

ASSOCIATION AND DISSOCIATION OF AGGREGATED IgG FROM RAT PERITONEAL MACROPHAGES*

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Mononuclear phagocytes in the reticuloendothelial system (RES)¹ can clear certain soluble antigen-antibody complexes from the circulation of experimental animals (1-4). The chief clearance mechanism is thought to be for complexes containing intact IgG molecules (4) to bind to Fc receptors known to be present on the surface of many mononuclear phagocytes (5, 6). Such binding of immune complexes to macrophages can be inhibited *in vitro* with monomeric IgG and with free Fc fragments, but not with Fab fragments (7, 8). Changes in the contact angles of antigens caused by combination with antibodies may contribute to binding (9, 10), and complement (C) and specific receptors for C3b and C3d (11, 12) may also play a role *in vivo*.

By measuring the small quantities of monomeric IgG bound at different concentrations by macrophages, the number and affinity of Fc receptors has been estimated for rabbit alveolar macrophages (5) and for mouse macrophages (13). Unkeless and Eisen have also examined association and dissociation kinetics of various IgG subtypes attaching to mouse macrophages (13). However, precise kinetic studies with immune complexes have not been possible because stable immune complexes of known size (and number) are not available.

Heat-aggregated IgG (A-IgG) possesses many biological activities similar to immune complexes. A-IgG can fix C (14), induce an Arthus reaction (15), and is cleared from the circulation of mice (16) and rats (17)² by the RES in a manner similar to immune complexes. A-IgG can also compete with immune complexes in binding assays used to detect immune complexes (18). A-IgG therefore can be used as a model of soluble immune complexes.

Stable A-IgG of various molecular weights have recently been isolated by ultracentrifugation in sucrose gradients containing supporting protein.³ Homologous A-IgG prepared in this manner have been used in the present studies to examine the kinetics of A-IgG binding to peritoneal macrophages from normal male rats. The findings suggest that attachment of larger A-IgG at multiple sites by Fc receptors on mononuclear phagocytes can account for more avid binding and slower dissociation of larger A-IgG.

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¹ *Abbreviations used in this paper:* A-IgG, aggregated IgG; PBS-BSA, phosphate-buffered saline containing 0.5% bovine serum albumin; RES, reticuloendothelial system; TBH, Tris-buffered Hanks' solution; TBH-RSA, TBH containing 0.5% rabbit serum albumin.

² Kijlstra, A., A. Van der Lelij, D. W. Knutson, and L. A. van Es. 1977. Glomerular localization of aggregated IgM in rats after administration of carbon. Manuscript submitted for publication.

³ Knutson, D. W., A. Kijlstra, H. Lentz, and L. A. van Es. 1977. Isolation of stable aggregates of IgG by zonal ultracentrifugation in sucrose gradients containing protein. Manuscript submitted for publication.

Materials and Methods

Preparation of A-IgG. IgG was isolated from normal rat serum by 50% saturated ammonium sulfate precipitation followed by column chromatography with DEAE Sephadex. IgG was radiolabeled with ^{125}I to a specific activity of 0.1 mCi/mg by the chloramine-T method (19). Concentrations of IgG were determined by optical absorption at 280 nm using an extinction coefficient ($E_{1\%}^{1\text{cm}}$) of 14.7. Aggregation of ^{125}I -IgG was performed by heating 6.88 mg for 25 min at 63°C in 0.8 ml of 0.1 M borate-buffered saline (pH 7.4).

Aggregated ^{125}I -IgG (A-IgG) were separated according to size by ultracentrifugation for 2 h at 269,000 g in 12.0-ml 10–30% wt/vol sucrose gradients. The homogeneity of the various sized A-IgG from different fractions was preserved by isolating A-IgG in gradients made with Tris-buffered Hanks' solution (TBH) containing 0.5% bovine serum albumin (pH 7.4 at 4°C) and then storing A-IgG in the same protein-containing buffer at -20°C .³ The approximate mean number of IgG molecules per aggregate for A-IgG in each different fraction was calculated from the sedimentation coefficient (S rate) as previously described (20).³ The molar concentration of A-IgG was then calculated for each fraction from the molecular weight and specific activity. All of the data presented in this paper were obtained using aggregates pooled from two gradients of a single ultracentrifugation.

A-IgG were thawed the day before use and dialyzed against TBH for 12 h to remove sucrose present from ultracentrifugation and to further reduce the small amounts of free ^{125}I present. Greater than 97% of ^{125}I was thus TCA precipitable in all preparations at the time they were used. A-IgG were always centrifuged at 3,000 g for 15 min immediately before any use to insure that only soluble material was present. The size distribution, homogeneity, and stability of the various A-IgG isolates were checked by periodic reultracentrifugation which always showed single narrow peaks in identical position in the gradient from where the A-IgG had originally been isolated. There was minimal formation of pelletable material and/or dissociation of A-IgG into free IgG (usually <5% total).

Macrophage Isolation and Characterization. Peritoneal cells were obtained from normal, young, male Wistar rats (225–275 g) by lavaging the peritoneal cavity of freshly killed animals with 15.0 ml of TBH. Rats were killed by opening their chests while under ether anesthesia, and 5–15 rats were used for each experiment. Cells were centrifuged at 1,000 rpm for 10 min and resuspended in TBH containing 0.5% rabbit serum albumin (TBH-RSA) (pH 7.4 at 37°C). Preparations showing any red color due to contaminating red cells were discarded.

The pooled cells were counted in a hemocytometer. Mean cells per rat varied from 1.25×10^7 to 2.1×10^7 in different experiments. Cells were serially diluted (maximum binding experiment only) or adjusted to 1.0×10^6 cells/ml in TBH-RSA. In all experiments, 1.0-ml samples were then preincubated for 2 h at 37°C in new, washed, lightly siliconized autoclaved, round-bottom 12-ml glass tubes to allow macrophages to adhere. Tubes were lightly siliconized to prevent nonspecific sticking of A-IgG to glassware.³

After preincubation, tubes were agitated and nonadherent cells were aspirated. Periodic counts of aspirates revealed that 40% of cells adhered. Studies with similarly prepared glass cover slips showed that greater than 90% of the adherent cells ingested latex particles and were esterase positive with α -naphthyl butyrate substrate (21). Control tubes, which were included in all experiments, were preincubated with buffer alone or with 5×10^6 sheep red blood cells and were otherwise treated exactly as were the macrophage-containing tubes.

Experimental Protocol. The protocol in all experiments was to add 50 μl of TBH-RSA (pH 7.4 at 4°C) to aspirated cell-containing tubes and place the tubes on ice. A-IgG (0.01–10.0 μg in 50 μl TBH-RSA) were then added and the tubes were incubated for appropriate times with continuous shaking at 4°C. After incubation, phosphate-buffered saline and 0.5% bovine serum albumin (PBS-BSA) (pH 7.4) was added and the tubes were centrifuged at 3,000 rpm for 10 min. The tubes were aspirated and the supernates saved. Cells were then washed with 0.5 ml PBS-BSA followed by 1.0 ml, again saving the washes. Control experiments showed that additional washes removed negligible quantities of ^{125}I from both cell-containing and control tubes. All tubes were counted for ^{125}I activity in an automatic well-type gamma counter along with appropriate standards. Nonspecific sticking was less than 0.5% in all control tubes; never-the-less, the cell-associated counts were always corrected for the nonspecific counts found in their controls. All cell tubes were in triplicate while controls were either single or duplicate.

Equilibrium experiments were done with incubations of 24 h. Preliminary experiments showed

that equilibrium between bound and free A-IgG was clearly reached by this time. TCA precipitation performed on representative samples in each experiment showed no increase in nonprecipitable ^{125}I , which indicates that no catabolism of A-IgG had occurred during the experiment (22).

Calculation of the Area on a Cell Surface Occupied by A-IgG at Cell Saturation. Polyethylene cylinders cut to have the approximate proportions of an IgG molecule (23) were placed in distensible plastic bags in a random fashion and the bag was manipulated so that the aggregate shape of each bag of cylinders approximated a sphere. The volume of each sphere was then measured by fluid displacement. A linear relationship was observed between total volume and numbers of cylinders. The proportionate volume of A-IgG containing different numbers of IgG molecules were then calculated assuming that IgG molecules had a cylindrical shape 105 Å long and 40 Å in diameter (23) and assuming that the space between IgG molecules was proportionate to that observed with the model. The area occupied on the cell surface by each A-IgG was assumed to be the area subtended by each sphere, i.e., the area of a circular plane passing through the center of a spherical A-IgG. This was calculated for each different size A-IgG studied from the assumed volume. The percent cell surface occupied by various A-IgG at cell saturation was then calculated from the measured maximum number of A-IgG bound per cell and an estimated total surface area of an unstimulated peritoneal macrophage (1,000 μm^2) (13).

Results

Binding of A-IgG by Excess Cells. In order to assess the maximum percent of A-IgG which could be bound by cells, small equimolar doses of A-IgG isolates were incubated for 24 h at 4°C with 0.1×10^6 to 2.0×10^6 adhering peritoneal macrophages from normal rats. The percent cell associated at the end of this time is plotted against the number of cells in Figs. 1 A and B. Numerical data from these experiments are summarized in Table I.

Larger aggregates had higher percents bound (Fig. 1 A). This was true despite the fact that the doses of larger aggregates contained greater quantities of IgG protein. As cell number increased, the percent bound seemed to approach a maximum for all four A-IgG, the larger A-IgG having the higher maximum percents.

In Fig. 1 B, the straight line extrapolations to the ordinate (infinite cell concentration) further supported these impressions though differences were small between the three larger A-IgG (Table I). Clearly, large proportions of all four aggregate preparations were "bindable."

Equilibrium Constants (K_e) and Maximum Number of A-IgG Bound Per Cell. Adherent cells (0.4×10^6 per tube) were incubated for 24 h at 4°C with various A-IgG isolates in doses ranging from 3.0 to 2,000 fmol (0.01–8.0 μg of IgG). Cell-associated ^{125}I corrected for nonspecific sticking ranged from 55% maximum for low doses down to 6% as saturating doses of A-IgG were approached. Scatchard plots of the moles A-IgG bound/ 0.4×10^6 macrophages (r) and the molar concentration of free bindable A-IgG (c) were constructed from the relation $r/c = n \cdot K_e - r \cdot K_e$ (24). The value n represents the limiting value for r as c becomes very large, i.e., the maximum A-IgG bound at saturation. Plots for four different A-IgG are seen in Fig. 2 and derived values for K_e and n are summarized in Table II. Values for c were corrected for maximum A-IgG bindable as determined in the preceding experiments. The differences among the various K_e 's were diminished by this correction. K_e increased and the maximum number of aggregates bound per cell decreased as a function of aggregate size. The heterogeneity indices (Sips coefficients) were calculated from the slopes of plots of $\log r/n - r$ vs. $\log c$ according to the expression $\log (r/n$

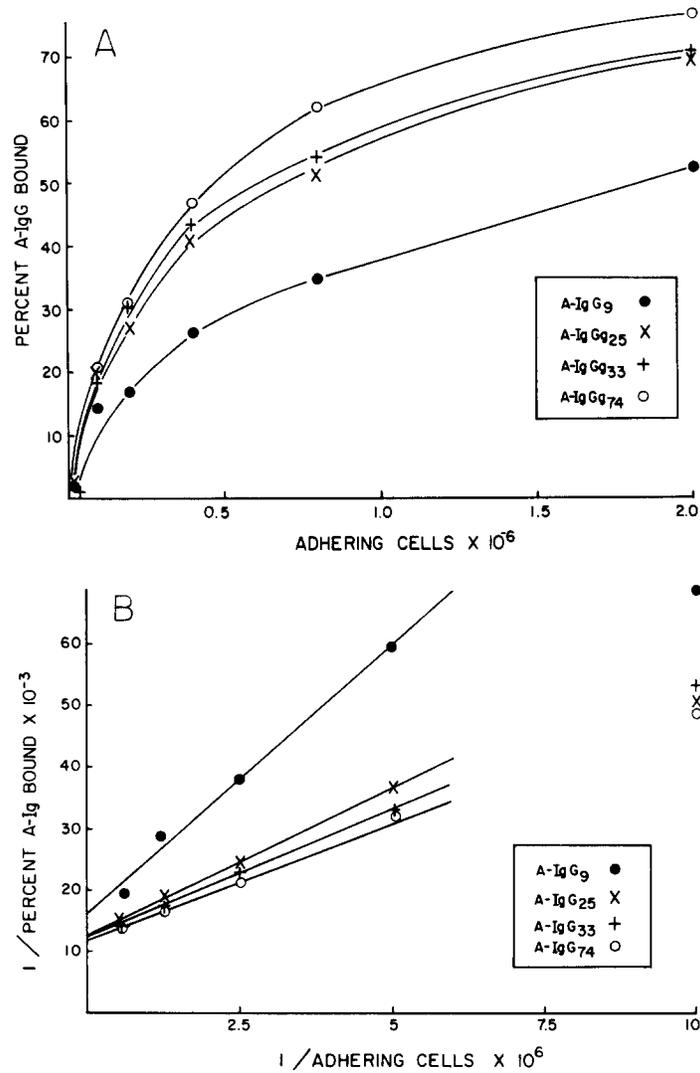


FIG. 1. Maximum A-IgG bound at equilibrium by increasing numbers of macrophages. Equimolar doses containing 0.03–0.22 μg of A-IgG (25 f-mol) were incubated for 24 h at 4°C with adhering macrophages. The percent “bindable” A-IgG was determined from the double reciprocal plot as the percent bound at infinite cell concentration (ordinate intercept). Numerical data are summarized in Table I.

$-r) = a \log K_e + a \log c$, where a is the heterogeneity index. Values were close to 1.0, which is the theoretical value for perfect homogeneity of binding (24).

Dissociation of A-IgG from Macrophages. Doses of A-IgG in mild A-IgG excess were incubated with cells at 4°C for 24 h and triplicate tubes of each dose (and their controls) were harvested to obtain equilibrium values. For various doses of A-IgG, from 8.3 to 68.0% of bindable A-IgG were bound at equilibrium. Cold (i.e., nonradioactive) A-IgG was added to all remaining tubes so that the final concentration of cold A-IgG was 250–1,000 times that of the radioactive A-

TABLE I
Binding of A-IgG by Excess Cells and Theoretical Binding of A-IgG by Infinite Cells

	Dose of A-IgG		A-IgG bound by 2×10^6 cells	Maximum A-IgG bindable
	μg	$f\text{-mol}$	%	%
A-IgG ₉	0.03	25	52.1	62
A-IgG ₂₅	0.07	25	69.6	80
A-IgG ₃₃	0.11	25	70.4	84
A-IgG ₇₄	0.22	21	77.7	86

Data are summarized from Figs. 1 A and 1 B. Bindable A-IgG was determined by extrapolating the lines in Fig. 1 B to infinite cell concentration (ordinate intercept).

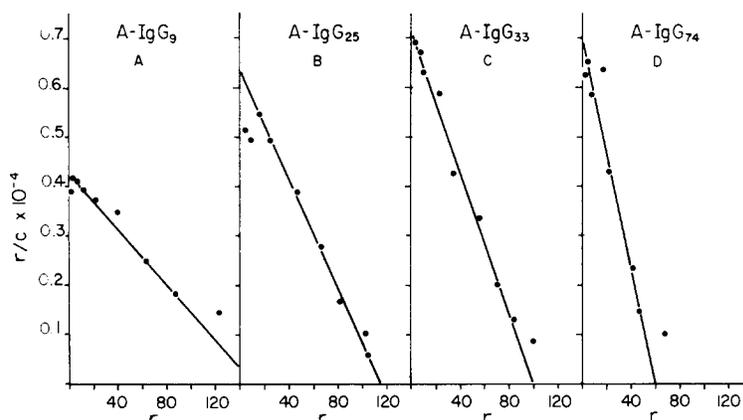


FIG. 2. Equilibrium binding at 4°C of various size A-IgG to adhering macrophages where r is moles $\times 10^{-15}$ A-IgG bound per 0.4×10^6 macrophages and c is unbound A-IgG in moles per liter. Numerical data are summarized in Table III.

IgG present. Tubes were then harvested at intervals over the following 32 h. Dissociation was expressed as $r_t'/r_e \cdot 100$ plotted vs. time, where r_e and r_t' are bound A-IgG at equilibrium and at time t' after excess nonradioactive A-IgG were added.

Dissociation of A-IgG after binding to macrophages was slow and appeared to be both size and dose dependent. Larger A-IgG were less readily dissociated than smaller ones (Fig. 3) and higher doses showed flatter dissociation curves and longer extrapolated $t^{1/2}$'s than lower doses of the same A-IgG (Fig. 4). Data are summarized in Table III.

Dissociation of A-IgG did not appear to follow simple first order kinetics in that semi-log plots of dissociation vs. time produced curves with at least two components. The later component, which in all cases was the major component, appeared linear. However, because of the shallow slope of the curve (Figs. 3 and 4) the question arose whether all cell-associated A-IgG bound at 4°C were located on the exterior of the cell membrane and were thus available to dissociate. The following experiment was done to answer this question.

TABLE II
 Maximum Number of Aggregates Bound Per Cell, Mean
 Equilibrium Constants (K_e) and Sips Heterogeneity Indices for
 Binding of Various Sized A-IgG with Macrophages

	Maximun A-IgG bound per cell	Equilibrium constant (K_e)	Heterogeneity index (Sips coefficient)
		$10^8 M^{-1}$	
A-IgG ₉	230,000	2.8	1.05
A-IgG ₂₅	173,000	5.4	1.04
A-IgG ₃₃	151,000	7.3	0.97
A-IgG ₇₄	90,000	11.7	1.00

Values were derived from Scatchard plots in Fig. 2 and from plots of the Sips function. The maximum number of A-IgG bound per cell was calculated by multiplying the moles A-IgG bound at saturation (abscissa intercept) by Avagadro's number and dividing by the number of cells. K_e 's were determined from the slope of the straight line fitted to the data. Heterogeneity indices were calculated as described in the text.

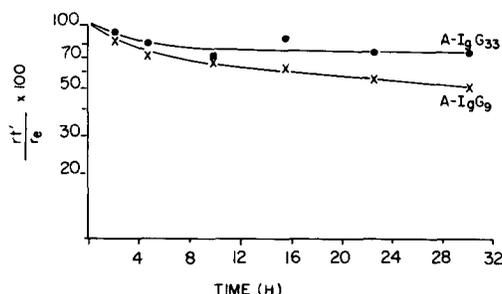


FIG. 3. Effect of A-IgG size on dissociation of A-IgG from macrophages expressed as $r_t'/r_e \cdot 100$, where r_e and r_t' are bound A-IgG at equilibrium and at time t' after excess nonradioactive A-IgG were added.

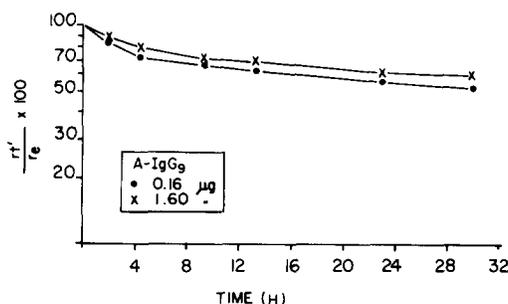


FIG. 4. Effect of A-IgG dose upon subsequent dissociation of A-IgG from macrophages. Data are plotted as in Fig. 3.

Effect of Trypsin Upon Macrophage-Bound A-IgG. Four groups of cells were brought into equilibrium with 0.4 mg A-IgG₅₈ by incubating A-IgG and cells at 4°C for 24 h. One group was then harvested to assess the amount of A-IgG bound at equilibrium and excess cold A-IgG were added to the three remaining groups

TABLE III
Dissociation Rates of Different Size A-IgG Bound to Peritoneal Macrophages*

Size of A-IgG	Dose of Bindable A-IgG		% Bindable A-IgG [†] bound at equilibrium	Dissociation $t^{1/2}$	Dissociation constant (k_d) [§]
	mg	μ -mol	%	h	min ⁻¹
A-IgG ₉	0.023	18.8	40.6	26	4.5×10^{-4}
	0.090	83.7	36.9	36	3.2×10^{-4}
	1.080	895.0	14.5	49	2.4×10^{-4}
A-IgG ₂₅	0.100	34.4	54.8	34	3.4×10^{-4}
A-IgG ₃₃	0.189	42.0	52.9	46	2.5×10^{-4}
	0.757	169.0	32.1	55	2.1×10^{-4}
A-IgG ₇₄	0.168	16.3	57.9	53	2.2×10^{-4}

* Data are derived from experiments done as in Figs. 3 and 4.

[†] The % bindable A-IgG was measured in Fig. 1 and Table I.

[§] The k_d 's were calculated as $\ln 2/t^{1/2}$ dissociation (25).

as in the above dissociation experiments. After an additional 24 h of shaking at 4°C, cells were washed with TBH and the supernates saved. One group was suspended in TBH containing 1.0 mg/ml trypsin and the remaining two groups were suspended in buffer alone. The trypsin group and one buffer-only group were then incubated for 15 min with shaking at 37°C. The remaining buffer only group was incubated for 15 min at 4°C. Cells were harvested in the usual manner and TCA precipitation was performed on the combined supernates and washes of each tube. The results expressed as percent of cell associated ¹²⁵I released are seen in Fig. 5.

Incubation with only cold A-IgG resulted in 34.6% dissociation of bound A-IgG, all of which were intact A-IgG, i.e., TCA precipitable. Cold A-IgG followed by trypsin caused an additional 38.8% release for a total of 73.4%. However 14.6% of these A-IgG were TCA nonprecipitable, which might have been due to cellular metabolism and extrusion of metabolic products of A-IgG during the 37° incubation. That this was not the case is shown by the last group of cells. Incubation at 37°C without trypsin caused a slight increase in A-IgG dissociation (15.8% more than 4°C or 50.4% total) but no increase in TCA nonprecipitable A-IgG. The flattening of the dissociation curves, therefore, cannot be due to A-IgG being "unavailable" to dissociate since 73% of A-IgG was released from cells after incubation with cold A-IgG and trypsin.

Association of A-IgG with Macrophages. The rates of binding of various A-IgG with macrophages were studied early in the association reaction so that the (slower) dissociation reaction could be neglected. Association $t^{1/2}$'s to equilibrium were determined graphically from the disappearance of A-IgG from the supernate, by plotting $r_e - r_t/r_e \cdot 100$ vs. time, where r_e and r_t are A-IgG bound at equilibrium and at time t . The $t^{1/2}$'s were read directly from these plots or (occasionally) from a short extrapolation of the observed line through the 50% value. Representative experiments are plotted in Figs. 6 and 7 and data are summarized in Table IV.

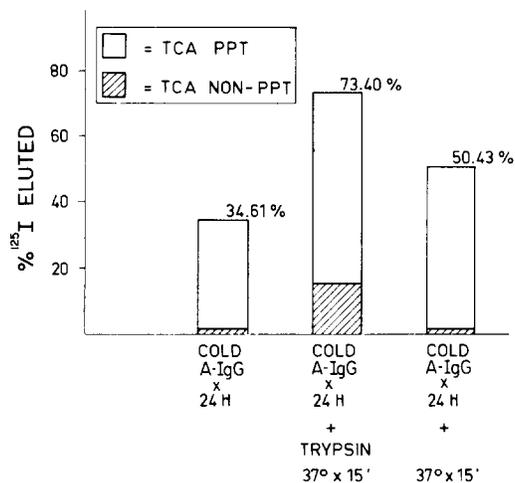


FIG. 5. Removal by trypsin of A-IgG remaining on macrophages after partial dissociation. The removed A-IgG were separated into TCA precipitable and nonprecipitable fractions.

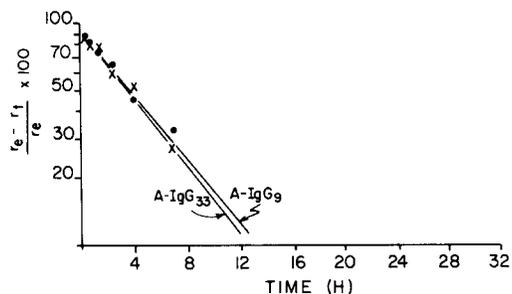


FIG. 6. Effect of A-IgG size upon uptake (association) of A-IgG by 0.4×10^6 adhering macrophages where r_e and r_t are bound A-IgG at equilibrium and at time t .

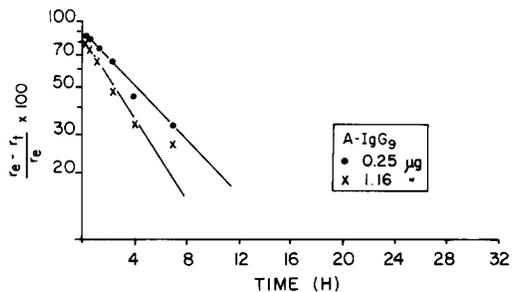


FIG. 7. Effect of A-IgG dose upon uptake (association) kinetics for A-IgG and macrophages. Data are plotted as in Fig. 6.

Binding of A-IgG with macrophages was clearly more rapid than the dissociation reaction ($t^{1/2}$, 2.1–4.5 h vs. 26–55 h). Association appeared to follow pseudo first order kinetics in that disappearance of A-IgG from the media was log-linear with time. The $1/2$'s were similar for different size A-IgG and no consistent trend

TABLE IV
*Association Rates of Different Size A-IgG Binding at Various Doses with Peritoneal Macrophages**

Size of A-IgG	Dose of bindable A-IgG		% Bindable A-IgG‡ bound at equilibrium	Association $t^{1/2}$	Pseudo first order association constant (k_a)§
	μg	$f\text{-mol}$			
A-IgG ₉	0.036	30.3	35.0	4.1	2.7×10^{-3}
	0.153	132	32.0	3.9	2.9×10^{-3}
	0.722	592	16.8	2.7	4.2×10^{-3}
A-IgG ₂₅	0.214	72.8	40.0	4.3	2.7×10^{-3}
A-IgG ₃₃	0.178	39.7	44.7	4.3	2.7×10^{-3}
	0.804	179	27.0	3.3	3.5×10^{-3}
A-IgG ₅₉	0.220	26.8	47.5	4.9	2.3×10^{-3}
	0.913	110	26.0	3.13	3.8×10^{-3}

* Data are divided from experiments done as those described for Figs. 6 and 7.

‡ The bindable A-IgG₆ was calculated from data presented in Table I.

§ The k_a 's were calculated as $\ln 2/t^{1/2}$ for association (25).

could be demonstrated relating A-IgG size and association $t^{1/2}$ (Fig. 6 and Table IV). Increasing the dose of A-IgG markedly decreased the association $t^{1/2}$ (Fig. 7). While not unexpected, this result underscores the importance of keeping the molar dose of bindable A-IgG constant when attempting to determine the effect of A-IgG size upon association $t^{1/2}$.

Inhibition of Binding by Monomeric IgG. IgG was isolated by DEAE chromatography and cleared of aggregates by ultracentrifugation in sucrose gradients containing 0.5% rabbit albumin. Adhering macrophages were then incubated for 30 min at 4°C in 50 μl TBH-RSA containing various concentrations of monomeric IgG. Equimolar doses of 70 fmol A-IgG₇₂ or A-IgG₁₈ in 50 μl TBH-RSA were then added and the percent inhibition of binding assessed after 160 min at 4°C. Specific binding of both A-IgG₇₂ and A-IgG₁₈ was 50% inhibited by a final concentration of 4 mg/ml of monomeric IgG (Fig. 8).

Effect of Pretreatment of Macrophages with Trypsin. Macrophages were incubated at 37°C for 30 min with 500 μl TBH containing trypsin, 10 mg/ml. Cells were washed and the binding of 0.1 mg A-IgG₂₁ after 2 h at 4°C was assessed. Pretreatment with trypsin reduced binding approximately 35%.

Discussion

In this report, soluble immune aggregates of homologous IgG (A-IgG) were reacted at 4°C with adherent macrophages from normal rats to compare the binding kinetics for A-IgG of different sizes. Such measurements were possible for two reasons. First, as previously shown,³ A-IgG are both homogeneous in size and stable when isolated by ultracentrifugation in gradients containing supporting protein. Such A-IgG could thus be measured in terms of moles of aggregates. Second, A-IgG isolated in this manner possess high biological activity in that 62–86% of each different A-IgG isolate could be bound by macrophages (Table I).

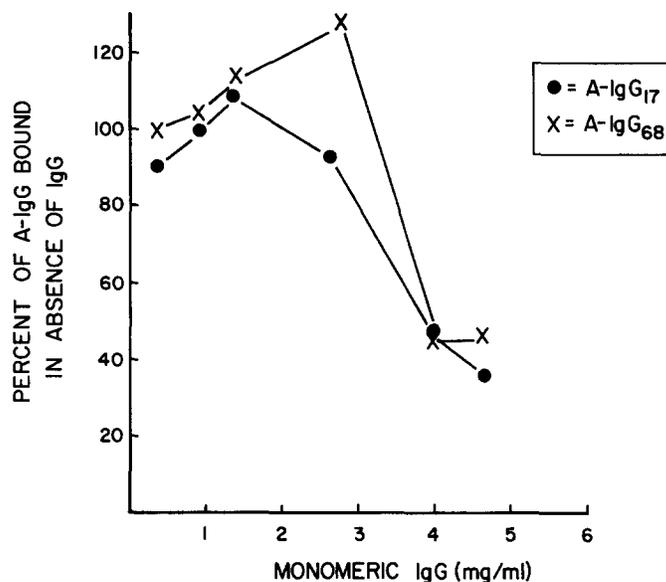


FIG. 8. Inhibition of macrophage uptake of A-IgG by monomeric IgG. The percent of A-IgG uptake in the absence of monomeric IgG is plotted as a function of IgG concentration for equimolar doses of A-IgG₁₇ and A-IgG₆₈.

Equilibrium constants (K_e) in these studies ($2.18-11.7 \times 10^8 \text{ M}^{-1}$) exceed published values for the binding of homologous monomeric IgG by stimulated rabbit alveolar macrophages ($0.5-1.5 \times 10^6 \text{ M}^{-1}$) (5) and IgG2a by mouse macrophages ($0.3-1.1 \times 10^6 \text{ M}^{-1}$) (13). More avid binding of A-IgG is probably due to attachment of A-IgG at multiple sites to Fc receptors on the macrophage as has been postulated for immune complexes (7). The finding that monomeric IgG in physiological concentrations could inhibit binding of A-IgG supports this hypothesis. This hypothesis could also explain why K_e values in the present studies increased with aggregate size. Larger A-IgG containing more IgG molecules per aggregate, and therefore more Fc fragments, could have increased opportunities to bind at greater numbers of sites. Alternatively, larger aggregates could bind more avidly because of an increased likelihood of having chance configurations more favorable for multisite binding.

The maximum number of A-IgG bound per cell decreased as aggregate size increased. Thus, fewer larger A-IgG were necessary to saturate a cell. If larger A-IgG occupy more Fc receptors per aggregate than smaller ones, fewer larger A-IgG would be necessary to saturate the available receptors.

On the other hand, bound larger A-IgG could prevent the binding of other A-IgG by steric effects. This possibility seems less likely in view of our calculations that only 4-7% of the cell surface area is occupied by A-IgG at cell saturation. However, steric hindrance could play a role if Fc receptors were polyvalent or concentrated in certain areas instead of being randomly distributed over the surface of the cell. Since A-IgG₇₄ was calculated to occupy the greatest surface area, the data are consistent with greater steric hindrance by larger aggregates being at least in part responsible for the smaller total number of larger A-IgG being bound at cell saturation.

Our estimates of 4-7% of the cell surface being occupied at saturation are

higher than the 1% figure for the surface area occupied by monomeric IgG at saturation calculated by Unkeless and Eisen (13). This is as expected since it is unlikely that all of the Fc fragments of an aggregate are bound to Fc receptors so some areas of the cell membrane devoid of specific receptors are undoubtedly "covered".

The number of Fc receptors present on macrophages has been estimated at about 100,000 for resting mouse peritoneal macrophages (13). Estimates are higher for stimulated rabbit alveolar macrophages (5) and stimulated guinea pig macrophages (2.5×10^6 sites per cell) (26, 27). The maximum numbers of A-IgG bound per cell in this study (90,000–230,000) are in better agreement with the higher estimates in that sufficient Fc receptors would thus be available for multisite binding of A-IgG.

The number of Fc fragments per aggregate available for binding and the actual number of attachment sites on the cell occupied per aggregate could not be measured in our studies. Therefore, the number of Fc receptors per cell that were occupied or available as well as the binding energy of each bond could not be calculated. However, since the equilibrium constants were only of the order of 10^8 M^{-1} it is likely that A-IgG were bound at only a few sites, probably less than six.

The $t^{1/2}$'s for dissociation of A-IgG from the cell surface which exceeded 26 h in our studies differ markedly from reported values for IgG dissociation $t^{1/2}$'s measured in minutes (13). Also, larger A-IgG were slower to dissociate than smaller ones. These two observations are again in keeping with an expected bonus effect when an aggregate or immune complex is attached at multiple sites.

Semi-log plots of the dissociation of A-IgG from macrophages with time showed curves with more than one component suggesting heterogeneous binding of A-IgG isolates. Such heterogeneous binding might be due to difference in the configuration of A-IgG of similar size. Since significantly less than 100% of each A-IgG isolate tested was practically (and theoretically) bindable, some functional heterogeneity was present. Alternatively, chance rearrangements limited by the availability and mobility of Fc receptors on the cell membrane might have contributed to more avid binding of certain A-IgG. Heterogeneity of binding was also suggested by the finding that after initial equilibrium, higher doses of the same A-IgG dissociated more slowly than lower doses. This suggests a competitive selection of more avidly bound A-IgG at the higher doses. However, such heterogeneity was not detected in the equilibrium experiments, where remarkably high Sips coefficients were observed.

Association (i.e., binding) of A-IgG was 10 times more rapid than dissociation and seemed to follow pseudo first order kinetics since semi-log plots of the disappearance of A-IgG from the supernate were linear. However, association $t^{1/2}$'s for A-IgG were much slower than published reports for IgG binding to macrophages (13) and for other bimolecular reactions such as the combining of antibody with antigen (28). This suggests that only a small percentage of aggregate-cell collisions results in A-IgG being avidly bound.

Association $t^{1/2}$'s were shorter for higher doses of A-IgG as expected. More concentrated A-IgG should more rapidly fill the available binding sites. Associa-

tion $t^{1/2}$'s were similar for comparable doses of different size A-IgG and small inconsistencies in the data were sufficient to preclude establishing with certainty whether association rates are different for different size A-IgG. Some, but not all of such inconsistencies could be explained by the difficulty encountered in administering exactly comparable doses of A-IgG. However, if size of A-IgG influences the rate of association, we were unable to demonstrate it.

Our model for A-IgG binding based upon all of the above data is as follows. A-IgG are initially loosely bound to a single Fc receptor. Because of the low binding energy of this single bond, most of the A-IgG again escape into the surrounding media. A few, however, are bound for sufficient time so that additional Fc receptors in the cell membrane can also attach; thus, some A-IgG become firmly bound. It is the rate of this firm attachment, and not the rate of initial binding, that determines the measured disappearance of A-IgG from the supernate. Thus, the reaction is not diffusion rate limited, but rather limited by the availability and mobility of Fc receptors in the membrane. However, since the instantaneous concentration of loosely bound A-IgG in the cell membrane is dependent upon the concentration of A-IgG in the media, the disappearance of A-IgG from the media follows pseudo first order kinetics. Further, since the initial binding equilibrium constant is low, very high concentrations of free A-IgG are necessary to "saturate" this initial binding step, and the reaction remains first order over a wide range of doses. Conversely, since the K_e 's of multiple site binding are high, given sufficient time, cells will approach saturation with doses that do not saturate the initial binding step.

Once A-IgG are firmly bound, the binding can undergo internal rearrangement and so A-IgG become even more firmly bound. This possibility is more likely for larger aggregates and so K_e 's are higher and dissociation rates slower for larger A-IgG. Functional heterogeneity among A-IgG, probably due mainly to configurational differences, causes chance rearrangements to bind certain A-IgG more firmly than others. Hence, dissociation does not follow simple first order kinetics.

While the association (k_a) and dissociation (k_e) rate constants measured in these studies can be grossly compared, their relationship is not a mathematical one, i.e., $K_e \neq k_a/k_d$. This is true because k_a is dependent upon concentration while k_d is not. This difficulty is minimized by studying association under "standard conditions" of mild aggregate excess and studying dissociation after an equilibrium has been established at this same concentration. In our data, dissociation $t^{1/2}$'s studied over a wide range of concentrations were all about 10-fold longer than their paired dissociation $t^{1/2}$'s and no overlap was even approached. Therefore, our conclusions concerning the relative rates are warranted.

Partial inhibition of A-IgG binding by near physiological concentrations of monomeric IgG cannot be used to argue against the possible importance of Fc binding as a mechanism for the removal of A-IgG and immune complexes from the circulation in vivo. On the contrary, the high equilibrium constants measured and the kinetic data of these studies show that low concentrations of A-IgG can effectively compete with IgG and displace it from the surface of the cell even in the absence of subsequent endocytosis/metabolism.

Summary

Stable aggregated IgG (A-IgG) of various sizes, having high biological activity, were incubated at 4°C with adhering peritoneal macrophages from normal rats and the kinetics of A-IgG binding to the cell surface were studied. Equilibrium constants were high ($2.8\text{--}11.7 \times 10^8 \text{ M}^{-1}$) and varied as a function of aggregate size. The maximum number of A-IgG bound per cell varied from 230,000 for A-IgG₉ to 90,000 for A-IgG₇₄. Binding was 50% inhibited by near physiological concentrations of monomeric IgG. These data suggest that A-IgG are bound at multiple sites by attachment of Fc fragments to Fc receptors present on the macrophage surface with larger A-IgG being more avidly bound.

Dissociation was slower for larger A-IgG while no clear trend was seen relating associating rates and aggregate size. Thus, differences in the avidity of binding of A-IgG are due primarily to slower dissociation of larger A-IgG.

Dissociation of A-IgG was slower from cells exposed initially to higher doses of A-IgG and dissociation did not follow simple first order kinetics. Thus, the avidity of binding appears to be heterogeneous in a population of similar sized A-IgG. As expected, association was dose-dependent, more rapid than dissociation, and followed pseudo first order kinetics. Based on all of the above data, it is proposed that binding of A-IgG proceeds in two steps. First, A-IgG are loosely bound to perhaps a single Fc receptor. Then, depending upon the availability and mobility of Fc receptors, additional Fc fragments are attached and the A-IgG becomes more firmly attached. Thus binding is slow, but once attached A-IgG are avidly held.

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