# INTERACTION OF AGGREGATED γ-GLOBULIN WITH B LYMPHOCYTES*

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A number of different types of apparent receptor sites have been demonstrated on the surface membrane of lymphocytes. These include immunoglobulin receptors for a variety of antigens (1), a receptor for the C3 component of complement (2), and a possible receptor site for the Fc portion of the immunoglobulin molecule through which antibody alone or antigen-antibody complexes adhere to mouse lymphocytes (3, 4). Attempts in the present study to demonstrate such a receptor for antigen-antibody complexes on human lymphocytes using fluorescein-labeled antigen or antibody presented a number of difficulties with different complexes. One particular antigen-antibody system consistently labeled a population of lymphocytes while several other systems were completely negative. The reason for this discrepancy was not apparent. As an alternative approach, aggregated γ-globulin was employed for the study of this interaction. Such preparations of γ-globulin are known to possess many of the properties of antigen-antibody complexes and are considerably more stable.

## *Materials and Methods*

*Aggregated Human Immunoglobulin.*—Fraction II human γ-globulin (Lederle Laboratories, Pearl River, N. Y.) was dissolved in phosphate-buffered saline (PBS),[1] pH 7.2, at a concentration of 50 mg/ml. This was heat inactivated at 56°C for 30 min, and fluorescein conjugated as previously described (5). Conjugated and unconjugated preparations were heat aggregated at 63°C for 15 min, pelleted, and homogenized in PBS, pH 8.0. Just before use, aggregates were centrifuged at 1000 *g* for 30 min and adjusted to 1–3 mg/ml. Aggregates of isolated myeloma proteins were prepared in a similar manner except lower concentrations of protein were used to form the aggregates. Aggregates of specific size and 7S IgG were obtained by preparative ultracentrifugation.

*Anti-Immunoglobulin Antiserum.*—A polyvalent anti-human immunoglobulin antiserum was raised in rabbits and conjugated with tetramethylrhodamine isothiocyanate, as described by Pernis et al. (6), by Dr. F. P. Siegal. The antiserum (Rho-anti-Ig) was specific for μ-, γ-, kappa, and lambda determinants. It was centrifuged to remove aggregates immediately before use.

*Lymphocyte Separation.*—Mononuclear cells were isolated from the peripheral blood of

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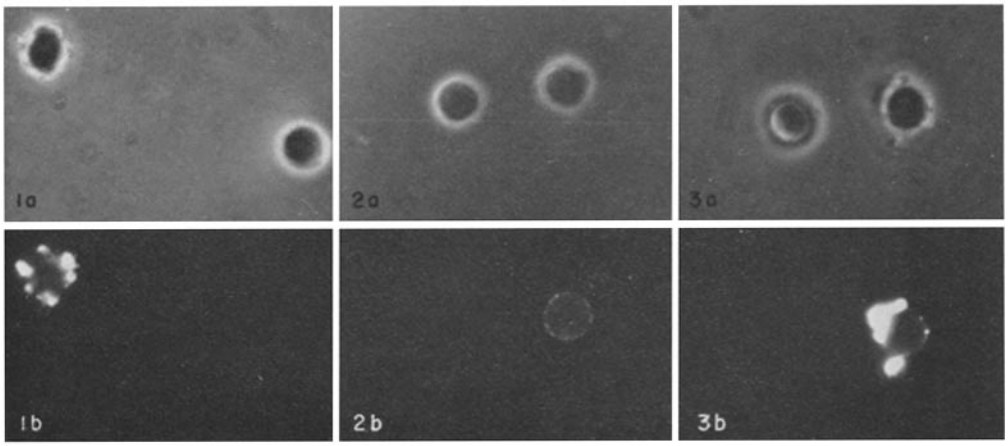
[1] *Abbreviations used in this paper*: Agg, unlabeled aggregated IgG; BSA-PBS, 2% bovine serum albumin in phosphate-buffered saline, pH 7.2, 0.02% Na azide; Fl-Agg, fluorescein-conjugated aggregated IgG; PBS, phosphate-buffered saline.

normal humans and rabbits as described by Siegal et al. (7). The cells were then incubated on nylon, washed, and resuspended in 2% bovine serum albumin in PBS, pH 7.2, 0.02% Na azide (BSA-PBS) at a concentration of 15–20 x $10^6$ cells/ml. The final preparation contained >98% small lymphocytes which were 98–99% viable. BSA-PBS, pH 8.0, was used in experiments with aggregates of specific size.

*Fluorescence.*—Immunofluorescent staining of lymphocyte surface Ig was performed as described by Pernis et al. (6). Two methods were used for aggregates. In the direct method, fluorescein-conjugated aggregated IgG (Fl-Agg) and lymphocytes (0.05 ml of each) were incubated at 4°C for 30 min. Cells were washed in BSA-PBS, resuspended in the same medium, and wet mounts prepared. Slides were kept at 4°C until read. The indirect method was similar to the direct method except unlabeled aggregates (Agg) were used and cells were stained after washing with Rho-anti-Ig. Ig spots were always uniformly small and differentiable from aggregates if the Rho-anti-Ig was centrifuged, Na azide used, and slides allowed only minimal time at room temperature. Microscopy was performed as described by Pernis et al. (6). A minimum of 200 cells per preparation were counted with reproducibility of ± 2%.

## RESULTS

*Binding of Aggregates to Normal Human Lymphocytes In Vitro.*—When peripheral blood lymphocytes from normal human subjects were incubated with fluorescein-conjugated heat-aggregated human IgG (Fl-Agg), a subpopulation of approximately 22% bound the aggregates. This binding is demonstrated in Fig. 1. The control experiment using fluorescein-conjugated 7S human IgG was completely negative. Further, binding of aggregates could also be demonstrated using an indirect method in which unlabeled heat-aggregated human IgG (Agg) was incubated with the cells, which were then




FIGS. 1–3. Demonstration by direct and indirect fluorescence of IgG aggregates binding to human peripheral lymphocytes, and comparison with immunofluorescent staining of surface Ig. (1 *a*) Phase contrast; (1 *b*) visualization of fluorescein-labeled aggregates on one of the cells. (2 *a*) Phase contrast; (2 *b*) visualization of one of the lymphocytes stained with Rho-anti-Ig showing small, uniform, punctate spots. (3 *a*) Phase contrast; (3 *b*) visualization of one of the lymphocytes reacted with aggregates and then stained with Rho-anti-Ig. All photographs × 900.

washed and stained with rhodamine-conjugated anti-human Ig (Rho-anti-Ig). The results were identical to those with the direct method and are illustrated in Fig. 3. The aggregates bind to the lymphocyte surface in an irregular, patchy distribution, and the aggregates bound to any particular cell varied in both size and number. The pattern of fluorescence was quite distinct from that of surface Ig (Fig. 2) provided that staining was carried out at 4°C in the presence of Na azide immediately after centrifugation of the antiserum. Thus, differentiation between membrane Ig and cell-bound aggregates could be made even when both were present on the same cell. Aggregates of isolated myeloma proteins of the γG1 and γG2 subgroups gave the same results as fraction II aggregates. This ruled out the possibility that a contaminant in fraction II was responsible for the binding to lymphocytes.

*Conditions for the Binding of Aggregates to Lymphocytes.*—The percentage of lymphocytes binding aggregates was not altered by temperature (4°, 23°, and 37°C), pH (6.0–8.2), time of incubation (10 min to 4 hr), presence or absence of protein in the incubation medium (media tested include Hanks' balanced salt solution, Medium 199, 2 and 5% BSA-PBS), presence of 0.02% Na azide, or presence of 0.02 M $Na_3$ H ethylenediaminetetraacetate. In contrast to the binding of antibody alone (3), aggregate binding to lymphocytes was not affected by repeated washing (one to five washes). Passage over a nylon column did not significantly alter the percentage of stainable Ig bearing small lymphocytes (24% pre- and 23% postnylon) or of lymphocytes bearing the aggregate receptor (23.5% pre- and 22% postnylon). Aggregate binding was found to be complement independent. The aggregate preparations (Fl-Agg and Agg) contained no complement activity, and the addition of fresh normal human serum as a source of complement did not enhance aggregate binding.

The maximum percentage of lymphocytes binding aggregates was obtained at a protein concentration of 0.2 mg/ml. Increasing concentrations stepwise up to 15 mg/ml did not enhance the number of lymphocytes binding aggregates although more aggregates were bound per cell. At still higher concentrations the preparations were less reliable because of clumping.

*Characterization of the Lymphocyte Subpopulation.*—The peripheral blood lymphocytes from nine normal subjects were tested in parallel for Ig staining and aggregate binding (Table I). For each individual and for the group as a whole the percentage of lymphocytes binding aggregates, as determined by either the direct or indirect method, closely matched the percentage of lymphocytes with stainable surface Ig. Normal peripheral lymphocytes from rabbits also had equal percentages of Ig-staining and aggregate-binding cells. These results strongly suggest that the aggregate receptor is carried by B lymphocytes. This conclusion was supported by scoring lymphocytes prepared with the indirect method for the presence of Ig staining, aggregate staining, or both. (As noted above, characteristic staining allowed for differentiation on single cells.) Virtually all positive cells showed both Ig patches and bound aggregates.

TABLE I

*Surface Immunoglobulin Staining and Aggregate Binding of Normal Human Peripheral Lymphocytes*

| Subject | Lymphocytes with stainable surface Ig* | Lymphocytes binding aggregates by direct method‡ | Lymphocytes binding aggregates by indirect method§ |
|---|---|---|---|
| | %‖ | %‖ | %‖ |
| R.M. | 13 | 12 | 11 |
| C.D. | 14 | 14 | 15 |
| R.D. | 15 | 12 | 17 |
| R.B. | 23 | 22 | 24 |
| F.S. | 24 | 21 | 21 |
| P.W. | 24 | 23 | 22 |
| H.D. | 25 | 27 | 27 |
| E.K. | 27 | 25 | 29 |
| S.F. | 40 | 38 | 40 |
| Mean | 22.8 | 21.8 | 22.7 |

* Stained with Rho-anti-Ig.

‡ Incubated with Fl-Agg.

§ Incubated with Agg, then washed × 3 and stained with Rho-anti-Ig.

‖ Minimum of 200 cells counted for each preparation.

0–1% had only Ig staining while 1–3% showed aggregates only. To confirm these results double staining experiments were performed. Lymphocytes were incubated with Rho-anti-Ig, washed × 3, incubated with unlabeled 7S IgG to saturate remaining anti-Ig sites, washed × 3, and incubated with either Fl-Agg or, as a control, Fl-7S IgG (Table II). These results showed that Ig-staining cells carried the aggregate receptor. That Fl-Agg were not bound by the Rho-anti-Ig was shown by lack of fluorescence with the Fl-7S IgG control. Additionally, these experiments provide evidence that the surface Ig and the receptor for aggregates are separate entities. Even when the surface Ig was blocked by anti-Ig there was no decline in the percentage of cells binding aggregates.

In certain pathologic conditions we have noted significant numbers of peripheral lymphocytes which bind aggregates but do not stain for Ig. The question of whether these are B cells which do not have Ig on their surface remains to be answered.

*Studies of the Aggregate Receptor.*—Experiments were performed to determine whether any previously described surface component and/or any substance known to interact with aggregated IgG was acting as the aggregate receptor. As previously noted, surface Ig was unlikely to be the receptor because blocking with anti-Ig did not diminish binding. Similarily, any rheumatoid factor adherent to the lymphocyte was unlikely to be the receptor as this would also be blocked by anti-Ig. The C3 receptor (2) and the aggregate receptor also

TABLE II

*Double Labeling of B Lymphocytes with Rhodamine-Labeled Anti-Ig and Fluorescein-Labeled Aggregates*

| 1st Incubation | 2nd Incubation | 3rd Incubation | Percentage lymphocytes positive for | | |
|---|---|---|---|---|---|
| | | | Rho and Fl | Rho only | Fl only |
| Rho-anti-Ig | 7S IgG* | Fl-Agg | 23 | 1 | 2 |
| Rho-anti-Ig | 7S IgG* | Fl-7S IgG | 0 | 24 | 0 |

* Used to saturate remaining anti-Ig sites.

appeared to be distinct since complement was not necessary for aggregate binding and did not enhance the reaction. C1q is known to interact strongly with aggregated immunoglobulin (8). Therefore a search was made for this protein either adsorbed to or as part of the lymphocyte membrane by staining with fluoresceinated anti-C1q. These experiments were entirely negative under physiologic conditions. However, it was noted in the course of these experiments that isolated Clq did bind firmly to lymphocytes. This is currently under further study.

It seemed probable that the Fc receptor described by Basten and associates (3, 4) was responsible for the aggregate binding observed. Therefore, attempts were made to block Fl-Agg binding with 7S IgG. There was no inhibition of the binding of aggregates despite 10-fold greater concentration of 7S IgG even when the cells were not washed before the addition of aggregates. Despite these experiments, it appears likely that the aggregates had a much higher binding affinity than 7S IgG because of the greater number of Fc determinants present and were therefore able to displace the 7S IgG and bind to the cells.

Experiments were carried out to determine the minimum size of complexes which bind to lymphocytes and are visualized by the fluorescence procedures utilized. Aggregate fractions obtained by preparative ultracentrifugation were not detectable with aggregate sizes below 200S, and optimal visualization was obtained with fractions containing aggregates >300S. Recent experiments indicate that smaller aggregates do bind to lymphocytes but require special procedures for their detection.

## SUMMARY

Specific binding of aggregated γ-globulin to a subpopulation of lymphocytes was demonstrated. This subpopulation was identified as the Ig-staining or B lymphocytes. The binding was irreversible and independent of complement, pH, temperature, protein content of the medium, and divalent cations. Aggregates of large size were needed for optimal visualization. Evidence was obtained that the site on the lymphocyte membrane responsible for binding aggregates was distinct from surface Ig.

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