

MECHANISMS UNDERLYING BINDING OF IMMUNE COMPLEXES TO MACROPHAGES*

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(Received for publication 18 August 1970)

Antigen-antibody complexes bind to the surface of macrophages even when little or no binding of antibody alone can be demonstrated (1-5). Binding of complexes is known to be mediated by the Fc region of the antibody molecules (2), and is inhibited by -Fc but not -F(ab')₂ fragments (6).

It has been postulated that complex formation might cause an allosteric change in the antibody molecule, analogous to that observed in electron micrographs by Feinstein and Rowe (7) and Valentine and Green (8) and inferred from physical data by others (9-12). This might result in exposure of a binding site for the macrophage surface, the implication being that combination of the -Fab portions of the antibody molecule with antigen produces a conformational change in the -Fc region. An alternative hypothesis, considered here, is that "free" antibody molecules may already have a binding site for the macrophage surface, but that binding is weak. Formation of a complex containing more than one antibody molecule, and thus more than one potential site of attachment, increases the strength of binding.

This paper reports our studies on this problem using monovalent, divalent, and polyvalent haptens and labeled purified antibody to the hapten. It was found that monovalent hapten and polyvalent hapten in antigen excess were unable to enhance the binding of antibody to macrophages, whereas polyvalent hapten and divalent hapten at equivalence could do so. Binding of complexes was inhibited by normal rabbit gamma globulin previously cleared of aggregated molecules. Bound complex was eluted from macrophages less easily than was bound antibody alone. These results support the hypothesis that the enhancement of antibody binding to macrophages in the presence of antigen is due to increased energy of binding resulting from summation of individual binding sites, rather than to the occurrence of allosteric change. In addition, saturation studies using ultracentrifuged normal

* Supported by grants from the National Institutes of Health (AI-09647, T01-AM-05604 and AI-0834) and by Contract Da-DA 17-67-7119 from the U.S. Army Medical Research and Development Command. Franco Quagliata is an Arthritis Foundation postdoctoral fellow.

rabbit gamma globulin permitted an estimation of the average number of binding sites for rabbit gamma globulin per alveolar macrophage. Preliminary reports of some of our findings have appeared (13, 14).

Materials and Methods

New Zealand rabbits were injected two to five times intravenously over a period of from 1 to 5 months with 0.1 ml of complete Freund adjuvant (Difco Laboratories, Detroit, Mich.) 4-6 days after the last injection, they were exsanguinated from the heart, their lungs were removed and immediately washed out with 300 ml of ice-cold 0.85% w/v sodium chloride solution (saline). For some experiments peritoneal macrophage-rich exudates were induced with mineral oil as previously described (1, 14). No significant difference in the capacity of these and of lung macrophages to bind antibody or antigen-antibody complexes was noted, but rigorous comparative experiments pertaining to this point were not made.

The cells were washed three times and suspended in medium 1066 (Grand Island Biological Co., Grand Island, N.Y.) buffered with 0.01 M tris (hydroxymethyl) aminomethane (Tris) phosphate, pH 7.2, containing 20% fetal calf serum (inactivated at 56°C for 30 min). Suspensions of 2×10^7 macrophages/ml were 70-85% pure; they also contained 10-20% small lymphocytes and 2-10% polymorphonuclears. The contribution of antibody binding made by nonmacrophages was ignored not only because of their small numbers but also because only 10-20% of small lymphocytes bind complexes (1, 3) and polymorphonuclear cells bind about half as much complex per cell as macrophages (14).

Purified rabbit anti-benzylpenicilloyl antibody (anti-BPO)¹ was prepared as previously described (14, 15). It was 90% specifically precipitable and showed a line of precipitation corresponding to IgG only on immunoelectrophoresis against goat anti-rabbit whole serum. The purified antibody was labeled with ¹²⁵Iodine (New England Nuclear Corp., Boston, Mass.) by the iodine monochloride method (16) using 1 mCi/mg of protein; approximately one atom of iodine was bound per molecule. Unbound radioactivity was removed by passage through Dowex 1- X 4 followed by dialysis against saline. 96-98% of the radioactivity was precipitable in cold 5% trichloroacetic acid. The specific activity of the ¹²⁵I anti-BPO varied from 20,000 to 50,000 cpm/ μ g at the time of use.

Normal rabbit gamma globulin (RGG) (Pentex Biochemical, Kankakee, Ill.) was dissolved in saline. Both the labeled antibody and the RGG were centrifuged at 84,000 g_{max} for 1 hr immediately before use in order to remove any possible aggregated material. The supernatant was removed and the bottom 0.2-0.3 ml was discarded. The protein concentrations were determined by optical density measurement at 280 $m\mu$ using $E_{1\%}^{1\text{cm}}$ RGG of 15.

A polyvalent hapten, succinylated benzylpenicilloyl-poly-L-lysine (BPO₉₃-PLL₄₀₂S); a divalent hapten, benzylpenicilloyl-hexamethylenediamine (BPO₂HMD); and a monovalent hapten, benzylpenicilloyl-propylamine, (BPO₁Prop) were all prepared as described previously (17, 18). An oligovalent hapten benzylpenicilloyl-heptalysine (BPO₆Lys₇) was prepared as described (18).

The haptens were diluted in buffered saline containing a chelating agent to prevent rearrangement and aggregation, 0.14 M NaCl, 0.02 M Tris, 10^{-4} M ethylenediaminetetraacetic acid, pH 8.2, (TBS-EDTA) (18). The labeled purified antibody was diluted in medium containing 20% fetal calf serum. The latter was present in all experiments both to prevent loss

¹ *Abbreviations used in this paper:* BPO, benzylpenicilloyl; BPO₉₃-PLL₄₀₂S, succinylated benzylpenicilloyl-poly-L-lysine; LYS₇, heptalysine; HMD, hexamethylenediamine; Prop, propylamine; RGG, rabbit gamma globulin.

of the very dilute antibody by denaturation and adsorption to glass, and to limit its non-specific adsorption to the cell surface so that it was bound at sites specific for gamma globulins.

The experiments were carried out as follows: duplicate or triplicate 1 ml volumes of medium containing macrophages, or an equal packed cell volume of three times washed packed cell sheep erythrocytes (from the N.Y.C. Department of Health), or no cells were incubated with 0.1 ml of hapten solution or diluent alone and 0.1 ml containing 2 μ g of ^{125}I anti-BPO. In experiments in which RGG was used to inhibit binding, 0.1 ml volumes of RGG and of anti-BPO were mixed before addition to the cells. Incubation was for 10 min at 37°C or for 20 min at 4°C as described in the text. For low temperature incubations, the haptens and ^{125}I anti-BPO were preincubated at 37°C for 10 min and then cooled in ice water before addition to the cells. The cells were maintained in suspension by periodic shaking. After incubation, the mixtures were diluted by the addition of 2 ml of cold medium and were centrifuged at 1200 g at 4°C for 6 min. The supernatant was removed and the tubes were washed four times with 2 ml of cold medium. This was sufficient to reduce the count to background in the control tubes containing erythrocytes. Other washing procedures are described in the text. The sedimented cells were counted in a Nuclear Chicago autogamma counter (Nuclear-Chicago, Des Plaines, Ill.) whose background in the ^{125}I channel was 14 cpm. Counts bound were not considered significantly different from one another unless the difference for each member of a pair of duplicates was greater than twice the standard deviation of the difference.

RESULTS

Effect of Hapten on the Binding of Antibody to Macrophages.—In the first experiments, the binding of ^{125}I anti-BPO to macrophages in the presence of various haptens was studied. Representative results are shown in Table I.

Incubation of macrophages with ^{125}I anti-BPO alone resulted in significant binding of radioactivity, detectable after four washes, which were sufficient to remove the antibody from an equal packed-cell volume of sheep red blood cells. The amount bound varied from 6.3 to 33.1 ng of antibody per 2×10^7 macrophages in seven experiments, that is between 0.3 and 1.6% of the gamma globulin available. Binding was observed whether the incubation was carried out at 37°C or at 4°C. A marked increase in the amount of antibody bound was observed when polyvalent hapten and antibody were present at equivalence, and a smaller increase was observed when divalent hapten was present at equivalence. However, when divalent hapten was present at 1000-fold excess, the increase in binding was only slight (experiment C) or did not occur (experiment A). The presence of monovalent hapten at various concentrations (from 0.1 to $1000 \times \text{mEq}$) did not increase antibody binding. The increase in binding of antibody caused by the presence of polyvalent hapten was to a considerable extent inhibited by the addition of either monovalent or divalent hapten at 1000-fold molar excess. Thus, the failure of the monovalent hapten to enhance the binding of antibody to macrophages was not due to a failure of antibody-hapten interaction. The monovalent hapten did not interfere with the binding of antibody to macrophages, since the amount of antibody bound was not less than that which bound in the complete absence of hapten.

These results favor the idea that the increased binding of antibody in the

presence of polyvalent hapten is due to lattice formation, and that this can be prevented by the addition of excess specific monovalent or divalent hapten.

To investigate further the effect of lattice formation on the binding of antibody, different concentrations of hapten were tried. For these studies an oligovalent hapten, BPO₆Lys₇ was used, as precipitation studies (19) had shown that a very sharp equivalence point could be obtained with this hapten and anti-BPO antibody.

TABLE I
The Binding of ¹²⁵I Anti-BPO to Macrophages in the Presence or Absence of Polyvalent, Divalent, and Monovalent Hapten

Antibody added 2 μg ¹²⁵ I anti-BPO	Hapten added						Nanograms of antibody bound		
	BPO ₉₃ PLL ₄₀₂ S		BPO ₂ HMD		BPO ₁ Prop		Experiment		
	0.02*	2.0	1.0‡	1000	2.0	2000	A§	B	C
+	—	—	—	—	—	—	13.8	6.3	31.0
+	+	—	—	—	—	—	178.0	168.0	171.0
+	—	+	—	—	—	—	N.D.	N.D.	83.0
+	—	—	+	—	—	—	29.0	N.D.	129.0
+	—	—	—	+	—	—	13.0	N.D.	48.0
+	—	—	—	—	+	—	N.D.	7.2	32.0
+	—	—	—	—	—	+	N.D.	N.D.	33.0
+	+	—	—	+	—	—	N.D.	N.D.	62.0
+	+	—	—	—	—	+	N.D.	39.5	92.0

* Hapten-antibody molar ratio at equivalence as determined from quantitative precipitation curves.

‡ Hapten-antibody molar ratio.

§ In experiments A and B, peritoneal macrophages were used and incubation was for 10 min at 37°C. In experiment C, alveolar macrophages were used and incubation was at 4°C for 20 min.

|| N.D., not done.

From Fig. 1 it can be seen that the binding of antibody to macrophages was optimal in the region of slight antibody excess. These conditions favor lattice formation. In regions of extreme antigen excess, antibody binding occurred to no greater extent than when hapten was absent from the system. Thus, when an oligovalent hapten was used in extreme antigen excess where it combined with antibody as though it were monovalent, it behaved like a monovalent hapten in failing to enhance the binding of antibodies to macrophages.

Inhibition of Binding of Complexes by Monomeric Immunoglobulin.—The next experiment concerned the ability of unaggregated RGG to inhibit the binding of complexes to macrophages. If the enhanced binding of complexes was due to a summation of binding sites already present on the antibody molecule,

then RGG would be expected to inhibit the binding of complexes to macrophages. If the enhancing effect of polyvalent hapten was through induction of allosteric change, then RGG would be expected to be relatively ineffective in inhibition.

Various concentrations of RGG, ultracentrifuged to remove aggregates, were therefore mixed with 2 μg of ^{125}I anti-BPO and 6×10^{-13} moles

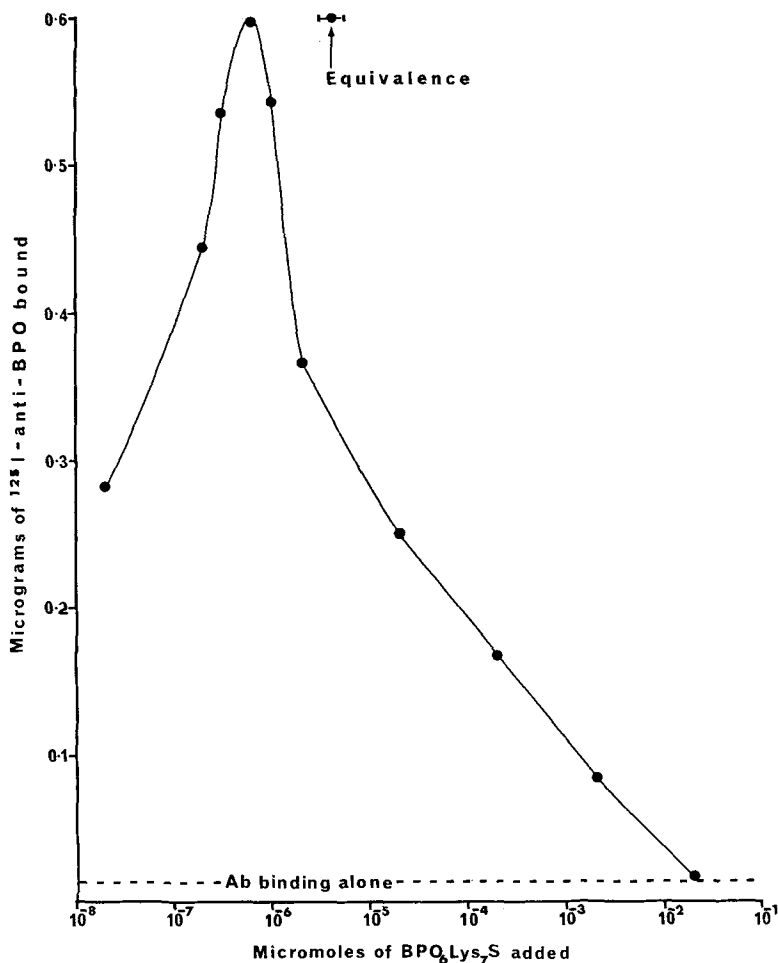


FIG. 1. Effect of different concentrations of oligovalent hapten on the binding of ^{125}I anti-BPO to rabbit alveolar macrophages. 2 μg of antibody and 2×10^7 macrophages were present in the 1.2 ml of reaction mixture. Incubation was at 37°C for 10 min and the cells were washed four times. Points represent averages of duplicate values. The region of equivalence, as determined from quantitative precipitation curves (19) is also shown.

of BPO₆Lys₇, and incubated at 37°C with 2×10^7 alveolar macrophages. The results are shown in Fig. 2.

It was found that almost complete inhibition of binding of the complex was obtained in the presence of sufficient RGG. Note that 50% inhibition was achieved with only 50 μ g of RGG. Thus, it was concluded that unaggregated RGG molecules have an exposed binding site for macrophages.

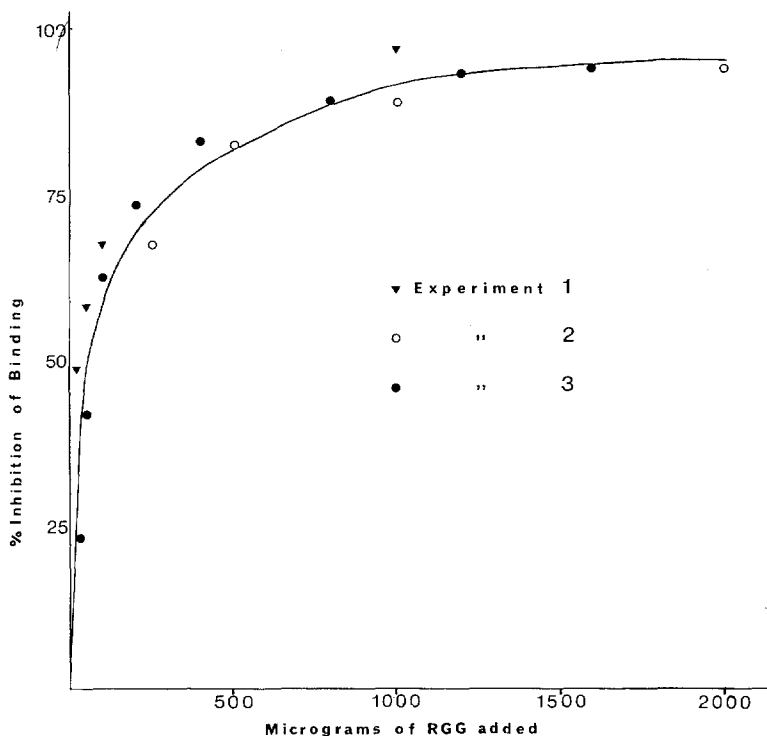


FIG. 2. Inhibition of binding of hapten-antibody complex to alveolar macrophages by ultracentrifuged RGG. 2 μ g of 125 I anti-BPO and 6×10^{-13} moles of BPO₆Lys₇ were present in the reaction mixtures which were incubated at 37°C for 10 min. The cells were washed four times.

The next and subsequent experiments were all conducted at 4°C to minimize possible interiorization of antibody and complexes.

Dissociability of Complexes or Antibody from Macrophages.—It was next investigated whether antigen-antibody complexes were less easily dissociable from macrophages than uncomplexed antibody. If so, it would be expected that repeated washes would elute antibody from the cells more rapidly than complexes.

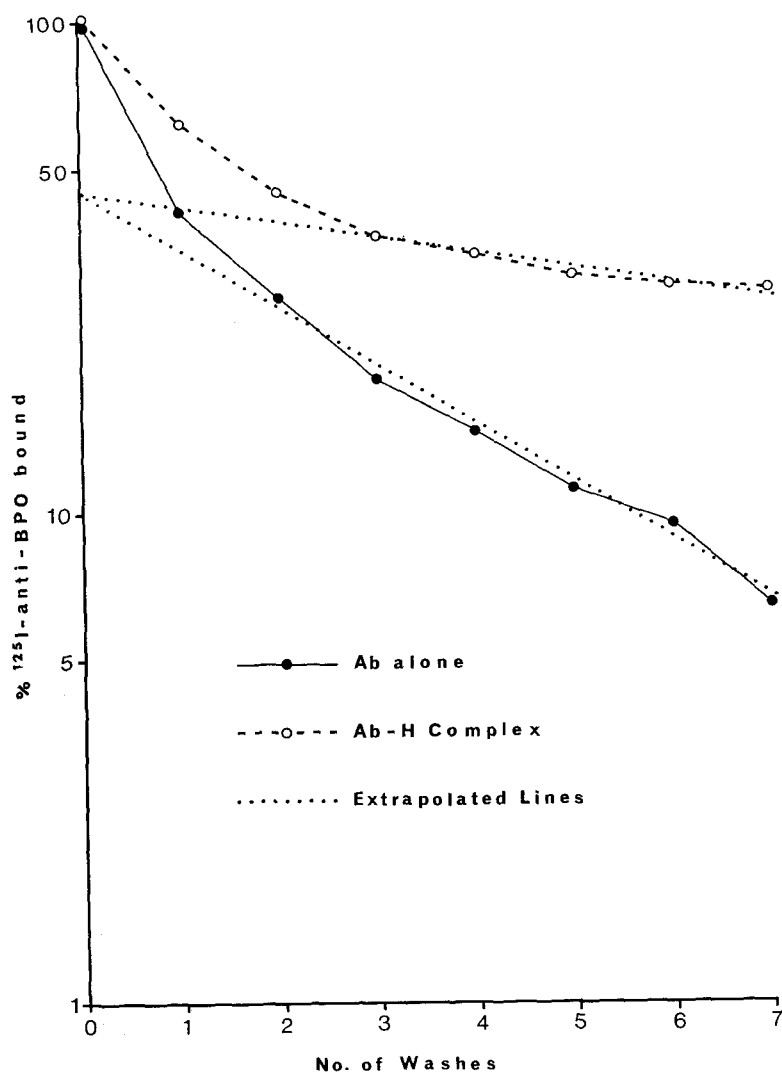


FIG. 3. Elution of antibody and BPO_6Lys_7 -antibody complex bound to 2×10^7 alveolar macrophages at 4°C by different numbers of washes. The radioactivity remaining in tubes not containing cells has been subtracted. The points represent averages of duplicate values.

Tubes containing 2×10^7 alveolar macrophages were incubated at 4°C for 20 min with either $2 \mu\text{g}$ of ^{125}I anti-BPO and 6×10^{-13} moles BPO_6Lys_7 , or $10 \mu\text{g}$ ^{125}I anti-BPO and diluent. (The amount of antibody in the latter tubes was increased 5-fold to ensure that there would be amounts of radioactivity adequate for assay still bound after several washes). The cells were

then washed from 0 to 7 times with 1 ml of cold medium and the radioactivity associated with the cell pellets counted. Radioactivity adherent to glass in tubes not containing cells was subtracted. More than 90% of this glass-bound radioactivity was removed by the first wash. The counts per minute remaining after each wash were expressed as a percentage of those associated with unwashed cell pellets. As can be seen in Fig. 3, the proportion of complex removed as a function of washing was less than that of antibody bound alone. Thus, the complex was bound more strongly. After the third wash, the proportion of either complex or antibody alone removed at each wash tended to be constant, as indicated by the straightness of the lines in the semilogarithm plot, i.e., a first order decay curve. These lines can be extrapolated back to the ordinate

TABLE II
Absorption of ^{125}I Anti-BPO by Successive Incubations with Rabbit Alveolar Macrophages

Absorption	cpm bound	Per cent of available radioactivity
Cell pellet	51,972	48.4
Control	7,958	7.4
Cell pellet	19,759	35.7
Control	6,476	6.5
Cell pellet	14,462	40.6
Control	8,865	9.5

Input cpm 107,343 = 2 μg of ^{125}I anti-BPO. Final supernatant (cells), 18,960 cpm; final supernatant (control), 80,457 cpm.

where they meet at the 45% point, indicating that approximately 55% of the activity associated with the unwashed cell pellet was probably not bound tightly by the membrane, or that there was a heterogeneity of binding sites varying in affinity for the antibody site.

Heterogeneity of Antibody Molecules with Regard to Binding to Macrophages.—The next question considered was whether all, or only a subpopulation of ^{125}I anti-BPO molecules could bind to macrophages. If all the molecules could bind, it should be possible to absorb out all the radioactivity by adding sufficient numbers of macrophages. 2 μg of ^{125}I anti-BPO was therefore incubated at 4°C for 20 min with 2×10^8 packed alveolar macrophages or in a control tube without cells. After centrifugation the supernatants were decanted into similar tubes and the incubation was repeated. After a third similar absorption, the radioactivity in supernatants, sedimented cells, and control tubes were counted. The results are shown in Table II.

As can be seen, approximately the same proportion, i.e. 35.7–48.4% of the available radioactivity, was absorbed by the cells and 6.5–9.5% by the glass

at each transfer. Overall, 82.3% of the radioactivity initially added was removed by the cells, and 25% by the glass. It thus appears that if the absorption had been carried out a sufficient number of times, all the antibodies could have been absorbed. However, trapping of antibody between cells may have accounted for some of the antibody apparently bound by the cells.

Number of Binding Sites for IgG on Alveolar Macrophages.—Finally, an attempt was made to determine the number of sites per macrophage capable of binding antibody. To do this it was necessary to saturate all the binding sites

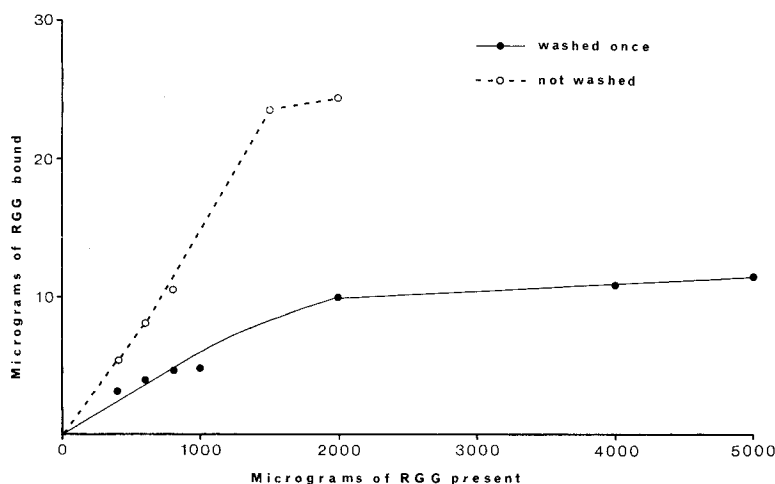


FIG. 4. Saturation of binding sites for RGG on alveolar macrophages, 2×10^7 cells were used; incubation was at 4°C . In one experiment the cells were washed once after centrifugation and in the other they were not washed.

on the cells and to calculate the numbers of molecules of antibody bound when the cells were saturated. This depended on the assumption that all molecules in normal rabbit gamma globulin preparations are potentially capable of binding. The previous experiment gave support for this assumption. To find the concentration of RGG at which all the binding sites on macrophages were saturated, several different concentrations of ultracentrifuged RGG mixed with ^{125}I anti-BPO (to act as a radioactive marker for bound gamma globulin) were incubated at 4°C with macrophages or in control tubes without cells. After centrifugation, the supernatants were removed and in one experiment the tubes were allowed to drain on to absorbent paper. In the other experiment the tubes were washed once with 1 ml of medium and recentrifuged before draining. The counts per minute in the control tubes (without cells) were subtracted from those in the respective experimental tubes. It was assumed that the ^{125}I anti-BPO molecules would not be distinguished from

normal RGG molecules, thus the per cent of radioactivity bound represented the per cent of total RGG available in each tube. The results of the two experiments are plotted in Fig. 4.

It can be seen that at very high concentrations of RGG, plateaux of IgG bound were reached. In the experiment in which the cells were not washed, the highest point on the plateau showed that 24.3 μg of RGG were bound by 2×10^7 macrophages. From the extrapolated lines derived in the washing experiment (Fig. 3), only 45% of this should be considered bound. In the second experiment, the highest point reached on the plateau was 11.4 μg bound. From the washing experiment, after one wash 41% of the amount bound without washing was obtained. Thus by multiplying $24.3 \times 45/100$ and $11.4 \times 45/41$, the amounts bound specifically were calculated. In the two experiments, 10.9 and 12.5 μg of IgG were bound by 2×10^7 macrophages. From these figures, using Avogadro's number and taking the molecular weight of RGG as 160,000, 2.05 and 2.35 million molecules were calculated to be bound per cell.

DISCUSSION

The evidence presented in this paper supports the concept that the increased binding of antibody to macrophages in the presence of antigen is due to a summation effect and not to the exposure of new binding sites on the antibody molecule as a result of allosteric change. This evidence consists of the following observations: (a) Antigen-antibody interaction per se did not enhance antibody binding to macrophages. Binding was enhanced only when hapten of more than two valences was present. (b) Monovalent hapten not only failed to enhance binding, but inhibited the enhancement due to polyvalent hapten. (c) Enhancement was maximal when oligovalent hapten and antibody were mixed in the region of equivalence to slight antibody excess, that is under conditions which favor lattice formation, and was markedly diminished in antigen excess, a situation in which the hapten behaves as if it were monovalent. (d) Binding of complexes appeared to be stronger than that of antibody alone as demonstrated by the slower rate of elution by washing.

The idea that the enhancement of binding in the presence of antigen is due to summation of binding sites on the surface of antigen-antibody complexes is reinforced by the demonstration that both ^{125}I anti-BPO and normal RGG molecules, ultracentrifuged to remove aggregates, can bind. From this we conclude that they already have exposed binding sites for macrophages. While we cannot absolutely exclude that aggregates could have reformed after centrifugation, the fact that the ^{125}I anti-BPO washed off the cells at a greater rate than hapten-antibody complex suggests that if there were any aggregates involved they must have a lower energy of binding than antigen-antibody complexes.

Although our evidence supports the idea that the enhancement of antibody binding to macrophages after complex formation results from summation of

individual binding sites, we cannot exclude the possibility that an allosteric change may take place within a complex, thus exposing additional binding sites. The fact that both normal gamma globulin and labeled antibody display binding suggests that new binding sites are not essential. This is not to deny that conformational changes do take place within antibody molecules upon combination with antigen; indeed they have been observed in electron micrographs (7, 8) and can be inferred from physical data (9, 10) and from the appearance of new properties of the molecule (11, 12). The question is whether these changes have any significance for the binding of antibody to macrophages. Similar questions have been raised with respect to the requirement for two adjacent 7S antibody molecules for the fixation of complement (20-22). We have no information pertaining to the suggestion that such conformational changes of cell-bound antibody are important in the induction of immunity or paralysis in lymphocytes (23).

We do not know whether all or only some rabbit gamma globulin molecules have a binding site for macrophages. Existing evidence on the structure of rabbit 7S IgG suggests that most of it belongs to the same subclass at least with respect to intrachain disulphide bonds (24), but a distinct IgG₁ subclass has been described (25). The known difference in galactosamine residues (30% of rabbit IgG has a galactosamine bound to a threonine on the Fd side of the hinge region [26]) is unlikely to be related to binding to macrophages, since this is a function of the Fc part of the molecule (2, 6). We studied the question by absorption of anti-BPO with macrophages. While our results are consistent with the idea that the major portion of the IgG can bind, they do not prove the point. However, assuming both homogeneity of rabbit IgG with respect to its ability to bind to macrophages, and a binding site on all macrophages, calculation of the average number of binding sites per macrophage was possible. Using macrophages from two different rabbits, the number of sites per alveolar macrophage was approximately 2×10^6 .

SUMMARY

The mechanism of binding of immune complexes to macrophages was investigated using purified antibody and haptens of different valences. Antibody alone bound to macrophages; enhancement of binding occurred when polyvalent and divalent haptens were present at equivalence but did not occur in great antigen excess. Monovalent hapten did not increase the binding of antibody at any concentration ratio tried, though it inhibited the enhancement due to oligovalent hapten. Ultracentrifuged normal rabbit globulin also inhibited the binding of complexes indicating the presence of exposed binding sites on the uncomplexed molecules. Complexes bound more strongly than antibody alone as determined from elution studies. These results support the hypothesis that the enhancement of antibody binding to macrophages in the presence of

antigen is due to increased energy of binding resulting from summation of individual binding sites already exposed on the antibody molecules.

It was also possible, by saturating the macrophages with gamma globulin, to estimate the number of binding sites per cell; this was calculated to be approximately 2 million per alveolar macrophage.

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