A Monoclonal IgM Protein with Antibody-like Activity for Human Albumin

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ABSTRACT The serum of a patient (L'ec) with an IgM lambda monoclonal protein was noted to bind albumin on immunoelectrophoresis. Analytical ultracentrifugation of the L'ec serum demonstrated 23S and 12S peaks, but no 4S (albumin) boundary. Immunologically identical 20S and 9S IgM proteins were isolated from the serum and the addition in vitro of either the patient's albumin or albumin isolated from normal serum was shown to reconstitute the 23S and 12S boundaries. The binding of high molecular weight IgM to albumin was demonstated by Sephadex G200 chromatography with ^{125}I-labeled albumin and isolated IgM. Immunoelectrophoresis of the L'ec IgM developed with aggregated albumin (reverse immunoelectrophoresis) also demonstrated the binding of albumin to IgM. That all of the patient's IgM complexed with albumin was shown by affinity chromatography employing an aggregated albumin-immunoadsorbent column. Binding was shown to be of the noncovalent type by polyacrylamide gel electrophoresis in 8 M urea. With hot trypsin proteolysis, Fabμ and Fcμ5 fragments were isolated, and monomer albumin was shown to complex only with the Fabμ fragment by both analytical ultracentrifugation and molecular sieve chromatogaphy employing ^{125}I-labeled Fab fragments. 1 mol of Fabμ fragment bound 1 mol of monomer albumin.

Polymers of human albumin, produced by heat aggregation, precipitated with the isolated L'ec protein on gel diffusion analysis and, when coated on sheep red blood cells, gave a hemagglutination titer greater than 1 million with the whole L'ec serum. 50 additional monoclonal IgM, 33 IgA, and 80 IgG sera failed to show precipitation or hemagglutination with aggregated albumin. Native monomer albumin inhibited precipitation only at high concentrations ($>$ 50 mg/ml); dimer albumin or fragments of albumin produced by trypsin digestion inhibited at low concentrations (0.4 mg/ml). No reactivity occurred with the albumin of five other mammalian species, including bovine.

The L'ec protein has the characteristics of an antibody against aggregated albumin, which also has reactivity with native (monomer) albumin. This system shares many similarities with the reaction of IgM human rheumatoid factors with IgG antigen.

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INTRODUCTION

In man, monoclonal immunoglobulins of the IgM class have been described as having antibody-like activity toward bacterial polysaccharides, lecithin, and nitrophenol compounds, as well as rheumatoid factor and antinuclear antibody (1–8). Monoclonal IgG or IgA immunoglobulins or both have also been recognized with activity toward serum proteins such as heterologous $\alpha 2$ macroglobulin, normal IgG, transferrin, lipoprotein, and coagulation factors (9–12). However, only a few of these have conformed to established criteria for an antibody (1). Two proteins have been shown to possess antibody activity toward endogenous serum proteins, i.e., transferrin and rheumatoid factor. The monoclonal IgG antibody to transferrin (13) was shown to complex via the Fab_2, and activity was not demonstrated in the Fab fragment. It is now well established that rheumatoid factors are antibodies with activity most commonly directed against an antigenic determinant on the Fc fragment of IgG.

Several reports (14–16) have described albumin covalently bound to monoclonal IgM and IgA proteins. There have also been studies showing kappa chains complexing covalently to albumin (17) and reports that the normal IgA in commercial preparations of gamma globulin is, at least in part, complexed with albumin (18). The covalent complexes are thought to be unrelated to antibody activity, since the albumin is disulfide-bonded, presumably to the Fc part of the molecule, although this has not been directly demonstrated. We report here a

monoclonal IgM protein with antibody-like activity toward native as well as denatured albumin.

METHODS

The patient (L'ec) was a 70-yr-old woman, who 10 yr previously had been diagnosed as having chronic lymphocytic leukemia. Her primary clinical manifestations included weakness, eccymosis, purpura, gastrointestinal bleeding, recurrent infections, and hepatosplenomegaly. There was no history of previous exposure to human or animal proteins, i.e. transfusions or injections of antitoxins. Serum electrophoresis showed a homogeneous spike in the α region that was typed by immunoelectrophoresis as IgM lambda. The patient died as a result of gastrointestinal hemorrhage and at autopsy showed features most consistent with a reticulum cell sarcoma. Immunofluorescence studies were not performed, but on routine sections, there was no evidence of arteritis, or "immune complex" disease.

Isolation of IgM. When L'ec serum was chromatographed on Sephadex G200 (see Fig. 1) two peaks were obtained, corresponding in position to 19S and 4S markers. However, the 4S area was considerably smaller than that obtained from normal serum and the 7S peak seen in gel filtration of normal serum was absent. This was verified by quantitative Ouchterlony on the whole L'ec serum, which showed markedly decreased levels of IgA and IgG. Albumin was detected in the 4S area and the void volume by gel diffusion analysis. Rechromatography of the ascending limb (pool I) of the void volume dissociated some albumin but in order to remove all residual albumin it was necessary to carry out chromatography on Bio-gel P200 (Bio-Rad Laboratories, Richmond, Calif.) in the presence of 6 M urea. After this, the void volume contained only IgM and a trace of albumin in the γ migrating region by immunoelectrophoresis. The albumin could be dissociated by reduction and was considered to represent a small amount of covalently bound protein.

Low molecular weight IgM was partially isolated from the descending limb (pool II) of the void volume (see Fig. 1) by density gradient ultracentrifugation. The IgM reacting material in the 8–10S region of the gradient (marked with a 10S dimer IgA) was pooled and designated as low molecular weight IgM. These fractions gave a reaction of immunological identity with the 19S IgM but were not entirely free of albumin. Due to the small amount of material available, further separation of IgM and albumin by chromatography in urea was not performed. Fab and Fc fragments of the L'ec 20S IgM were produced by the hot trypsin technique as described by Plaut and Tomasi (19).

Albumin preparations. Monomer albumin was isolated from L'ec serum by starch block electrophoresis. The anodal fractions containing albumin were pooled and treated with 50% ammonium sulfate and the supernate was dialyzed against 0.15 M sodium chloride and then chromatographed on Sephadex G200. The albumin-containing fractions (determined immunologically) in the "4S region" of the column were pooled and concentrated. Immunologic analysis with anti-whole antisera by Ouchterlony and immunoelectrophoresis as well as polyacrylamide gel electrophoresis and analytical ultracentrifugation revealed a homogeneous protein.

A dimer of albumin was isolated from a commercial albumin preparation (Hoechst Corporation, Woodbury, N. Y.) by chromatography on Sephadex G200 equilibrated in 0.15 M sodium chloride. The 7S region of the column

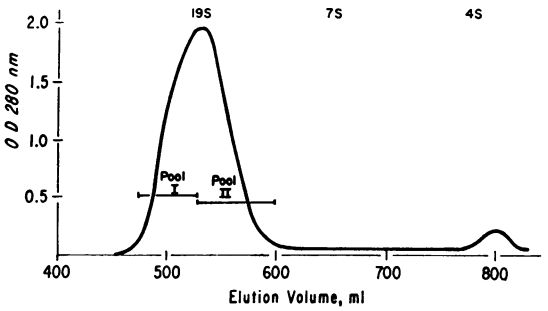

FIGURE 1 Sephadex G200 chromatogram of whole L'ec serum. The positions of marker 19S, 7S, and 4S proteins are shown on top. Note that the 7S peak seen with normal serum is essentially absent and the 4S boundary markedly depressed. Position of pools used in subsequent studies (see text) are illustrated.

(marked with human IgG) was rechromatographed twice on the same column. Analytical ultracentrifugation revealed a single boundary with a sedimentation coefficient at infinite dilution of 6.6S.

Aggregated albumin was prepared by dissolving commercial albumin in 0.05 M Tris-HCl buffer at pH 8.1 and heating at 65°C for 7 min.

Albumin preparations were also digested with trypsin (5% enzyme by weight) in 0.05 M Tris-HCl buffer, pH 8.1, containing 0.01 M calcium chloride. Digestion was performed at 65°C for 7.5 min.

Other techniques. Analytical ultracentrifugation was performed in the Spinco Model E ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 52,640 rpm at 20°C. The results were corrected to water at 20°C and extrapolated to zero concentration to obtain the $s_{20,w}$ values.

Polyacrylamide disc electrophoresis was performed on 5% polyacrylamide gels as described by Ornstein and Davis (20, 21). Samples were applied in either aqueous buffer or, in some experiments, 8 M urea.

Radiolabeling of albumin and Fabμ fragments were performed with ^{125}I according to the methods of Greenwood and Hunter as modified by McConahey and Dixon (22, 23). The specific activity of the labeled albumin and Fabμ was 4 Ci/μg and 2.5 Ci/μg, respectively.

Immunoadsorbent columns were prepared with Sepharose 4B activated with cyanogen bromide and coupled to heat-aggregated albumin according to the method described by Mannik and Stage (24). The columns were first eluted with 0.15 M NaCl and subsequently with glycine-HCl buffer at pH 2.3.

Hemagglutination was performed in 12 × 75-mm test tubes, with tanned sheep red blood cells treated with heat-aggregated albumin according to the method of Boyden (25). Titers were expressed as the reciprocals of the highest dilution giving definite agglutination. Preparations to be tested were first inactivated at 56°C for 30 min and then absorbed with sheep red blood cells. Inhibition of hemagglutination was carried out in the following manner: The titer of a standard 20S L'ec IgM preparation was determined by direct hemagglutination. The next lowest dilution was then chosen for inhibition studies with various trypsin fragments of albumin or fragments of the L'ec protein. The concentration producing complete inhibition was recorded in micrograms per milliliter. Indirect hemag-

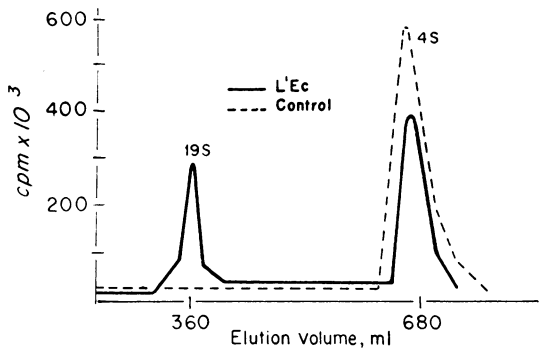

FIGURE 2 Sephadex G200 chromatogram of L'ec IgM after incubation with radiolabeled albumin (solid line) and a control 19S IgM incubated with labeled albumin (dotted line) under similar conditions. The positions of 19S and 4S marker proteins are shown. Note that a shift in the position of the labeled albumin indicative of binding occurs only with the L'ec IgM.

glutination was performed by incubating the tanned sheep red blood cells coated with aggregated albumin with double dilutions of the L'ec Fab for 30 min at 37°C. After the cells were washed three times, hemagglutination was recorded after the addition of an anti-Fab antiserum (26).

Starch block electrophoresis and sucrose density gradient ultracentrifugation were done according to previously described methods (27).

RESULTS

Evidence of albumin binding to the L'ec IgM. Immunoelectrophoresis of pool I (see Fig. 1) with specific anti-albumin antiserum revealed two distinct precipitin bands: a slow-migrating albumin component in the gamma globulin region corresponding to the IgM precipitin line, and another band corresponding to free albumin in its usual anodal position.

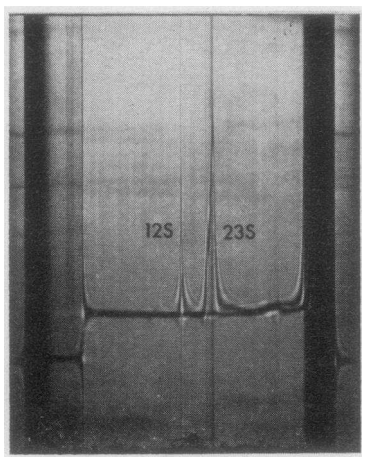

FIGURE 3 Analytical ultracentrifugation of L'ec serum at a dilution of 1:8. Frame taken 64 min after full speed (52,640 rpm) was reached. Direction of sedimentation is from left to right.

To determine whether the albumin found in pool I resulted from albumin complexed to IgM or aggregates of albumin, the L'ec albumin was radiolabeled with ^{125}I. When the isolated L'ec IgM was incubated with labeled albumin for 1 h at 37°C and chromatographed on Sephadex G200, radioactivity was demonstrated in two major peaks, the void volume and the 4S region (see Fig. 2). A control (nonbinder) monoclonal 19S IgM treated similarly demonstrated a single peak of radioactivity in the 4S region. Therefore, the shift of the label into the void volume seen with L'ec IgM was thought to be indicative of complex formation between L'ec IgM and albumin.

Demonstration of complex formation in the ultracentrifuge. Analytical ultracentrifugation of L'ec serum at a dilution of 1:8 demonstrated two rapidly sedimenting peaks with sedimentation coefficients at infinite dilution of 12 and 23S, while the 4S boundary usually seen at this dilution was absent (Fig. 3). Experiments were undertaken to determine the origin of these peaks. The isolated L'ec IgM (2.5 mg/ml), L'ec low molecular weight IgM (2.5 mg/ml), and a control 19S IgM (2.5 mg/ml) were each incubated with 8 mg/ml of monomer albumin at 37°C for 1 h and sedimentation analysis was performed. Each of the preparations were analyzed at four different dilutions and s vs. c plots extrapolated to zero concentration. The following sedimentation coefficients were obtained: The L'ec IgM had a sedimentation coefficient of 20S, which after incubation with albumin increased to 23S, and no residual 20S IgM was noted (Fig. 4). Incubation of albumin with the low molecular weight IgM (9S) caused an increase in the sedimentation coefficient to 11.3S. As

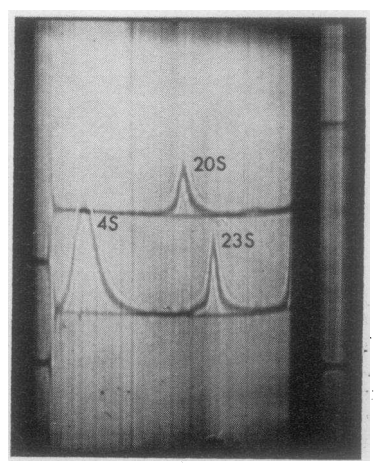

FIGURE 4 Analytical ultracentrifugation of isolated L'ec 20S IgM (upper sector) and the same preparation incubated with monomer albumin (lower sector). Frame taken 64 min after full speed (52,640 rpm) was reached. Direction of sedimentation is from left to right. The s rate of L'ec IgM increased from 20S to 23S on addition of albumin.

TABLE I

Sedimentation Coefficients of L'ec IgM before and after Addition of Albumin

Preparation	No albumin	Albumin added
L'ec high mol wt IgM	20.3s	23.5s
L'ec low mol wt IgM	9.3s	11.3s
Control IgM	19.1s	18.0s

Sedimentation coefficients (extrapolated to zero concentration) of L'ec high and low molecular weight IgM and a control monoclonal IgM before and after incubation with monomer albumin (see test for details). This table demonstrates increase in sedimentation coefficient indicative of binding on adding albumin to both L'ec proteins but not the control.

shown in Table I, the addition of albumin to a control 19S IgM caused a decrease in the sedimentation coefficient to 18S. These findings suggest that the 23 and 12S boundaries in the L'ec serum were due to complexing between high molecular weight IgM and albumin, and low molecular weight IgM and albumin, respectively. The sedimentation coefficient of 9S found for the low molecular weight IgM may be due to residual albumin complexed to the 7S IgM molecule that was not completely removed by the isolation procedures employed. The slightly higher sedimentation coefficient (20.3S) obtained for L'ec IgM compared to the control IgM (19.1S) could be similarly explained.

Type of bonds between albumin and L'ec IgM. The results discussed above suggest that the L'ec IgM binds albumin in a reversible fashion and that partial dissociation of the complex occurs on electrophoresis but not on ultracentrifugation. In order to determine the types of bonds involved, experiments were performed with polyacrylamide gel electrophoresis. As shown in Fig. 5, the 20S L'ec IgM in aqueous buffer does not penetrate the gel (gel *a*) as would be expected for a high molecular weight polymer. Gel *b* illustrates the L'ec IgM in 8 M urea and shows the dissociation of albumin, while gel *c* represents isolated L'ec albumin for comparison. Gel *d* shows the effect of reduction with 0.01 M dithiothreitol and alkylation with 0.023 M iodoacetamide on the 20S IgM, demonstrating 7S IgM and no release of albumin. After reduction and alkylation, the sample was applied in 8 M urea and two bands can be seen corresponding to IgM subunits (7S) and albumin as shown in gel *e*. Typical J chain bands appeared when a higher concentration of L'ec IgM was applied to the gels. The results of the gel experiments as well as the previously mentioned ability to separate albumin from IgM by chromatography in urea suggest that the majority of albumin was bound to the IgM by noncovalent forces. However, some experiments demonstrated that a small amount of albumin was released only after reduction

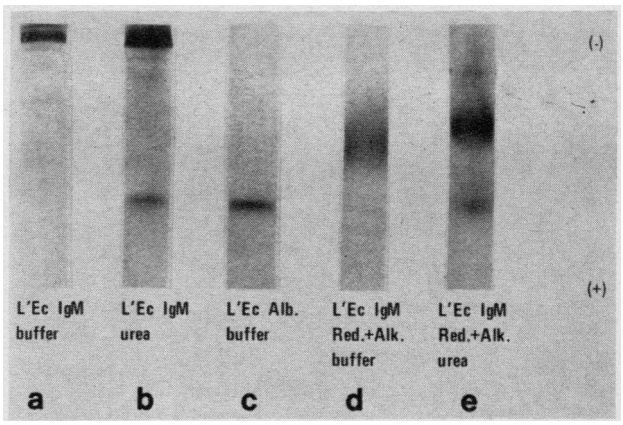

FIGURE 5 Polyacrylamide gel electrophoresis (5%) anode (+) and cathode (−). After treatment of L'ec IgM (gel *a*) with 8 M urea, albumin dissociates as shown in gel *b*. The dissociated albumin has the same electrophoretic mobility as isolated L'ec albumin (*c*). Reduction (0.01 M dithiothreitol) and alkylation (gel *d*) does not release albumin unless it is electrophoresed in 8 M urea as shown in gel *e*.

and was, therefore, probably bound to the L'ec IgM by covalent disulfide bonds.

Albumin binding to fragments of L'ec IgM. Trypsin digestion of isolated L'ec 19S IgM at 65°C was employed to determine which region of the molecule was responsible for the albumin binding. The fragments were isolated by chromatography on Sephadex G200 and studied for their ability to bind monomer or dimer albumin in the ultracentrifuge. The sedimentation coefficient of each of these mixtures was calculated by plotting *s* vs. *c* at four different dilutions and extrapolating to zero concentration. When the Fabμ (2.0 mg/ml) was incubated with monomer albumin (6 mg/ml) at 37°C for 1 h, the sedimentation rate increased from 3.7 to 4.7 (see Table II). Albumin at the same concentration (8 mg/ml) had

TABLE II

Sedimentation Coefficients of L'ec Fab and Fc before and after Addition of Albumin

Preparation	No. albumin	Monomer (3.9S) albumin added	Dimer (6.6S) albumin added
L'ec Fab	3.7s	4.7s	7.6s
Control Fab	3.2s	3.1s	ND
L'ec Fc	11.2s	11.1s	10.2s

Sedimentation coefficients (extrapolated to zero concentration) of L'ec Fab, L'ec Fc, and a control Fab, before and after incubation with monomer or dimer human albumin (see text text for details). This table demonstrates increase in sedimentation coefficient indicative of complex formation when albumin (either monomer or dimer) is added to L'ec Fab but no increase with control. The L'ec Fc shows no binding of albumin.

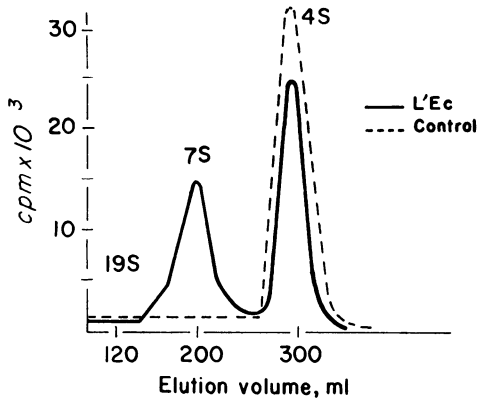

FIGURE 6 The L'ec Fabμ was labeled with ^{125}I, incubated with dimer albumin, and then chromatographed on Sephadex G200 (solid line). A control Fabμ treated similarly is also illustrated (dotted line). The positions of 19S, 7S, and 4S marker proteins are shown.

a sedimentation coefficient of 3.9S. Upon the addition of albumin to a control Fabμ and the L'ec Fc, there was a decrease in the sedimentation coefficient due to concentration dependence. Because of the overlapping boundaries of Fabμ and monomer albumin in the ultracentrifuge, dimer albumin was employed to facilitate separation of the two peaks. When the Fabμ (2.2 mg/ml) was incubated with dimer albumin (5 mg/ml) at 37°C for 1 h and subjected to analytical ultracentrifugation, the sedimentation coefficient of the complex was shown to be 7.6*2*, while that of the dimer albumin was 6.6S. In separate experiments, increasing amounts of dimer albumin were added to a fixed concentration of L'ec

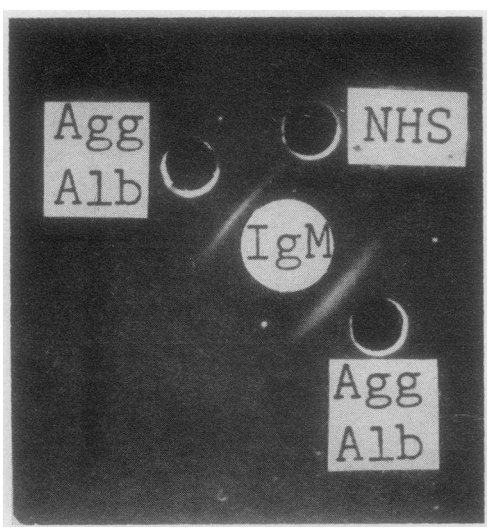

FIGURE 7 Ouchterlony analysis. L'ec IgM is in the center well, aggregated albumin in both lateral wells, and normal human serum in the top well. Normal human serum fails to precipitate and does not clearly inhibit precipitation of L'ec IgM with aggregated albumin.

Fab. When 1 mol of dimer albumin was added to 2 mol of Fab there was a complete disappearance of the Fab peak on analytical ultracentrifugation. These experiments suggest that 1 mol of L'ec Fab bound 1 mol of monomer albumin.

Binding could also be demonstrated with radiolabeled Fabμ. L'ec Fabμ and a control Fabμ at 1 mg/ml were labeled with ^{125}I and incubated with dimeric albumin. The dimer was used to facilitate the separation of the Fabμ from albumin after chromatography on Sephadex G200 (see Fig. 6). The L'ec Fabμ preparation demonstated two peaks corresponding to the 7S and 4S regions of the column, while the control demonstrated one peak coinciding with the 4S marker location. The shift of the label into the 7S region is indicative of complex formation between the L'ec Fabμ and dimer albumin. The labeled Fc of the L'ec protein showed no ability to complex in similar experiments.

Specificity of L'ec IgM. In analogy with rheumatoid factor it was thought reasonable that the specificity of the L'ec protein might be directed toward an altered form of albumin. Consequently, native albumin was heated to 65°C and analyzed by gel diffusion. As illustrated in Fig. 7, a precipitin line was noted between aggregated albumin and isolated L'ec IgM, although no reaction was observed between the L'ec IgM and normal human serum, monomer 4S albumin, or dimer albumin at an equivalent albumin concentration. However, concentrations of monomer albumin above 50 mg/ml and of dimer albumin in excess of 0.4 mg/ml inhibited precipitation of L'ec IgM with heat-aggregated albumin. Reverse immunoelectophoresis with the L'ec IgM in the well and aggregated albumin in the trough, as shown in Fig. 8, revealed a precipitin band corresponding to the migration of IgM.

The specificity of the L'ec IgM for altered albumin could also be demonstrated with hemagglutination and inhibition of hemagglutination of sheep red blood cells coated with aggregated albumin. Whole L'ec serum had a hemagglutination titer greater than one million, while the low molecular weight L'ec IgM preparation had a titer greater than one thousand. Inhibition of hemagglutination occurred with a concentration of monomer albumin greater than 50 mg/ml or of dimer albumin in excess of 0.4 mg/ml. The controls of seven normal human sera, two cord sera, two hypogammaglobulinemic sera, ten macroglobulinemic sera, and eight IgA myeloma sera were all negative in hemagglutination tests. Indirect hemagglutination, with the L'ec Fabμ and sheep red blood cells coated with aggregated albumin, demonstrated a titer of greater than four thousand with an anti-Fab antiserum.

The isolated L'ec 20S IgM at 7.5 mg/ml was shown to have a hemagglutination titer of three million. When

this preparation was passed over an aggregated albumin immunoadsorbent column, all of the L'ec IgM, as well as the hemagglutinating activity, adsorbed to the column. Essentially all of the activity could be recovered by acid elution. An isolated control IgM at a similar concentration was not adsorbed to the affinity column. This experiment demonstrated that all of the L'ec IgM protein possessed antibody-like activity.

Heat-aggregated albumin was proteolyzed with trypsin at 65°C and then chromatographed over Sephadex G200 with the results illustrated in Fig. 9. Each peak was tested for its ability to inhibit hemagglutination and gel precipitation. The void volume and monomer albumin (4S) were unable to inhibit hemagglutination or precipitation up to 50 mg/ml but a fragment of the trypsinized albumin, estimated from its Sephadex G200 elution position to be smaller than albumin (shown as a cross-hatched area in Fig. 9) inhibited hemagglutination at 100 μg/ml. This fragment of albumin also inhibited the precipitation of L'ec IgM with aggregated albumin in gel diffusion.

We have analyzed by gel diffusion 44 macroglobulinemic, 62 IgA myeloma, and 76 IgG myeloma sera, and no precipitation reaction was seen between these sera and aggregated albumin. Some of these proteins were selected because they showed a small amount of binding to albumin, but this binding was of the covalent type as described by Mannik (14). Also, no precipitation occurred between the L'ec IgM and heat aggregated bovine, rabbit, mouse, or goat albumin. Aggregation similar to that seen with heated human albumin was demonstrated by ultracentrifugation only for the bovine albumin. The albumins of the other species were not subjected to ultracentrifugation and, therefore, it is not certain that these preparations were indeed aggregated.

DISCUSSION

The criteria discussed by Metzger (1) for establishing antibody activity are: (*a*) the protein should resemble known immunoglobulins; (*b*) antigen-binding activity should be localized to the Fab fragment; (*c*) the combining ratio of antigen to antibody should be well defined and limited, i.e., a ratio of one site per heavy-light polypeptide chain pair; (*d*) the interaction of the protein with its antigen should express a clearly defined specificity; (*e*) the entire population of antibody should be active. By these criteria, the L'ec IgM molecule has the characteristics of an antibody. The protein is an immunoglobulin that binds albumin noncovalently. The binding is via the Fab portion of the molecule and the molar ratio of Fab to albumin is 1:1. As to the antigen specificity, the L'ec IgM seems to have a well-defined specificity for human albumin. However, there was a spectrum of activity with the highest towards heat-aggregated human

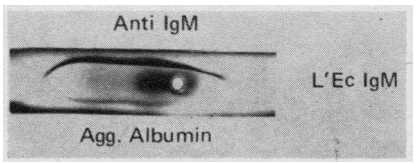

FIGURE 8 Reverse immunoelectrophoresis: cathode is to the left. Shows precipitation of L'ec IgM by heat-aggregated albumin.

albumin and the lowest for monomeric albumin. Whether this was due to antigenic multivalency, exposure of a hidden determinant upon heating, or both has not been established in this study. The fifth criterion was established by affinity chromatography with an aggregated-albumin immunoadsorbent column. The entire L'ec IgM was adsorbed to the column and could be recovered in an active form after acid elution.

The L'ec protein has many features in common with rheumatoid factor. In rheumatoid sera there are 22S complexes consisting of 19S rheumatoid factor and 7S IgG (28, 29). In the L'ec sera there are 23S complexes consisting of 20S IgM and 4S endogenous albumin. In urea or acid the 22S rheumatoid complex dissociates into 19S and 7S units (29, 30), while in our study the 23S complex dissociates in urea into two major constituents, 20S and 4S. Rheumatoid factor is thought to be an antibody formed in response to altered IgG, particularly in the form of antigen-antibody complexes. This has been postulated to involve the exposure of a hidden determinant. Rheumatoid factor also reacts with native 7S IgG as shown by the 22S complexes seen on ultracentrifugation. The binding phenomenon observed with the L'ec protein leads us to speculate that, like rheumatoid factor, the L'ec IgM may have formed initially against altered albumin but also shows activity with native monomer albumin, thus forming the 23S complex observed. Certain rheumatoid sera also have intermediate

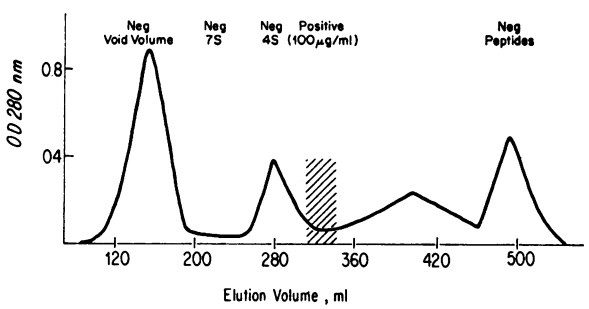

FIGURE 9 Sephadex G200 of trypsinized (65°C) albumin. The positions of the 7S and 4S marker proteins are shown. The results of hemagglutination inhibition of the various pools are designated negative or positive (μg/ml) at the top of the illustration. The elution position of the pool that was positive in inhibition is illustrated by the cross-hatched area.

complexes with a sedimentation coefficient between 9S and 17S. These dissociate in urea or acid to 7S units and are composed of anti-IgG and the reactant monomer IgG, both of which are 7S (31–33). Some complexes are also composed of low molecular weight IgM reacting with IgG. The L'ec sera displayed an intermediate 12S complex that dissociated into 9S and 4S components. The 12S boundary could be reconstituted by the addition of 4S albumin to 9S IgM and thus could be analogous to rheumatoid intermediates.

The question of antigenic specificity deserves further emphasis. Heating produces aggregates of human albumin that can be detected ultracentrifugally or by polyacrylamide gel electrophoresis. Antigenic multivalency is suggested by the finding that aggregation of albumin enhances precipitation with the L'ec IgM and native monomer fails to inhibit except at very high concentrations. This is similar to the rheumatoid system, where native IgG does not inhibit agglutination by rheumatoid factor of sheep red blood cells coated with aggregated IgG globulin. Normansell (34) was able to show by analytical ultracentrifugation that the binding constant of aggregated, altered, and native 7S IgG for IgM rheumatoid factor subunits was approximately the same (i.e., 4×10^5 liter/mol). He suggested that the inability of native IgG to inhibit was due to an equilibrium that favored the rheumatoid factor-cell agglutinate due to the formation of multiple bonds between individual rheumatoid factor molecules and the multivalent aggregates of IgG. Therefore, multivalency of antigen rather than a hidden determinant was postulated to account for the apparent discrepancy in the ability to demonstrate 7S IgG complexing to 19S rheumatoid factor in the ultracentrifuge, and the inability of 7S IgG to precipitate or inhibit precipitation of rheumatoid factor.

With hot trypsin digestion of albumin, inhibition of precipitation and hemagglutination was found both with the whole digest and a fragment of albumin, which, judging from its position on Sephadex, is smaller than the 4S monomer. This suggests that heat aggregation may also reveal a hidden determinant, although it has not been excluded that this fragment represents aggregates of smaller subunits, thereby creating antigenic multivalency. These data are consistent with those obtained by Henney in the rheumatoid system (35). Using radiolabeling techniques, he was unable to show that IgG monomer complexed with rheumatoid factor. Also, the monomer did not inhibit rheumatoid factor precipitation with aggregated IgG. He invariably found denaturation accompanied by aggregate formation and increased rheumatoid factor precipitability. He interpreted these data to indicate that rheumatoid factor had specificity toward a hidden determinant revealed as a result of the structural unfolding accompanying denaturation of IgG.

Heremans and Heremans (36) have documented that 74% of IgM and 53% of IgA monoclonal proteins complex covalently to albumin. Although it is possible that a small number of these might have specific antibody activity, we do not believe that the majority represent antibodies to albumin. In this study, we have tested by gel precipitation a large number of monoclonal proteins of the IgM and IgA classes and have been unable to detect another protein that precipitated or showed hemagglutination with heat-aggregated albumin. Some of these proteins were selected because they demonstrated a small amount of covalently bound albumin, as previously described (14).

The L'ec IgM could be representative of a population of antibodies present in normal sera. These "normal" antibodies might function in the catabolism of macromolecules such as albumin. Similar considerations have been raised about the function of papain and pepsin agglutinators found in many normal human sera (37–39). In this regard, we have recently shown that cirrhotic sera contains significant titers of "antibody-like" activity in the Fab fragment towards heat-aggregated albumin. This will be the subject of a future communication.[1] According to this speculation, antigenic determinants normally sequestered in macromolecules could become exposed by denaturation resulting from ingestion, senescence, or metabolism of these molecules. Alternatively, cross-reactions between human and bovine or other species of albumin could occur as a result of enzymatic degradation of heterologous albumin in the gastrointestinal tract revealing one or more sequestered antigens. In this regard, Wright and Rothberg (40) have shown that new sites are exposed with pepsin digestion of bovine albumin and these determinants could show cross-reactions with those on the surface of native human albumin. There is precedence for such a hypothesis in the results of Milgrom and Witebsky (41). These workers demonstrated that injection of altered rabbit gammaglobulin gave rise to antibodies that cross-reacted with native human gamma globulin. Although we were unable to show cross-reactions with heat-aggregated bovine albumin or heated albumin of other species, additional methods such as proteolysis may be required and these are currently being pursued.

Our studies also raise the question of whether a significant proportion of human monoclonal proteins may have antibody activity toward other endogenous serum or tissue proteins. In investigating this question, it would be important to employ altered or aggregated anti-

[1] Tomasi, T. B., Jr. and S. Hauptman. Manuscript in preparation.

gens or both, since reactivity could be specific for the altered form of the antigen and may be difficult to demonstrate with the native antigen because of antigen univalency.

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