STUDIES OF THE HUMAN LYMPHOCYTE RECEPTOR FOR HEAT-AGGREGATED OR ANTIGEN-COMPLEXED IMMUNOGLOBULIN*

By HOWARD B. DICKLER

(From the Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014)

(Received for publication 6 May 1974)

A mouse lymphocyte receptor for antibody complexed to antigen has been described by several laboratories (1–5). The receptor appeared to be characteristic of bone marrow-(or nonthymus)-derived (B) lymphocytes; binding to the receptor was apparently complement independent; the receptor formed a stable bond to antibody only when the latter was complexed to antigen; binding to the receptor was not inhibited by antimouse immunoglobulin; and the receptor appeared to have binding specificity for the Fc portion of the antibody molecule.

In what appears to be an analogous observation, a human lymphocyte receptor for heat-aggregated immunoglobulin (Ig) complexes has been described (6–9). In normal human peripheral blood, the receptor was found on both B cells and a small subpopulation of lymphocytes (mean 3.6%) which bore both thymus-derived (T) and B-cell markers. In certain cases of chronic lymphocytic leukemia or hypogammaglobulinemia, the receptor was detected on some peripheral blood lymphocytes (PBL)[1] which appeared to be B cells but which lacked easily detectable surface Ig. The binding of aggregated Ig to this receptor was not dependent on complement, divalent cations, or temperature, and was not inhibited by polyvalent (γ, μ, κ, λ) anti-Ig.

The present studies were carried out to further characterize the human lymphocyte receptor for complexed Ig. Data from these studies indicate that: (*a*) the receptor has specificity for the Fc portion of complexed Ig, and it appears that the site on the Fc is dependent on disulfide bond(s); (*b*) heat-aggregated Ig and antigen-complexed Ig bind to the same receptor; (*c*) the receptor for complexed antibody is distinct from surface Ig and appears to be a trypsin-resistant protein or glycoprotein; (*d*) membrane-bound Ig complexes do not readily accumulate at one pole of the cell (cap formation) unless they are subsequently cross-linked with anti-Ig; (*e*) the receptor is necessary for antibody-

* A portion of this work was supported by contract no. NIH-N01-CB-02207.

[1] *Abbreviations used in this paper:* BSA-PBS, 2% bovine serum albumin in phosphate-buffered saline, pH 7.2 or 8.0 with or without Na Azide 0.02%; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; f/p, absorption ratio (495 nm or 515 nm/280 nm); HSA human serum albumin; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline, pH 7.2 or 8.0, 0.05 M PO_4 and 0.15 M NaCl; TMRITC, tetramethylrhodamine isothiocyanate.

dependent lymphocyte-mediated cytotoxicity; and (*f*) there may be more than one kind of lymphocyte receptor for complexed antibody and/or lymphocyte subpopulations which bear the receptor.

Materials and Methods

Aggregated Human Ig.—Cohn Fraction II human Ig and IgG prepared by DEAE-cellulose chromatography (both from Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill.) were dissolved in phosphate-buffered saline 0.05 M PO_4, 0.15 M NaCl (PBS), pH 7.2, at concentrations of 10–50 mg/ml. For conjugation of Ig to fluorescein isothiocyanate (FITC), 10% (vol/vol) carbonate bicarbonate buffer, 0.5 M, pH 9.0, was added, the pH corrected to 9.0 with 1 N NaOH, and FITC (Baltimore Biological Laboratories, Cockeysville, Md.), 20 μg/mg, protein added. The preparation was incubated and stirred for 18 h at 4°C, and then exhaustively dialyzed with PBS, pH 7.2, to remove unconjugated FITC. The absorption 495 nm/280 nm (f/p) ratios of conjugated preparations were between 1.0 and 1.1. Conjugated and unconjugated preparations were heat aggregated at 63°C for 20 min and pelleted at 145,000 *g* for 1 h at 4°C. The supernate was discarded; the pellet (~20–25% of the starting material) homogenized in PBS, pH 8.0, in a 7-ml Dounce homogenizer (Kontes Glass Co., Vineland, N. J.) and the pH corrected to 8.1 with 0.1 N NaOH. Just before use aggregates were centrifuged at 600 *g* for 15 min at 4°C, and adjusted to appropriate concentrations (see below) with PBS, pH 8.0. For certain experiments, aggregates of specific size and deaggregated 7S Ig and IgG were obtained by preparative ultracentrifugation as described by Trautman and Cowan (10).

Aggregated Proteins. –Crystallized bovine serum albumin (BSA), crystallized human serum albumin (HSA), and human transferrin (all from Miles Laboratories, Inc., Miles Research Div.) were dissolved in PBS, pH 7.2, at 20 mg/ml and conjugated to FITC by the same methods as those used for Ig except that 10 μg FITC/mg protein was used for BSA and HSA. f/p ratios were: BSA, 1.2; HSA, 1.0; and transferrin, 1.2. In order to produce the same degree of aggregation as obtained with Ig (as judged by sedimentation rate during subsequent centrifugation), the proteins were heated as follows: BSA, 80°C, 20 min; HSA, 70°C, 20 min; and transferrin, 70°C, 20 min. After heating, the proteins were further processed as described for aggregated Ig. Protein aggregates similar in size to Ig aggregates were obtained by preparative ultracentrifugation (10).

Chemically Modified Ig.—Chemical modifications were performed on Cohn Fraction II Ig. $F(ab')_2$ fragment was prepared by pepsin digestion (1:50 ratio pepsin to protein) by the published methods (11), and separation by Sephadex G-150 gel filtration (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). Mild oxidation was performed by adding H_2O_2 (1.0 mM final concentration) to Ig (10 mg/ml PBS, pH 7.2) for 2 h at 23°C followed by dialysis with PBS, pH 7.2. Reduction and alkylation was performed as described by Wiedermann et al. (12). Modified Ig preparations were conjugated to FITC and heat aggregated as described for Ig, and aggregates similar in size to Ig aggregates were obtained by preparative ultracentrifugation (10).

Antigen-Antibody Complexes.—Keyhole limpet hemocyanin (KLH) and rabbit antibody to KLH purified by affinity chromatography were kindly provided by Dr. Michael Blaese, Metabolism Branch, National Cancer Institute, Bethesda, Md. Both KLH and anti-KLH were dialyzed into PBS, pH 8.0, and KLH was conjugated to FITC as described for Ig. Preliminary experiments showed that neither KLH nor anti-KLH alone would bind to lymphocytes, and that complexes preformed at equivalence or in antigen excess caused clumping of cells. Complexes were therefore preformed in antibody excess (molar basis) by combining equal amounts of KLH (0.4 mg/ml) and anti-KLH (0.4 mg/ml) and incubating for 30 min at 23°C.

Anti-Ig Antiserum.—A polyvalent (μ, γ, α, κ, λ), unabsorbed, antihuman Ig antiserum raised in rabbits and conjugated to tetramethylrhodamine isothiocyanate (TMRITC) was

obtained from Cappel Laboratories, Downingtown, Pa. (lot no. 6481). The antiserum was tested for specificity by immunoelectrophoresis and Ouchterlony double diffusion. Specificity was also demonstrated by loss of activity after absorption with insolubilized purified Ig (μ, γ, α, κ, λ). The antiserum was reconstituted with PBS, pH 7.2, had an f/p absorption 515 nm/280 nm ratio of 0.36, and was deaggregated by preparative ultracentrifugation immediately before use (10).

Lymphocyte Separation.—Mononuclear cells were isolated from the peripheral blood of normal humans by density flotation as described by Böyum (13). The mononuclear cells (up to 10^8) were washed three times with PBS, pH 7.2, and, in order to remove monocytes, resuspended in 5 ml of 100% autologous serum, passed onto 200 mg of nylon fiber (Fenwal Laboratories, Morton Grove, Ill.), loosely packed to the 8 ml mark of a 10 ml disposable glass syringe (Becton, Dickinson & Co., Rutherford, N. J.), and incubated at 37°C for 30 min. Cells were then eluted with 25 ml of PBS, pH 7.2, washed once, and again resuspended in 100% autologous serum (2×10^7/ml). In order to assess residual phagocytic cells, 1-μm polystyrene beads (Dow Diagnostics, Indianapolis, Ind.) were added (2 mg/ml); the cell suspension was mixed and incubated at 37°C for 30 min. The cells were then washed three times with PBS, pH 7.2, and resuspended as required for various assays (see below). Final preparations contained 99.7% lymphocytes (as determined by morphology and phagocytosis) and were >99% viable (trypan blue exclusion). Yields were ~60% and minimal selective loss of B lymphocytes was noted (8).

Fluorescence.—For immunofluorescent staining of lymphocyte surface Ig, isolated lymphocytes were suspended at 20×10^6/ml in 2% BSA-PBS, 0.02% Na azide, pH 7.2 (BSA-PBS, pH 7.2), and TMRITC-conjugated anti-Ig antiserum was used at 1.2 mg/ml. Antiserum and cells (0.05 ml of each) were mixed and incubated at 4°C for 30 min. The cells were then washed three times with 10 vol excess of BSA-PBS, pH 7.2, vigorously resuspended (to prevent clumping) in the same medium, and wet mounts prepared.

Fluorescent detection of complex binding was performed as described for Ig staining except that BSA-PBS, pH 8.0, was used and the incubation was at 23°C.[2] Preparations of FITC-conjugated and heat-aggregated Ig, chemically modified Ig, and other proteins (see above), were used at 1.0 mg/ml; preformed FITC-conjugated KLH-anti-KLH complexes were used at 0.4 mg/ml.

Microscopy was performed on a Leitz orthoplan microscope (E. Leitz, Inc., Rockleigh, N. J.) equipped with a Ploem vertical illuminator, D. C. power source and H. B. O. 100 W mercury arc lamp, Heine phase condenser and 90×/1.32 N. A. apochromatic oil immersion objective, and a monocular head. The excitation filters for FITC were: one BG 38, two KP 490's, and one K475; and for TMRITC: one BG 38 and three S 546's (Type AL). Dichroic mirror/suppression filters for FITC were: TK 510/K515, and for TMRITC: TK 580/K 580. These filter combinations allowed detection of the indicated fluorochrome with absolute selectivity. Microscopic fields were read alternately under phase and UV illumination for the percentage of fluorescent-positive lymphocytes. The criterion for positive fluorescence with anti-Ig was uniform punctate surface staining, and for complexes was 3 or more fluorescent complexes bound/cell. Only cells which appeared to be lymphocytes by morphologic criteria and which had neither phagocytized nor adhered polystrene beads were counted. A minimum of 200 lymphocytes were counted/preparation and reproducibility was ±2%.

Alterations of Cell Surface Antigens.—

Modulation: Isolated lymphocytes were labeled with either TMRITC-conjugated anti-Ig or FITC-conjugated heat-aggregated Ig as described above except no Na azide was used. After washing three times the cells were resuspended (10^6/ml) in Eagle's no. 2 medium con-

[2] Binding of complexes at pH 8.0 and 23°C produces a more uniform staining pattern with small complexes being distributed over the entire cell surface. This is in contrast to the clumpy pattern of binding obtained at pH 7.2 and 4°C as first described (6).

taining 20% (vol/vol) heat-inactivated fetal calf serum (FCS). After incubation at 37°C for various periods of time, the cells were washed with BSA-PBS containing Na azide and wet mounts were prepared. In certain experiments samples of the modulated cells were restained with the same reagent or tested with the second reagent.

Enzymatic Treatment: Isolated lymphocytes (10^7/ml in Hanks' balanced salt solution) were incubated for 20 min at 37°C in the presence of trypsin, 2 or 10 mg/ml, (Grade V, Miles Laboratories, Inc., Miles Research Div.), or pronase, 1 mg/ml, (Type VI, Sigma Chemical Co., St. Louis, Mo.). The cells were then washed three times and assayed for surface Ig and aggregated Ig binding as described above.

Antibody-Dependent Lymphocyte-Mediated Cytotoxicity.—The assay utilized has been described in detail elsewhere (14). Briefly, isolated nonimmune lymphocytes (attackers) were mixed in suspension with ^{51}Cr-labeled lymphocytes coated with antibody from a multiply transfused patient (targets). After a 4-h incubation at 37°C the suspensions were centrifuged and supernatant samples counted for ^{51}Cr. Immune activity was gauged by "% lysis", the amount of ^{51}Cr released from target cells by attacking lymphocytes relative to maximal releasable ^{51}Cr as determined by freeze-thaw of the targets. Results were corrected for spontaneous release of ^{51}Cr from targets incubated with media alone. Quadruplicate samples were used and standard errors of the mean (SEM) calculated.

The effect of lymphocyte-bound Ig complexes on this immune activity was evaluated by preincubating attacker lymphocytes with various amounts of heat-aggregated IgG (or, as controls, 7S IgG or medium) as described above, for the binding of fluorescent complexes. No Na azide was present and the lymphocytes were thoroughly washed to remove unbound complexes before use in the cytotoxic assay.

RESULTS

Specificity of the Receptor.—To determine whether the property of binding of heat-aggregated Ig to the lymphocyte receptor was shared by other proteins, FITC-conjugated and heat-aggregated HSA, BSA, and human transferrin were prepared so as to be equivalent to the Ig aggregates (size, concentration, and f/p ratio). The various protein aggregate preparations were then assayed for binding to normal human PBL (Table I). Aggregated Ig bound to the same percentage of lymphocytes as that which bore surface Ig. These two markers have previously been shown to be on the same cells, which are primarily B lymphocytes (6, 8). In contrast, only a rare lymphocyte bound any of the other aggregated proteins, indicating that the receptor would not bind every type of aggregated protein and appeared to be specific for complexed Ig.

The nature of the site(s) on the Ig molecule recognized by the receptor was examined by assaying the binding to PBL of aggregates prepared from Ig which had been chemically modified. As shown in Table II, lymphocytes did not bind aggregated Ig which lacked the Fc portion of the molecule ($F(ab')_2$), or aggregates prepared from Ig with modified disulfide bonds (reduced and alkylated Ig, oxidized Ig).[3] These studies provided evidence that the receptor recognizes a site on the Fc portion of complexed Ig, and that the integrity of this site is dependent upon intact disulfide bond(s).

[3] Hydrogen peroxide is a relatively nonspecific oxidizing agent capable of modifying several amino acid residues including disulfide bonds (15), whereas reduction and alkylation with mercaptoethanol and iodoacetamide specifically cleaves disulfide bonds (16).

TABLE I

Binding of Heat-Aggregated Proteins to Normal Human Peripheral Blood Lymphocytes

Protein*	% Positive‡	
	Exp. 1§	Exp. 2
Human Ig	16.5	24.5
HSA	<0.1	0.1
Human transferrin	0.3	0.5
BSA	0.2	0.6
Antihuman Ig	16.0	25.0

* Proteins were conjugated to FITC, heat aggregated, and preparatively ultracentrifuged (see Materials and Methods) so as to be equivalent (size, f/p ratio, and concentration).

‡ 300-1,000 cells evaluated/preparation.

§ Different preparations of each of the proteins as well as a different blood donor were used in the two experiments.

TABLE II

Binding of Chemically Modified and Heat-Aggregated Ig to Normal Human Peripheral Blood Lymphocytes

Preparation*	% Positive‡	
	Exp. 1§	Exper. 2
Ig	24.0	15.5
$F(ab')_2$	0.5	<0.5
Reduced and alkylated Ig	0.5	1.0
Oxidized Ig	1.5	0.5
Antihuman Ig	23.5	16.5

* For details of chemical modifications see Materials and Methods. Preparations were conjugated to FITC, heat aggregated, and preparatively ultracentrifuged (see Materials and Methods) so as to be equivalent (size, f/p ratio, and concentration).

‡ Minimum of 200 cells evaluated/preparation.

§ The two experiments used different blood donors and separately modified Ig's.

To assess whether the same receptor bound both heat-aggregated Ig and antigen-antibody complexes, the latter were preformed with affinity chromatography purified rabbit anti-KLH and FITC-conjugated KLH. When such complexes were examined for binding to lymphocytes (Table III), the same percentage of PBL bound KLH-anti-KLH as bound heat-aggregated Ig and stained for surface Ig. In addition, inhibition studies were performed, in which lymphocytes were incubated with unconjugated KLH-anti-KLH or heat-aggregated Ig, washed thoroughly, and then incubated with the alternate complex conjugated to FITC. The results are presented in Table IV. The binding of aggregated Ig inhibited the binding of KLH-anti-KLH (exp. 8 vs. 3) and vice versa (exp. 10 vs. 2). Inhibition was never complete (~70%), but a similar

TABLE III

Comparison of Heat-Aggregated Ig and Antigen-Antibody Complex Binding to Normal Human Peripheral Blood Lymphocytes

Donor	% Positive*		
	Surface Ig	Aggregated Ig binding	Anti-KLH-KLH binding‡
J. C.	30.0	29.0	29.0
P. H.	24.0	23.5	25.5
H. D.	14.0	15.5	15.0
R. H.	9.0	9.0	9.5

* Minimum of 200 cells evaluated/preparation.

‡ KLH-Anti-KLH complexes were preformed (see Materials and Methods).

TABLE IV

Cross Inhibition of Binding to Lymphocytes Between Heat-Aggregated Ig and Antigen-Antibody Complexes

Exp.	1st incubation*	2nd incubation‡	% Positive§
1	Medium	Anti-Ig	24.5
2	Medium	Aggregated Ig	24.0
3	Medium	KLH-anti-KLH	26.5
4	Anti-KLH	Aggregated Ig	23.5
5	KLH	Aggregated Ig	26.0
6	KLH-anti-KLH	Anti-Ig	25.0
7	Aggregated Ig	Aggregated Ig	6.5
8	Aggregated Ig	KLH-anti-KLH	8.0
9	KLH-anti-KLH	KLH-anti-KLH	8.5
10	KLH-anti-KLH	Aggregated Ig	6.0

* Reagents for 1st incubation were not conjugated with a fluorescent isomer.

‡ Lymphocytes washed three times between incubations. Reagents for 2nd incubation were conjugated to FITC (aggregated Ig and KLH-anti-KLH) or TMRITC(anti-Ig).

§ Minimum of 200 cells evaluated/preparation.

lack of complete inhibition was noted when homologous complexes were used (exps. 7 and 9). This was presumed to be due to the heterogeneity in the size of the complexes such that some small complexes bound during the first incubation could be displaced by larger complexes during the second incubation. Other controls showed that neither antigen or antibody alone inhibited the binding of aggregated Ig (exps. 4 and 5), and that the binding of antigen-antibody complexes did not interfere with the ability to detect surface Ig (exp. 6). Thus, it appeared that the same receptor was responsible for binding both antigen-antibody complexes and heat-aggregated Ig.

Chemical Nature of the Receptor.—In order to examine the question of whether the receptor for complexed Ig and lymphocyte surface Ig were associated, capping experiments were performed. PBL were labeled with TMRITC-con-

jugated anti-Ig, washed, and then incubated in medium containing FCS for 2 h at 37°C. After such treatment fluorescence was usually seen as a cap at one pole of the cell as described by Taylor et al. (17). If capped cells were relabeled with the same reagent, no staining was seen outside the cap. In contrast, when capped cells were allowed to bind FITC-conjugated heat-aggregated Ig, the complexes were found to be bound randomly to the lymphocyte surface, not just the portion bearing the Ig cap (Fig. 1). This result provided strong evidence that surface Ig and the receptor for complexed antibody were independent. The reverse experiment could not be performed because of the failure of bound complexes to cap (see below).

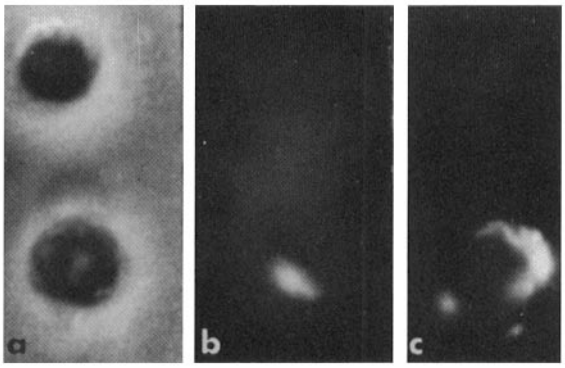

FIG. 1. Demonstration of the independence of surface Ig and the receptor for aggregated Ig. (*a*) Phase-contrast illumination of two lymphocytes. One lymphocyte, under selective illumination for rhodamine (*b*) reveals cap formation with TMRITC-conjugated anti-Ig, and under selective illumination for fluorescein (*c*) reveals binding of FITC-conjugated aggregated Ig. The aggregated Ig is randomly bound to the cell surface, not just the portion bearing the surface Ig cap. × 900.

Additional information on the nature of the receptor was obtained by studying the ability of lymphocytes which had been treated with enzymes to bind aggregated Ig (Table V). Lymphocytes treated with trypsin, even at concentrations as high as 10 mg/ml, retained their ability to bind aggregated Ig, whereas no complexes were bound when the cells had been treated with pronase. This suggested that the receptor was a trypsin-resistant protein or glucoprotein. These experiments also provided further evidence of the independence of the receptor and surface Ig, since the latter was trypsin sensitive.

Cap Formation of Lymphocyte-Bound Heat-Aggregated Ig Complexes.— Lymphocytes were allowed to bind FITC-conjugated heat-aggregated Ig, washed, and then incubated in medium containing FCS for varying time periods at 37°C (Table VI). The complexes appeared to be relatively stable on the cell surface under these conditions, and only a minority of the cells showed cap formation. The paucity of capping was not due to a lack of mobility of the

receptor since if lymphocytes bearing FITC-conjugated heat-aggregated Ig complexes were exposed to unconjugated anti-Ig and then allowed to incubate at 37°C, cap formation was readily apparent (>90% of aggregate-positive cells). Thus, it appeared that the Ig complexes alone did not produce sufficient cross-linking of the receptor to allow cap formation.

Role of the Receptor in Antibody-Dependent Lymphocyte-Mediated Cytotoxicity.—Lymphocytes were allowed to bind heat-aggregated IgG and then were assayed for their ability to lyse antibody-coated target cells (Table VII). Lymphocytes which had bound IgG complexes were markedly inhibited in this immune activity as compared to lymphocytes pretreated with medium or native IgG. In addition, lymphocytes which had bound aggregated IgG did not show nonspecific toxicity for targets which had not been coated with antibody. These results provided strong evidence that the receptor for complexed Ig plays a necessary role in antibody-dependent lymphocyte-mediated cytotoxicity, and that binding of nonspecific complexes to the receptor is not sufficient to activate cytotoxic activity under these conditions.

The quantity of aggregated IgG necessary to inhibit this immune activity was investigated by varying the amount of complexes preincubated with the

TABLE V

Effect of Enzymatic Pretreatment of Lymphocytes on their Ability to Bind Heat-Aggregated Ig

Enzymatic treatment*	% Positive‡			
	Exp. 1§		Exp. 2	
	Aggregated Ig binding	Surface Ig	Aggregated Ig binding	Surface Ig
None	19.5	21.0	17.0	16.5
Trypsin (2 mg/ml)	19.0	0.5	15.5	<0.5
Trypsin (10 mg/ml)	20.0	<0.5	16.0	0.5
Pronase (1 mg/ml)	0.5	<0.5	1.0	<0.5

* For details see Materials and Methods.

‡ Minimum of 200 cells evaluated/preparation.

§ Different blood donors and enzyme preparations used in each experiment.

TABLE VI

*Cap Formation of Lymphocyte-Bound Heat-Aggregated Ig Complexes**

Hours at 37°C	% of PBL binding aggregated Ig	% of PBL with cap formation‡
0	21.0	0.5
1	18.5	2.0
2	18.0	3.5
4	13.5	4.5

* For details see Materials and Methods. Minimum of 200 cells evaluated/preparation.

‡ Cap formation defined as surface fluorescence over <50% of cell surface.

attacking lymphocytes (Fig. 2). A minimum of 0.06 mg/2 × 10^7 lymphocytes was necessary to produce detectable inhibition, and maximal inhibition was obtained with 2 mg/2 × 10^7 lymphocytes. Total inhibition was never obtained. This may have been due to the fact that during the 4-h incubation attacking cells may have generated new receptors and/or IgG complexes may have dissociated from some receptors. Nevertheless, the possibility that some lytic

TABLE VII

*Effect of Preincubation of Nonimmune Lymphocytes with Heat-Aggregated Ig on their Ability to Lyse Antibody-Coated Target Cells**

Preincubation‡	Antibody-coating of target cells	% lysis ± SEM§
Medium	No	−0.8 ± 0.5
IgG	No	−1.1 ± 0.4
Heat-aggregated IgG	No	0.6 ± 0.4
Medium	Yes	52.1 ± 2.8
IgG	Yes	56.8 ± 2.0
Heat-aggregated IgG	Yes	16.7 ± 1.6

* For details see Materials and Methods.

‡ Both IgG preparations used at 2.0 mg/ml.

§ % lysis of target cells incubated in medium alone was 9.9% and 9.7% for coated and uncoated targets, respectively. This value was substracted from lysis values in test groups.

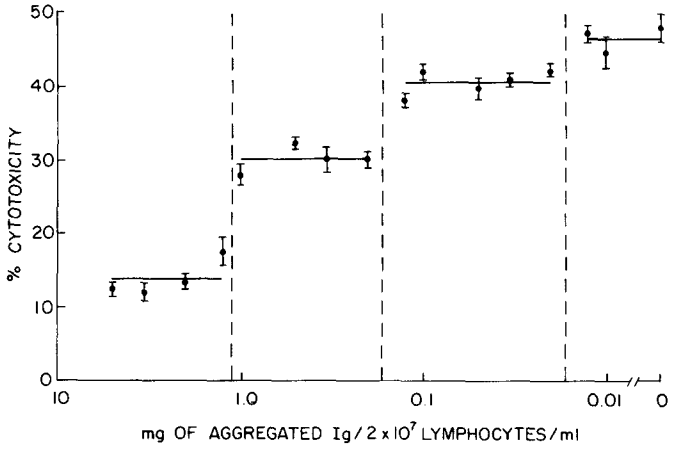

FIG. 2. Effect of preincubation of nonimmune lymphocytes with various amounts of heat-aggregated Ig on their ability to lyse antibody-coated target cells. Each point represents the mean of quadruplicate samples with SEM indicated. Vertical dashed lines are drawn at concentrations of aggregated IgG above and below which plateaus were predicted on the basis of an identical and independent previous experiment. Horizontal solid lines represent the means of points between those concentrations. Each of these means was significantly different from each of the other means ($P < 0.005$) as measured by Student's t test.

activity was mediated by another mechanism not involving the receptor for complexed Ig could not be ruled out.

Between the concentrations necessary to produce perceptible and maximal inhibition, the dose-response curve was not linear but rather two plateaus were seen. That this observation was not artifactual was suggested by: (*a*) the same phenomenon was observed in three completely independent trials; (*b*) the concentrations above and below which plateaus were seen did not vary and were predictable from experiment to experiment; and, (*c*) the mean percent cytotoxicity for each of the predicted plateaus was significantly different ($P < 0.005$) from the means of every other plateau as measured by Student's t test. These data were consistent with the possibility that there may be more than one kind of receptor which may vary in affinity for Ig complexes and/or different populations of lymphocytes which vary in the number of receptors which they bear or in the topographical location of the receptors on their cell surface.

DISCUSSION

The human lymphocyte receptor for complexed Ig has been further characterized in the present studies. The specificity of the receptor was investigated by studying the binding of heat-aggregated preparations of both other proteins and Ig's which had undergone chemical modifications. Such preparations were equivalent (size, f/p ratio, and concentration) to heat-aggregated Ig complexes. Aggregates of HSA, BSA, and transferrin did not bind to lymphocytes, which suggested that the receptor is specific for complexed Ig. In addition, the site recognized by the receptor appears to involve the Fc portion of the Ig molecule, since aggregated $F(ab')_2$ did not bind. Studies in the murine system (2, 3) are in agreement with this latter result. Further, intact disulfide bond(s) appear to be required for the integrity of the site recognized by the receptor, since aggregates of mildly oxidized or reduced and alkylated Ig did not bind. Finally, inhibition experiments indicated that the same receptor binds both heat-aggregated Ig and Ig complexed to antigen. Thus, evidence was obtained that at least some of the changes produced in the Ig molecule by heat aggregation mimic those effected by complexing antibody to antigen. This provides a basis for the use of heat-aggregated Ig complexes as a model to study the receptor.

The evidence that lymphocyte surface Ig and the lymphocyte receptor for complexed Ig are distinct is now substantial. In addition to the present studies that showed independent capping of these two surface moieties and differential sensitivity to trypsin, previous reports have indicated that anti-Ig does not inhibit complexed Ig binding (2, 6), and that in certain disease states some PBL with no detectable surface Ig can nevertheless bind heat-aggregated Ig (7, 9).

The inability of trypsin to affect the binding of complexed Ig, previously reported by Eden et al. (5), has now been confirmed. Furthermore, the present

studies have shown that lymphocytes treated with pronase are unable to bind such complexes. This result suggested that the receptor is a protein or glycoprotein. However, the possibility that the receptor is attached to the membrane by a pronase-sensitive protein could not be ruled out by the present studies.

It is of interest to compare the lymphocyte binding of Ig complexes with that reported for macrophages (18). The binding of Ig complexes to both lymphocytes and macrophages was inhibited by reduction and alkylation of the Ig, and not inhibited by pretreatment of the cells with trypsin. In contrast, lymphocytes pretreated with pronase were unable to bind Ig complexes, which was not the case for macrophages (although in the macrophage studies a much lower concentration of enzyme was used). Also, the macrophage receptor for complexed Ig appeared to have relative species specificity, whereas this has not been found for lymphocytes (binding and inhibition studies utilizing rabbit antibody-antigen complexes and heat-aggregated human Ig complexes are shown in Tables III and IV. Also, see reference 1). However, differences in experimental design might account for these contrasting results. Thus, it remains unclear whether the receptor sites for complexed Ig on lymphocytes and macrophages are similar or different.

The hypothesis has been put forward that capping of receptors may play a role in lymphocyte triggering (17). Therefore, the ability of lymphocyte-bound heat-aggregated Ig complexes to cap was investigated. Only a minority of cells with bound complexes showed cap formation when incubated for several hours at 37°C in FCS-supplemented tissue culture medium. However, if such complexes were further cross-linked with anti-Ig, almost all cells showed capping of the complexes. Thus, since movement of the receptor could be induced, this suggested that complexes alone do not produce sufficient cross-linking to allow cap formation. This could be explained by the receptor being univalent, or by lymphocytes having few receptors and/or the receptors being far apart on the cell surface. The present studies do not distinguish between these possibilities. However, the results do imply that whatever the function(s) of the receptor, marked movement of the receptor and/or the bound complexes is not required.

Data from several laboratories have suggested that the receptor for complexed Ig plays a role in antibody-dependent lymphocyte-mediated cytotoxicity (19–23). This was directly evaluated in the present studies by assaying lymphocytes which had bound heat-aggregated IgG for their ability to lyse antibody-coated target cells. Complexes bound under these conditions only affect the receptor for complexed Ig in that no steric inhibition of other surface molecules, e.g. surface Ig (see Table IV, exp. 6), and the C3 receptor (5), has been detected. Therefore, the inhibition of antibody-dependent lymphocyte-mediated cytotoxicity which was produced by the bound complexes provides strong evidence for the requirement of the receptor for complexed Ig in this immune activity. Further, the binding of nonspecific complexes (heat-aggregated IgG) to the receptor was not sufficient to elicit cytotoxic activity in the cells bearing

the receptor. A possible interpretation of this latter result is that the receptor serves as a recognition and attachment mechanism but that cytotoxic activity is activated by some other means.

When the amount of heat-aggregated IgG needed to inhibit cytotoxic activity was investigated, an unexpected observation resulted: Between the concentrations of complexes needed to produce perceptible and maximal inhibition the dose-response curve was not linear but rather showed distinct plateaus. This suggested that there may be more than one kind of receptor for complexed Ig and/or different subpopulations which bear the receptor. The previous observation that normal lymphocytes which bind complexed Ig are very heterogeneous in the amount bound (6), whereas "clonal" lymphocyte populations are relatively homogenous (7), is consistent with these two possibilities but does not distinguish between them. With regard to the possibility of different subpopulations bearing the receptor, the responsible cytotoxic cell in the test system used in these studies has been defined as an Ig-bearing, nonphagocytic, nonadherent lymphocyte.[4] Such lymphocytes could be either B cells or lymphocytes bearing both B- and T-cell markers (8). While the exact nature of these latter cells is unknown, it is conceivable that they may be activated T cells since some T lymphocytes can absorb cytophilic antibody (24), and activated T cells apparently bear the receptor for complexed Ig (25, 26). Thus, the different lymphocyte populations involved in antibody-dependent lymphocyte-mediated cytotoxicity may be B cells and activated T cells. Alternatively, subpopulations of B lymphocytes may exist. Studies are in progress using a fluorescent-activated cell sorter in an attempt to answer these questions.

While the receptor for complexed Ig appears to perform a necessary function in antibody-dependent lymphocyte-mediated cytotoxicity, it cannot be assumed that this is the only or even the most important role of this receptor. In view of the accumulating evidence for the existence and functional properties of T-cell Ig (27–30), the possibility should be considered that the lymphocyte receptor for complexed Ig plays a role in cell-cell cooperation and regulation of the immune response.

SUMMARY

The lymphocyte receptor for complexed immunoglobulin was shown not to bind heat-aggregated human serum albumin, bovine serum albumin, transferrin, $F(ab')_2$, reduced and alkylated Ig, and mildly oxidized Ig, which indicated that the receptor is specific for a site dependent on disulfide bond(s) on the Fc portion of complexed Ig. Inhibition experiments provided evidence that the same receptor binds both heat-aggregated Ig and antigen-antibody complexes.

Lymphocytes treated with pronase were no longer able to bind Ig complexes, which suggested that the receptor is a protein or glycoprotein. Additional

[4] Yust, I., N. F. Adkinson, Jr., H. B. Dickler, R. W. Smith, R. H. Schwartz, S. O. Sharrow, H. W. Tyrer, D. L. Mann, and J. R. Wunderlich. Manuscript in preparation.

evidence was obtained that lymphocyte surface Ig and the receptor for complexed Ig are distinct since the former could be capped without affecting the distribution of the latter, and surface Ig was not detectable after trypsinization of lymphocytes, whereas the binding of Ig complexes was unaffected by such treatment. Incubation of lymphocytes which had bound Ig complexes in tissue culture medium at 37°C revealed that the complexes remained on the surface membrane for several hours, and that only a minority of lymphocytes binding complexes showed cap formation.

Lymphocytes which had heat-aggregated IgG specifically bound to their receptors for complexed Ig were markedly inhibited in their ability to mediate antibody-dependent cytotoxicity, thus providing strong evidence for the necessity of the receptor in this immune activity. Titration of this inhibition with varying amounts of complexes revealed distinct plateaus in the dose-response curve. This suggested that there may be more than one kind of receptor and/or different populations of lymphocytes which bear the receptor.

Grateful acknowledgment is made to Doctors R. M. Blaese, H. P. Brusman, P. A. Henkart, H. G. Kunkel, W. D. Terry, and J. R. Wunderlich for their help and advice in various phases of these studies; and to M. Kubicek and J. Smith for their invaluable technical assistance.

REFERENCES

1. Basten, A., J. F. A. P. Miller, J. Sprent, and J. Pye. 1972. A receptor for antibody on B lymphocytes. I. Method of detection and functional significance. *J. Exp. Med.* **135**:610.
2. Basten, A., N. L. Warner, and T. Mandel. 1972. A receptor for antibody on B lymphocytes. II. Immunochemical and electron microscopy characteristics. *J. Exp. Med.* **135**:627.
3. Paraskevas, F., S.-T. Lee, K. B. Orr, and L. G. Israels. 1972. A receptor for Fc on mouse B-lymphocytes. *J. Immunol.* **108**:1319.
4. Paraskevas, F., K. B. Orr, E. D. Anderson, S.-T. Lee, and L. G. Israels. 1972. The biologic significance of Fc receptor on mouse B-lymphocytes. *J. Immunol.* **108**:1729.
5. Eden, A., C. Bianco, and V. Nussenzweig. 1973. Mechanism of binding of soluble immune complexes to lymphocytes. *Cell. Immunol.* **7**:459.
6. Dickler, H. B., and H. G. Kunkel. 1972. Interaction of aggregated γ-globulin with B lymphocytes. *J. Exp. Med.* **136**:191.
7. Dickler, H. B., F. P. Siegal, Z. H. Bentwich, and H. G. Kunkel. 1973. Lymphocyte binding of aggregated IgG and surface Ig staining in chronic lymphocytic leukemia. *Clin. Exp. Immunol.* **14**:97.
8. Dickler, H. B., N. F. Adkinson, Jr., and W. D. Terry. 1974. Evidence for individual human peripheral blood lymphocytes bearing both B and T cell markers. *Nature (Lond.).* **247**:213.
9. Dickler, H. B., N. F. Adkinson, Jr., and W. D. Terry. 1974. Lymphocytes in patients with variable immunodeficiency and panhypogammaglobulinemia: evaluation of B and T cell surface markers and a proposed classification. *J. Clin. Invest.* **53**:834.

10. Trautman, R., and K. M. Cowan. 1968. Preparative and analytical ultracentrifugation. *Methods Immunol. Immunochem.* **2:**81.
11. Nisonoff, A., F. C. Wessler, and L. N. Lipman. 1960. Properties of the major component of a peptic digest of rabbit antibody. *Science (Wash. D. C.).* **132:** 1770.
12. Wiedermann, G., P. A. Miescher, and E. C. Franklin. 1963. Effect of mercaptoethanol on complement binding ability of human 7S gamma globulin. *Proc. Soc. Exp. Biol. Med.* **113:**609.
13. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest. Suppl.* **21:**97.
14. Wunderlich, J., E. Rosenberg, J. Smith, and J. Parks. 1974. Cytotoxic human lymphocyte dependent antibody. *J. Natl. Cancer Inst.* In press.
15. Neumann, N. P. 1972. Oxidation with hydrogen peroxide. *Methods Enzymol.* **25:**393.
16. White, F. H., Jr. 1972. Reduction and reoxidation at disulfide bonds. *Methods Enzymol.* **25:**387.
17. Taylor, R. B., W. P. H. Duffus, M. C. Raff, and S. dePetris. 1971. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nat. New Biol.* **233:**225.
18. Arend, W. P., and M. Mannick. 1972. In vitro adherence of soluble immune complexes to macrophages. *J. Exp. Med.* **136:**514.
19. Larsson, Å., and P. Perlmann. 1972. Study of Fab and $F(ab')_2$ from rabbit IgG for capacity to induce lymphocyte-mediated target cell destruction in vitro. *Int. Arch. Allergy Appl. Immunol.* **43:**80.
20. Möller, G., and S.-E. Svehag. 1972. Specificity of lymphocyte-mediated cytotoxicity induced by in vitro antibody-coated target cells. *Cell. Immunol.* **4:**1.
21. MacLennan, I. C. M., A. Howard, F. M. Gotch, and P. G. Quie. 1973. Effector activating determinants on IgG. I. The distribution and factors influencing the display of complement, neutrophil and cytotoxic B-cell determinants on human IgG sub-classes. *Immunology.* **25:**459.
22. Larsson, Å., P. Perlmann, and J. B. Natvig. 1973. Cytotoxicity of human lymphocytes induced by rabbit antibodies to chicken erythrocytes. Inhibition by normal IgG and by human myeloma proteins of different IgG subclasses. *Immunology.* **25:**675.
23. Wisløff, F., T. E. Michaelsen, and S. S. Frøland. 1974. Inhibition of antibody-dependent human lymphocyte-mediated cytotoxicity by immunoglobulin classes, IgG subclasses, and IgG fragments. *Scand. J. Immunol.* **3:**29.
24. Webb, S. R., and M. D. Cooper. 1973. T cells can bind antigen via cytophilic IgM antibody made by B cells. *J. Immunol.* **111:**275.
25. Yoshida, T. O., and B. Andersson. 1972. Evidence for a receptor recognizing antigen complexed immunoglobulin on the surface of activated mouse thymus lymphocytes. *Scand. J. Immunol.* **1:**401.
26. Van Boxel, J. A., and D. L. Rosenstreich. 1974. Binding of aggregated γ-globulin to activated T lymphocytes in the guinea pig. *J. Exp. Med.* **139:**1002.
27. Marchalonis, J. J., R. E. Cone, and J. Atwell. 1972. Isolation and partial characterization of lymphocyte surface immunoglobulins. *J. Exp. Med.* **135:**956.

28. Marchalonis, J. J., and R. E. Cone. 1973. Biochemical and biological characteristics of lymphocyte surface immunoglobulin. *Transplant. Rev.* **14:**3.
29. Feldman, M. 1972. Cell interactions in the immune response in vitro. V. Specific collaboration via complexes of antigen and thymus-derived cell immunoglobulin. *J. Exp. Med.* **136:**737.
30. Feldmann, M., R. E. Cone, and J. J. Marchalonis. 1973. Cell interactions in the immune response in vitro. VI. Mediation by T cell surface monomeric IgM. *Cell. Immunol.* **9:**1.