RECEPTORS FOR IgG: SUBCLASS SPECIFICITY OF RECEPTORS ON DIFFERENT MOUSE CELL TYPES AND THE DEFINITION OF TWO DISTINCT RECEPTORS ON A MACROPHAGE CELL LINE*

By CHRISTOPH H. HEUSSER,‡ CLARK L. ANDERSON,§ AND HOWARD M. GREY

(From the National Jewish Hospital and Research Center, Denver, Colorado 80206)

Fc receptors have been found on many different cells including B and T lymphocytes, macrophages, neutrophils, and K cells, as well as cells not involved in the immune system. Some cellular functions such as phagocytosis of IgG-coated particles (1, 2) and the antibody-dependent cell-mediated cytotoxicity reaction (3) can clearly be attributed to this surface activity. On the other hand, the functional significance of Fc receptors on other cells such as B and T lymphocytes has not as yet been elucidated. As part of our investigations of the possible function of Fc receptors, we have studied the specificity of receptors for immunoglobulins of different IgG subclasses, as well as the degree of polymerization of the immunoglobulin capable of binding to these receptors. For this purpose myeloma proteins of different IgG subclasses were chemically cross-linked by bis-diazotized benzidine (BDB).[1] Monomer IgG and IgG aggregates of comparable size, representative of the different subclasses, were examined for their capacity to bind to receptors on different established cell lines and normal cells. Our findings indicate that the binding of myeloma proteins is dependent both upon subclass and degree of polymerization. Furthermore, studies on a macrophage-like cell line (P388) strongly suggest the presence of at least two different receptors for IgG on this cell, one characterized by its reactivity with monomeric IgG_{2a} and the other for its activity with aggregated IgG of all subclasses.

Materials and Methods

Myeloma Proteins. The following IgG myeloma proteins were used in this study: MOPC-21 (IgG_1), PC-5, and HOPC-1 (IgG_{2a}), MOPC-141 (IgG_{2b}), and J606 (IgG_3). The myeloma proteins were isolated by starch block electrophoresis. Only the peak tubes of the myeloma protein-containing fractions were used. The myeloma proteins were cross-linked with BDB according to a modification of the method of Ishizaka (4) resulting in soluble complexes with a wide range of size.

---

[1] *Abbreviations used in this paper:* BDB, bis-diazotized benzidine; BSS, balanced salt solution; BSS-BSA-NaN₃, BSS plus 2.5 mg/ml bovine serum albumin and 0.1% sodium azide; FCS, fetal calf serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Although several attempts were made, we were unable to produce soluble aggregates of the IgG$_3$ protein available to us (J606). All BDB-treated proteins were dialyzed overnight against phosphate-buffered saline (pH 7.0). Insoluble material was removed by centrifugation at 1,500 g for 15 min. The soluble aggregates were subsequently fractionated on Sepharose 4B or Bio-gel A50m. Calculations of the molecular weight of the aggregates were based on their elution volumes from these columns. Aggregated and monomeric myeloma proteins were iodinated by the chloramine-T method (5). To prepare monomeric IgG, unaggregated protein was either centrifuged for 2.5 h at 165,000 g and the top one-half of the samples used, or it was filtered through a Sephadex G-200 column and the descending portion of the IgG peak was used as the monomer preparation.

Cells. Cell lines were grown in spinner cultures in RPMI-1640 containing penicillin, streptomycin, and 10% heat-inactivated fetal calf serum (FCS). Peritoneal macrophages were obtained from BDF$_1$ mice 4 days after injection of 1 ml of 3% thioglycolate broth. Spleen cells and thymocytes were obtained from 8- and 4-wk-old DBA/2 mice, respectively. Trypsinization was carried out by incubating 10^7 cells in 1 ml of balanced salt solution (BSS) with 0.1-1 mg trypsin (TPCK treated, Worthington Biochemical Corp., Freehold, N. J.) for 15-30 min at 37°C. After being washed, cells were resuspended in BSS with 0.2 mg/ml soybean trypsin inhibitor.

Spleen cells were fractionated into T-enriched ("T") and B-enriched ("B") populations by the nylon wool method (6). T and B cells were enumerated by determining the percentage of fluorescent cells after staining with either fluoresceinated rabbit anti-mouse Ig (to detect B cells) or anti-Thy-1 and fluoresceinated rabbit anti-mouse Ig (to detect T and B cells). The T-cell fraction contained 68% Thy-1-positive cells and 13% Ig-positive cells; the B-cell fraction contained 13% Thy-1-positive cells and 52% Ig-positive cells.

Assays for IgG Binding to Cells. To 0.1 ml BSS-BSA-NaN$_3$ in 5-ml polyethylene tubes was added 0.1 ml of inhibitor Ig or buffer and 0.1 ml of ^{125}I-IgG (usually at a concentration of 0.01 mg/ml). To this mixture, 3-10 × 10^6 cells in 0.3 ml were added. Incubation with intermittent agitation proceeded for 60 min at 4°C for macrophage-like cells and at 37°C for all other cells (7). The amount of radiolabeled IgG bound by the cells was determined by layering 0.2 ml of the reaction mixture on top of 1 ml cold fetal calf serum (FCS) in 1.5 ml conical polyethylene tubes and separating unbound from cell-associated IgG by centrifuging the cells through the FCS at 350 g for 8 min. Tubes were then frozen in a bath of dry ice and ethanol, and the bottoms of the tubes containing the cell pellets were cut off with a hot wire and assayed for radioactivity. In control experiments without cells it was shown that only 0.01% of ^{125}I-IgG was found in the pellet portion of the tubes. Cells were also analyzed for bound ^{125}I-IgG by autoradiography as previously described (7).

Results

I. Receptor Activity for IgG on Different Mouse Cells and Cell Lines. Subclass Specificity and Aggregate Size Requirements. The binding capacity of the IgG subclasses for different cell lines was tested using IgG myeloma proteins in three different polymeric forms: monomers, (IgG)$_1$; tetramers, (IgG)$_4$; and high molecular weight aggregates, (IgG)$_{>100}$. The size of the latter preparations was estimated by their elution from agarose columns and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8). Representative results of one of three such experiments using these preparations are shown in Table I. IgG binding was studied with the following cell lines: P388 and J774, which are macrophage-like cell ines (9, 10); P815, a mastocytoma; D2N, an Ia-positive Friend leukemia virus-induced cell line (11); and S49, a Thy-1-positive lymphoma. If monomer binding were analyzed for subclass specificity, (IgG$_{2a}$)$_1$ was preferentially bound by the macrophage-like cell lines and by P815. The lymphoid cell lines, D2N and S49, did not express detectable (IgG$_{2a}$)$_1$ binding capacity.

The extent of membrane binding of aggregated IgG varied among the cell types analyzed. The differences observed were dependent on both the subclass and the degree of aggregation. Polymerization to a mean size of four (range two

TABLE I

Binding of IgG of Different Subclasses and Different Degrees of Aggregation to Mouse Cell Lines

IgG preparation	Percent of ^{125}I-IgG bound to different cell lines				
	P388	J774	P815	D2N	S49
$(IgG_1)_1$	0.4	0.9	0.6	0.1	0.2
$(IgG_{2a})_1$	4.5	6.9	2.6	0.2	0.3
$(IgG_{2b})_1$	0.4	1.0	0.7	0.2	0.5
$(IgG_3)_1$	0.1	0.2	0.2	0.1	0.2
$(IgG_1)_4$	41.3	52.1	32.1	5.8	12.6
$(IgG_{2a})_4$	41.7	55.8	29.8	2.0	3.7
$(IgG_{2b})_4$	20.5	36.3	19.5	1.9	3.7
$(IgG_1)_{>100}$	54.1	62.6	22.7	15.0	32.9
$(IgG_{2b})_{>100}$	57.2	58.8	51.5	17.3	31.0

P388, J774, and P815 cells were incubated at 4°C to prevent pinocytosis. D2N and S49 cells were incubated at 37°C (7). P815 cells were also studied at 37°C and essentially the same binding was obtained at this temperature as that given at 4°C. Nonspecific uptake of IgG was assayed by measuring the amount of radioactivity in the cell pellet when a large excess of unlabeled IgG was added to the incubation mixture of ^{125}I-IgG and cells. This nonspecific uptake was 0.2% or less for $(IgG)_1$ and was 1% or less for aggregated IgG.

to seven) increased remarkably the capacity of IgG_1, IgG_{2a}, and IgG_{2b} proteins to bind to these cells compared with monomer IgG. This was most marked with the $(IgG_1)_4$ protein, which bound to the cell types studied as well as or better than $(IgG_{2a})_4$ and better than $(IgG_{2b})_4$. When $(IgG)_{>100}$ aggregates were used there was little or no increase in the uptake of IgG_1 protein, whereas IgG_{2b} now bound as well as IgG_1, and in one case better (P815). Although aggregates of the IgG_{2a} protein of this high molecular weight were not available for study, results obtained using 10×10^6 dalton aggregates, $(IgG)_{65}$, indicated that in a highly aggregated state IgG_{2a} was usually the weakest binding protein (see below).

The capacity of cells to bind high molecular weight aggregates was further analyzed using aggregates with a mol wt of approximately 10^7 daltons, $(IgG)_{65}$. To study normal populations of cells with considerable heterogeneity of Fc receptor activity, we analyzed aggregate binding by autoradiography. Table II shows these results. It is apparent from these data that normal macrophages and the cell lines studied in Table I were capable of binding 10–100-fold more aggregated IgG than normal T cells, B cells, thymocytes, and the Thy-1-positive tumors, EL4 and L5178. The cells studied demonstrated three fairly distinct patterns of reactivity with aggregates of the IgG subclasses used. Macrophages and macrophage-like cell lines bound the aggregates of the three subclasses to a similar extent. In contrast, the group represented by D2N, S49, and P815 bound the IgG_{2a} protein less efficiently than either IgG_{2b} or IgG_1. The third group of cells which included normal T cells, B cells, and thymocytes, together with the EL-4 and L5178 cell lines, bound IgG_1 two to fourfold more efficiently than either IgG_{2a} or IgG_{2b} aggregates.

II. Characterization of the Specificity of Monomer and Aggregated IgG Binding by P388 Cells. As shown in Table I, the macrophage-like cell lines (and to

TABLE II

Binding of ^{125}I-$(IgG)_{65}$ of Different Subclasses to Mouse Cell Lines and Normal Cells

Cell type	Binding of ^{125}I-aggregated myeloma proteins		
	MOPC-21 IgG$_1$	PC-5 IgG$_{2a}$	MOPC-141 IgG$_{2b}$
	grains/cell per day		
J774	74	102	101
P388	103	118	ND
Macrophages	100	96	130
P815	89	48	89
D2N	101	40	93
S49	83	33	78
EL4	9	2	4
L5178	1.2	0.4	0.5
Splenic T	7.2	1.8	2.8
Splenic B	8.2	2.5	3.5
Thymus	3.1	1.4	1.6

Macrophages and macrophage-like cell lines (J774 and P388) were incubated at 4°C, all other cell types at 37°C. Autoradiographic preparations of these cells were exposed for different lengths of time and the grain distribution per cell was normalized for 1 day exposure. One of two similar experiments is shown. ND, not done.

a lesser extent, P815) differed from the other cell types studied in that they were capable of binding significant quantities of (IgG$_{2a}$)$_1$. The preferential binding of monomer IgG$_{2a}$ contrasted with the equal binding capacity of all three subclasses when aggregates were tested. These differences suggested the possibility that two distinct receptors for IgG were present, one capable of binding (IgG$_{2a}$)$_1$ preferentially to other monomer proteins, and one capable of binding only aggregated IgG and exhibiting no subclass specificity. In order to further explore this possibility, the specificity of (IgG$_{2a}$)$_1$ and aggregated IgG binding was investigated by determining the patterns of inhibition obtained with unlabeled monomeric and aggregated proteins of the different subclasses.

(a) INHIBITION OF BINDING OF AGGREGATED IgG TO P388 CELLS. The capacity of a 600-fold excess of unlabeled aggregates of the three subclasses to inhibit the binding of radiolabeled aggregates to P388 was determined. Inhibition of (IgG)$_4$ binding by unlabeled aggregates was high, varying from 73–99%, and no striking degree of selectivity for the aggregates of any one particular subclass was revealed by this analysis. In this experiment, however, since a single large excess of unlabeled aggregated protein was used, the inhibition by aggregates was studied in a more quantitative way by comparing inhibitory capacities over a wide range of inhibitor concentrations. These data are presented in Fig. 1. Tetramers of IgG$_1$ and IgG$_{2a}$ inhibited the uptake of radiolabeled IgG$_{2a}$ in a quantitatively similar fashion. 50% inhibition was obtained with 13.5 μg of IgG$_{2a}$ and 17.5 μg of IgG$_1$. In experiments not shown, when inhibition of IgG$_1$ aggregate binding was studied, both IgG$_{2a}$ and IgG$_1$ inhibited well (50% inhibition being obtained with 21.5 and 12.5 μg, respectively). In both experiments the tendency was noted for the homologous aggregates to inhibit somewhat more
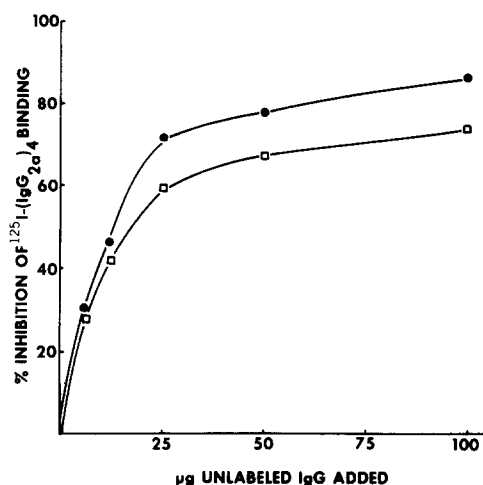
FIG. 1.   Inhibition of aggregate binding by aggregates. Binding by P388 cells of ^{125}I-$(IgG_{2a})_4$ was inhibited by varying amounts of $(IgG_{2a})_4$ (●—●) and $(IgG_1)_4$ (□—□). Similar inhibition was noted.

efficiently than heterologous aggregates. From these studies it was concluded that IgG aggregates of the different subclasses could effectively inhibit the binding of the other subclasses and therefore most likely bound to the same receptor structure on the cell surface.

Next, inhibition of aggregate binding by monomeric proteins was studied. Using a 600-fold excess of inhibitor, the percentage inhibition obtained with monomers was as follows: $(IgG_{2b})_1$, 61-84%; $(IgG_1)_1$, 42-51%; $(IgG_{2a})_1$, 16-42%; $(IgG_3)_1$, 12-19%. Further, dose-response experiments using $(IgG)_1$ obtained by Sephadex gel filtration were performed. $(IgG_{2a})_1$ and $(IgG_{2b})_1$ were compared for their capacity to inhibit the binding of both IgG_1 or IgG_{2a} aggregates. 30% inhibition of aggregate binding was obtained with 60 μg of $(IgG_{2b})_1$, whereas 122-400 μg of $(IgG_{2a})_1$ were needed for the same degree of inhibition. Thus, these data indicate that $(IgG_{2b})_1$ was the best inhibitor of $(IgG)_4$ binding.

The results described above suggest that the receptor capable of binding IgG aggregates, although not demonstrating subclass specificity when aggregates were used for inhibition studies, did express some degree of specificity for IgG_{2b} when monomeric proteins were studied for inhibition.

(b) INHIBITION OF BINDING OF MONOMERIC IgG_{2a} TO P388 CELLS.   The inhibition of binding of radiolabeled $(IgG_{2a})_1$ to P388 cells by other monomeric proteins is shown in Table III. The two IgG_{2a} proteins tested were efficient inhibitors of $(IgG_{2a})_1$ binding, whereas the IgG_1, IgG_{2b}, and IgG_3 proteins demonstrated negligible inhibitory capacity.

To study the quantitative aspects of this inhibition, we compared in a dose-response experiment the inhibitory capacity of G-200 fractionated $(IgG_{2a})_1$ and $(IgG_{2b})_1$. These data are shown in Fig. 2. $(IgG_{2a})_1$ was a much more efficient inhibitor than $(IgG_{2b})_1$ achieving 40% inhibition with 4 μg protein, whereas 150 μg of $(IgG_{2b})_1$ were needed to achieve the same degree of inhibition. These observations of $(IgG)_1$ inhibition of monomer IgG_{2a} binding contrast with the data of inhibition of aggregate binding described in section IIa in that strong

TABLE III

Inhibition of Radiolabeled $(IgG_{2a})_1$ Binding to P388 Cells by Unlabeled $(IgG)_1$

Inhibitor protein	Percent inhibition of ^{125}I-$(IgG_{2a})_1$ binding	
	Exp. 1	Exp. 2
$(IgG_1)_1$	22	5
$(IgG_{2a})_1$ (PC-5)	80	57
$(IgG_{2a})_1$ (HOPC-1)	ND	46
$(IgG_{2b})_1$	20	5
$(IgG_3)_1$	0	ND

40-fold weight excess of unlabeled to labeled protein was added in experiment 1, and a 10-fold excess was added in experiment 2. ND, not done.
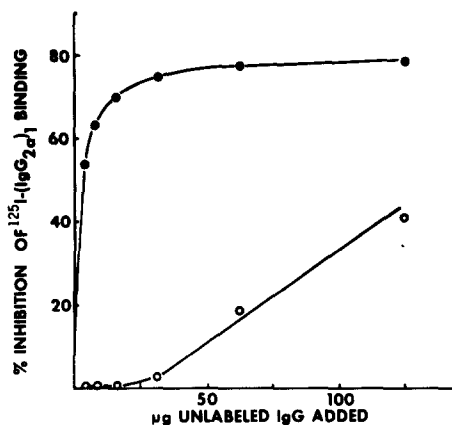

FIG. 2. Inhibition of IgG_{2a} monomer binding by monomers. P388 uptake of ^{125}I-$(IgG_{2a})_1$ was inhibited by varying amounts of $(IgG_{2a})_1$ (●—●) and $(IgG_{2b})_1$ (○—○). Subclass specificity of inhibition was noted.

IgG_{2a} specificity of inhibition was shown for $(IgG_{2a})_1$ binding, whereas aggregated IgG binding was preferentially inhibited by $(IgG_{2b})_1$.

Next, the capacity of IgG aggregates to inhibit the binding of IgG_{2a} monomer was measured. These data, shown in Fig. 3, indicate that aggregates of the IgG_{2a} subclass were efficient inhibitors of monomer IgG_{2a} binding, whereas IgG_1 and IgG_{2b} aggregates inhibited very poorly. The studies showing absence of inhibition with the $(IgG_{2b})_4$ and $(IgG_1)_4$ preparations were performed over a concentration range that in previous experiments demonstrated equivalent binding to P388 cells of ^{125}I-$(IgG_{2a})_4$, ^{125}I-$(IgG_{2b})_4$, and ^{125}I-$(IgG_1)_4$. With these quantities of protein (0.5–2.5 μg) saturation of binding sites was obtained and indicated that P388 cells were capable of binding approximately 0.15 μg $(IgG)_4/10^6$ cells. These inhibition data indicate another difference between the binding of IgG aggregates and monomer to P388 cells. Whereas aggregate binding was inhibited in a similar fashion by all aggregates, $(IgG_{2a})_1$ binding was inhibited to a significant degree only by aggregated IgG_{2a}. The inhibition studies detailed above strongly suggest the presence of two distinct receptors for IgG on P388 cells, one reactive with both monomer and aggregated IgG_{2a}, and another receptor reactive with aggregates of all three subclasses as well as with monomer IgG_{2b}.
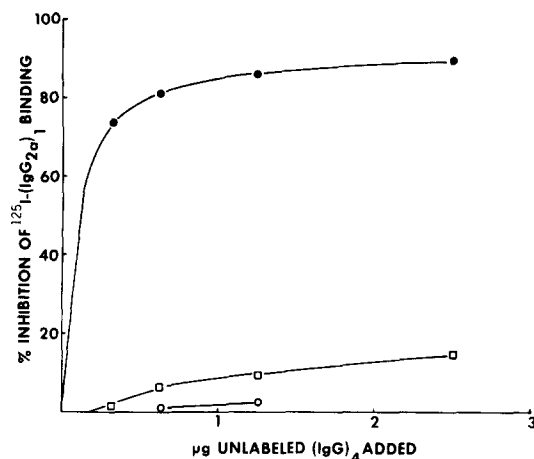
FIG. 3. Inhibition of IgG_{2a} monomer binding by various aggregates. P388 uptake of ^{125}I-$(IgG_{2a})_1$ was inhibited by varying amounts of $(IgG_{2a})_4$ (●—●), $(IgG_1)_4$ (□—□), and $(IgG_{2b})_4$ (○—○). IgG_{2a} monomer binding was inhibited most efficiently by the homologous aggregate.

(c) INHIBITION OF BINDING OF MONOMERIC IgG_{2b} TO P388 CELLS. The possible binding of monomer IgG_{2b} to the same site to which the aggregated proteins bound was further investigated by studying the specificity of inhibition of the binding of radiolabeled IgG_{2b} monomer. First, when monomeric proteins were used to inhibit the uptake of $(IgG_{2b})_1$, it was found that at 10-fold molar excess, unlabeled IgG_{2b} inhibited the binding of labeled IgG_{2b} by 60%, whereas IgG_{2a} proteins inhibited the binding by only 25%, and IgG_3 and IgG_1 proteins did not inhibit at all. The ability of IgG_{2a} and IgG_{2b} proteins to inhibit the binding of IgG_{2b} monomer was further studied in the experiment shown in Fig. 4. The uptake of monomer IgG_{2b} was selectively inhibited by $(IgG_{2b})_1$ compared to $(IgG_{2a})_1$ proteins and contrasts markedly with the selective inhibition by $(IgG_{2a})_1$ of monomer IgG_{2a} binding shown in Fig. 3. This indicates that $(IgG_{2a})_1$ and $(IgG_{2b})_1$ bind to different structures on the cell surface.

Next, the capacity of aggregated proteins to inhibit the uptake of monomer IgG_{2b} was examined. The results of these experiments indicated that IgG_{2a} and IgG_1 aggregates inhibited the uptake of monomer IgG_{2b} as efficiently as aggregated IgG_{2b}. 50% inhibition was obtained with 0.7–1.7 μg protein. These data further strengthen the data presented above that suggest that the site to which monomer IgG_{2b} binds is most likely the same site to which aggregated proteins bind.

III. Trypsin Sensitivity of Receptors for IgG. Because of previously published data (12–16), it was considered likely that the two IgG receptors characterized in section II might also be distinguishable from one another on the basis of differences in sensitivity to tryptic digestion. To analyze this, P388 cells were treated with trypsin and subsequently tested for their capacity to bind monomer IgG_{2a} or aggregated IgG_{2b}. The results of two experiments are shown in Table IV. Whereas the uptake of $(IgG_{2b})_4$ was not inhibited (and was actually enhanced significantly), the uptake of $(IgG_{2a})_1$ was markedly inhibited (61–81%) under the conditions used. Although alternative explanations are possible, the
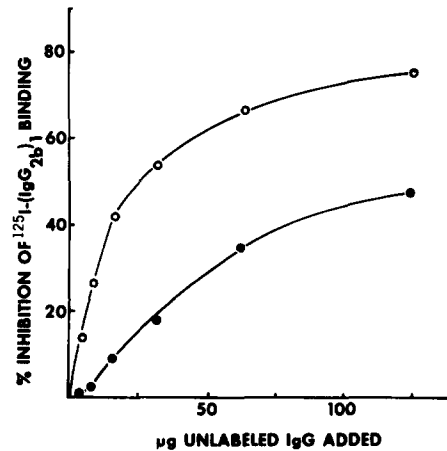
FIG. 4. Inhibition of IgG_{2b} monomer binding by monomers. P388 uptake of ^{125}I-$(IgG_{2b})_1$ was inhibited by various amounts of $(IgG_{2b})_1$ (O—O) and $(IgG_{2a})_1$ (●—●). Significant homologous specificity of inhibition was noted.

TABLE IV

Effect of Trypsinization of P388 Cells on Their Capacity to Bind IgG

Experiment	Trypsin treatment*	Percent binding of IgG	
		$(IgG_{2b})_4$	$(IgG_{2a})_1$
1	−	44	4.6
	+	49	1.8
2	−	53	3.1
	+	63	0.6

* P388 cells $(10^7/ml)$ were treated with 0.3 mg/ml trypsin for 20 min at 37°C.

results are consistent with the concept of two distinct receptors for IgG, one relatively resistant to trypsinization and the other relatively sensitve.

Discussion

The present study on the subclass specificity of receptors for IgG on different cell types was undertaken for two reasons: (a) the accumulating data that immunoglobulins of different subclasses have markedly different biologic activity which may be related to their differential capacities to interact with cell-bound receptors; and (b) to attempt to clarify some of the conflicting reports on the subclass specificities of Fc receptors. With respect to the latter point, several technical reasons may be responsible for these discrepancies. First, in several of the test systems, an EA-rosette assay and its inhibition by myeloma proteins was used. The subclass of the antibody present in the anti-erythrocyte antiserum was not, in general, characterized, and therefore different laboratories may have been utilizing antibodies of different subclasses. The pattern of inhibition observed with myeloma proteins may have reflected to some extent the subclass of the anti-erythrocyte antibody, especially if multiple Fc receptors with different subclass specificity exist. Second, there has been great variation in the form of the myeloma proteins used to inhibit the assay systems. In some cases the

myelomas were unaggregated, although the presence of small amounts of aggregates was not specifically tested for. In other systems the myeloma proteins were heat aggregated but no rigorous analysis of the degree of aggregation was performed. As shown in Table I, degree of aggregation is an important factor in determining the binding capabilities of the myeloma proteins and may well be responsible, in part, for the conflicts noted in the literature.

To avoid the problems stated above, in the present study we have utilized well-defined sizes of chemically aggregated myeloma proteins and have measured the direct binding and the inhibition of binding of these proteins onto various IgG receptor-bearing cells. Although the choice of BDB-aggregated myeloma proteins eliminates some of the technical problems present in the studies mentioned above (viz., the use of antibody of undefined subclass as a test system and the use of myeloma proteins with undefined degrees of aggregation) there are also certain possible disadvantages to the use of BDB-aggregated myeloma proteins. First, although there is no data to support the possibility, the capacity of IgG to interact with an Fc receptor may be altered as a result of the conjugation of gamma globulin with BDB. Second, since the aggregation induced by BDB occurs via Fc as well as Fab fragments, the orientation of IgG within aggregates is not likely to be the same as that which occurs in immune complexes. Despite these theoretical considerations, BDB aggregates have proven to be very useful in the study of the biologic functions of immunoglobulins (4).

When aggregates of the same molecular size were compared for their capacity to bind to different cell types, three patterns of reactivity were demonstrated (Table II). One group, which consisted of macrophage cell lines, bound all subclasses in a similar fashion. Another group bound IgG_1 and IgG_{2b} proteins to a similar degree but bound IgG_{2a} proteins to a lesser extent. A third group of cells bound IgG_1 preferentially to either IgG_{2a} or IgG_{2b} proteins. It is possible that this pattern of reactivity may be simpler than that described, in that the difference in the capacity to bind IgG_{2a} proteins between the macrophage cell lines and the second group of cells represented by D2N and S49 may be due to the presence of two Fc receptor sites on macrophages: one receptor that is similar in its pattern of specificity to that present on S49 and D2N cells (i.e., $IgG_1 = IgG_{2b} > IgG_{2a}$); and a second receptor that specifically reacts with monomeric and aggregated IgG_{2a}. The net result of these two receptor activities would give the observed pattern of aggregated IgG uptake by macrophages (i.e., $IgG_1 = IgG_{2b} = IgG_{2a}$).

The possibility that there are two distinct types of Fc receptors on macrophages initially was evaluated because of the subclass specificity observed when monomer and aggregated proteins were compared; that is, monomer uptake indicated IgG_{2a} specificity, whereas aggregate uptake did not suggest any subclass specificity. The evidence obtained, both by enzymatic degradation studies as well as by specificity of inhibition, strongly supports the hypothesis that two distinct receptors for IgG are present on the surface of macrophages and macrophage-like cell lines. Trypsinization studies demonstrated that under conditions that led to strong inhibition of the binding of monomeric IgG_{2a} to the surface of cells, there was no significant effect on the uptake of radiolabeled

aggregates by these same cells. The inhibition of the binding of monomer IgG_{2a} by trypsin treatment of cells is in keeping with the studies of Unkeless and Eisen on P388 cells, (14) but conflicts with the data of Walker on another macrophage-like cell line, IC-21 (17). Whether this conflict is due to the different cell lines used or to technical differences in the trypsinization procedure is not known.

Inhibition studies gave independent evidence for the presence of two receptors on macrophages. The most striking differences in inhibition patterns between monomer IgG_{2a} binding and aggregate IgG binding which support the two-receptor hypothesis are: (a) when inhibition of binding of monomer and aggregates was investigated utilizing monomeric proteins as inhibitors, $(IgG_{2a})_1$ binding was preferentially inhibited by $(IgG_{1a})_1$, whereas the binding of all subclasses of aggregates was preferentially inhibited by $(IgG_{2b})_1$; (b) when inhibition by aggregates was investigated, the binding of $(IgG_{2a})_1$ was preferentially inhibited by aggregated IgG_{2a}, whereas the binding of aggregated proteins was equally inhibitable by IgG_1, IgG_{2a}, or IgG_{2b} aggregates. The observed heterogeneity of binding was not due to different populations of cells in the P388 cultures bearing single receptors with different specificities since autoradiograms demonstrated that aggregated IgG (of all subclasses) and monomer IgG_{2a} each bound to greater than 95% of the P388 cells.

It was also shown in this study that $(IgG_{2b})_1$ proteins were bound to P388 cells at a site distinct from the $(IgG_{2a})_1$ site since the uptake of $(IgG_{2b})_1$ protein could be inhibited preferentially by $(IgG_{2b})_1$ and not by $(IgG_{2a})_1$ in contrast to the preferential inhibition of $(IgG_{2a})_1$ binding by $(IgG_{2a})_1$. Because of the capacity of $(IgG_{2b})_1$ to inhibit the binding of all aggregates, it is considered most likely that monomer IgG_{2b} binds to the surface of P388 cells at the same site to which aggregates bind.

Our results confirm and extend previous studies by Unkeless and Eisen on the binding of monomeric proteins to macrophages (14). These authors have shown preferential binding of monomer IgG_{2a} proteins to membrane receptors which are trypsin sensitive. Askenase and Hayden also have presented evidence for trypsin-sensitive sites on mouse macrophages which bind oxazolone-specific cytophilic antibody of the IgG_{2a} subclass (15). In a recent report, Walker described IgG binding and inhibition studies similar to ours using an SV-40 transformed macrophage line, IC-21 (17). Although his basic conclusion of the presence of two distinct receptors is in accord with our appraisal, certain differences exist in the pattern of reactivity obtained with IC-21 and the data presented in our study. Walker observed significant binding only with $(IgG_{2a})_1$ and aggregated IgG_{2a} and IgG_{2b}, whereas we found equivalent binding of all aggregated IgG subclasses as well as significant binding of $(IgG_{2b})_1$. Further evidence for dual Fc receptors on mouse macrophages is found in the work of Kossard and Nelson (16) who noted that anti-erythrocyte cytophilic antibody in mouse serum taken soon after sensitization binds to trypsin-sensitive sites on macrophages, whereas antibody obtained long after primary sensitization binds to trypsin-resistant sites.

Although it is difficult to explain the data that we have presented in this study by postulating a mechanism other than there being two distinct Fc

receptors on P388 cells, it is to be stressed that the evidence to support this concept is basically indirect and proof of the existence of two receptors must await their biochemical isolation and characterization.

Summary

To evaluate subclass specificity and aggregate size requirements of IgG receptors on mouse cells, we measured binding of radiolabeled monomeric and BDB-aggregated mouse myeloma proteins fractionated into various sizes by means of gel filtration. Monomers, tetramers, and high molecular weight ($\sim 10^7$ daltons) aggregates were used. The various cells and cell lines studied could be segregated into three patterns of reactivity: (a) Macrophage and macrophage-like cell lines bound monomer IgG_{2a} preferentially; high molecular weight IgG aggregates bound as follows: $IgG_1 = IgG_{2b} = IgG_{2a}$. (b) Lymphoid lines D2N and S49 showed no capacity to bind monomer IgG_{2a}; high molecular weight aggregates bound as follows: $IgG_1 = IgG_{2b} > IgG_{2a}$. (c) Other Thy-1-positive lymphoid cell lines (EL4 and L5178) and normal T and B cells showed no capacity to bind monomer IgG; high molecular weight IgG aggregates bound to a lesser extent than to cells of the first two categories in the following manner: $IgG_1 > IgG_{2b} \geq IgG_{2a}$.

The variable pattern of reactivity of the macrophage-like cell lines with monomer and aggregated IgG suggested that two distinct receptors for IgG were present: one capable of binding IgG_{2a} and another capable of binding all aggregates. Further evidence for this hypothesis was obtained by analysis of the inhibitory capacity of different IgG subclasses on the binding of aggregated IgG and monomer IgG_{2a} to P388 cells. Inhibition of monomer IgG_{2a} binding was effected only by monomer or aggregated IgG_{2a}, whereas inhibition of binding of aggregated IgG_1 or IgG_{2b} was noted with aggregates of all three subclasses with some preferential inhibition by monomer IgG_{2b} being observed. Furthermore, monomer IgG_{2b} binding was preferentially inhibitable by monomer IgG_{2b}. It is postulated from these data that two receptor sites are present on this macrophage-like cell line, one reactive with aggregates of all three subclasses as well as monomer IgG_{2b}, and another receptor specific for monomer IgG_{2a} which also binds aggregated IgG_{2a}. Support of this concept was obtained by trypsinization experiments in which the binding of monomer IgG_{2a} was markedly decreased by trypsin treatment of cells, whereas the binding of aggregated IgG_{2b} was unaffected by this treatment.

References

1. Cline, M. J., N. L. Warner, and D. Metcalf. 1972. Identification of the bone marrow colony mononuclear phagocyte as a macrophage. *Blood.* **39**:326.
2. Rabinovitch, M. 1967. Studies on the immunoglobulins which stimulate the ingestion of glutaraldehyde-treated red cells attached to macrophages. *J. Immunol.* **99**:1115.

3. MacLennan, I. C. M. 1972. Antibody in the induction and inhibition of lymphocyte cytotoxicity. *Transplant. Rev.* **13**:67.

4. Ishizaka, K., and T. Ishizaka. 1960. Biologic activity of aggregated gamma globulin. II. A study of various methods for aggregation and species differences. *J. Immunol.* **85**:163.

5. Klinman, N. R., and R. B. Taylor. 1969. General methods for the study of cells and serum during the immune response: the response to dinitrophenyl in mice. *Clin. Exp. Immunol.* **4**:473.

6. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3**:645.

7. Anderson, C. L., and H. M. Grey. 1974. Receptors for aggregated IgG on mouse lymphocytes. Their presence on thymocytes, thymus-derived, and bone-marrow-derived lymphocytes. *J. Exp. Med.* **139**:1175.

8. Maizel, J. V., Jr. 1969. Acrylamide gel electrophoresis of protein and nucleic acids. *In* Fundamental Techniques in Virology. K. Habel and N. P. Salzman, editors. Academic Press, Inc., New York. 334.

9. Koren, H. S., B. S. Handwerger, and J. R. Wunderlich. 1975. Identification of macrophage-like characteristics in a cultured murine tumor line. *J. Immunol.* **114**:894.

10. Ralph, P., J. Prichard, and M. Cohn. 1975. Reticulum cell sarcoma: an effector cell in antibody-dependent cell-mediated immunity. *J. Immunol.* **114**:898.

11. Chesebro, B., K. Wehrly, K. Chesebro, and J. Portis. 1976. Characterization of Ia8 antigen, Thy-1.2 antigen, complement receptors, and virus production in a group of murine-virus-induced leukemia cell lines. *J. Immunol.* **117**:1267.

12. Lay, W. H., and V. Nussenzweig. 1968. Receptors for complement on leukocytes. *J. Exp. Med.* **128**:991.

13. Steinman, R. M., and Z. A. Cohn. 1972. The interaction of particulate horse-radish peroxidase (HRP)-anti-HRP immune complexes with mouse peritoneal macrophages in vitro. *J. Cell Biol.* **55**:616.

14. Unkeless, J. C., and H. N. Eisen. 1975. Binding of monomeric immunoglobulins to Fc receptors of mouse macrophages. *J. Exp. Med.* **142**:1520.

15. Askenase, P. W., and B. J. Hayden. 1974. Cytophilic antibodies in mice contact-sensitized with oxazolone. Immuno-chemical characterization and preferential binding to a trypsin-sensitive macrophage receptor. *Immunology.* **27**:563.

16. Kossard, S., and D. S. Nelson. 1968. Studies on cytophilic antibodies IV. The effects of proteolytic enzymes (trypsin and papain) on the attachment to macrophages of cytophilic antibodies. *Aust. J. Exp. Biol. Med. Sci.* **46**:63.

17. Walker, W. S. 1976. Separate Fc receptors for immunoglobulins IgG_{2a} and IgG_{2b} on an established cell line of mouse macrophages. *J. Immunol.* **116**:911.