CELL SURFACE IMMUNOGLOBULIN

II. Isolation and Characterization of Immunoglobulin from Mouse Splenic Lymphocytes*

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There is accumulating evidence that the capacity of lymphocytes to bind antigen specifically (1–6) is due to Ig on the cell surface: anti-Ig causes the transformation of lymphocytes (7–12) and other changes in membrane properties (13, 14); anti-L chain and anti-Ig inhibit both binding of specific antigen and stimulation of cell-mediated immune reactions by antigen (15–18); anti-Ig of allotype or class specificity binds to lymphocytes (19–23); "rosette" formation around lymphocytes occurs when hybrid antibody to both Ig and specific antigen is added along with red cells coated with antigen (24, 25); lymphocytes treated with anti-Ig form rosettes with Ig-coated red cells (26, 27); and specific adherence of lymphocytes to antigen-coated columns is blocked by pretreatment of cells with anti-Ig serum (28). There is conflicting evidence, however, as to whether the major class of cell surface immunoglobulin on normal splenic lymphocytes is IgM (12, 20) or IgG (7, 21), as well as the distribution of Ig on cells (20, 21).

In evaluating the above results, It should be emphasized that the methodologies used rely on the capacity of anti-Ig antibody to make effective contact with antigenic determinants of the receptor. Such contact depends upon the presence of determinants on portions of the receptor available to anti-Ig antibody in the medium and retention of antigenicity when the receptor is bound to the plasma membrane. These problems may account for the several contradictory reports mentioned above and the finding that individual antisera directed against the same immunoglobulin can give conflicting results, presumably depending on differences in the specificities of the antisera (20, 29).

For these reasons we developed an approach in which surface Ig could be studied by standard immuno- and biochemical techniques after its removal from the cell surface (30). This was accomplished by radioiodinating the surface of

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living splenic lymphocytes, using a lactoperoxidase, as has been done for red blood cells by Phillips and Morrison (31). After radioiodination, cells were separated into one fraction containing almost exclusively small lymphocytes and another containing large lymphocytes and plasma cells. From each cell fraction, radiolabeled surface Ig was released by treatment with detergent and precipitated with specific antiserum. Heavy (H) and light (L) chains were identified from reduced and alkylated precipitates by electrophoresis on acrylamide gels. 95% of the radioactivity of H chain from the small lymphocyte fraction and 90% from the large lymphocyte–plasma cell fraction were identified as μ by antigenicity and by molecular weight.

Materials and Methods

Preparation of Iodinated Spleen Cells.—Adult BALB/c mice were killed by cervical dislocation and perfused with 5–10 ml of 0.15 M NaCl in 0.01 M phosphate-buffered saline, (PBS),[1] pH 7.2. After excision of the spleens, cells were teased into PBS, filtered through a stainless steel screen, washed four times, and refiltered into 1–2 ml of PBS. Cell counts and per cent viability were determined in 0.05% trypan blue-PBS. Iodination was performed using the method of Marchalonis (32) as applied to intact cells by Phillips and Morrison (31). Washed spleen cell suspensions containing approximately 10^8 cells (viability of 95%) were suspended in 1 ml of PBS. 200 μg of lactoperoxidase (prepared according to Morrison and Hultquist [33] and assayed according to Polis and Shmukler [34]), 1 mCi of carrier-free $Na^{125}I$ (New England Nuclear Corp., Boston, Mass.), and 25 μl of 0.03% H_2O_2 were added. The reaction mixture was incubated at room temperature for 10 min, during which time two more 25 μl samples of 0.03% H_2O_2 were added. The iodination was terminated by the addition of 10 volumes of cold 5 mM L-cysteine-hydrochloride in PBS. Cells were pelleted in the cold at 1000 *g* for 15 min, washed once in 10 ml of 5 mM L-cysteine-hydrochloride, and twice in PBS.

Cell Separation on Albumin Gradients.—The washed, radioiodinated cell pellet was suspended in 3 ml of 35% bovine serum albumin (BSA) (Pentex-Miles, Kankakee, Ill.), again filtered through a stainless steel screen, and distributed into three 5 ml cellulose nitrate tubes. Discontinuous layers of 29, 26, 23, and 10% BSA were pipetted above the cell-containing layer (35) and tubes were centrifuged at 4°C for 30 min at 15,000 *g* in a SW-39 rotor in a Spinco ultracentrifuge. After centrifugation the layers of cells (designated as A-D from the top to bottom at each interface [35]) were removed by a pasteur pipette, diluted with 5 volumes of PBS, pelleted at 1000 *g* for 15 min, and washed twice with PBS. In most experiments the A and B layers were pooled (top) as were the C and D layers (bottom). The pellet was discarded.

Preparation of Cell Lysates.—Washed cells from the gradients were suspended in 1–2 ml of 0.5% Nonidet P_{40} (Shell Chemical Corp., New York) and allowed to stand for 10 min at room temperature. The nuclei were removed by centrifugation at 1500 *g* for 15 min and proteins from small samples of the cell lysates were precipitated for 15 min at 4°C in 10% trichloroacetic acid (TCA) containing 5 mM NaI. Precipitates were collected on Millipore filters (type HA, white, plain), washed five times with 10% TCA-5 mM NaI, two times with 10% TCA, and two times with abolute ethanol. Millipore filters were then dissolved in 1 ml of 1:10 methanol: 1,4 dioxane (Fisher Scientific Co., Fairlawn, N. J.) and counted in a Packard Model 3001 Tri-carb scintillation spectrophotometer.

Immunoprecipitation.—For direct precipitation of radioiodinated Ig, 25 μg of mouse Ig and

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; 2-EM, 2-mercaptoethanol TCA, trichloroacetic acid.

excess of goat anti-mouse Ig were added to each milliliter of lysate. Control precipitations were done with the same amount of rabbit Ig (Pentex-Miles) and an excess of goat anti-rabbit Ig. Reaction mixtures were incubated at 37°C for 30 min and overnight at 4°C. For "sandwich" precipitation, 20 μl of either rabbit or goat antimouse Ig was added to each milliliter of lysate. Normal or hyperimmune serum to an unrelated antigen was added to controls. After incubation at 37°C for 15 min, an excess of goat anti-rabbit Ig or rabbit anti-goat Ig was added, and incubations continued for 30 min at 37°C and overnight at 4°C. Preliminary experiments had shown that results of direct and sandwich precipitations were identical.

Precipitates were pelleted at 1500 *g* for 15 min at 4°C, washed four to five times with cold PBS, and solubilized by incubation for 30 min at 37°C with 0.05 M tris (hydroxymethyl) aminomethane (Tris) buffer, pH 8.4, containing 1% sodium dodecyl sulfate (SDS) and 8 M urea. Small samples of the dissolved immunoprecipitates were reprecipitated in TCA as described above and counted. For reduction and alkylation of precipitates, 2-mercaptoethanol (2-ME) was added to a final concentration of 0.2 M and incubation was continued for 30 min at 37°C. A 0.5 M solution of iodoacetamide adjusted to pH 8.4 with solid Tris base (Fisher Scientific Co.) was added to a final concentration of 0.25 M and incubation continued for another 30–60 min. Precipitates were dialyzed overnight at room temperature against 0.01 M phosphate buffer, pH 7.2, containing 0.1% SDS and 0.5 M urea. Reduced and alkylated samples were dialyzed against the same buffer with 0.006 M 2-ME. 0.2–0.5 ml samples of dialyzed material were electrophoresed in polyacrylamide gels (36) at 15 ma/gel for 2.5 hr and fractionated on a Savant auto-gel divider (37). Samples were collected in plastic tubes (Falcon Plastics, Los Angeles, Calif.) and counted on a Packard Model 3001 Tri-carb scintillation spectrophotometer.

Preparation of Antisera Specific to μ-Chain.—Goat anti-mouse IgM had been prepared (Pentex-Miles) against a purified IgM from the mouse MOPC-104E plasmacytoma. To remove antibodies to L chain, the antiserum was completely adsorbed with acetyl cellulose (Miles-Yeda, Rehovot, Israel) conjugated (38) to immunoelectrophoretically pure mouse IgG (prepared from mouse Ig by chromatography on diethylaminoethyl [DEAE]-Sephadex with 0.01 M pH 8.0 Tris buffer). Rabbit anti-mouse μ-chain was a gift from Dr. F. Miller of the State University of New York at Stoney Brook, N. Y. It has been prepared against μ-chains from the IgM of mouse MOPC-104E tumors. The specificity of both antisera was determined by immunoelectrophoresis against mouse serum and their capacity to bind radiolabeled mouse Ig in a sandwich precipitation with goat anti-rabbit Ig or rabbit anti-goat Ig.

Preparation of ^{3}H-IgM.—MOPC-104E tumors, kindly provided by Dr. Michael Potter, National Institutes of Health, Bethesda, Md., were maintained by subcutaneous transplantation of BALB/c mice. 2 or 3 wk after transplantation, tumors were removed and cell suspensions prepared in Eagle's minimal essential medium lacking tyrosine. Cells were labeled for 1–3 hr at 37°C with 50 μCi/ml of L-tyrosine-3-5-H^3 (specific activity 25 Ci/mM; New England Nuclear Corp.). The cells were pelleted at 1000 *g* for 10 min, washed three times in PBS, and lysed in 0.5% Nonidet P_{40}. Both cell lysates and secretion (extracellular medium) were dialyzed against PBS and precipitated with carrier mouse Ig and excess goat anti-mouse Ig. Precipitates were solbuilized, reduced and alkylated, and electrophoresed on SDS-acrylamide gels as previously described. Samples were dissolved in Beckman Cocktail D and counted in a Beckman Model LS-250 liquid scintillation spectrophotometer. IgM monomer was obtained from the cell lysates and 19S IgM from the secretions.

RESULTS

Cell Fractionation and Recovery of Radioactive Protein and Ig.—Cell viability was 95% before and after radioiodination. After gradient centrifugation of both

iodinated and control cells, two to four times as many cells were found in the pooled C and D layers (bottom) as in the pooled A and B layers (top). Phase-contrast and light microscopy indicated that 95% of the cells in the bottom layer were small lymphocytes. The top layers contained 85–90% large lymphocytes and plasma cells and 10–15% small lymphocytes. The cells lost in the pellet (40% of the original population) consisted of red cells, dead and aggregated lymphocytes, and a number of viable lymphocytes. These observations are consistent with previous reports (35, 39).

The amount of acid-precipitable radioactivity from the cell lysates varied in individual experiments, but in general, 2–3% of the radioactivity from both the top and bottom layers could be precipitated with antisera to mouse Ig. In contrast, only 0.4–0.6% of the acid-precipitable radioactivity from the lysate was coprecipitated with control antisera.

Are Intracellular Proteins Radiolabeled?—Studies by Phillips and Morrison (31) using human erythrocytes and by Baur et al. (30) using mouse myeloma and human lymphoma cells have shown that only molecules on the exterior of the cell surface are iodinated by the methods used here. To test further for intracellular iodination of intact splenic lymphocytes, the following experiments were performed:

(*a*) *Subcellular fractionation:* 2×10^8 spleen cells, prepared, iodinated, and washed in the usual manner were suspended in 0.88 M sucrose and disrupted with 30–40 strokes of a tight-fitting Dounce homogenizer. The homogenate was diluted with PBS to a final concentration of 0.25 M sucrose and the whole cells, nuclei, organelles, and membranes were pelleted by centrifugation at 100,000 *g* for 120 min at 4°C in a No. 40 rotor in the Spinco Model L ultracentrifuge. The supernatant (cell cytoplasm) was removed and the pellet homogenized in PBS containing 1% sodium deoxycholate. The two fractions were dialyzed for 16 hr at 4°C against PBS and the acid-precipitable counts determined for each. Less than 3% of the acid-precipitated radioactivity was found in the cytoplasm, and none of this radioactivity was precipitated with antiserum to mouse Ig.

(*b*) *Presence of intracellular peroxidases:* Since it is probable that both $Na^{125}I$ and H_2O_2 can enter spleen cells during the iodination reaction, the question arises as to whether intracellular iodination might occur because of endogenous peroxidases. Therefore, cells were incubated without lactoperoxidase in the presence of $Na^{125}I$ and H_2O_2 for 10 min at room temperature. Reactions were stopped with cysteine, cells were washed, centrifuged on albumin gradients, lysed, and immunoprecipitates prepared. Such immunoprecipitates were found to contain less than 2% of the radioactivity of those obtained from cells incubated in the usual manner.

(*c*) *Transport of lactoperoxidase into spleen cells:* To investigate whether lactoperoxidase could enter cells and thereby catalyze iodination of intracellular proteins, cells were incubated for 10 min at room temperature with lacto-

peroxidase, washed three times in PBS, and then incubated in the presence of $Na^{125}I$ and H_2O_2. The iodination was terminated with cysteine, cells were pelleted, washed, centrifuged on albumin gradients, lysed, and immunoprecipitated as above. The precipitates contained less than 1% of the radioactivity incorporated into Ig when lactoperoxidase is maintained in the reaction mixture, indicating that lactoperoxidase is not transported into or adsorbed onto the cells in significant amounts.

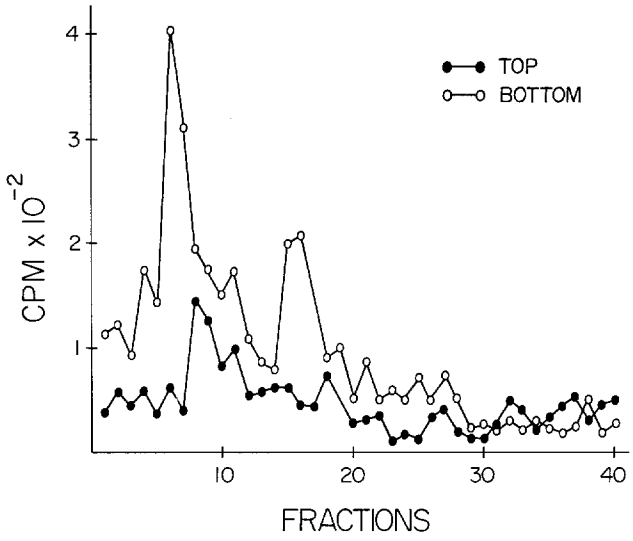

FIG. 1. Acrylamide gel electrophoresis of radiolabeled Ig from the surface of small and large splenic lymphocytes. Radioiodinated cells were separated by density gradient centrifugation into a fraction containing small lymphocytes (bottom) and one enriched in large lymphocytes and plasma cells (top). Cell lysates were precipitated with anti-Ig and the washed precipitates were dissolved in 1% SDS and 8 M urea. Electrophoresis was performed in 0.1% SDS and 0.5 M urea.

These findings indicate that over 97% of the cell-associated molecules iodinated by the methods employed here are on the plasma membrane. Since controls for acid and immunoprecipitation were not done in the experiments described above, it is probable that the 2–3% of radioactivity recovered in acid or immune precipitates represent free $Na^{125}I$ (fractionation experiment) or nonimmunoglobulin protein (experiments without enzyme or with removal of enzyme).

Acrylamide Gel Electrophoresis of ^{125}I Cell Extracts Precipitated with Anti-Ig.— From both top and bottom cell layers, a major peak was observed in fraction 8 of the acrylamide gel and minor ones in 2, 4, 12, and 17 (Fig. 1). (In this and subsequent figures, the position of the peaks given in the text is an average

position based on several experiments [ranges are shown in Fig. 3].) The relative areas under the minor peaks varied considerably in individual experiments, but the predominant peak was always found in fraction 8. The position of the peak is that of a molecule slightly larger than IgG.

In order to identify the radioactive material further, the specific precipitates were reduced, alkylated, and electrophoresed in SDS-acrylamide gels. As can be seen in Fig. 2, major peaks appeared in fractions 20 and 33. A minor shoulder

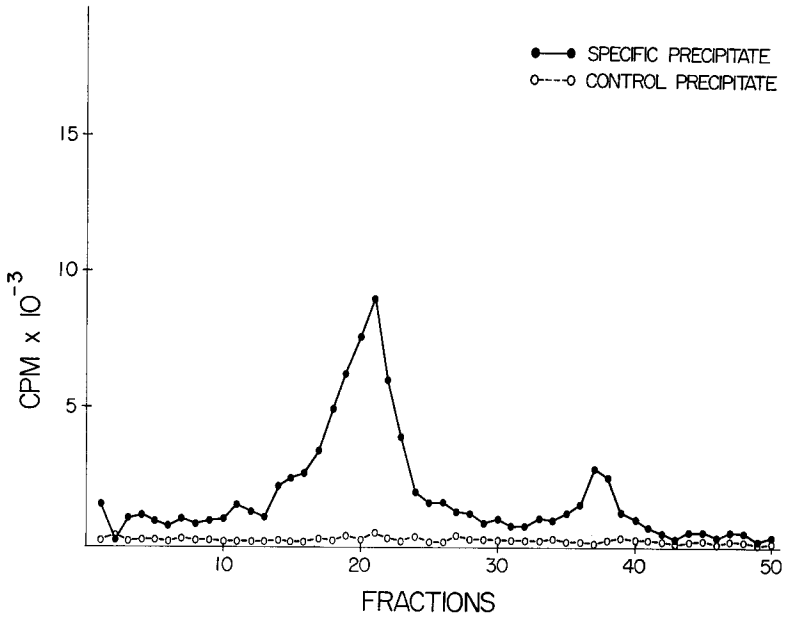

FIG. 2. Radiolabeled Ig from the surface of lymphocytes. Radioiodinated cells from the bottom and top of the gradient were pooled. Cell lysates were precipitated with anti-Ig or an unrelated immune complex (control). Precipitates were reduced and alkylated before electrophoresis on acrylamide gels.

was occasionally seen in fraction 25. Control precipitates of rabbit Ig and excess goat anti-rabbit Ig gave no discernible peaks on the gels despite the precipitation of 0.4–0.6% of the acid-precipitable radioactivity from the lysates (Fig. 2). Molecular weights of the above peaks were determined by plotting a curve of relative mobilities of mouse proteins of known molecular weights (L chain,γ-chain, μ-chain, IgM monomer, and IgG) in 5% acrylamide gels according to Shapiro et al. (36) (each point represents 10–20 independent "runs" by different investigators in our laboratory). As can be seen in Fig. 3, the peaks in fractions 20 and 33 correspond to the positions of mouse μ-chain and mouse L chain respectively, and the shoulder in fraction 25 to mouse γ-chain. The calculated

molecular weight of the unreduced material in fraction 8 (Fig. 1), corresponding to an IgM monomer (40), is 214,000 ± 31,500 (Fig. 3).

Acrylamide Gel Electrophoresis of ^{125}I Cell Extracts Precipitated with Anti-μ Sera.—In order to determine whether the material in fraction 8 has the antigenicity of IgM, cell lysates were precipitated with each of two anti-μ sera. These sera were shown to be monospecific for μ by immunoelectrophoresis and also by the failure to bind IgG labeled with ^{125}I (Fig. 4). The resultant precipitates accounted for 2–3% of the acid-precipitable radioactivity, a percentage similar to that obtained with anti-Ig sera. Moreover, the gel patterns appeared similar (Fig. 5). In addition, gel patterns of reduced and alkylated precipitates

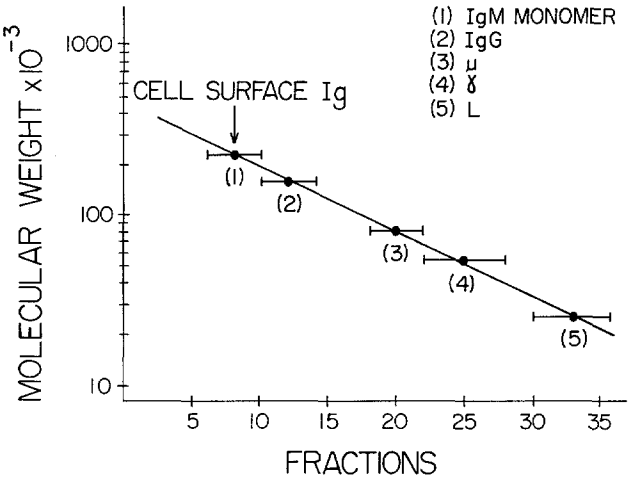

FIG. 3. Molecular weight determinations of mouse proteins on acrylamide gels. Each position represents an average of 15–20 experiments.

again had major peaks in fractions 20 and 33 (Figs. 6, 7), further strengthening the conclusion that the majority of the H-chain is μ.

In order to determine more precisely the proportion of γ- and μ-chain in the small and large lymphocyte fractions of the gradient, the following experiment was performed: 1 ml samples of lysate from cells in the top and bottom of the gradient were precipitated with goat anti-mouse-μ and an excess of rabbit anti-goat Ig. The washed precipitates were reduced and alkylated and prepared for acrylamide gels. The supernatants of the precipitates were then precipitated with goat anti-mouse Ig and excess rabbit anti-goat Ig. Acrylamide gel patterns of the first (IgM) and second (Ig minus IgM) precipitates (Figs. 8, 9) indicate H_μ/H_γ radioactivity ratios of 10 and 25 from the large and small lymphocyte fractions respectively. It is unlikely that this is due to preferential labeling of IgM compared to IgG because Marchalonis (32) has shown that IgG is more

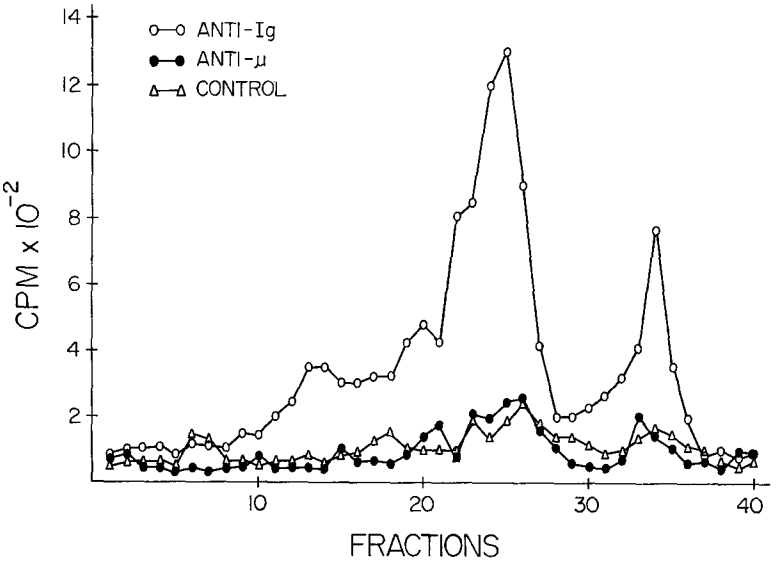

FIG. 4. Demonstration of the inability of goat anti-μ to bind radiolabeled IgG. Mouse IgG (Pentex-Miles) was enzymatically radiolabeled with ^{125}I and was mixed with either anti-Ig, anti-μ, or an unrelated antiserum, and the antibody was then precipitated by a sandwich technique. Precipitates were reduced and alkylated before electrophoresis.

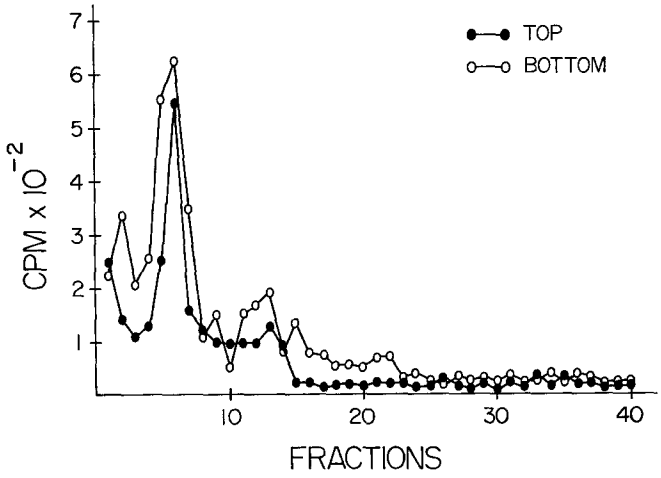

FIG. 5. Radiolabeled IgM from the surface of small (bottom) and large (top) splenic lymphocytes. Radiolabeled cell lysates were precipitated with anti-μ and the dissolved precipitates were electrophoresed on acrylamide gels.

readily labeled than 19S IgM in solution. Thus, the surface Ig of both types of lymphocytes is almost exclusively IgM. It is therefore unlikely that the small amount of IgG associated with the small lymphocyte population is from trace contamination with large lymphocytes and/or plasma cells.

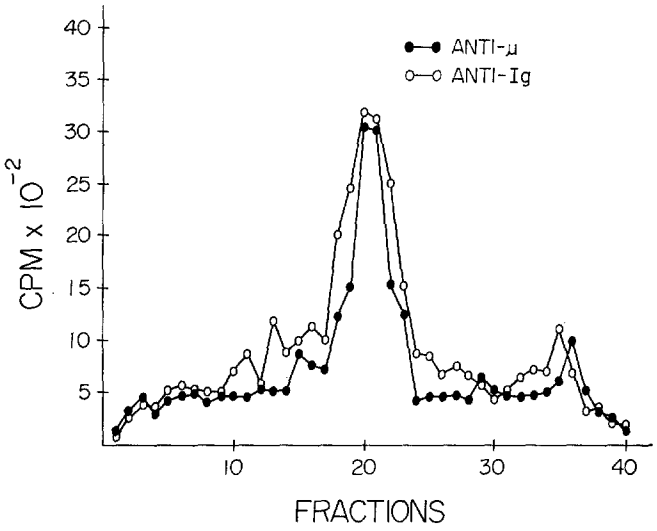

FIG. 6. Radiolabeled IgM and total Ig from the surface of small splenic lymphocytes Lysates were precipitated with either anti-μ or anti-Ig. Precipitates were reduced and alkylated before electrophoresis on acrylamide gels.

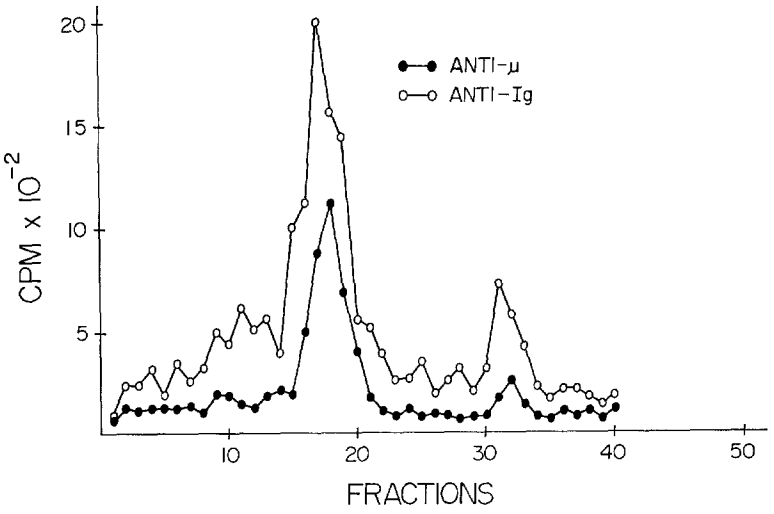

FIG. 7. Radiolabeled IgM and total Ig from the surface of plasma cells and large splenic lymphocytes. (See Fig. 6)

Molecular Weight of the Cell Surface IgM.—Several experiments were performed to determine whether the IgM monomer found in the acrylamide gels represents the in vivo form of the molecule or whether alterations in size have taken place in vitro:

(*a*) *Isolation under nonreducing conditions:* Because concentrations of L-cysteine used to inactivate the lactoperoxidase after iodination might reduce polymeric IgM on the cell membrane or during detergent lysis, experiments were done in which no cysteine was used. The gel patterns obtained from experiments with and without cysteine were identical.

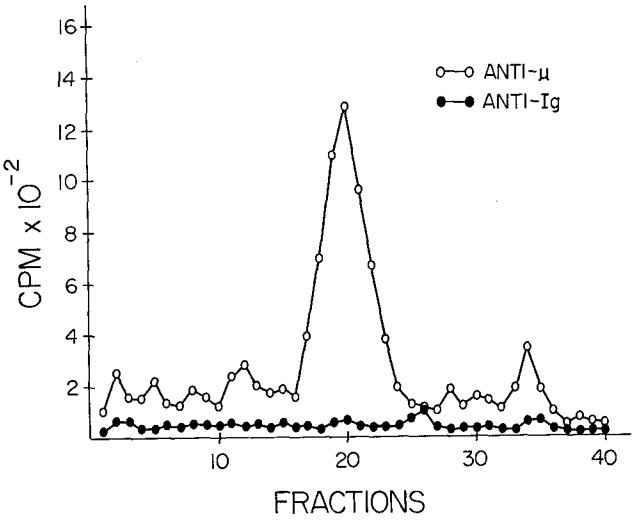

FIG. 8. Comparative amounts of radioactive IgM and other classes of Ig from the surface of small splenic lymphocytes. After precipitation of IgM only, the supernatant was treated with anti-Ig and immunoprecipitated by a sandwich technique. Precipitates were reduced and alkylated before electrophoresis on acrylamide gels.

(*b*) *Self-assembly of μ and L chains:* To exclude the possibility that disulfide bonding of chains was occurring during detergent lysis, cells were disrupted and simultaneously alkylated by lysis in 0.05 M phosphate buffer, pH 7.3, containing 0.5% Nonidet P_{40} and 0.5 M recrystallized iodoacetamide. (Control experiments had demonstrated that alkylation occurs under these conditions.) The acrylamide gel patterns of immunoprecipitates from cells lysed in this manner were identical to those of cells lysed in the absence of iodoacetamide, indicating that the IgM monomer had not been assembled in the lysate.

(*c*) *Depolymerization of IgM under conditions of iodination, detergent treatment, or solubilization of immunoprecipitates:* A small sample of hyperimmune BALB/c mouse serum was enzymatically iodinated and exhaustively dialyzed against PBS. It was then brought to a final concentration of 0.5% Nonidet P_{40} in PBS

and precipitated with an excess of anti-μ serum. The precipitate was washed, solubilized, and divided into two samples. One was reduced and alkylated, and portions of both samples were then electrophoresed in SDS-acrylamide gels. The remainder of the unreduced sample was layered on a 5–20% sucrose gradient containing 1% SDS and 0.5 M urea and centrifuged at 25,000 rpm for 17 hr at 30°C in a SW-56 rotor of the Spinco Model L-2 ultracentrifuge. ^{125}I-human IgM and ^{125}I-mouse IgG served as 19S and 7S markers in a separate gradient tube. After centrifugation, tubes were punctured and 0.1 ml samples collected and counted. The unreduced material, which remained at the origin of the acrylamide gel (Fig. 10), cosedimented with 19S IgM in the sucrose gradient

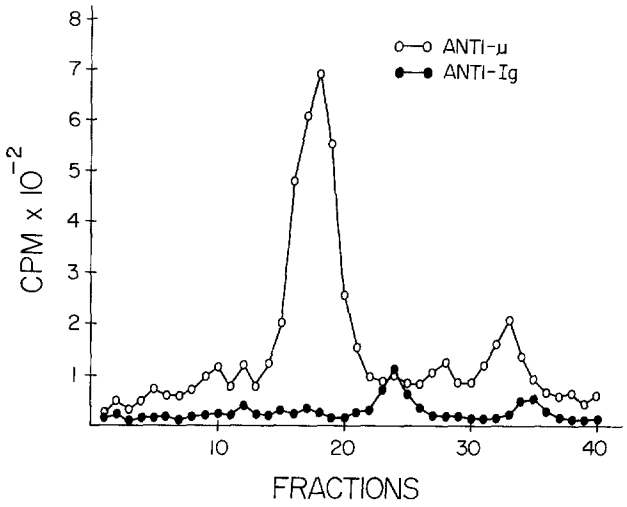

FIG. 9. Comparative amounts of radioactive IgM and other classes of Ig from the surface of large splenic lymphocytes and plasma cells. (See Fig. 8)

(Fig. 11). In contrast, cell surface IgM cosedimented with the 7S marker. After reduction and alkylation of this 19S material, characteristic μ and L chain peaks were observed on the acrylamide gel (Fig. 10). This experiment suggests that conditions of iodination, detergent treatment, and solubilization of immunoprecipitates do not cause depolymerization of 19S IgM.

(*d*) *Depolymerization of IgM in the cell lysate:* In order to demonstrate that IgM was not being degraded during incubation in the cell lysate, ^{3}H-mouse 19S IgM was incubated for 30 min at 37°C and overnight at 4°C in: (*a*) 0.5% Nonidet P_{40} in PBS and (*b*) lysate of spleen cells which had been enzymatically iodinated with nonradioactive NaI and centrifuged on BSA gradients. ^{3}H-IgM was specifically precipitated from both solutions. The washed, solubilized precipitates were electrophoresed in acrylamide gels and centrifuged on sucrose

gradients. Both samples were recovered primarily as 19S IgM, indicating that no degradation had occurred in the lysate.

Relationship of the Cell Surface IgM to the Plasma Membrane.—Ig receptors that bind antigen presumably have their Fab portions fully exposed to the media. It is possible, however, that the Fc portion is "buried" within the plasma membrane. If a significant portion is buried, radioactivity of μ:L should be higher when IgM monomer is radioiodinated in solution rather than on the

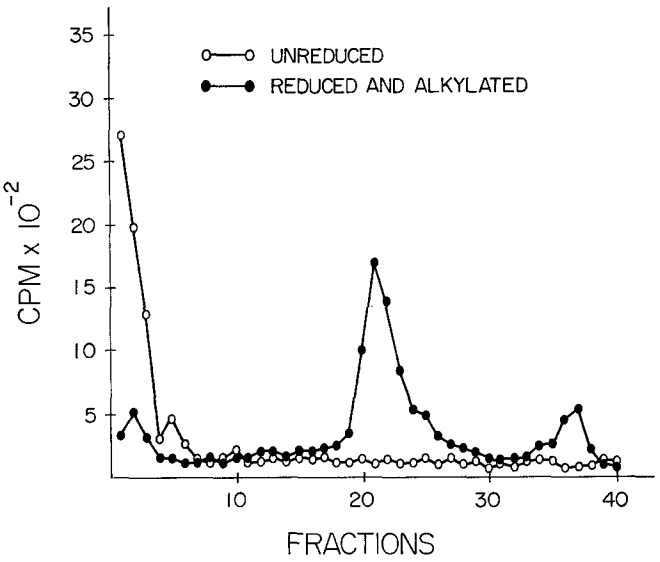

FIG. 10. Acrylamide gel electrophoresis of radiolabeled IgM from the serum of hyperimmunized mice. Anti-μ was used to precipitate serum that had been labelled enzymatically with ^{125}I. One portion of the dissolved precipitate was reduced and alkylated before electrophoresis.

cell surface (provided there are tyrosine residues in the buried portion of the Fc fragment).

To test this possibility, IgM was radioiodinated in solution (serum IgM) and on the cell surface. In addition, IgM monomer (MOPC-104E lysate) was internally labeled with ^{3}H-tyrosine. All three samples were specifically precipitated. After reduction and alkylation of the immunoprecipitates, μ:L chain radioactivity was calculated. The ratio in each case was 3.9 ($\pm$0.3):1. The simplest explanation is that the tyrosine residues are equally available to the lactoperoxidase whether the IgM molecule is in solution or on the cell surface. This finding is consistent with the hypothesis that a substantial portion of the Fc fragment of the monomer is not buried within the cell membrane.

Origin of the Surface Immunoglobulin.—In order to determine if the Ig re-

covered from normal lymphocytes had been adsorbed onto the cell surfaces either in vivo or in vitro, several experiments were performed:

(*a*) *In vitro binding of mouse serum to rat spleen cells:* Rat spleen cells were teased into unlabeled mouse serum, washed, iodinated, lysed, precipitated with mouse Ig carrier and goat anti-mouse Ig (adsorbed with rat serum), reduced and alkylated and electrophoresed on SDS-acrylamide gels. As can be seen in Fig. 12, rat spleen cells did not adsorb mouse Ig.

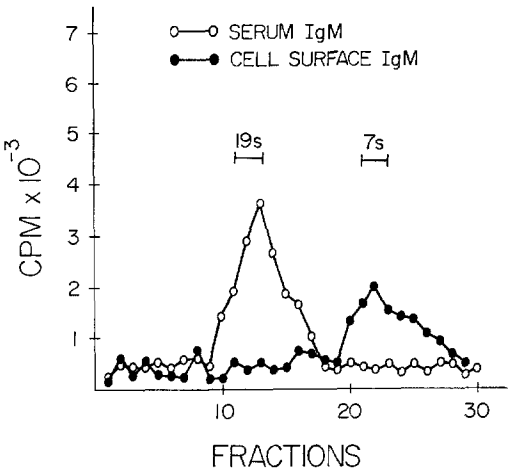

FIG. 11. Sedimentation in sucrose gradients of radiolabeled IgM from serum of hyperimmunized mice and from surface of splenic lymphocytes. Linear gradients containing 5–20% sucrose in 1% SDS and 0.5 M urea were employed. A control tube contained radioiodinated human IgM and mouse IgG as markers.

(*b*) *In vitro binding of radioiodinated mouse serum to mouse spleen cells:* Unlabeled mouse spleen cells were teased into enzymatically iodinated and dialyzed mouse serum (containing labeled IgM and IgG). Cells were washed and centrifuged on albumin gradients as usual, the four cell layers from the gradient were pooled, washed, lysed, and specifically precipitated as previously described. As indicated in Table I, virtually none of the acid-precipitable serum counts was recovered in immunoprecipitates of the lysates.

(*c*) *In vivo binding of radioiodinated mouse serum to mouse spleen cells:* Dialyzed, iodinated mouse serum was injected intravenously into three groups of mice. Groups were sacrificed at 2, 24, and 96 hr after injection. Spleen cells were prepared as usual (without iodination), centrifuged on albumin gradients, and the four cell layers pooled, lysed, and precipitated with anti-Ig. Results (Table I) indicate that there is no significant binding of radiolabeled Ig to the spleen cells. Moreover, the trace amounts of radioactivity recovered could represent catabolic products of the radiolabeled serum within the cells.

(*d*) *In vivo binding of mouse anti-BSA antibodies to mouse spleen cells:* 0.5 ml of mouse anti-BSA serum (obtained 3 wk after immunization) containing 900 μg of antibody was injected intravenously into each of four mice. A control group received the same amount of normal mouse serum. 24 hr later the spleens from each group were removed, the cells prepared, washed, iodinated, and lysed as usual. Lysates from the two groups were exhaustively dialyzed at 4°C against

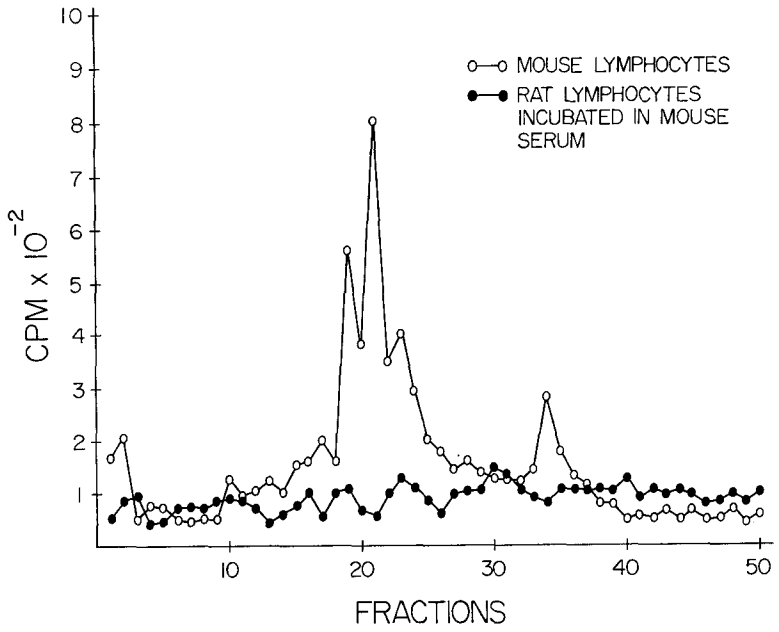

FIG. 12. Comparative amounts of radioactive mouse Ig precipitated from radioiodinated rat splenic lymphocytes previously incubated in mouse serum and radioiodinated mouse splenic lymphocytes. Samples were precipitated with mouse Ig and excess goat anti-mouse Ig (previously adsorbed with rat serum). Precipitates were reduced and alkylated before electrophoresis on acrylamide gels.

PBS along with another control preparation containing ^{125}I rabbit Ig anti-BSA in 0.5% Nonidet P_{40}. After dialysis each of the three preparations was incubated with acetyl cellulose-BSA (38) for 30 min at 37°C. The immunoadsorbent was then pelleted at 10,000 *g* for 30 min and washed three times in PBS. Adherent material was eluted with 2 ml of 0.25 N acetic acid at 37°C for 10 min. The immunoadsorbent was removed by centrifugation and the supernatants dialyzed overnight at 4°C against PBS. Of the total ^{125}I rabbit anti-BSA bound to the immunoadsorbent (80–90% of the input), 80% was eluted and 69% was recovered after dialysis (Table II). 85–90% of the dialysate was found to be acid or immunoprecipitable. Thus, anti-BSA was easily detected and measured by

this method. The radiolabeled material from the two lysates that was eluted from the immunoadsorbent was completely dialyzable (Table II). Thus, passively immunized animals did not have detectable antibody to BSA associated with spleen cells.

TABLE I

Binding of Ig to Spleen Cells

Protein added or injected		Conditions	No. spleen cells	^{125}I-Ig recovered from cells*
Source	Acid-precipitable radioactivity			
	cpm			*cpm*
^{125}I-mouse serum	1.8×10^7	Incubation in vitro	2×10^8	32‡
		Injected i.v.; spleens removed at		
^{125}I-mouse serum	4.7×10^6	2 hr	1×10^8	320§
	4.7×10^6	24 hr	1×10^8	83§
	4.7×10^6	96 hr	1×10^8	357§
^{3}H-mouse IgM	2.1×10^5	Incubation in vitro	1×10^8	0§
^{3}H-mouse IgM	2.1×10^5	Injected i.v.; spleens removed 20 hr postinjection	1×10^8	0§

* Radioiodination of 1–2 $\times$ 10^8 spleen cells yielded 50,000–100,000 immunoprecipitable cpm.

‡ Difference between specific and control precipitates.

§ Control precipitations not performed.

TABLE II

Binding of Anti-Bovine Serum Albumin Antibody to Spleen Cells In Vivo

Radioactive material added to acetyl-cellulose bovine serum albumin			Radioactivity		
Source	Amount of acid-precipitable radioactivity	Radioactivity in Ig	Adsorbed	Eluate before dialysis	Eluate after dialysis
	cpm	*cpm*	*cpm*	*cpm*	*cpm*
Lysate of ^{125}I spleen cells from mice injected with mouse anti-BSA	2.1×10^6	74,990*	2,863	310	49
Lysate of ^{125}I spleen cells from mice injected with normal mouse serum	2.3×10^6	79,251*	2,098	315	39
^{125} rabbit Ig-anti BSA	3.3×10^5	260,000‡	230,000	190,000	160,000§

* Precipitated with carrier mouse Ig and excess goat anti-mouse Ig.

‡ Precipitated with carrier rabbit Ig and excess goat anti-rabbit Ig.

§ Eluted counts were 85–90% acid and immunoprecipitable (with carrier rabbit Ig and excess goat anti-rabbit Ig).

(*e*) *In vitro and in vivo binding of ^{3}H-labeled mouse IgM to spleen cells:* A dialyzed secretion (from MOPC-104E tumors incubated in vitro with ^{3}H-tyrosine) containing IgM and L chains was incubated with mouse spleen cells in order to investigate IgM cytophilia. Results (Table I) again indicate no immunoprecipitable radioactivity associated with spleen cells from the four pooled layers of the gradient. In addition, ^{3}H-labeled mouse IgM was injected into several mice; 20 hr later spleens were removed and cells prepared as usual (without iodination) for BSA gradients. Pooled gradient layers were then washed, lysed, and precipitated. Again, results indicate no adherence of IgM to spleen cells in vivo (Table I).

All of the preceding experiments strongly suggest that the surface Ig of the splenic lymphocytes is not adsorbed from the serum but represents proteins synthesized by the cells themselves.

DISCUSSION

The studies described above indicate that the surface of living lymphocytes from the spleens of normal mice can be radioiodinated enzymatically without labeling intracellular molecules. Thus, subcellular fractionation of iodinated cells indicated that over 97% of the acid-precipitable radioactivity and all of the immunoprecipitable radioactivity was associated with membranes. Additional experiments excluded iodination of intracellular protein by endogenous peroxidases or by the added lactoperoxidase.

In lymphocytes from spleens of normal mice, regardless of the subpopulation obtained from density gradients, 2–4% of the total radiolabeled material on the cell surface was Ig. Experiments performed in vivo and in vitro provided no evidence that this Ig was adsorbed to the cells from the serum or medium. It is therefore concluded that the Ig, which presumably represents the antigen-specific receptor, was formed by the cells on which it resides. Identification of this Ig on acrylamide gels after immunoprecipitation with class-specific antiserum indicates that over 95% of the radioactive Ig from the small lymphocyte fraction is IgM. The large lymphocytes and plasma cells also have the majority of radioactivity in IgM (90%) and only 10% in IgG. Therefore the small percentage of IgG molecules on small lymphocytes is probably not due to contamination with large lymphocytes and/or plasma cells. These results are in good agreement with previous studies of Warner et al. (18), in which IgM was shown to be the dominant antigen-specific receptor on mouse splenic lymphocytes, and with Pernis (20) who demonstrated that IgM is the major class of Ig on rabbit splenic lymphocytes. In addition, Bosman and Feldman (41) have shown that 90% of the lymphocytes making antibody in immunized rabbits are synthesizing IgM rather than IgG.

Virtually all of the cell surface IgM was recovered under nonreducing conditions as monomer. Since monomers of mammalian IgM are held together by disulfide bonds (rather than noncovalent bonds) (40), the above finding suggests

that monomer is the form of the molecule on the cell surface. The possibility that the monomer is generated by self-assembly of H and L chains after detergent lysis of cells was excluded by the results of lysing cells in the presence of alkylating agents. Degradation of polymeric IgM by conditions in the lysate or by iodination, detergent treatment, or solubilization of immunoprecipitates was not supported by appropriate control experiments using 19S serum IgM and mouse tumor (MOPC-104E) IgM. However, it could be argued that 19S IgM on the cell surface is more susceptible to depolymerization than IgM in solution. For this reason, the interpretation that the monomer is the major form of the molecule must be considered as a working hypothesis only.

Although this is the first reported finding of monomeric IgM on the surface of normal lymphocytes, such monomers have been described as the major class of immunoglobulin in the sera of some lower vertebrates (42–48) and occasionally in trace amounts in normal mammalian sera (49–51) or in association with immunological deficiencies (52–56), myeloma (57), connective tissue diseases (58), and parasitic or other infections (59, 60). IgM monomers have also been described intracellularly in a mouse myeloma tumor that secretes polymeric IgM (61).

The relationship of the IgM molecule to the plasma membrane has not yet been elucidated. The release of IgM monomer of the expected molecular weight by detergent lysis is presumptive evidence that the molecule is bound noncovalently to the plasma membrane. Of particular interest is the finding that the ratio of μ:L chain radioactivity is similar in radioiodinated IgM from spleen cell surfaces or serum as well as ^{3}H-tyrosine–labeled monomeric IgM (MOPC-104E). These findings suggest that the tyrosine residues of μ and L chain are available to the lactoperoxidase even when the IgM is attached to the plasma membrane and argue against a model in which a large portion of the Fc fragment is buried in the membrane. This supports a suggestion that the receptor molecule is entirely on the outside of the plasma membrane and is attached to it by its Fc fragment (62), i.e., Ig molecules destined for secretion or to act as receptors become bound to the inside of Golgi vesicles by the Fc fragments, are transported to the plasma membrane within such vesicles, and are exteriorized by reverse pinocytosis.

The spleen contains both thymus-derived and bone marrow-derived lymphocytes (63). Although both can bind antigen specifically (64, 65), Ig has been demonstrated convincingly only on bone marrow-derived cells;[2] thymus-derived cells have less or no Ig (66). Thus, the IgM molecules isolated in our studies probably come from the surface of bone marrow-derived lymphocytes primarily. These cells are the precursors of antibody-secreting cells (67–70). In most immune responses, however, the vast majority of antibody-secreting cells (and

[2] Unanue, E. R., E. Rabellino, and H. M. Grey. 1971. Immunoglobulins on the surface of lymphocytes. II. The bone marrow as the source of detectable surface-bound immunoglobulins. Submitted for publication.

serum antibody) is eventually IgG. There are two major possibilities to explain these findings. The first is that lymphocytes that have synthesized IgM "switch" to IgG synthesis after stimulation by antigen. This could occur in cells secreting IgM antibody as suggested by others (71–74) or in lymphocytes that have only synthesized receptor. Thus, IgG-secreting cells could have IgM receptors.[3] The second is that IgG-synthesizing cells have a selective advantage so that with time after immunization, these clones eventually predominate in an immune response. These possibilities are currently being evaluated by the study of cell surface antibody at various stages of the immune response.

SUMMARY

The proteins on surfaces of living splenic lymphocytes from normal BALB/c mice were iodinated enzymatically. Such cells were fractionated into two subpopulations: one composed almost exclusively of small lymphocytes and the other mainly of large lymphocytes and plasma cells. Specific immunoprecipitation of radiolabeled surface Ig obtained from lysates of these cell populations indicated that approximately 2–3% of the acid-precipitable radioactivity from the cell surface is Ig. Moreover, 95% of the H chain radioactivity from the Ig of the small lymphocyte fraction and 90% from the large lymphocyte–plasma cell fraction was characterized as μ by precipitation with anti-μ sera as well as by molecular weight determination on polyacrylamide gels in sodium dodecyl sulfate. The Ig was recovered from the cell surface in the form of an IgM monomer. Control experiments suggested that the monomer did not result from depolymerization of 19S IgM by the methods used to radiolabel and isolate the molecule. ^{3}H-tyrosine labeling of IgM produced by meyloma cells and radioiodination of IgM in solution gave the same ratios of μ:L radioactivity as radiolabeling of IgM on cells, indicating that the tyrosine residues of L and μ-chains of cell surface IgM are available to the lactoperoxidase during the iodination. This is consistent with the hypothesis that cell surface IgM is entirely on the outside of the plasma membrane presumably attached to it by its Fc fragment. These results, together with previous reports by others, suggest that IgM, in its monomeric form, is the main antigen-specific receptor on lymphocytes of normal mice.

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[3] *Note Added in Proof.*—Pernis, B. S. (*Ann. N. Y. Acad. Sci.* In press.) has recently demonstrated the existence of plasma cells in immunized rabbits with IgM on the cell surface and IgG inside. His finding supports the concept of a switch-mechanism.

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