

# IMMUNOGLOBULINS ON THE SURFACE OF LYMPHOCYTES

## I. DISTRIBUTION AND QUANTITATION\*

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According to the clonal selection theory, antigen selects out cells genetically pre-committed to make antibody directed toward it, and stimulates them to produce antibody of that particular specificity. The simplest mechanism by which this selection might occur would be to postulate the presence of preformed antibody as the recognition unit. This prediction has stimulated several laboratories to investigate the presence of these recognition antibody molecules on cell surfaces. The first indirect demonstration of immunoglobulin on the surface of lymphoid cells was the study of Sell and Gell (1) who used a variety of anti-immunoglobulin antisera to stimulate blast transformation of normal lymphoid cells. Subsequently, the capacity of a small portion of a lymphocyte population to react with specific antigen has been demonstrated by means of antigen-coated bead columns (2) and by the use of highly labeled radioactive antigen and radioautographic techniques (3, 4). Finally, recent studies using radioiodinated and fluorescein-conjugated anti-immunoglobulin antisera have shown the presence of detectable amounts of immunoglobulin on the surface of some small lymphocytes (5).

The purpose of the present study was to confirm and extend these latter observations by studying the distribution of the known classes of mouse immunoglobulins in the various lymphoid organs under different experimental conditions. Also, by use of a sensitive radioactive antigen-antibody assay system the amount of surface immunoglobulin present on the lymphoid cells was quantitated.

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### Materials and Methods

*Mice*—A/J strain mice of 8–10 wk of age and of either sex were used throughout most of this study, with BALB/c, CBA/St, and outbred Swiss Webster mice being used in one experiment.

*Preparation of Cell Suspensions*—Suspensions of spleen or thymus were made by gently pressuring the minced organ through a stainless steel wire mesh into tissue culture dishes with 2.5 ml of Hank's balanced salt solution (HBSS)<sup>1</sup> (Microbiological Associates Inc., Bethesda, Md.). Cells were further dissociated by means of suction into a syringe fitted with a 27 gauge needle. Bone marrow was obtained from the femur and tibia. The bones were split open with scissors and the marrow flushed into the culture dishes with 2.5 ml of HBSS. Lymph nodes were obtained from the inguinal, axillary, and submaxillary areas. The nodes were isolated, cut in half, and squeezed through a stainless steel wire mesh into dishes with 2.5 ml of HBSS. Peripheral blood lymphocytes were separated from erythrocytes and neutrophils by centrifugation in a Ficoll solution according to the procedure of Noble et al. (6). Before use the Ficoll was rid of salt by dialysis against water and by subsequent lyophilization. 5 ml of blood was collected directly into 5 ml of HBSS containing 5 mg of disodium EDTA as anticoagulant. The diluted blood was gently mixed and layered over 9 ml of 35% Ficoll in HBSS, and centrifuged at 10,800 g for 30 min. A grayish white band appeared in the middle of the tube which was recovered by puncturing the side of the tube using an 18 gauge needle and syringe and gentle aspiration. Cells were dispersed in HBSS and found to be composed of erythrocytes, lymphocytes, and monocytes in a ratio of 20:1:0.1. Peritoneal cells were harvested from the peritoneal cavity after an intraperitoneal injection of 2 ml of HBSS with heparin (10 units/ml). The peritoneal exudate contained about 50–60% macrophages, the rest being lymphocytes of varying sizes and occasional mast cells. No attempt was made to separate the different cells of the exudate. All procedures for isolation of cells were carried out at 4°C and under sterile conditions.

*Preparation of Fluorescein-Conjugated Antisera*—All anti-mouse immunoglobulin antisera were produced in rabbits. Rabbits were injected intradermally and subcutaneously with a 1 mg dose of the corresponding protein in complete Freund's adjuvant every 2 wk for a period of 3–6 months. Purified mouse myeloma proteins representative of the different classes of immunoglobulins were used as antigens. Each rabbit antiserum was treated as follows: The antiserum was dialyzed against 0.0175 M sodium phosphate buffer, pH 7, and the  $\gamma$ G fraction was obtained by passage over DEAE-cellulose equilibrated with the same buffer. This  $\gamma$ G preparation was then made specific for a particular immunoglobulin by passing it through one or more immunoadsorbent columns composed of different individual myeloma proteins coupled to Sepharose 4B by the procedure of Cuatrecasas (7). The purity of these preparations was checked by immunoelectrophoresis. The specific anti-mouse immunoglobulin, at a concentration of 10 mg/ml in a 0.5 M sodium carbonate buffer, pH 9.5, was fluoresceinated, using fluorescein-isothiocyanate (FITC) (Cal Biochem, Los Angeles, Calif.) (8, 9). A fluorescein-protein weight ratio of 5:100 was used. The pH of the reaction mixture was maintained at 9.5 by the addition of NaOH during the first hour of reaction. The reaction was allowed to continue at 4°C for 24 hr with constant stirring. Unreacted dye was removed by gel filtration through G-25 Sephadex. The conjugated protein was fractionated by column chromatography on DEAE-cellulose, using a step-wise elution with sodium phosphate buffers, pH 7.4, of 0.01, 0.05, and 0.1 M, and finally with 1 M sodium chloride containing 0.01 M sodium phosphate, pH 7.4. Each chromatographic fraction was characterized with respect to its staining char-

<sup>1</sup> Abbreviations used in this paper: FCS, fetal calf serum; FITC, fluorescein-isothiocyanate; HBSS, Hank's balanced salt solution; NMS, normal mouse serum; PBS, phosphate-buffered saline.

acteristics and the amount of fluorescein conjugated to the  $\gamma$ G. For control studies rabbit anti-mouse albumin and anti-mouse beta 1C globulin were used. These antisera had been prepared for unrelated experiments and were monospecific. The fluorescein-protein ratios of the antisera used for this study varied between 2-3:1.

*Immunofluorescence Staining*—Cells were washed three times in HBSS before their reaction with fluoresceinated antisera. Portions of  $5-10 \times 10^6$  cells, in about 0.1 ml, were added to 0.1 ml of fluoresceinated antiserum (at a concentration of 1-3 mg/ml) and the reaction mixture was incubated at room temperature (or at 4°C in occasional experiments) for 30 min with occasional gentle shaking. Cells were then washed three times with HBSS and resuspended in 0.5 ml of HBSS. A drop of the cell suspension was placed on a slide, covered with a cover slip, and the edges were sealed with paraffin. The slides were examined with a Zeiss ultraviolet microscope equipped with an Ossran HBD 200 mercury arc lamp as a light source.

*Quantitative Radio-Immunoassay for Cellular Immunoglobulins*—An antiserum specific for  $\gamma$ G2 heavy chains was used together with an  $^{125}\text{I}$ -labeled (10) preparation of a  $\gamma$ G2 myeloma protein. The assay system was based on the capacity of unlabeled immunoglobulin to inhibit the reaction between labeled  $\gamma$ G2 protein and anti- $\gamma$ G2. The radioactive antigen-antibody complexes were precipitated with an excess amount of sheep anti-rabbit  $\gamma$ G which had been previously absorbed with mouse  $\gamma$ G to render it specific for rabbit immunoglobulin.

First, the amount of antibody needed to complex with 50% of the radioactive antigen was determined by testing serial dilutions of the antibody. For this procedure, 20  $\mu\text{l}$  of  $^{125}\text{I}$ - $\gamma$ G2 at a concentration of 1  $\mu\text{g}$  N per ml (containing 50% normal rabbit serum in phosphate-buffered saline [PBS]) was added to 100  $\mu\text{l}$  of serial dilutions of the rabbit anti- $\gamma$ G2 antiserum. After an overnight incubation in the cold, the radioactive antigen-antibody complexes were precipitated by the addition of 0.2 ml of sheep anti-rabbit  $\gamma$ G. This precipitate was incubated in the cold for 15 min, followed by centrifugation at 700  $g$  for 15 minutes at 4°C. This precipitate was then washed with 3 ml of PBS and counted in a well-type gamma scintillation counter. To determine the amount of immunoglobulin on the surface of lymphocytes, 0.5 ml of the dilution of antiserum necessary to complex with 50% of the radioactive antigen was added to a tube containing a known amount of cells (usually between 5 and  $40 \times 10^6$  cells). The antiserum was diluted in 10% fetal calf serum (FCS) in HBSS. This mixture was allowed to incubate in the cold for 16 hr with gentle shaking to maintain the cells in suspension. After this period of incubation the cells were pelleted by centrifugation at 1000 rpm for 10 min in a PR-2 cold centrifuge. The supernatants were poured off and two 100  $\mu\text{l}$  fractions of the supernatant were placed into fresh test tubes. 20  $\mu\text{l}$  of the radioactive  $\gamma$ G2 was then added and this reaction mixture was incubated for a further 24 hr. The radioactive antigen-antibody complexes were then precipitated with sheep anti-rabbit  $\gamma$ G and the amount of radioactive antigen in the precipitates counted. A standard inhibition curve was constructed by use of varying quantities of soluble unlabeled  $\gamma$ G2 antigen in the place of cells in the first incubation step. The amount of cell-bound immunoglobulin was then quantitated by comparison with the standard inhibition curve. Controls which contained cells plus normal rabbit serum, antibody without any inhibitor, and antigen alone, were included in each experiment.

## RESULTS

*Pattern of Staining with Fluoresceinated Anti-Immunoglobulin Antisera.*—A selected number of lymphocytes from the spleen exhibited a positive reaction with fluoresceinated anti-immunoglobulin sera. This reaction was specific. Lymphocytes that were reacted with anti- $\gamma$ G absorbed with  $\gamma$ G protein did not show fluorescence. Furthermore, lymphocytes did not show fluorescence if reacted with fluoresceinated antisera to other proteins such as beta 1C

globulin or albumin. It was apparent from these controls that the positive reaction did not represent a nonspecific attachment of heavily conjugated  $\gamma$ G molecules to the surface of these live cells. Several technical aspects were of importance in showing the specific presence of immunoglobulin on lymphocytes. First, it was crucial that the lymphocytes were living at the time of reaction with the fluoresceinated conjugate. Live lymphocytes did not show nonspecific fluorescence and exhibited no "background" reaction. Dead cells, on the other hand, reacted heavily and nonspecifically with all fluorescein-conjugated antigens. The reaction of the dead cells was homogeneous and diffuse and could thereby be differentiated from the reaction exhibited by the live cells. Thus, we would not have been able to use our fluorescein conjugates for the routine immunocytologic detection of intracellular immunoglobulins in smears or tissue sections of lymphoid cells because of the high nonspecific attachment of the fluorochrome to dead cells. Secondly, the volume of the total reaction mixture appeared to be of importance with some conjugates: a 10-fold increase in volume markedly reduced the positive reaction. Finally, it was apparent that the positive reaction represented immunoglobulins at the surface and not in intracellular vesicles of the lymphocyte. At 4°C, a temperature at which there is little if any pinocytosis by cells, there was still a positive staining of lymphocytes. More importantly, trypsin treatment of the lymphocytes, which removes some protein attached to the plasma membrane, greatly decreased the staining reaction (see last section).

Three patterns of distribution of immunoglobulin were observed and these are illustrated in Fig. 1. The most prevalent pattern was one in which the immunoglobulin was limited to a small portion of the edge of the cell with little or no staining in other areas (Fig. 1 *a, d*). The second pattern was an extension of the previous one. In it immunoglobulin was distributed almost entirely, or entirely, around the edge of the lymphocyte. Usually there were some stippled positive areas elsewhere on the surface of the cell as well (Fig. 1 *b, e*). The third pattern was a more homogeneous stippling of immunoglobulin throughout the surface of the cell (Fig. 1 *c, f*). This latter pattern resembles the pattern shown for H2 antigens on the surface of lymphocytes (8). It was important in evaluating the pattern of staining to focus at a variety of planes since the pattern of fluorescence on one plane may be that of a crescent, whereas when the focusing was changed to bring other planes into view, a more homogeneous pattern might be obtained. This phenomenon is illustrated in Fig. 1 *j-l* in which different planes of a single cell are shown. The first focal (crescent) pattern was obtained in approximately 50% of the positive cells, whereas the other two patterns were found in approximately equal frequencies of 25% each. This pattern of staining was found with all fluoresceinated antisera regardless of their specificity for immunoglobulin class. The homogeneous stippled staining and the continuous rim staining seemed to be somewhat more

prevalent with the more highly conjugated antisera. Hence, a weaker antiserum with the same specificity as a strong antiserum would have a greater percentage of crescent patterns than the stronger antiserum. The pattern of staining was also independent of the tissue of origin, in that spleen, peripheral blood, lymph nodes, and bone marrow all contained cells with the different staining patterns in roughly the same proportion as described above.

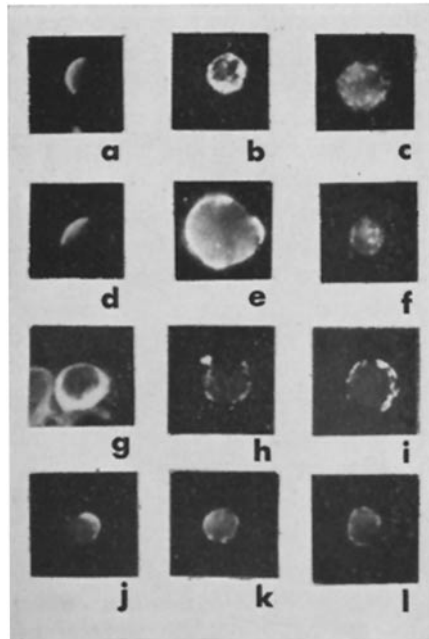


FIG. 1. Immunofluorescent pattern of surface immunoglobulin on lymphocytes. *a* and *d*, crescent pattern; *c* and *f*, homogeneous pattern; *b*, rim pattern; *e*, plasma cell from a mouse plasmacytoma showing intense rim pattern; *g*, homogeneous cytoplasmic staining of plasma cell stained after fixation; *h* and *i*, appearance of surface staining on cell smear (live cells were stained and then smeared and fixed); *j* and *l*, staining pattern of a single cell at different focal planes.

Plasma cells obtained from a plasmacytoma also reacted with anti-immunoglobulin antisera (Fig. 1 *e*). The staining reaction was a continuous rim pattern with surface stippling. It was, however, considerably more intense than that seen with lymphocytes.

Macrophages did not have immunoglobulin on their surfaces. Some macrophages exhibited some positive reaction in occasional experiments. This reaction could be observed with any antiserum regardless of its specificity and probably represented molecules pinocytosed by this cell.

*Tissue Distribution of Cells Containing Surface Immunoglobulin.*—In several experiments cell suspensions were made from spleen, thymus, bone marrow, and peripheral lymph nodes. These tissue sources together with peripheral blood leukocytes were examined for the presence of surface immunoglobulin with a fluoresceinated polyvalent anti-immunoglobulin antiserum which contained antibodies to light chains as well as to heavy chains of the known classes of immunoglobulins. The percentage of positive nucleated cells obtained from these different tissues is shown in Table I. For the spleen, thymus, lymph node, and peripheral blood the percentage is an accurate estimation of the distribution among lymphocytes. More than 90% of cells were in fact lymphocytes. This was not the case for the bone marrow since many hematopoietic cells were included in the population of nucleated cells. 40–50% of

TABLE I  
*Distribution of Lymphocytes Containing Surface Immunoglobulin in Different Lymphoid Tissue*

Lymphocyte source	Positive nucleated cells	Lymphocytes	Positive lymphocytes*
	(%)	(%)	(%)
Spleen	48, 49, 47, 40	93	49
Lymph nodes	6, 8	97	7
Peripheral blood	13	90	14
Thymus	<0.01, <0.01, 0.1	95	<0.1
Bone marrow	9, 8	18†	47
Peritoneal exudate	4	40	10

\* Assuming all positive cells are lymphocytes.

† Taken from reference 11.

lymphocytes from the spleens of normal adult mice had immunoglobulin on their surface. There was little variation in the number of positive cells among spleens from different mice of the same strain or of other strains. Thymus cells were essentially negative, the per cent of positive cells being consistently less than 1% and usually in the range of 0.01%. Peripheral lymph nodes contained 6–8% of positive cells, the bone marrow 8–9% of positive cells, and peripheral blood contained 13% of positive cells. When calculations were made on the basis of the percentage of lymphocytes present, the per cent of positive bone marrow lymphocytes equalled that of the spleen.

*Distribution of Different Immunoglobulins on the Surface of Splenic Cells.*—Antisera specific for the following immunoglobulin classes and subclasses were tested:  $\gamma$ G1,  $\gamma$ G2,  $\gamma$ G3,  $\gamma$ A,  $\gamma$ M,<sup>2</sup> and kappa light chains. The proportion of cells that had surface staining with the above antisera is shown in Table II.

<sup>2</sup>  $\gamma$ G1 =  $\gamma$ 1,  $\gamma$ F;  $\gamma$ G2 =  $\gamma$ 2a, b,  $\gamma$ G,  $\gamma$ H;  $\gamma$ G3 is a newly described  $\gamma$ G subclass.<sup>3</sup>

<sup>3</sup> Grey, H. M., J. W. Hirst, and M. Cohn. 1970. A new mouse immunoglobulin: IgG3. *J. Exp. Med.* In press.

The percentage of cells that reacted positively with anti-kappa chain antiserum was about 45%, which is in close agreement with the results obtained with the polyvalent antiserum (Table I). This is not unexpected since this is the predominant light chain type in the mouse. On the average, 19% of the cells stained with anti- $\gamma$ G2 antisera, 12% with  $\gamma$ G1, 6% with  $\gamma$ A, 10% with  $\gamma$ M antiserum, and 1% with antiserum directed against  $\gamma$ G3. In the lymph nodes, bone marrow, and peripheral blood the different immunoglobulins were represented in roughly the same proportions as found in the spleen, with the exception that there was a moderate increase in the relative number of  $\gamma$ M-containing cells in blood ( $\sim$ 30% of the total positive cell population). Due to the low percentage of positive cells in these other lymphoid tissues, exact quantitation was not possible. The total number of cells that reacted with each different immunoglobulin antiserum, if added, was 48%, a figure very close

TABLE II

*Distribution of the Different Immunoglobulin Classes on the Surface of Splenic Lymphocytes*

	$\gamma$ G1	$\gamma$ G2	$\gamma$ G3	$\gamma$ A	$\gamma$ M	$\kappa$ light chains
Individual determinations %	16, 15, 13, 13, 13, 12, 12, 11, 11, 11, 10	26, 24, 20, 16, 16, 16, 15, 14, 12	2, 1, 1, 0.5	9, 8, 8, 6, 4, 3	11, 11, 10, 8, 8	46, 44, 44, 45
Average %	12.4	19	1.1	6.3	9.6	44.8

to that found when the polyvalent antiserum was used. These results suggest that a positive lymphocyte carries a single immunoglobulin class on its surface, since, if there were a significant number of cells with more than one immunoglobulin, the per cent of positive cells obtained by adding the results from the different monospecific antisera would be significantly greater than that obtained with the polyvalent antiserum.

*Quantitation of Immunoglobulin on the Surface of Cells.*—In order to quantitate the amount of immunoglobulin on the surface of cells, a radioactive antigen-antibody inhibition assay was used, as described in Materials and Methods. The inhibition curve using soluble  $\gamma$ G2 protein as an inhibitor is shown in Fig. 2. As can be seen, easily measurable inhibition was obtained with as little as 0.005  $\mu$ g nitrogen of  $\gamma$ G2. Cells incubated overnight in the cold with the standard antiserum retained their viability as estimated by their exclusion of trypan blue. Greater than 90% of the cells were viable by this test following the period of incubation with the antiserum. Table III summarizes the results of a series of experiments utilizing this technique. The amount of  $\gamma$ G2 found in different experiments ranged from 0.39 to 1.08 ng

of nitrogen per  $10^6$  cells. These data expressed in terms of molecules of  $\gamma$ G2 per cell are shown in the second column. On the basis of 19% of all lymphocytes possessing  $\gamma$ G2 on their surface (Table II), a further calculation can

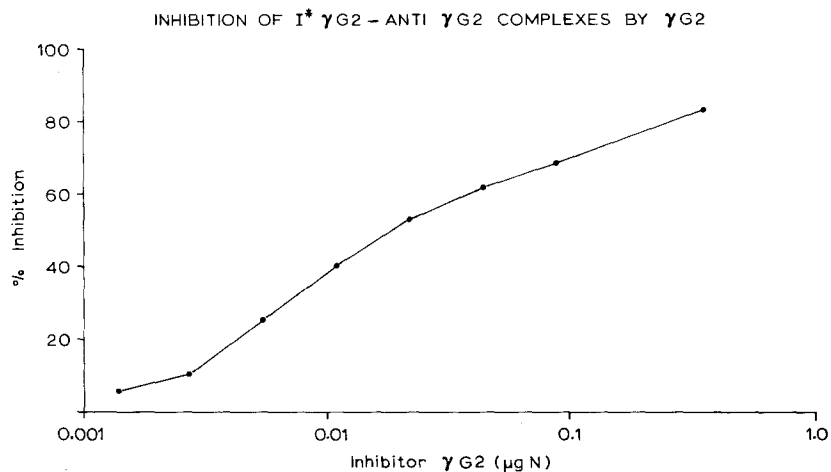


FIG. 2. Standard inhibition curve of  $^{125}I$   $\gamma$ G2-anti- $\gamma$ G2 complexes using unlabeled  $\gamma$ G2 as inhibitor.

TABLE III  
*Quantitation of Mouse  $\gamma$ G2 Associated with Splenic Lymphocytes*

	N/ $10^6$ Cells	Molecules/Cell	Molecules/ $\gamma$ G2 positive cell
	( $\mu$ g)		
Mouse spleen 1	0.00039	9,800	51,600
2	0.00051	12,800	67,400
3	0.00053	13,300	70,000
4	0.00108	27,100	143,000
5	0.00055	13,800	72,600
Mouse spleen, living	0.00108		
Mouse spleen, freeze-thawed	0.00192		
Mouse spleen	0.00055		
Mouse spleen, trypsin	0.00016		

be made in terms of the number of molecules present per  $\gamma$ G2 positive cell. This is shown in the last column, the range being 51,000–143,000 molecules per cell. As a control for these experiments rabbit spleen cells were handled in the same way. The rabbit spleen cells inhibited the assay system at approxi-



mately 1% of the level of mouse spleen cells, indicating that there was little nonspecific inhibition. In order to determine how much of the total immunoglobulin measured in this system could be due to release of immunoglobulin from nonviable cells, the immunoglobulin present in a freeze-thawed preparation of cells was also measured. Approximately twice as much immunoglobulin was present in the freeze-thawed preparation as in the intact preparation. On the basis of a maximum of 10% dead cells in the cell suspension used in these studies, approximately 10% of the immunoglobulin titrated in the cell suspension could be due to release of immunoglobulin from the interior of dead cells.

*Removal of Surface-Bound IgG by Trypsin.*—It could be shown both by immunofluorescence and by the quantitative immunoglobulin assay that the immunoglobulin on the cell surface was to a large extent removable by treatment with trypsin. Immunofluorescent staining with a polyvalent antiserum indicated that the intensity of fluorescence on trypsinized lymphocytes markedly diminished. The percentage of fluorescent cells also decreased from 47% in controls to 20% in trypsinized cells. In one experiment, after trypsin treatment, the spleen cells were incubated for 8 hr (in Eagle's medium containing either 10% FCS or normal mouse serum [NMS]). During this time there was no loss in viability but there was no reappearance of the immunoglobulin on the surface. On quantitative assay for immunoglobulin it was found that approximately 70% of the surface immunoglobulins were released into the supernatant following trypsin digestion for 1 hr (Table III).

#### DISCUSSION

The present experiments demonstrate that lymphocytes carry immunoglobulin on their surface membranes that can be identified by a suitable immunofluorescent technique and that can be quantitated by means of an antibody-inhibition test using radioactive immunoglobulin and a standard antibody. A series of experimental observations led us to these conclusions. First, absorption of the anti-immunoglobulin antibodies from the fluorescent-conjugated antiserum led to complete abolition of the reaction. Second, antibodies to other serum proteins gave negative reactions. Third, the reaction was carried out on live lymphocytes which are cells that have little pinocytotic activity; moreover, it could be carried out at temperatures where endocytosis by cells is markedly diminished. This indicates to us that the reaction was taking place on the surface of the cell and was not representative of uptake and endocytosis of antibody which was heavily conjugated with FITC. Lastly, the fluorescent reaction was greatly decreased by trypsinizing the lymphocytes before their reaction with the fluorescent antibody. The quantitative  $\gamma$ G2 assay confirmed the presence of surface immunoglobulin as well as its partial removal by trypsin. Taken together, the diverse experiments indicate

that the positive fluorescent reaction was indicative of the presence of immunoglobulin molecules bound to the plasma membrane of many lymphocytes.

Three experimental conditions led to the demonstration by immunofluorescence of surface-bound immunoglobulin on lymphocytes: the use of live cells, the use of antibody heavily conjugated to a fluorochrome, and a reaction mixture kept at relatively high concentrations. The whole procedure essentially followed the one that was previously used by Cerottini and Brunner (8) to demonstrate histocompatibility antigens on lymphocytes. It became apparent that in smears of cells it would not have been possible to identify immunoglobulin on the lymphocytes since: (a) it was impossible in smears to differentiate between fluorescence derived from the cytoplasm and the discrete fluorescence that is derived from the membrane; and (b) the use of heavily conjugated fluorochrome led to high nonspecific reactions on smeared cells. Live cells apparently did not pick up immunoglobulin molecules nonspecifically and therefore showed a completely dark background under the ultraviolet microscope, thus allowing small discrete positive foci to stand out clearly.

The present observations raise the obvious question of the significance of these lymphocytes with surface immunoglobulin. Is it an actual product of the cell or is it simply extracellular immunoglobulin that happens to attach to the outside of the lymphocyte? In other words, is the surface-bound immunoglobulin equatable with cytophilic antibody? Although direct proof is still lacking, the behavior of known cytophilic systems is sufficiently different from that of the lymphocyte surface immunoglobulin that our tentative conclusion is that this surface-bound immunoglobulin is in fact an actual product of the lymphocytes. The major differences between the two systems are: (a) Cytophilic antibodies of all immunoglobulin classes tested so far have not been shown to bind to lymphocytes unless there is C' in the reaction mixture (12). (b) The cytophilic antibody will bind to macrophages, precisely the cells that in the fluorescent antibody test had no detectable antibody on their surfaces. Our inability to detect immunoglobulin on macrophages in the present study is most likely due to its being present in lower concentration than that present on the lymphocyte. (c) If the immunoglobulin found on lymphocytes is cytophilic antibody, it would be a yet undescribed antibody type represented by all classes of immunoglobulin, and preferentially binding only to certain lymphocytes and not to others. (d) When lymphocytes were trypsinized and placed in culture fluid with mouse serum there was no reappearance of immunoglobulin on their surface, indicating that the putative antibody was not present in mouse sera or, if present, could not bind to a trypsinized lymphocyte. The receptor for cytophilic antibodies on macrophages, on the other hand, is resistant to trypsin. Apart from these considerations, further experiments in progress support the hypothesis that the reaction of anti-immunoglobulin antibody on the lymphocyte surface is of biological importance. Thus, lymphocytes treated with anti-immunoglobulin sera were not able to react

with radioactive antigen such as hemocyanin nor were they able to adoptively transfer an immune response in irradiated mice; moreover, trypsinized lymphocytes had a reduced capacity for making antibody in adoptive transfer experiments.<sup>4</sup> Comparable results to these have already been published by other investigators (4). Hence, on the basis of these series of considerations, we tend to believe that the surface-bound immunoglobulin is most likely an important and intrinsic product of the lymphocytes.

If the above hypothesis is accepted, then certain salient features should be briefly analyzed. First, among the lymphocytes that carried immunoglobulin on their surface the majority were the typical small inactive lymphocytes that presumably are resting cells until their interaction with antigen. It was previously known that small lymphocytes could secrete antibody, but the idea that in a normal spleen a rather large percentage of the small lymphocytes would actually have immunoglobulin was not realized until the observations of Raff et al. (5) and the present ones. This observation on the one hand is not unexpected since by the clonal selection theory the genetically selected lymphocytes would be present at all times, ready to react with antigen. On the other hand, it is also quite possible that the positive lymphocytes are cells that have already reacted at least once with antibody, i.e., they are memory cells. Whether these lymphocytes are actually in a slow process of synthesis of immunoglobulin or whether they are truly in a resting stage that may last for long times following an initial synthesis of this immunoglobulin is not known.

Perhaps the most interesting aspect of the present studies is the finding that morphologically similar lymphocytes appear to be very different from one another with regard to the presence or absence of immunoglobulin on their surface. If the surface immunoglobulin serves as a receptor for an antigen to allow the initiation of antibody formation in this cell population, the question arises as to the function of those cells which do not contain immunoglobulin on their surface. Whether these represent uncommitted cells or cells whose function has nothing to do with that of the positive cells is an area of investigation which we are currently pursuing. Obviously the possible importance of this cell population with regard to the various cell-to-cell interactions which are involved in antibody formation is one of the major areas to be investigated.

Recent experiments performed in our laboratory have shed light on the source of the surface immunoglobulin-containing cells. The data, obtained in thymectomized animals, indicate that the bone marrow is the major source of the positive cell population and that most of the negative cell population is made up of thymus-derived cells. Also quantitative immunoglobulin assays indicate that if any light chain containing immunoglobulin is present on the surface

<sup>4</sup> Unanue, E. R., and H. M. Grey. Experiments in progress.

<sup>5</sup> Unanue, E. R., E. Rabellino, and H. M. Grey. Immunoglobulins on the surface of lymphocytes. II. The bone marrow as the source of detectable surface-bound immunoglobulin. Submitted for publication.

of thymocytes it is present at a level of about 1% or less of that present on spleen cells.<sup>5</sup>

#### SUMMARY

The distribution and quantity of immunoglobulins on the surface of lymphocytes has been studied by means of immunofluorescence and a quantitative radio-immunoassay. Surface immunoglobulins were found on approximately 45% of spleen and marrow lymphocytes and 7-14% of lymphocytes from lymph nodes, peripheral blood, and peritoneal exudate. Thymic lymphocytes contained undetectable amounts of immunoglobulin. In the spleen the different immunoglobulins were present in the following order:  $\gamma G2 > \gamma G1 > M > \gamma A > \gamma G3$ . The surface immunoglobulin was largely removable by brief treatment with trypsin. Quantitative analysis indicated that 50,000-150,000 molecules of immunoglobulin were present on an individual cell. A variety of observations make it likely that this lymphocyte-associated immunoglobulin is a product of the cell to which it is attached rather than a form of cytophilic antibody.

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