-cellulose, was prepared as described before (12).

Ribonuclease A (250 mg) in borate buffer (pH 8.6) was added to a slurry of PAP-cellulose which contained 1 g of dry solid. The immunoadsorbent, conjugated with ribonuclease A, was then suspended in potassium phosphate (I = 0.2, pH 7.4) and stored at 4°C. Isolation of specific antibodies was carried out by batch adsorption and elution. Hyperimmune sera (40 ml) were added to the antigen-conjugated PAP-cellulose and the mixture was kept overnight at 4°C, with constant stirring. It was then centrifuged at 2°C and 21,000 × G for 30 min. While the supernatant was set aside for a second adsorption, the precipitate was suspended in glycine-HCl buffer (I = 0.1, pH 3.0) and was stirred at 25°C for 1 hr. Eluted antibodies were separated from the immunoadsorbent by centrifugation at 2°C and 21,000 × G. Thereafter, the supernatant was treated twice more by the previously described adsorption procedure. The eluted antibodies from three successive adsorptions and the supernatant from the third adsorption were dialyzed at 4°C for 2 days with two daily changes of Tris-HCl buffer (I = 0.02, pH 8.0, containing 1 × 10⁻³ M NaN₃). They were then concentrated by pervaporation. The isolation of antibodies from the serum was nearly completed by these procedures. Antibodies of IgG type were separated from antibodies of IgM type by gel filtration on Sephadex G-200 and were then fractionated at pH 8.0 by DEAE Sephadex A-50 as described above for whole serum. Elution patterns are shown in Figure 2.

Assay of allotypic specificity. Fractionations were followed by assays of protein content and of allotype content. Allotype content gave a more detailed resolution of fraction than did protein content. All antibody donors were homozygous (A⁺⁺/A⁺⁺). Concentration of A⁺ was measured by a single diffusion-ring test, as previously described in detail (13, 14). Antisera against A⁺ (0.38 ml) and glycine buffer (1.12 ml) were mixed at 56°C and added to glycine agarose (1.5 ml). The carefully mixed fluid was poured into the agar diffusion plate and allowed to set. For this purpose "Immuno-Plates," supplied by Hyland Laboratories, Los Angeles, Calif., were used. A series of 24 evenly spaced wells (diameter: 3.7 mm) were

Figure 1. Elution from DEAE-Sephadex A-50 of rabbit serum. Antisera against ribonuclease A were obtained from 16 rabbits on the 255th day after starting immunization. Normal rabbit sera were obtained from 10 rabbits. All sera were of allotypic specificity A(1⁺4⁺). - - - , pool of ribonuclease (rabbit) antisera; - - - - , pool of normal rabbit sera.
Figure 2. DEAE-Sephadex A-50 chromatograms of normal rabbit serum (N79-10) and of ribonuclease antiserum (No. 973). Normal serum (allotype A(1+4+); 90 ml) was used for the fractionation (upper panel). Antisera against ribonuclease A (76 ml) were obtained from one rabbit on the 56th, 81st and 110th days after the initiation of immunization (bottom panel). -O-O-, absorption at 280 nm; -C-C-, % allotype (taking the starting material as 100%).
punched into the gel and were filled with antigen to the rim. The agar diffusion plates were sealed, kept for 4 hr at 20.8 ± 0.2°C and were then photographed in the Cordis immuno-diffusion camera. Photographs were projected and the ring-widths of the opaque antigen-antibody rings were determined and corrected for enlargement. The resulting measurements were evaluated by comparison with a calibration curve in which ring-widths were plotted against the logarithm of various concentrations of a standard preparation. Each set of fractions was calibrated against the starting material as the standard.

Electrophoresis. Electrophoresis on cellulose acetate was carried out with a Gelman electrophoresis instrument by the zonal procedure. Vertical starch gel electrophoresis was performed as described by Smithies (15). Acid urea starch gel electrophoresis was carried out by Poulak's method (16). The technique employed for immunoelectrophoresis was that of Scheidegger (17).

Separation of heavy (H) and light (L) chains. Separation was carried out by the method of Fleischman et al. (18). Preparations of IgG (5 to 10 mg) were reduced with 0.1 M mercaptoethanol (4 hr, 25°C, pH 8.0) and were then alkylated with 0.11 M iodoacetamide for 30 min (pH 8.0). The reduced-alkylated samples were dialyzed against propionic acid (1 N) at 4°C for 2 days with two daily changes. Thereafter the preparations were fractionated by passing them through columns (3 by 45 cm) of Sephadex G-100, which had been previously equilibrated with propionic acid (1 N). The yield of L-chains from

Figure 3. Distribution of allotype content (A 4) among fractions of specifically purified ribonuclease antibody (IgG) and of an IgG preparation obtained after removal of ribonuclease-antibody. Antisera were obtained from a rabbit (No. 972) immunized with ribonuclease A on the 56th, 81st, 110th and 116th days after the initiation of the immunization. The specifically purified antibody preparation was obtained from 40 ml of antiserum by immunoadsorption on a conjugate of ribonuclease A with p-aminophenacyl ether of cellulose (PAP-cellulose), followed by elution at pH 3.0. Specifically purified antibody and the antibody-free supernatant were passed through Sephadex G-200. IgG was thus separated from other immunoglobulins and was applied to DEAE-Sephadex A-50 and eluted with salt-gradients (see Materials and Methods). The concentration (absorption and allotype-content) was plotted as a function of fraction-number. The top panel shows the elution of the specifically purified antibody; the bottom panel shows elution of IgG from which the ribonuclease antibody has been removed. •••••, allotype content (starting material being 100%); -O-O-, absorption at 280 nm.
Figure 4. Immunoelectrophoretic patterns of various antibody fractions of IgG type. Electrophoresis in Veronal buffer (pH 8.6) was continued for 60 min at a voltage gradient of 12 volts/cm. Fractions of immunoglobulin were obtained as shown in Figure 1 (top panel) and were placed in the cylindrical wells, goat antiserum against normal rabbit γ globulin was placed in the horizontal troughs.

Various reduced-alkylated preparations was 22% to 26% of the starting material as judged by absorption at 280 nm. Heavy chains were resolved in two distinct fractions as observed by Koshland et al. (19); these are designated as H and H'. The relative yield of these two fractions of H-chain varied depending on the IgG preparation. Antibody fraction P1 contained only one of these two heavy chain-fractions (H').

Removal of barium from the barium salt of uridine 2',3' cyclic phosphate. The barium salt of uridine 2',3' cyclic phosphate was converted to the sodium salt by precipitation of the barium by the addition of powdered anhydrous sodium sulfate. The solution was stirred mechanically during all additions and the precipitate was removed by centrifuging at 4°C. Sodium sulfate was added until there was no free barium, as shown by the spot test with rhodizonic acid. The supernatant was tested for sulfate by a spot test with barium chloride (20).

Nucleic acid solution. Ribonucleic acid was dissolved in dilute NaOH and was dialyzed against glass-distilled water; the solution was finally adjusted to pH 7.5 with 0.1 N NaOH.

Ribonuclease assay. All fractions, obtained from Sephadex A-50, were tested for ribonuclease activity and were found to be devoid of such activity.

Manometric method (20-22): Sodium salts of cyclic cytidylic acid, uridylic acid and ribonucleic
Acid were employed as substrate and hydrolytic splitting was followed in the Warburg apparatus. Flask content was 2 ml and side-arm content was 0.1 ml. The final mixture contained 0.018 M NaHCO₃ and was kept in an atmosphere of 5% CO₂ and 95% N₂ at a temperature of 37°C, thus being at pH 7.5. The salt concentration in both flask and side-arm mixture was adjusted to 0.12 M with KCl. Final substrate concentration was 0.029 M.

**RESULTS**

It was our aim to separate and characterize antibody fractions, to assess their effect on enzyme activity and to define the substrate specificity of antibody-activated enzyme.

Sera from rabbits, immunized with bovine pancreatic ribonuclease, were separated into 12 fractions on DEAE Sephadex A-50 (pH 8.0). Fractionation was followed by measurement of protein concentration and of allotypic specificity (A*) on the light chain. The expected complexity of fractions was observed by this technique (Fig. 2). The complexity of the immunoglobulins was not reduced by prior separation of the ribonuclease-antibody on immunoabsorbents (Fig. 3). Clearly the antibody was distributed throughout the chromatographic fractions.

The arbitrary division of the heterogeneous population of immunoglobulins (Fig. 1) resulted in a series of fractions with progressively increasing but overlapping ranges of electrophoretic mobilities (Fig. 4). The distribution of heavy chain polymers and monomers (after reduction and alkylation) differed from fraction P1 to P10 in electrophoretic mobility and complexity. Fraction P1 was less heterogeneous than other fractions and showed unique mobilities of heavy chains. P1 also differed from other fractions in that its heavy chain showed a minimal tendency to polymerize (Fig. 5).

A pool of antisera was selected which gave the classical decrease of enzyme activity with increasing antibody concentrations (23–27) and which led to residual activity which was not decreased by further 20-fold increase in antibody; the antiserum was less inhibitory in the presence of cyclic cytidylic acid than in the presence of nucleic acid (broken lines in Fig. 6). Ten fractions (P3–P12), obtained from this antiserum, inhibited catalytic activity of ribonuclease, when nucleic acid was employed as substrate (Fig. 7). The extent of inhibition, in antibody excess, showed a progressive increase from 70% for P3 to more than 99% for P9 and P10. With fractions 11 and 12, the residual activity in antibody excess was 10% and 30%, respectively. Thus, the inhibitory effect of fractions differed from one another and from the starting material in residual activity in antibody excess: some fractions left more and others less enzyme activity in antibody excess than did the starting material. The properties of antibody fractions showed a qualitative departure from those of the unfractioinated serum when cyclic cytidylic acid served as enzyme substrate (full lines in Fig. 7).

P1 showed a clear-cut activating effect and increased by 50% the rate of hydrolytic splitting. Enzyme-activation was observed not only in the presence of cyclic cytidylic but also in the presence of uridylic acid. The activity of ribonuclease goes through a maximum as the concentration of fraction P1 is increased (Fig. 7). Was this a property of activating antibody or was it attributable to the presence in P1 of small quantities of inhibiting antibody? If such an impurity was of different charge, it could be separated by chromatography. Fraction P1 was therefore rechromatographed over DEAE Sephadex A-50. Protein was eluted as a double peak. The material in the front-peak was pooled and its effect on enzyme activity was examined (Fig. 8). The rechromatographed fraction P1' resembled P1 in not affecting catalytic activity of ribonuclease in the presence of nucleic acid, and in activating enzyme by 47% in the presence of cyclic cytidylic acid. Unlike P1, it did not show reduced activation, at higher antibody concentration. It follows that activating antibody leads to a constant level of increased activity which, in antibody excess, is independent of antibody concentration.

Fractions such as P5 and P6 led to an initially steep decrease in enzyme activity as a function of antibody concentration. This was followed, at higher antibody concentrations, by an increase of activity before a constant relatively high level of residual activity was reached upon the addition of yet larger quantities of antibody. A subsequent publication will present evidence for the presence of activating antibody in these fractions. Some intermediate fractions, P3 and P4, showed a tendency to affect enzyme activity in a similar way but did not contain sufficient antibody to allow detection of a final constant level of activity.
Figure 6. Neutralization of ribonuclease by a pool of antisera. The enzyme was bovine pancreatic ribonuclease A. The pool of antiserum was from 16 hyperimmunized rabbits and was obtained on the 245th day after the start of immunization. Neutralization capacity of antiserum was measured in the presence of yeast ribonucleic acid, \( \Delta \), and cyclic cytidylic acid, \( \bigcirc \).

Figure 7. Neutralization of ribonuclease by chromatographic fraction of antiserum. A pool of antisera (see legend of Fig. 6) was fractionated by chromatography over DEAE-Sephadex A-50 (see Fig. 1). Neutralization capacity of individual fractions was measured, using yeast ribonucleic acid, \( \Delta \), and cyclic cytidylic acid, \( \bigcirc \), as substrates.
Figure 8. Activation of ribonuclease by antibody-fractions P1' using cyclic cytidylic acid as substrates. Constant quantities of enzyme were mixed with varying quantities of antibody fraction and the hydrolytic activity of the mixtures was determined manometrically in the Warburg apparatus.

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<th>Symbol</th>
<th>Substrate</th>
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<td></td>
<td>Cyclic cytidylic acid</td>
<td>P1' from pool of ribonuclease antisera (Fig. 1, Fig. 6).</td>
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<td>Cyclic uridylic acid</td>
<td>P1' from two individual sera (972, 973) from hyperimmunized animals, bled 150th-300th day after beginning of immunization.</td>
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<tr>
<td></td>
<td>Yeast ribonucleic acid</td>
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<td>Cyclic cytidylic acid</td>
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Only some of the terminal fractions, particularly P9 and P10, resembled the unfractionated antisera in their inhibitory capacity. It thus appeared that antisera contained activating antibody and that this activating antibody occurred in its purest form in fraction P1'.

The activating effect of fraction P1 was not due to contaminating ribonuclease. Fraction P1 did not have ribonuclease-activity. It remained possible that the fraction contained some inactive ribonuclease which was activated by the in vitro addition to the native enzyme. We therefore attempted to separate this hypothetical molecule from the antibody. P1' was passed through Sephade G-100 at pH 2.5 and found to migrate as a symmetrical peak without a discernible component in the position in which ribonuclease migrates (Fig. 9).

It thus appeared improbable that inactive ribonuclease was combined with P1 and was responsible for the increase in catalytic activity, upon the addition of P1 to ribonuclease A. Employing DEAE-Sephadex chromatography, P1 fractions were separated from 16 different individual antisera and were screened for their effect on enzyme activity. Five of these fractions augmented the activity of ribonuclease. Clearly, antisera to ribonuclease frequently contain an antibody-fraction which augments rather than inhibits the activity of the enzyme towards cyclic cytidylic acid. The greatest activation found with any of these fractions was twofold.

DISCUSSION

Until a few years ago, all described antibody activity appeared to be inhibitory in nature. It
became a tacit assumption of immunology that neutralization was a characteristic property of antisera. The discovery that antibodies could enhance tumor growth shattered this view. However, it soon became apparent that tumor enhancement was not due to a direct effect of antibodies on tumor cells but was due to an antibody-mediated reduction in the activity of the cellular immune apparatus (28, 29). We have seen that direct enhancement of activity can be observed in another system: antibody-mediated increase in enzyme activity. This activation occurs in vitro in a chemically defined three component system (7, 27) and is attributable to antibody-mediated change in the reactivity of the catalytic site.

The antibody fractions that have been separated in this study differ from one another in the resultant of neutralization by several molecular entities contained in each fraction. The detailed analysis of this problem will be the subject of a subsequent paper. It was the aim of this paper to demonstrate that activating antibodies can be found in most antisera. Fraction P1 shows this activity but is not always homogeneous in terms of neutralization capacity. It contains some inhibitory antibody, which we have shown to be separable by refractionation. The observed incidence of activating P1 in 5 out of 16 antisera from different donors is, therefore, a minimum estimate of the number of antisera which contained activating antibody.

When activating antibody is the predominant component of a fraction, a constant level of enzyme activity is found in antibody excess and further increase in antibody concentration does not result in decreased enzyme activity. It is therefore apparent that activating and inhibiting effects are attributable to distinct species of antibody molecules.

The activating antibody described here differs from the activating antibody elicited by polyalanine-polytyrosine ribonuclease (7) in that its effect is only manifested in the presence of cyclic cytidylic and cyclic uridylic acid but not in the presence of ribonucleic acid. This difference is not necessarily attributable to steric hindrance and may be due to a change in enzyme specificity or to activation that affects the splitting of the cyclic bond but does not affect transphosphorylation: this remains to be examined in detail.

There is no reason to think that P1 is the only fraction that contains activating antibody, since other cathodic fractions (Fig. 1) may contain small quantities of similar antibodies. On the other hand, as an inspection of Fig. 7 indicates, the anodic fractions P8 to P12 almost certainly are largely composed of inhibiting antibody. It remains to be seen whether this distribution from P1 to P12 reflects a causal relation between activating capacity and charge of the antibody molecule (30).

Protein molecules are flexible and enzyme molecules are subject to reversible conformational changes. Antibody molecules may be able to stabilize one of a number of alternative conformations of the enzyme or may change the charge-distribution in the environment of the catalytic site. It will be intriguing to learn to understand the mechanism of activation, not only in terms of
antibody-mediated changes in the antigens but also in terms of the nature of biologic catalysis.

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REFERENCES