Immunochimical Cross-reactivity between Cyanogen Bromide Fragments of Human Serum Albumin*

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The antigenic structure of human albumin was investigated in order to establish whether or not there was any similarity between its antigenic sites. Using immunoadsorbent techniques, several antigenic sites on the albumin molecule were isolated. Measurement of the amount of the antibodies isolated and study of their specificity by inhibition techniques show that these subpopulations of antibodies reacted not only with the fragment used for their isolation (homologous) but also with other fragments (heterologous). Heterologous fragments were inhibiting only at a very high concentration with regard to the homologous ones. These results show that there is a weak cross-reactivity between different portions of the albumin molecule. This reaction is most probably due to the homology existing in the sequence of the human albumin molecule which has arisen by gene duplication. The same type of behavior can be predicted to extend to other molecules which have evolved by similar mechanisms.

Analysis of the antigenic structure of human serum albumin by degradation with several enzymes has demonstrated that the albumin molecule possesses several antigenic sites with distinct specificities (1, 2). These results have been confirmed and extended to bovine serum albumin by isolation of fragments reacting with only a portion of the antibodies directed against the parent albumin molecule (3-10) and by study of the cross-reactivity between albums of different species (11-15).

In contrast, Atassi et al. (16) and Habeeb and Atassi (17) found that the use of immunoadsorbent techniques, fragments of bovine albumin comprising the first or the last third of the molecule reacted with virtually all of the anti-albumin antibodies; it was concluded that bovine albumin carried repeating identical antigenic sites. In the present work we reinvestigated the antigenic structure of human albumin in order to establish whether or not there was any similarity between its antigenic sites. We used CNBr degradation to prepare large fragments which comprised the entire molecule. Antibody subpopulations were isolated with these fragments and their specificity ascertained by very sensitive methods.

CNBr degradation of human albumin gave rise to three fragments named, from the NH2 to COOH terminus, B, C, and A (18). An additional fragment, D, made of B and C previously isolated in this laboratory, was also used (19, 20). The location of the fragments within the albumin molecule was derived from the sequence information of Brown (21) and Meloun et al. (22) and they will be designated as follows: B1-123, C124-285, D1-298, and A299-500.

This study did not show repeating antigenic sites on the human albumin, but demonstrated a weak cross-reactivity between these fragments.

EXPERIMENTAL PROCEDURES

Materials—Human serum albumin was either fraction V from Squibb prepared by ethanol fractionation and kindly given by the American Red Cross or crystallized albumin 100% pure by electrophoresis from Mann.

Fragments B1-123, C124-285, D1-298, and A299-500 were prepared according to Lapresle and Doyen (19).

Rabbit antisera were prepared against Mann albumin by the same protocol described previously (19).

Buffer A contained 0.01 M potassium phosphate, pH 7.4, 0.15 M NaCl, and 0.1% (v/v) Tween 20. Buffer B, contained in addition to these components, 0.5% (v/v) gelatin. Titration plates were Linbro EIA micro-titration plates with 96 flat bottom wells (1.0 x 0.6 cm) purchased from Linbro Division, Flow Laboratories.

Polyacrylamide-agarose plates for electrophoresis (5.5%, w/v of acrylamide) were from Industrie Biologique Francaise, Paris, France.

Methods—Electrophoresis in polyacrylamide agarose plates was performed in Tris-glycine buffer, pH 8.7, and the peptides were detected by staining with Coomassie blue as described by Uriel (23)

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed on slab gels as described by Ames (24). The acrylamide concentration of the gel was 15%. The discontinuous sodium dodecyl sulfate buffer system of Laemmli (25) was used.

Immunoadsorbents were prepared by reacting 2 g of activated Sepharose 4B with 20 mg of anti-D1-298 or anti-A299-500 antibodies using the procedures given by the manufacturer (Pharmacia). Fragments B1-123, C124-285, or D1-298 were passed over the anti-A299-500 column. Fragment A299-500 was passed over an anti-D1-298 column. The material not absorbed was dialyzed against distilled water and lyophilized.

For isolation of antibodies, immunoadsorbents were prepared in the same manner as described above by reacting 2 g of Sepharose with 20 mg of human albumin or fragment. Anti-albumin serum (4-10 ml) was decomplemented by heating for 30 min at 56 °C and passed through immunoadsorbent columns prepared with the different fragments. Antibodies were eluted with 0.5 M glycine, pH 1, immediately neutralized with NaOH as described previously (4), dialyzed against 0.15 M NaCl and stored frozen in aliquots at -20 °C. Control experiments made by passing normal rabbit serum over the various immunoadsorbents showed that nonspecific absorption did not occur.

Proteins were labeled with alkaline phosphatase by the method of Avrameas (26). Albumin fragments and alkaline phosphatase were dialyzed against 0.1 M potassium phosphate buffer, pH 8.5. To 0.5 mg of albumin or fragments in 0.3 ml of buffer, 80 µl of a solution

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containing 2 mg of alkaline phosphatase and 10 μl of 1% solution of glutaraldehyde were added. After incubation at room temperature during 3 h, 50 μl of 1 M lysine, pH 7, were added. The solution was dialyzed overnight at 4 °C against 0.01 M phosphate buffered saline, pH 7.4. Insoluble material was removed by centrifugation at 20,000 rpm. The supernatant was mixed with an equal volume of glycerol and stored at −20 °C.

The inhibition assay using antibody fixed to a solid support was an enzyme-linked immunosorbent assay modified from Engvall and Perlmann (27). In each well of a Linbro EIA microtitration plate, 0.2 ml of antibodies (2.5 pg/ml) in 0.1 M sodium carbonate buffer, pH 9.5, was incubated at 37 °C for 1 h and then overnight at 4 °C. The coated plates were washed six times with buffer A. Various concentrations of inhibitor in 0.1 ml of buffer B were placed in wells and incubated for 1 h at 37 °C. Then 0.1 ml of a solution of 0.5 μg/ml of labeled albumin fragment was added. This corresponded to maximal binding of labeled antigen. The mixture was incubated for 2 h at 37 °C and the plate washed six times with buffer A. The amount of labeled antigen fixed on the plate was determined by reacting with 200 μl of 10−3 p-nitrophenyl phosphate in 0.1 M Tris-HCl buffer, pH 8.0, containing 1.5 M NaCl. The reaction was stopped by the addition of 50 μl of 1 M K2HPO4, and the color was read at 405 nm.

Passive hemagglutination was done with sheep red blood cells treated by glutaraldehyde (28) and sensitized with albumin under the conditions described by Lapsele and Goldstein (29). Albumin-sensitized red cells were suspended in 32 ml of saline containing 1% of normal rabbit serum. Hemagglutination was performed in microplates. Inhibition experiments were done by adding 50 μl of different concentrations of inhibitors to 50 μl of each dilution of purified antibodies. The initial concentration of antibody was 10 μg/ml. After incubation at 37 °C during 30 min, 50 μl of red cells sensitized with albumin were added.

In all these inhibition assays, specificity of the inhibition was validated by negative results with an unrelated protein, ribonuclease.

RESULTS

Purity of the Fragments of Human Albumin—Purity of the fragments was checked by electrophoresis in polyacrylamide agarose gel (Fig. 1), and in sodium dodecyl sulfate polyacrylamide gel (Fig. 2). No impurities were detected and contamination of one fragment by another could be eliminated. Frag-

![Fig. 1. Electrophoresis in polyacrylamide agarose gel of fragments A209-3R3, B1-123, C124-289, and D1-258. Duration of electrophoresis was 3 h at 10 V/cm.](image1)

![Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 1, B1-123; 2, C124-289; 3, A209-345; 4, D1-258; and 5, human serum albumin. The amount of each sample was 20 μg.](image2)

<table>
<thead>
<tr>
<th>Table I</th>
<th>Antibody recovery by immunoadsorbent isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>Serum</td>
</tr>
<tr>
<td></td>
<td>mg</td>
</tr>
<tr>
<td>Anti-albumin</td>
<td>12.4</td>
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<tr>
<td>Anti-B1-123</td>
<td>6.15</td>
</tr>
<tr>
<td>Anti-C124-289</td>
<td>5.16</td>
</tr>
<tr>
<td>Anti-A209-345</td>
<td>4.7</td>
</tr>
<tr>
<td>Anti-D1-258</td>
<td>10.6</td>
</tr>
<tr>
<td>Anti-(B1-123 + C124-289 + A209-345)</td>
<td>16.01</td>
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<tr>
<td>Anti-(D1-258 + A209-345)</td>
<td>15.3</td>
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</table>
Having a maximum absorbing capacity of 1.4 mg for fragment Am\(^{585}\) were passed over immunoadsorbent prepared with anti-Dl-\(z_{ss}\) antibodies. Four mg of fragments B1-123, C124-298, and Dl-298 and A299-585 which have similar mobility in sodium dodecyl sulfate-polyacrylamide electrophoresis are quite distinct in polyacrylamide agarose gel.

In addition, purity of the fragments was ascertained by immunoadsorption. Four mg of fragments B1-123, C124-298, and Dl-298 were passed over immunoadsorbent prepared with anti-A299-585 antibodies having a maximum absorbing capacity of 2 mg for fragment A299-585. 4 mg of fragment A299-585 were passed over immunoadsorbent prepared with anti-Dl-298 antibodies having a maximum absorbing capacity of 1.4 mg for fragment Dl-298. Only traces of material were retained.

**Measurement of Amount of Antibodies Isolated from Immunoadsorbent Columns Made with Albumin and Albumin Fragments**—Antibodies were isolated from albumin antisera with immunoadsorbent columns prepared with albumin and the different albumin fragments. The amount of antibody obtained was determined from absorbancy measurements at 280 nm of the proteins eluted with pH 1 buffer. Antibody recovery was calculated by adding the protein isolated from either B1-123, C124-298, and A299-585 columns or Dl-298 and A299-585 columns. Table I shows the results obtained with 4 sera. Except in one case, the sum of antibodies isolated with the fragments was always higher than the amount of antibodies isolated with albumin. The excess was within the range of 5 to 30% of the anti-albumin value. This observation indicated that some of the antibodies reacted with different fragments.

**TABLE II**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Antibody recovery upon Dl(-298)</th>
<th>Antibody recovery upon A299-585</th>
<th>Difference</th>
<th>Antibody recovery upon Dl(-298)</th>
<th>Antibody recovery upon A299-585</th>
<th>Difference</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Preabsorption by sequential</td>
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<tr>
<td>7</td>
<td>10.65</td>
<td>10.32</td>
<td>(2.03)</td>
<td>10.97</td>
<td>4.58</td>
<td>(6.39)</td>
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<tr>
<td>9</td>
<td>10.65</td>
<td>7.62</td>
<td>(3.03)</td>
<td>4.9</td>
<td>1.52</td>
<td>(3.38)</td>
</tr>
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<td>32</td>
<td>8.16</td>
<td>2.94</td>
<td>(5.3)</td>
<td>6.25</td>
<td>2.04</td>
<td>(4.2)</td>
</tr>
</tbody>
</table>

*Values in parentheses correspond to the difference in the amount of antibodies without and after absorption upon the heterologous fragment.

It should be noted that, in the case of one antiserum, number 9, it was possible to isolate 85% of the total antibody with the fragment Dl\(-123\) immunoadsorbent; in contrast, this antiserum contained less antibody to fragment A299-585 than the other sera tested.

**Antibody Recovery after Sequential Absorption on Immunoadsorbent**—In these experiments, two immunoadsorbents prepared with fragment Dl\(-298\) and A299-585 were used. The amount of antibodies isolated with one fragment was measured before and after absorption by the other fragment. Table II shows the results obtained with three different sera. The amount of antibodies isolated with one fragment was significantly decreased by previous passage over the other fragment. The difference between the two values corresponds to the amount of antibody reacting with both fragments. It should be noted that this value is roughly similar for each serum whether it is measured with either anti-Dl\(-298\) or anti-A299-585.

**Inhibition of Passive Hemagglutination**—Red cells were sensitized with albumin and agglutinated by either anti-Dl\(-298\) or anti-A299-585 antibodies. Inhibition of these reactions with albumin, Dl\(-298\), A299-585, B1-123, and C124-298 was studied. Fig. 3 shows the results of these experiments.

The antibodies were inhibited not only by the fragment which was used for their isolation (homologous) but also by the other fragments (heterologous). The ranking of inhibitory capacity was, with anti-Dl\(-298\), albumin, Dl\(-298\), and A299-585, and, with anti-A299-585, albumin, A299-585, and Dl\(-298\). Without inhibitor, the last positive dilution of antibodies was 1/120. To inhibit antibodies diluted 1/60, it was necessary to use about 15 to 50 times more of homologous fragment than of albumin and 100 to 200 times more of heterologous fragment than of the homologous one.

Fragments B1-123 and C124-298 had a weaker inhibiting capacity which is probably due to their lower molecular weight. This explanation is substantiated by the observation that A299-585 is a better inhibitor of anti-Dl\(-298\) than either B1-123 or C124-298 which are components of Dl\(-298\).

**Enzyme Immunoassay**—Plates were coated with anti-Dl\(-298\), anti-A299-585, anti-B1-123, and anti-C124-298. The corresponding fragments were labeled with alkaline phosphatase and allowed to react with the antibodies. Curves of inhibition of these reactions are presented in Fig. 4.

With anti-Dl\(-298\) and anti-A299-585, albumin was a slightly better inhibitor than the homologous fragment. To obtain 50% of inhibition, it was necessary to use about 6 times more...
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FIG. 4. Enzyme immunoassay inhibition curves. Polystyrene plates were coated with antibody specific for a given fragment. α, anti-D1-298; β, anti-A299-506; γ, anti-B1-123; δ, anti-C124-298. Inhibitor was added followed by the homologous fragment labeled with alkaline phosphatase. The per cent inhibition was calculated as follows for each inhibitor concentration:

\[
\text{\% inhibition} = \frac{I_0 - I_C \times 100}{I_0}
\]

where \( I_0 \) is the absorbancy in the absence of inhibitor. Inhibitors, ■ albumin; ▲ D1-298; △ A299-506; ● B1-123

○ C124-298.

of homologous fragment than albumin.

The heterologous fragments were inhibiting but only at a very high concentration. Within the experimental limits of the method, it was not possible to reach the maximum of inhibition; in several cases it was not even possible to obtain 50% of inhibition. If inhibition was compared at lower levels it was necessary to use roughly 1000 times more mole of heterologous fragment than of homologous to obtain the same degree of inhibition.

With anti-B1-123 and anti-C124-298 antibodies, albumin, fragment D1-298, and the homologous fragment gave inhibition curves close to each other. Only a slight inhibition was also observed with fragment A299-506 and with fragments B1-123 or C124-298 when they reacted with heterologous antibodies.

**DISCUSSION**

In order to study possible relationships between antigenic sites on the albumin molecule, CNBr was used because it gave rise to fragments constituting each half of the molecule (D1-298 and A299-506) or two quarters of the molecule (B1-123 and C124-298). Similarity between antigenic sites of albumin was more likely to be observed with such large fragments.

The sum of antibodies isolated by immunoabsorption with the fragments was greater than that isolated with albumin; this implied that a certain portion of the anti-albumin antibody could react with several fragments. Contrary to the observation of Atassi et al. (16) and Habeeb and Atassi (17), it was not possible to isolate all the anti-human albumin antibodies with the fragments corresponding either to the NH2- or COOH-terminal halves of the molecule. In one serum (number 9), 85% of anti-albumin antibodies were isolated with D1-298 fragment, but this probably reflected the asymmetry of antibody production because this particular rabbit made the smallest proportion of antibody to the fragment A299-506.

Specificity of antibodies isolated with the fragments was studied by two techniques. The first was the inhibition of passive hemagglutination; the second, more quantitative, was an enzyme immunoassay. The main difference between these techniques was a much better inhibition by albumin than by the homologous fragment in the hemagglutination assay. This was probably due to the fact that in this technique the antibodies were reacting with red cells sensitized with albumin while, in the enzyme immunoassay, antibodies were reacting with their respective homologous fragment.

With both techniques, the same general results were obtained. The antibodies were inhibited not only by the homologous fragment but also by the heterologous ones. It must be emphasized that to obtain the same inhibition, it was always necessary to use a much greater amount of heterologous fragment than of homologous one. This eliminated the possibility that the antigenic sites were identical as previously postulated for bovine serum albumin (16, 17).

Contamination of the heterologous fragments by trace amounts of albumin or homologous fragment, undetectable by chemical criteria, could explain these results. Each heterologous fragment was then passed through an antibody immunoabsorbent directed against the homologous fragment, under conditions that all contaminating homologous fragment or albumin would be removed. After this procedure, the inhibition capacity of the heterologous fragment was maintained, which ruled out this possibility.

The most likely explanation for our results is a similarity of structure between the different portions of the albumin molecule so that an antibody specific for an antigenic site would cross-react with another portion of the molecule.

This conclusion is in agreement with those of Peters et al. (10) who tested 14 fragments of bovine serum albumin in an inhibition assay and found cross-reactivity between two different fragments.

Benjamin and Teale (9) did not observe cross-reactivity between six different fragments of bovine serum albumin. It should be noted that, in general, antibody specific for a given portion of bovine albumin was obtained by subtractive immunoabsorption with fragments from the other portions of the molecule; cross-reacting antibody was likely to be removed by this procedure.

The existence of different antigenic determinants in human serum albumin has been demonstrated for a very long time by the following approaches: formation of multi-precipitating systems by degraded albumin reacting with anti-human albumin serum; isolation of two fragments, Inhibitor and F1, which react only with some of the anti-albumin antibodies and isolation with Inhibitor and F1 of antibodies reacting respectively with two or one site(s) of the albumin molecule (1-6). Moreover, it was observed that the two fragments D and A which formed a precipitate with anti-albumin sera gave, by the Ouchterlony method (results not shown), the classical pattern of completely unrelated antigens. Therefore, by these methods, no similarity between the antigenic sites of human albumin could be observed.

The present demonstration of a cross-reaction between fragments of human albumin depended upon special conditions not fulfilled in the previous results. Subpopulations of anti-albumin antibody were isolated by immunoadsorption. By this procedure, as pointed out by Peters et al. (10), all the antibodies of a given specificity are obtained whatever their affinity. Moreover, these antibodies were tested against differ-
ent fragments of the molecule by techniques which permitted use of a very high ratio of heterologous fragments with regard to albumin or homologous fragment.

The concept of uniqueness of the different antigenic sites of globular proteins is consistent with the finding that they do not have any repetition in their primary structure. However, human serum albumin which contains 585 residues is comprised of three domains of about 190 amino acids which evolved from gene duplication and have a homology of 18% (30). The CNBr fragments of human serum albumin contain homologies in sequence which could account for the observed cross-reactivity. This "intramolecular" cross-reactivity appears to be analogous to the cross-reaction observed between albumins of different species which reflects homology in their structure (31). This type of behavior can be predicted to extend to other molecules which have arisen by similar mechanisms of gene duplication.

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