SUMMARY

The interaction between human Clq and immunoglobulins was measured quantitatively by determining the ability of IgG, IgM, and (Fc)\(_\mu\) to inhibit the binding of \(^{125}\text{I}\)-labeled Clq to IgM covalently linked to cyanogen bromide activated Sepharose. The following inhibition constants were determined: \(K\text{, for } \text{IgM} = 6.42 \times 10^{-4} \text{M}\); \(K\text{, for (Fc)\(_\mu\)} = 4.35 \times 10^{-4} \text{M}\); and \(K\text{, for IgG} = 1.10 \times 10^{-3} \text{M}\). The heat aggregation of IgM and IgG increased the ability of these proteins to bind \(^{125}\text{I}\)-labeled Clq, but had no significant effect on the binding properties of (Fc)\(_\mu\). The binding between the \(^{125}\text{I}\)-labeled Clq and the IgM-Sepharose complex was inhibitable with aromatic and alkyl diamino compounds. The most potent inhibitor was 2,5-diaminotoluene.

The binding of the human complement protein Clq to immunoglobulins is the initiating event in the complement sequence. Previous studies have demonstrated that Clq binds to IgM and IgG, and the binding site is located in the Fe region of the immunoglobulins (1-4).

We were interested in measuring quantitatively the binding of IgM and IgG by Clq, and in analyzing various small molecular weight compounds for their ability to inhibit the interaction. We found that these phenomena could be studied by using a system in which one of the components, IgM, was insolubilized by covalently coupling to cyanogen bromide activated Sepharose. It was then possible to measure the binding of \(^{125}\text{I}\)-labeled Clq to this complex, and to inhibit the binding using monomeric and aggregated IgM, IgG, and (Fc)\(_\mu\). Inhibition constants and relative free energy changes were calculated for the interaction of Clq with monomeric IgM, IgG, and (Fc)\(_\mu\). With this system it was also possible to study the inhibition of Clq-immunoglobulin interaction by various small molecular weight diamino compounds. The ability of the diamines to inhibit the binding appears to be related to the aromatic and alkyl nature of the compounds.

EXPERIMENTAL PROCEDURE

Protein Preparations—Human Clq was purified according to the procedure of Sledge and Bing (5). Human IgG was isolated from pooled human serum by chromatography on DEAE-cellulose (6) and by precipitation with \((\text{NH}_4)_2\text{SO}_4\) at 40% saturation (w/v). Human Waldenstrom macroglobulin was kindly supplied by Dr. Poulik, Beaumont Hospital, Detroit, Michigan, and was purified by anion exchange chromatography (7) and ultracentrifugation in a 10 to 40% sucrose gradient. The (Fc)\(_\mu\) fragment of IgM was prepared according to the method of Plaut and Tomasi (8). The IgM was digested with trypsin at 63°C for 8 min and the (Fc)\(_\mu\) fragment isolated from the mixture by gel filtration chromatography on Sephadex G-200.

Heat aggregation of proteins was performed at 63°C for 10 min in phosphate buffered saline (1). Protein concentrations were determined according to the procedure of Lowry et al. (9).

Sucrose Gradient Ultracentrifugation—Ultracentrifugation was conducted in a SW-27 rotor with a Beckman L2-65B ultracentrifuge. The sample was layered on a linear 10 to 40% (w/v) sucrose gradient buffered with 0.15 ionic strength Tris-HCl, pH 8.1, and centrifuged for 15 hours at 27,000 rpm. Fifty drop fractions were collected.

Organic Compounds—Ethylenediamine was purchased from Eastman Kodak Co., and 1,4-diaminobutane from K and K Laboratories, 1,7-diaminohexane, 1,8-diaminoctane, 1,10-diaminodecane, 1,12-diaminododecane, 1,4-diaminopiperazine dihydrate, 3,5-diamino-1,2,4-triazole tech., and 2,5-diaminotoluene dihydrochloride were obtained from Aldrich Chemical Co. The following compounds were recrystallized as the hydrochloride salt: 1,7-diaminohexane, m.p. 283°C (m.p. 285°C lit. (10)); 1,8-diaminoctane, m.p. 275-277°C (m.p. 274°C lit. (10)); 1,10-diaminodecane, m.p. 294°C (m.p. 288-289°C lit. (10)); 1,12-diaminododecane m.p. > 300°C. The 2,5-diaminotoluene di-
hydrochloride, m.p. 300°, and the 3,5-diamino-1,2,4-triazole, m.p. 209° (m.p. 206° lit. (10)) were recrystallized five times from ethanol. Thin layer chromatography (butanol-acetic acid-water, 120:30:50 v/v/v) was performed on all compounds. The compounds were prepared at a final concentration of 10^{-3} \text{M} in 0.075 ionic strength Tris-HCl, pH 8.1. 1,4-[4-C]Diaminobutane dihydrochloride was obtained from A. Merck-Searle Corp. This was diluted with unlabeled 1,4-diaminobutane to yield a specific activity of 50 \mu Ci per \mu mol.

**Preparation of IgM Sepharose Resin**—The coupling of IgM to Sepharose was performed according to the procedure of Crouch (11). Fifteen milligrams of settled Sepharose 4-B were mixed with 4.0 g of cyanogen bromide dissolved in 15 ml of H_2O and the pH maintained at 11 by addition of 4 M NaOH for 15 min or until the pH remained constant. The cyanogen bromide activated Sepharose was then reacted with 168 mg of IgM for 36 hours at 4° in 0.1 M sodium phosphate buffer (pH 7.0). The macroglobulin-Sepharose resin was washed extensively with 0.05 M ionic strength Tris-HCl, pH 8.1. The absorbance at 290 nm of the wash was determined, and using a F_1251^1 for 1.2 for IgM the amount of IgM bound to the resin was calculated. A more analytical determination of the amount of IgM bound to the resin involved the analysis of 1 ml of lyophilized resin for protein using a modified ninhydrin procedure (12).

**Oxidation of Clq—Clq was iodinated with Na_2^{125}I (New England Nuclear) according to the method of Helmkamp et al. (13), and had a specific activity of 1.1 x 10^6 cpm per mg.

**Binding Assays**—The binding of ^{125}I-labeled Clq to the macroglobulin-Sepharose resin was assayed using the following procedure: 0.2 ml of macroglobulin resin was pipetted from a stock solution, which was kept continually stirring to maintain the homogeneity, into a test tube containing 0.1 ml of an appropriate dilution of ^{125}I-labeled Clq. The mixture was incubated for 30 min with continual shaking in a water bath maintained at 37 ± 0.1°. The entire sample was then transferred to a suction filter apparatus and filtered on Whatman No. 1 filter paper. The sample test tube was rinsed three to four times with a total of 1.5 ml of buffer. Finally, the resin trapped on the filter paper was washed with 1.5 ml of buffer to remove residual unbound ^{125}I-labeled Clq. Pilot binding experiments were conducted with incubation times of 15 min at 37°. Extended incubation periods up to 1 hour did not result in any increase in binding of ^{125}I-labeled Clq, and thus for technical convenience 30 min was chosen as the time of incubation in all binding experiments. The resin and filter paper were counted in a Packard Gamma Liquid Scintillation Counter.

**RESULTS**

**Binding of ^{125}I-labeled Clq to IgM-Sepharose Resin**—The amount of macroglobulin bound to the Sepharose resin was determined by two separate procedures and the values agreed quite well. Based on the amount of protein present in the wash from the resin it was calculated that 5.2 mg of IgM had been coupled per ml of Sepharose. Analysis of the resin using the modified ninhydrin procedure yielded a value of 5.9 mg of IgM per ml of resin.

The results of two separate experiments on the interaction of ^{125}I-labeled Clq with the IgM resin are illustrated in Fig. 1. The agreement between the two sets of data is good and dissociation constants were determined to be 8.60 x 10^{-7} M and 7.04 x 10^{-7} M.

**Inhibition of Binding of ^{125}I-labeled Clq to IgM-Sepharose Resin by Immunoglobulins**—Due to the fact that the IgM-Sepharose resin was not characterizable in terms of the molecular form of IgM present on the resin, it was decided not to assume that the binding properties of the macroglobulin-Sepharose complex were wholly characteristic of IgM. Instead, the ability

\[
\frac{1}{[\text{Clq}_b]} = \frac{1}{[\text{Clq}_t]} \times \frac{K_d}{[\text{IgM}]} + \frac{1}{[\text{IgM}]}
\]

where [Clq]_b = bound Clq, [Clq]_t = free Clq, [IgM] = total number of IgM sites available for binding, and \(K_d\) = dissociation constant of the complex (14).\(^8\) Plots of (bound Clq) versus (free Clq) were linear and the intercepts at the abscissa and ordinate represent \(K_d^{-1}\) and (total IgM sites)\(^-1\), respectively.

Inhibition of the binding of ^{125}I-labeled Clq to the macroglobulin-Sepharose resin by IgG, IgM (Fc)\(_s\), and the heat-aggregated forms of these proteins was studied by incubating various concentrations of the proteins with a constant amount of ^{125}I-labeled Clq for 30 min at 37°. IgM-Sepharose resin (0.2 ml) was added to the mixture and incubation proceeded at 37° for 30 min. The sample was then filtered and counted. The following two tubes were included as controls: (a) ^{125}I-labeled Clq plus 0.2 ml of cyanogen bromide activated Sepharose (blank); and (b) ^{125}I-labeled Clq plus 0.2 ml of IgM-Sepharose. The amount of ^{125}I Clq bound was obtained by subtracting the counts per min in the blank tube (a) from the counts per min in the control (b) and the experimental tubes. Assigning a 100% bound value to control (b) the percent inhibition values were calculated according to the equation:

\[
\%\text{Inhibition} = \left(1 - \frac{\text{cpm experimental tubes}}{\text{cpm control tubes}}\right) \times 100
\]
of known molecular forms of IgM, (Fc)μ, and IgG to inhibit the binding of 125I-labeled Clq to the IgM-Sepharose complex was determined and used as a quantitative measure of the interaction between Clq and immunoglobulins. The sedimentation properties of the monomeric proteins used in these experiments were determined in a sucrose density gradient and IgM, (Fc)μ, and IgG exhibited sedimentation coefficients of 19 S, 11 S, and 7 S, respectively, when compared to known standards (Fig. 2).

The ability of IgM, (Fc)μ, and IgG to inhibit the binding of 125I-labeled Clq to the IgM-Sepharose complex is shown in Fig. 3. Both IgM and (Fc)μ were quite effective in binding the radiolabeled Clq and thereby inhibiting its binding to the IgM resin. The (Fc)μ, however, was slightly more effective on a molar basis as a binding agent than the IgM. IgG was capable of interacting with the 125I-labeled Clq, but it was a much poorer binding agent than either IgM or (Fc)μ. When various concentrations of bovine serum albumin were substituted for the immunoglobulins in the inhibition assay, no inhibition was detected. This strongly suggested that the inhibition exhibited by the immunoglobulins was not due to nonspecific protein-protein interactions.

Inhibition constants (Kᵢ) were determined according to Dixon Webb (15) using a previously determined 1/[IgM]. The ΔF' was calculated using the relationship ΔF' = -RT ln Kᵢ. Inhibition constants were calculated for IgG, IgM, and (Fc)μ, respectively (Table I). The ΔF' values ranged from 5.60 Cal per mole for IgG to 7.60 Cal per mole for (Fc)μ.

Heat-aggregated IgM, (Fc)μ, and IgG were also tested for their ability to bind the radio labeled Clq (Fig. 4). The heat aggregated IgG was a considerably more effective binding agent than the monomeric protein. Heat aggregation of IgM also increased its ability to bind Clq, however, heat treatment of (Fc)μ did not considerably alter its binding affinity for Clq.

Inhibition constants were calculated for IgG, IgM, and (Fc)μ, respectively (Table I). The AF' values ranged from 5.60 Cal per mole for IgG to 7.60 Cal per mole for (Fc)μ.

FIG. 2. Sucrose gradient (10 to 40%) ultracentrifugation of IgM, (Fc)μ, and IgG. Ultracentrifugation was conducted under the conditions described under "Experimental Procedures." IgM, ▲; (Fc)μ, ■; IgG, ●. Arrows indicate the sedimentation behavior of the marker proteins 7 S IgG, and 10 S IgM. The fractions (1.0 ml) were analyzed in a Hitachi-Coleman 101 spectrophotometer for their absorbance at 280 nm. The fraction number is plotted on the abscissa, and the A$_{280}$ nm is represented on the ordinate.

FIG. 3. The inhibition of the binding of 125I-labeled Clq to the IgM-Sepharose complex by IgM, (Fc)μ, IgG, and bovine serum albumin. The methods described under "Experimental Procedures" were used for the assay and the determination of percent inhibition of binding. IgM, ▲; (Fc)μ, ■; IgG, ●; bovine serum albumin, ○.

Heat-aggregated IgM, (Fc)μ, and IgG were also tested for their ability to bind the radio labeled Clq (Fig. 4). The heat aggregated IgG was a considerably more effective binding agent than the monomeric protein. Heat aggregation of IgM also increased its ability to bind Clq, however, heat treatment of (Fc)μ did not considerably alter its binding affinity for Clq.

Inhibition of Binding of 125I-labeled Clq to IgM-Sepharose Resin by Small Molecular Weight Amino Compounds—Previous studies had demonstrated that 1,4-diaminobutane was capable of inhibiting the binding of both macromolecular C1 and Clq (5, 16) to γ globulin. To investigate the nature of this inhibition more carefully it was decided to determine with which of the two...
Inhibition constants ($K_i$) and relative free energy values ($\Delta F'$) for IgG, IgM, and (Fc)$_\mu$

The $K_i$ and $\Delta F'$ values were calculated according to the procedures described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_i$</th>
<th>$\Delta F'$</th>
</tr>
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<tbody>
<tr>
<td>IgG</td>
<td>$1.10 \times 10^{-4}$</td>
<td>5.60</td>
</tr>
<tr>
<td>IgM</td>
<td>$6.42 \times 10^{-6}$</td>
<td>7.35</td>
</tr>
<tr>
<td>(Fc)$_\mu$</td>
<td>$4.35 \times 10^{-6}$</td>
<td>7.60</td>
</tr>
</tbody>
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Fig. 4. The inhibition of the binding of $^{125}$I-labeled Clq to the IgM-Sepharose complex by monomeric and heat treated IgM, (Fc)$_\mu$, and IgG. The heat treatment of the proteins and the assays were carried out as described under "Experimental Procedures." IgG, A; heat-treated IgG, B; IgM, C; heat-treated IgM, D; (Fc)$_\mu$, E; heat-treated (Fc)$_\mu$, F. All proteins were analyzed for inhibition at a concentration of 2 mg per ml.

proteins the 1,4-diaminobutane was interacting. A series of experiments were conducted in which 1,4-[4-$^4$C]diaminobutane was allowed to interact directly with either Clq or IgM. The amount of labeled 1,4-diaminobutane bound to these proteins was determined by precipitating the complex with 50% saturated (NH$_4$)$_2$SO$_4$. The results (Fig. 5) showed that Clq was capable of binding the 1,4-[4-$^4$C]diaminobutane, whereas the same molar concentration of IgM was unable to bind the amine. However, the binding of the amine to Clq could be inhibited by the preincubation of IgM ($2.0 \times 10^{-6}$ M) with Clq ($2.0 \times 10^{-6}$ M).

A set of experiments were performed to measure the inhibitory effect of various diaminoalkyl compounds on the binding of $^{125}$I-labeled Clq to the IgM Sepharose resin. Of the diaminoalkyl compounds tested those having 8, 10, and 12 carbons are the most effective inhibitory of binding (Fig. 6). At a concentration of $10^{-3}$ M their inhibiting capacities are approximately equal, however, the 1,12-diaminododecane was a much better inhibitor at a concentration of $10^{-4}$ M. The inhibition observed with 1,4-diaminobutane and 1,7-diaminoheptane at $10^{-2}$ M was similar, while ethylenediamine was the poorest of the alkyl amines in inhibiting the binding.

The relative abilities of 3,5-diamino-1,2,4-triazole, 2,5-diaminotoluene, and 1,4-diaminopiperazine to inhibit the binding of $^{125}$I-labeled Clq to the IgM resin are shown in Fig. 7. The most effective inhibitor compound tested was 2,5-diaminotoluene. At a concentration of $10^{-2}$ M it inhibited 75% of the binding, while 3,5-diamino-1,2,4-triazole and 1,4-diaminopiperazine inhibited the binding by 41% and 30.5%, respectively.

**DISCUSSION**

The purpose of the present study has been to obtain quantitative data on the interaction between immunoglobulins and Clq, and to analyze the ability of various diamines to inhibit this
thus provides a highly efficient Clq binding site. The important factor is that the complex exhibits a specific affinity for Clq and of the resin may resemble an antigen-antibody complex, and on the other hand, it can be speculated that the molecular nature saturating conditions of Clq, and one other worker has determined that for some macroglobulins the ratio is 0.3:1 (2). On estimate since it is based on a Clq : IgM molar ratio of 1:1 at spite of the precautions, we have calculated that approximately on IgM, the coupling reaction was done at pH 7.0 (11). In (Fig. 1, Experiment A). However, this calculation is a lower 10% of the IgM attached to the resin is available for binding rose is via free amino groups on the molecule being attached. Other studies have indicated that free ε amino groups on the immunoglobulin molecule are important in the interaction with Cl (16). To avoid preferential coupling via the ε amino groups the aflinity of these monomeric immunoglobulins for Clq. The inhibition observed with the diamines has yielded original data on a series of inhibitors of immunoglobulin-Clq interaction, and has provided the first observations on the nature of the Clq binding site.

The chemical fixation of IgM to Sepharose apparently does block many of the complement binding sites on the molecule. It should be recalled that cyanogen bromide coupling to Sepharose is via free amino groups on the molecule being attached. Other studies have indicated that free ε amino groups on the immunoglobulin molecule are important in the interaction with Cl (16). To avoid preferential coupling via the ε amino groups on IgM, the coupling reaction was done at pH 7.0 (11). In spite of the precautions, we have calculated that approximately 10% of the IgM attached to the resin is available for binding (Fig. 1, Experiment A). However, this calculation is a lower estimate since it is based on a Clq: IgM molar ratio of 1:1 at saturating conditions of Clq, and one other worker has determined that for some macroglobulins the ratio is 0.3:1 (2). On the other hand, it can be speculated that the molecular nature of the resin may resemble an antigen-antibody complex, and thus provides a highly efficient Clq binding site. The important factor is that the complex exhibits a specific affinity for Clq and this has been used to determine the relative capacity of Clq to bind to known molecular forms of immunoglobulins.

The inhibition constants determined for IgM ($K_i = 6.42 \times 10^{-4}$) and for IgG ($1.10 \times 10^{-4}$) clearly demonstrate that the IgM molecule is more effective in binding Clq. This difference is a direct reflection of the number of sites on the molecules capable of binding Clq. Since Clq binding to immunoglobulins is via the Fc portion of the molecule (3), the arrangement of the five Fc regions within IgM must provide a more suitable binding site for Clq than the one Fc position contained in IgG. The ($Fe$)$_m$ ($K_i = 4.35 \times 10^{-6}$) was observed to be slightly more effective in binding Clq than the parent IgM molecule. This finding agrees with a recent report by Plaut et al. (17), and suggests that the Clq binding sites in the native IgM molecule are not totally exposed.

Heat aggregation of IgM and IgG increased the capacity of these immunoglobulins to bind Clq, and the most significant change occurred with the IgG. The heat treatment probably increased the proximity of the Fc regions in the molecule, thus making conditions more favorable for binding Clq. Heat aggregation of immunoglobulins under these conditions has previously been shown to affect only the Fab regions (18). This observation is relevant to the present study since the heat treatment of ($Fe$)$_m$ did not alter its binding properties.

The results obtained in the present study agree well with the findings of Augener et al. (1). These investigators used a complement fixation test to assay the ability of various immunoglobulins to bind whole Cl, and showed that on a weight basis monomeric IgM was approximately 20 times more effective at binding Cl than monomeric IgG. The heat aggregated forms of these immunoglobulins were comparable in complement binding efficiency.

The finding that 1,4-[14C]diaminobutane interacts directly with Clq and inhibits its binding to macroglobulin is of considerable interest. This explains the inhibition phenomenon with 1,4-diaminobutane observed by other investigators (5, 16, 19), and provides a basis for studying other diamine compounds. The fact that the binding is exponential (Fig. 5) indicates there is no discrete binding site on Clq for 1,4-diaminobutane. This suggests that its inhibition of Clq-IgM interaction is mediated by the titration of anionic groups on the Clq.

When various diaminoalkyl compounds were tested for their ability to inhibit the interaction between 125I-labeled Clq and the IgM-Sepharose complex it was observed that 1,12-diaminododecane was the most effective inhibitor (Fig. 6). The inhibiting ability of these compounds appeared to be a function of the length of the hydrocarbon chain.

Of all the diamino compounds studied, 2,5-diaminotoluene was the most potent inhibitor of Clq binding (Fig. 7). At a concentration of $10^{-5}$ M this compound inhibited 75% of the binding. These findings suggest that in addition to the anionic groups there are hydrophobic groups on Clq which contribute to the interaction with the immunoglobulins. The nature of this hydrophobic interaction is dependent on structure as the triazole, a five membered ring, is less effective.

It is interesting to note that on a molar basis the IgM was only 18 times more effective in binding Clq than IgG (Table 1), while in humans the mean circulating serum concentration of IgG is 60 times higher than IgM (20). This suggests that the differences observed in the binding efficiencies of these two molecules may be overcome in the biological system by increasing the concentration of the weaker binding IgG. The physical form of the antigen to which IgG or IgM is bound is also impor-
tant in determining which immunoglobulin is the most effective. Ishizaka et al. (21) have shown that the binding of Cl by IgG-particulate antigen complexes is less effective than IgM-particulate antigen complexes. This situation, however, is reversed when a soluble antigen is employed.

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