CELL SURFACE IMMUNOGLOBULIN

IX. A New Method for the Study of Synthesis, Intracellular Transport, and Exteriorization in Murine Splenocytes*

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It was previously hypothesized that Ig destined for the cell surface is synthesized and initially transported along a pathway similar to that of Ig destined to be secreted (1–3). In the Golgi complex both types of Ig become bound to the inner surface of the membrane. At this point, their pathways diverge: one type of Ig remains attached to the Golgi complex whereas the other type is released into the Golgi vesicle. Hence, after reverse pinocytosis of post-Golgi vesicles, attached Ig becomes cell surface Ig and released Ig becomes secretory Ig. This hypothesis received support from pulse-chase studies in a neoplastic line of human B lymphocytes (3, 4). The experiments established that within these cells, Ig was synthesized on membrane-bound polyribosomes and was subsequently confined to the microsomal compartment. It was not possible, however, to determine if this microsomal Ig was transported to the cell surface. It was desirable, therefore, to develop a method for internally labeling and then distinguishing cell surface Ig from Ig in other subcellular compartments.

In this paper, we describe such a method. It depends upon aggregating surface Ig on cells with specific antibody and subsequently removing the antigen-antibody complexes after cell lysis by centrifugation of large complexes and immunoprecipitation of remaining soluble complexes using antibody to the heterologous Ig. By means of this method, several fundamental questions concerning cell surface Ig have been approached: site of synthesis and intracellular transport; carbohydrate content and the rate of turnover. It has also been possible to confirm earlier conclusions based on experiments using cell surface iodination, that is, 8S IgM is the predominant Ig on murine splenocytes (5–7) and the molecule is attached to the cell surface by its μ-chains.

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Materials and Methods

Preparation of Cell Suspension.—Splenocytes from 4- to 8-wk old BALB/c mice were teased into phosphate-buffered saline (PBS),[1] pH 7.3, filtered through a stainless steel screen, and washed once.

Labeling with [^3H]Amino Acids.—Cells were washed in Eagle's minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, N. Y.) lacking either leucine or tyrosine and containing 10% fetal calf serum (FCS) and 2% antibiotic-antimycotic mixture. Cells were suspended at 10^7/ml; 20–30 μCi/ml L-tyrosine (3, 5-^{3}H) or L-leucine (4, 5-^{3}H) (New England Nuclear, Boston, Mass.) were added; the cultures were aliquoted into sampling volumes and incubated at 37°C in a moist CO_2 environment.

Labeling with [^3H]Sugars.—Cells were washed in MEM containing 20% of the normal glucose concentration, 10% FCS, and 2% antibiotic-antimycotic. 200 μCi/ml L-fucose (1, 5, 6, ^{3}H), D-galactose (1-^{3}H), or D-glucosamine hydrochloride (6-^{3}H) (New England Nuclear) were added and cultures were incubated for 5–6 h at 37°C in a moist CO_2 environment.

Labeling with ^{125}I.—Splenocytes were iodinated as previously described (5) at 10^8 cells/ml with 2 mCi ^{125}I, 50 μg lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.) and 25 μl of 0.03% hydrogen peroxide. The reaction was terminated after 5–10 min by the addition of 25–50 vol of cold PBS. Cells were centrifuged, washed once in PBS, once in MEM and FCS, and incubated for 20 min at 37°C in MEM and FCS. This incubation step removed the majority of the remaining free isotope without the need for additional centrifugations.

Aggregation of Cell Surface Ig Molecules.—Cells were centrifuged, washed twice in 10 ml of cold MEM and FCS, filtered through a stainless steel screen, and suspended in 2-ml aliquots each containing 5×10^7 cells. Each aliquot was treated for 15 min at 4°C with 100 μl of heat-inactivated rabbit antiserum containing specificities against either mouse Ig (RAMIg) (μ, γ, κ, λ), μ (5), γ (8), κ (9), or egg albumin (RIg) (control). The monospecific antisera to μ and γ were made using IgM isolated from MOPC 104E and mouse serum IgG (Pentex Biochemical Co., Kankakee, Ill.), respectively. Sera were exhaustively absorbed with myeloma proteins of other classes coupled to immunoabsorbants (8). Specificity was demonstrated by showing lack of binding of radioiodinated myeloma proteins of other classes of Ig using "sandwich" immunoprecipitation and analysis by acrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) (5, 10). After incubation, cells were centrifuged at 4°C, washed once in cold MEM and FCS, and resuspended in 2 ml of cold medium containing 50 μg mouse Ig (Pentex Biochemical Co.). When anti-μ serum was used for the first treatment, mouse IgM (MOPC 104E) was substituted for mouse Ig. Cultures were incubated for 15 min at 4°C, centrifuged, washed twice in 2-ml vol of cold PBS, and resuspended in 1.8 ml of PBS. The cell suspensions were then transferred to 5-ml cellulose nitrate tubes (Beckman Instruments, Inc., Fullerton, Calif.) and cells were lysed in a final volume of 4 ml of PBS containing 0.5% Nonidet P-40 (NP-40) (Shell Chemical Corp., New York) for 10 min at 4°C. Lysates were centrifuged at 100,000 *g* for 1 h at 4°C in the 50.1 rotor of the Model L ultracentrifuge (Beckman Instruments, Inc.). Lysates were dialyzed for 16 h at 4°C against 2–4 liters of PBS.

Identification of Immune Complexes in "Nuclear" Pellets.—The pellets from the high speed centrifugation were washed twice in 4 ml PBS by centrifugation at 100,000 *g* for 1 h. The pellets were transferrred to fresh tubes between washes with a plastic pipette. Washed pellets were treated with 0.5 ml of 1% SDS containing 8 M urea and 0.2 M mercaptoethanol (ME) for 60 min at 56°C. The entire volume, containing insoluble material as well as dissolved im-

[1] *Abbreviations used in this paper:* FCS, fetal calf serum; GARIg, goat antirabbit Ig; ME, mercaptoethanol; MIg, mouse Ig; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; RAMIg, rabbit antimouse Ig; RER, rough endoplasmic reticulum; RIg, rabbit antiegg albumin Ig.

mune complexes, was dialyzed for 16 h at 4°C against 0.1% SDS and 0.5 M urea, and electrophoresed on SDS 5% acrylamide gels (5, 10). Gel fractions were cut and counted as described previously (11).

Immunoprecipitation of Radiolabeled Ig from Cell Lysates.—After dialysis, lysates were centrifuged at 10,000 *g* for 30 min and small aliquots were precipitated in 10% trichloracetic acid (TCA) and counted (5). Ig was precipitated from the lysates as previously described using a sandwich procedure and suitable controls for nonspecific precipitation (12). Immune precipitates were washed three to four times in cold PBS and counted (5, 12). Values for control precipitates were subtracted from experimetal values. Dissolved precipitates were electrophoresed on SDS-agarose 2.5% acrylamide gels (1, 13), or were reduced and alkylated (5) and were electrophoresed on SDS 5% acrylamide gels. Markers were electrophoresed on companion gels.

Preparation of [^{125}I]rabbit or Mouse Ig.—Rabbit sera (RAMIg or RIg) were brought to 50% saturation with ammonium sulfate at 4°C. The precipitated material was dissolved in water, desalted on G25 Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) (14), lyophilized, reconstituted with phosphate buffer, and Ig prepared on DEAE-cellulose (15). The purified IgG was desalted on G25 Sephadex, lyophilized, and redissolved in PBS before use. The purity of the fractions was determined by immunoelectrophoresis against goat antirabbit serum and the antibody activity was determined by immunodiffusion against MIg or egg albumin (Ea). Small aliquots of the rabbit Ig or the mouse Ig (Pentex Biochemical Co.) were enzymatically iodinated (16) and exhaustively dialyzed against PBS at 4°C. Immediately before use, the ^{125}I-labeled samples were centrifuged at 100,000 *g* to remove aggregates and the specific activities were determined. All preparations were greater than 94% acid and immunoprecipitable with appropriate reagents.

RESULTS

Experimental Design.—(Fig. 1) The experimental plan was to label Ig by incubating cells with [^{3}H]amino acids or sugars for varying periods of time and to determine the amount of radioactive Ig on the cell surface by treating cells with rabbit antimouse Ig (RAMIg) to selectively aggregate cell surface Ig. Control cells were treated with rabbit antiegg albumin (RIg). Cells were then washed and treated with mouse Ig (MIg) in order to block remaining free combining sites on the RAMIg. After washing and lysis of cells in NP-40, the majority of the aggregates (consisting of surface MIg, RAMIg, and exogenous MIg) were pelleted along with nuclei and other cell debris by high speed centrifugation. Soluble complexes were then precipitated from the lysates with goat antirabbit Ig (GARIg). A comparison of radioactive Ig from lysates of cells treated in this manner with control cells indicated how much radioactive Ig had been pelleted by a combination of centrifugation and immunoprecipitation. This value represents the radioactive Ig that had been on the surface during treatment of cells with RAMIg. In the following sections, each aspect of this procedure is analyzed and quantified.

Binding by Cells of RAMIg and Exogenous MIg and their Fate after Cell Lysis.—In order to determine what percentage of the RAMIg that had bound to the cell surface could be pelleted after disruption of treated cells with NP-40, trace amounts of [^{125}I]rabbit Ig (RAMIg or RIg) were added to the corresponding antiserum. As seen in Table I, using RIg's of equal specific radioactivities

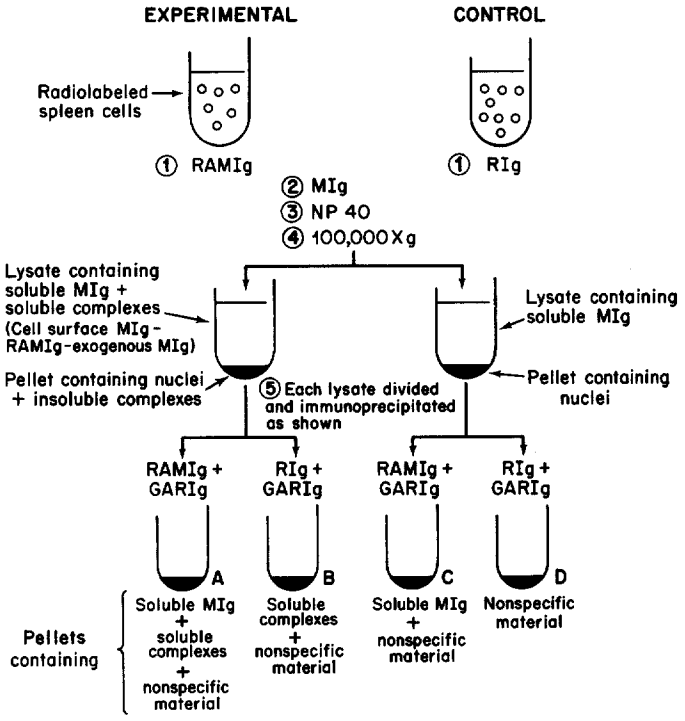

FIG. 1. Method for the detection of surface Ig on cells by anti-Ig-induced aggregation.

(cpm/μg Ig), approximately three times as much radioactivity was associated with the washed cell pellets of the cells treated with RAMIg as with those treated with RIg. When cells were incubated with unlabeled rabbit Ig's and then with [^{125}I]MIg, the ratio of specific to control radioactivity was approximately 20. Although both of the above ratios indicate specific binding of RAMIg to cell surface Ig, the lower ratio obtained when rabbit Ig's (rather than MIg) were labeled could be due to natural antibodies to mouse cells in the RIg. Such anticellular antibodies would not be expected to bind ^{125}I-labeled MIg, thereby accounting for higher ratios of specific to control radioactivity when MIg was labeled. The binding of [^{125}I]MIg to the cells which had been pretreated with RAMIg, indicates that free RAMIg-binding sites are present

TABLE I

Binding of RAMIg and Exogenous MIg to Splenocytes

Aliquot	Treatment of cells*		Radioactivity in washed cell pellet	% radioactivity in cell pellet recovered in		% radioactivity in lysate precipitated with		
	1st step	2nd step		Lysate	Nuclear pellet	RIg + GARIg	MIg + GAMIg	CIg + GACIg (control)‡
			cpm					
1	[^{125}I]RAMIg	MIg	168,618	5.2	94.8	98.2	97.5	7.1
2	[^{125}I]RIg	MIg	53,858	—	—	—	—	—
3	RAMIg	[^{125}I]MIg	500,401	6.3	93.7	96.4	100	10.3
4	RIg	[^{125}I]MIg	24,720	—	—	—	—	—

* Trace amounts of [^{125}I]RAMIg, [^{125}I]RIg, or [^{125}I]MIg were added to the corresponding unlabeled preparation. Input radioactivity, 4-7 × 10^7 cpm (>96% acid precipitable).

‡ Chicken Ig and goat antichicken Ig, control for nonspecific precipitation.

which could bind intracellular Ig after cell lysis. Therefore, treatment with unlabeled MIg was routinely performed.

After extensive washing of cells treated with either labeled RAMIg or MIg, lysis in NP-40, and centrifugation at 100,000 *g*, approximately 94% of the radioactivity specifically bound to the cells was pelletable (Table I). Under these conditions of detergent lysis, virtually all cells are disrupted. However, NP-40 does not dissociate antigen-antibody complexes. Hence, antigen-antibody aggregates containing cell surface Ig were pelleted by high speed centrifugation. The remaining radioactivity in the form of soluble complexes could be precipitated with GARIg (Table I).

Aggregation of ^{125}I-Labeled Cell Surface Ig by RAMIg.—The above experiments indicated that virtually all of the [^{125}I]RAMIg or the exogenous [^{125}I]-MIg which was bound by the cells was associated with the pellet after cell lysis and the combination of high speed centrifugation and immunoprecipitation. The question of what proportion of cell surface Ig is incorporated into the aggregate could then be approached. In these experiments, the cells were radioiodinated, washed, and incubated with appropriate concentrations of unlabeled RAMIg or RIg followed by soluble MIg as previously described. It should be noted that after radioiodination and lysis of untreated cells, approximately 10% of the total radioactivity is always found in the nuclear pellet. This nuclear radioactivity may represent adherent free isotope as well as denatured or insoluble surface proteins. The cells which had been treated with RAMIg contained additional radioactivity in the nuclear pellet.

As seen in Table II after treatment with RAMIg, Ig was the only molecule absent from the lysate, i.e., Ig accounted completely for the loss in acid precipitable radioactivity. Greater than 99% of radiolabeled *H-2* alloantigen, another surface protein, was recovered from lysates of such treated cells further indicating the specificity of the aggregation. Examination of the entire nuclear

TABLE II

Loss of Total Protein, Ig, and H-2 from Lysates of Radioiodinated Cells in which Cell Surface Ig was Aggregated

	Treatment	Protein*	Ig‡	*H-2*§ antigen
Radioactivity (CPM)	RIg	2,168,400	149,200	43,970
	RAMIg	2,042,800	23,740	43,856
Radioactivity lost‖ (CPM)		125,600	125,400	114
% of cell surface molecules bound by RAMIg¶		5.7	83	0.3

* Acid precipitable radioactivity.

‡ Radioactivity in anti-Ig precipitate minus radioactivity in control precipitate (anti-$\phi \times 174$).

§ Radioactivity in anti-$H\text{-}2^a$ precipitate minus radioactivity in control precipitate (anti-$H\text{-}2^b$).

‖ Subtraction value of RIg minus RAMIg.

¶ 100 (cpm in RIg-treated cells − cpm in RAMIg-treated cells)/ cpm in RIg-treated cells.

pellet by acrylamide gel electrophoresis revealed the presence of ^{125}I-labeled surface IgM only. No IgM was found in the pellet of cells treated with RIg (Fig. 2). In nine experiments, an average of 84% of the surface IgM radioactivity was complexed with antibody: 76–79% appeared in the nuclear pellet and 6–8% remained in the lysate in the form of soluble antigen-antibody complexes (RAMIg-MIg). This 6–8% could be removed from the lysate by treatment with carrier RIg and GARIg as indicated in the previous section when [^{125}I]-RAMIg or [^{125}I]MIg was used. MIg which was not complexed to rabbit antibody remained in the supernate. Hence, the amount of cell surface Ig was determined by subtracting the value obtained by precipitation of lysates in 1 aliquot with RAMIg and in another aliquot with RIg. In addition, this subtraction value also provided a control for nonspecific precipitation.

Attempts to Increase Proportion of Cell Surface Ig Aggregated by RAMIg.— It was unclear why 12–20% of surface Ig on cells was not bound to added RAMIg. It was possible that increasing the concentration of RAMIg might increase the proportion of cell surface Ig that was bound by antibody. As can be seen in Fig. 3, this was not the case. The possibility that the unbound cell surface Ig represented classes of Ig not recognized by the RAMIg was excluded by the observation that such unbound Ig could be precipitated from the lysate by the same RAMIg.

If some cell surface Ig molecules were sterically "hidden" by other Ig molecules, it might be possible to aggregate the available Ig molecules first, "cap" them at 37°C (17, 18), and then bind the remaining Ig molecules by additional treatment with RAMIg. The results of this experiment are shown in Table III.

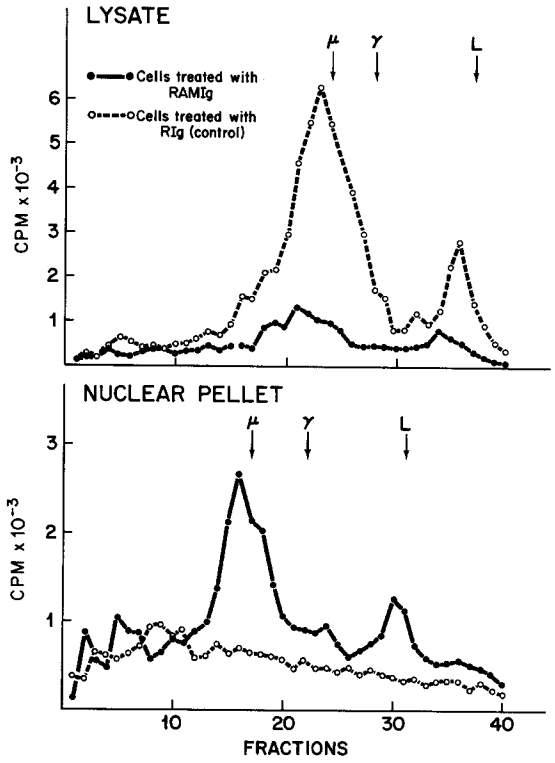

FIG. 2. IgM in the lysate (upper panel) or nuclear pellet (lower panel) of radioiodinated cells treated as described in Fig. 1. The immunoprecipitates from the lysate and the entire nuclear pellet were dissolved, reduced and alkylated, and electrophoresed on SDS 5% acrylamide gels with appropriate markers.

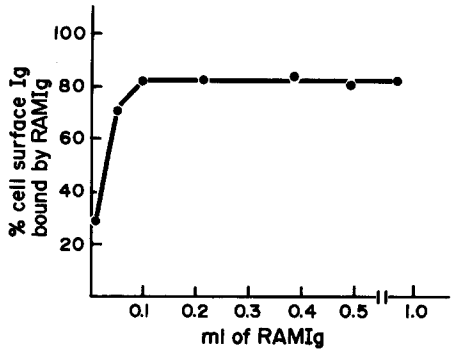

FIG. 3. Effect of increasing amount of RAMIg on binding of ^{125}I-labeled cell surface Ig.

TABLE III

Effect of Temperature and Additional Reagents on Binding of Cell Surface Ig on Radioiodinated Cells by RAMIg

Aliquot	Reagents*	Temperature	% of [^{125}I]Ig bound by RAMIg‡
		C°	
1	RAMIg + exogenous MIg	4	80
2	RAMIg + exogenous MIg	37	77
3	RAMIg + exogenous MIg (Sequence performed twice)	37	84
4	RAMIg + normal goat serum	4	86§
5	RAMIg + goat anti-RIg	4	86§
6	RAMIg + goat anti-RIg	37	88§

* All samples were compared to an aliquot of cells treated under the same conditions with RIg instead of RAMIg.

‡ Aliquots were incubated with rabbit antisera and with exogenous MIg or GARIg at temperatures indicated.

§ In aliquots 4–6, cells were not treated with exogenous MIg after RAMIg. Therefore, there were probably some unblocked sites on the RAMIg which could bind cell surface Ig after lysis. This might account for the higher values in these experiments.

There was no evidence of increased binding of cell surface Ig by such a sequential treatment compared to previous experiments. These results indicate that the surface Ig molecules which are not bound to RAMIg are not sterically blocked by other cell surface Ig molecules.

Table III also shows that the percentage of cell surface Ig bound by RAMIg was not increased by: (*a*) increasing temperature of incubation to 37°C, or (*b*) addition of a "piggyback" layer of antibody to RAMIg (GARIg) either at 37°C or 4°C. Since secretion does not occur at 4°C (1) this temperature was routinely used for treatments with RAMIg and MIg. This avoided the problem that ^{3}H-labeled Ig secreted during incubation (i.e., before MIg is added) could be bound by free RAMIg sites on the treated cells.

It was possible that the unbound Ig might be buried in the plasma membrane by its Fc portion (19, 20). If this were the case, the Fc portion might not be as readily iodinated as the Fab portion. Thus, the ratio of radioactivity in mu per light would decrease since the Fc portion contains only μ-chains. In order to investigate this possibility, the immunoprecipitate from the lysates of treated radioiodinated cells (i.e., the Ig not aggregated by RAMIg) was compared with the aggregated Ig (pellet obtained by high speed centrifugation). In these experiments, lysates were first precipitated with RIg and GARIg to remove soluble Ig-RAMIg complexes. The supernate was then used for immunoprecipitation with RAMIg. The two IgM preparations (aggregated and not aggregated) were then electrophoresed on SDS-agarose 2.5% acrylamide gels and the fractions coelectrophoresing with IgM monomer were eluted, reduced

and alkylated, and electrophoresed on SDS 5% acrylamide gels. The ratio of radioactivity in the nonaggregated 8S IgM monomer was similar to that of the aggregated monomer (4.5:1) suggesting that the former was not buried in the membrane by tyrosine-containing sequences of its Fc portion. These results suggest that the unbound surface Ig molecules are probably inaccessible to RAMIg because of steric hindrance by other surface macromolecules or structures.

Evidence that RAMIg does not Bind Intracellular Ig.—As mentioned previously, RAMIg which was bound to the cell could then bind [^{125}I]MIg, indicating that some of its antibody combining sites were free, *i.e.*, some RAMIg molecules had only one antibody site occupied by cell surfaces Ig. Exogenous unlabeled MIg was added, therefore, to block these free sites so that intracellular Ig would not be bound during cell lysis. In order to demonstrate that this blocking was complete, admixture experiments were performed. In a typical experiment, 5×10^7 unlabeled spleen cells were treated with RAMIg and MIg. This washed aliquot was admixed with an equal number of radioiodinated untreated cells and the mixture lysed in NP-40.

As seen in Table IV, the lysate of the admixture showed no evidence of binding of radioactive Ig from the labeled cells by unblocked sites of the RAMIg on unlabeled cells. Aliquots 3–6, Table IV, represent controls that indicate that the usual proportion of cell surface Ig was bound by RAMIg. The same experiments were performed with [^{3}H]tyrosine-labeled cells and the results were analogous.

The preceding experiments, therefore, characterized the method by establishing: (*a*) that appropriate concentrations of RAMIg bound an average of 84% of cell surface Ig on intact mouse splenocytes and this percentage could

TABLE IV

Demonstration that Splenocytes Treated Sequentially with RAMIg and MIg Cannot Bind Additional Ig after Cell Lysis

Aliquot*	Treatment of cells before admixture and lysis		% ^{125}I cell surface Ig bound by RAMIg‡
	^{125}I cells	Unlabeled cells	
1	No treatment	RAMIg + MIg	0 ± 4
2	No treatment	RIg + MIg	
3	RAMIg + MIg	No treatment	81 ± 3
4	RIg + MIg	No treatment	
5	RAMIg + MIg	No cells	80 ± 5
6	RIg + MIg	No cells	

* Aliquots 1–4, 5×10^7 ^{125}I cells + 5×10^7 unlabeled cells; aliquots 5–6, 1×10^8 ^{125}I cells.

‡ Average of three experiments ± range.

not be increased by altering several variables. (*b*) 90–93% of this bound Ig appeared in the pellet after detergent lysis and high speed centrifugation; the remaining 6–10% was present in the lysate in the form of soluble antigen-antibody complexes and could be immunoprecipitated with GARIg. (*c*) All antibody combining sites on the RAMIg were blocked by the addition of exogenous MIg before lysis. It was possible, therefore, to use this method to investigate questions related to the biochemistry and dynamics of cell surface Ig.

Effect of Specificity of RAMIg on its Capacity to Bind to Cell Surface Ig.—To investigate which portion of the cell surface IgM molecule is attached to the plasma membrane, RAMIg's of different specificities were employed. If IgM is attached by its Fc portion, it could be predicted that anti-κ would bind cell surface IgM more effectively than anti-μ because of steric considerations.

The results of such experiments are summarized in Table V. As can be seen,

TABLE V

Effect of Specificity of RAMIg on its Capacity to Bind Cell Surface Ig

Specificity of antiserum*	No. of experiments	% binding of cell surface Ig by RAMIg‡
μ γ κ λ	9	84 ± 6
μ	3	33 ± 5
κ	4	77 ± 3
γ	2	0

* Cells were treated with concentration of rabbit antibody which gave maximum binding of Ig.

‡ Average ± range.

anti-μ was far less effective in binding cell surface Ig than anti-κ. This same anti-μ sera could precipitate all the cell surface Ig after cell lysis. Anti-γ did not bind detectable amounts of cell surface Ig.

This experiment extends earlier results (5) and suggests that IgM is attached to the plasma membrane by its Fc fragment. The experiment also emphasizes the problems of establishing the presence of individual classes of Ig on cell surfaces by binding with monospecific anti-Ig reagents; such studies favor the detection of light chains rather than heavy chains when both are present in equimolar amounts as part of an Ig molecule.

Detection of Cell Surface Ig after Labeling with [^{3}H]Tyrosine.—In using [^{3}H]-amino acids which label the entire intracellular pool of Ig, it was important to demonstrate that the loss of Ig from the lysate was due to aggregation of cell surface Ig rather than lysis of Ig-bearing cells during treatment. Hence, total labeled protein from the cell suspensions of treated and control cells was compared before lysis with detergent. A typical experiment of several that were performed is shown in Fig. 4. There is no loss in total protein induced by the aggregation method indicating that cells have not been lysed by this method. In con-

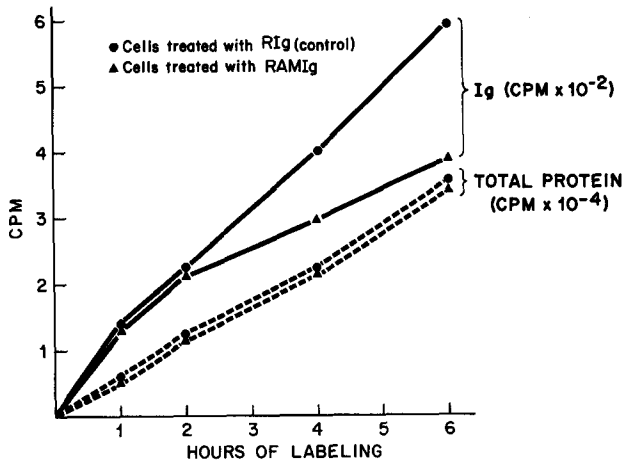

FIG. 4. Total protein and Ig in lysates of [^{3}H]tyrosine-labeled cells treated as described in Fig. 1.

trast, Ig was lost by this method beginning 2 h after labeling. It was, therefore, possible to label cells with [^{3}H]amino acids and study the biochemistry and kinetics of appearance of cell surface Ig. In all these experiments, the amount of radioactive Ig on the cell surface was corrected for the efficiency of detection (84%) (Fig. 1).

Molecular Form of [^{3}H]Tyrosine-Labeled Cell Surface Ig.—In order to determine the molecular forms of cell surface Ig, cells were labeled for 6 h with [^{3}H]-tyrosine and then treated as described in Fig. 1. As shown in Fig. 5, most of the 8S IgM and free chains were on the cell surface whereas virtually all 19S IgM and most of the IgG were not. Free chains were shown to be μ by eluting the appropriate fraction from the gel, reducing and alkylating the eluted proteins, and reelectrophoresing on SDS 5% acrylamide gels. This experiment confirms earlier results obtained by enzymatic radioiodination of cell surface Ig (5–7). It is noteworthy that 19S IgM (which is presumably destined for secretion) does not appear on the cell surface to any significant extent.

Carbohydrate Content of Cell Surface Ig. In order to investigate whether carbohydrates are present in cell surface Ig, cells were labeled for 6 h with [^{3}H]sugars and then analyzed for radioactive cell surface Ig. As seen in Table VI, Ig in splenocytes can be labeled with galactose, fucose, and glucosamine, as well as leucine. Fig. 6 shows acrylamide gel patterns for cell-associated and secreted Ig labeled by [^{3}H]fucose and leucine. As expected, only the μ-chains were labeled by fucose indicating that [^{3}H]fucose is probably incorporated in its precursor form. The percentage of cell-associated Ig that was on the surface after 6 h of incubation with particular ^{3}H precursors was 81 for fucose, 57 for galactose, 47 for glucosamine, and 43 for leucine. The different values obtained probably reflect the intracellular compartment in which individual sugars are added, and,

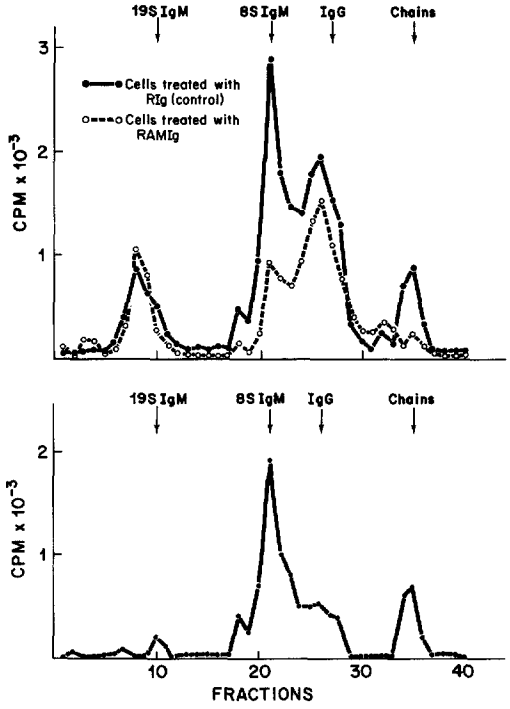

FIG. 5. Ig in the lysates of cells labeled for 6 h with [^{3}H]tyrosine and treated as described in Fig. 1. Immunoprecipitates were dissolved and electrophoresed on SDS-agarose 2.5% acrylamide gels with appropriate markers (upper panel). The lower panel shows the curve when the values of the experimental curve are subtracted from those of the control curve (upper panel); the resultant values represent radioactive Ig on the cell surface. (Since [^{3}H]tyrosine labels nucleoprotein as well as surface Ig, the high speed pellets of both experimental and control cells contain several radiolabled proteins. Hence, it is not possible to examine the nuclear pellet directly for the presence of aggregated surface IgM).

TABLE VI

Incorporation of ^{3}H Precursors by Murine Splenocytes into Intracellular, Surface and Secreted Ig

^{3}H isotope	Acid precipitable radioactivity/ 5×10^7 cells		cpm specific precipitate/cpm control precipitate		% acid precipitable radioactivity that was Ig‡		% cell-associated Ig that was on the surface
	Lysate	Secretion	Lysate	Secretion*	Lysate	Secretion	
	cpm						
Galactose	7,520	4,292	14.1	3.2	2.5	33.4	57
Fucose	20,800	5,104	193.6	9.7	7.4	100	81
Glucosamine	11,520	17,960	6.4	2.1	4.5	8.6	47
Leucine	827,360	31,900	4.3	11.2	2.1	74.1	43

* NP 40 added to secretions before immunoprecipitation.

‡ 100 (cpm specific ppt − cpm control ppt)/acid precipitable radioactivity.

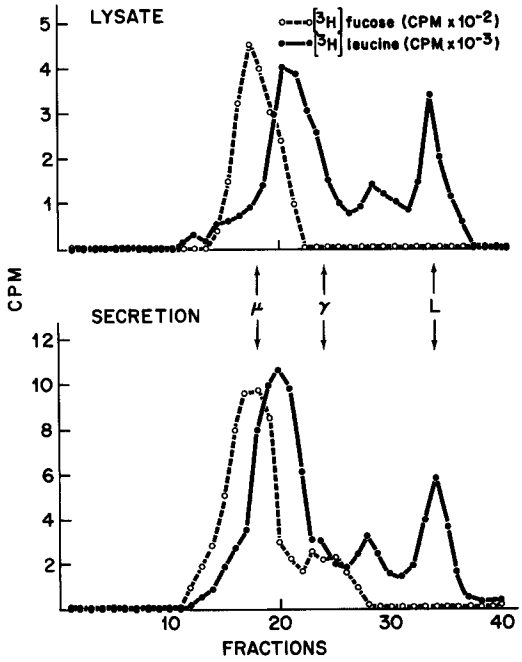

FIG. 6. [^{3}H]fucose- and [^{3}H]leucine-labeled Ig recovered from lysates (upper panel) or secretions (lower panel) of splenocytes. Immunoprecipitates were reduced and alkylated before electrophoresis on separate gels.

therefore, the duration of time between incorporation and exteriorization. Thus, when incorporation occurs near the time of exteriorization, the intracellular pool is small compared to the cell surface compartment.

Kinetics of Appearances of Cell Surface Ig after Labeling with [^{3}H]tyrosine.— Two major possibilities were considered for the sites of synthesis and intracellular transport of cell surface Ig. The first is that cell surface Ig follows the route for secretory molecules; that is, synthesis in the rough endoplasmic reticulum (RER), transport to the Golgi complex where sugars are incorporated, transport to the plasma membrane in post-Golgi vesicles, and exteriorization via reverse pinocytosis (2). The second possibility is that cell surface Ig is made on plasma membrane-bound polyribosomes (21). The first model predicts a long latent period between synthesis and appearance on the plasma membrane, because of the need for a succession of transport steps, some of which are time consuming (22–34). The second model predicts virtually no latent period since there is no transport; that is, synthesis occurs at the site at which the molecule will reside.

To differentiate between these two models, cells were labeled with [^{3}H]tyrosine and the time before the appearance of cell surface Ig was determined by the Ig aggregation method. The results are illustrated in Figs. 7 and 8. As can be

seen in a representative experiment (Fig. 7) labeled intracellular (nonsurface) Ig rises rapidly for a period of 2 h and then reaches a virtual plateau. In contrast, secreted and cell surface Ig appear after latent periods of approximately 1 and 2 h, respectively, and then both Ig's increase rapidly. The rate of increase is more rapid for secreted Ig. Fig. 8 summarizes six experiments and shows the percentage of cell-associated Ig on the surface as a function of time of labeling. These experiments confirm the existence of a latent period before detection of cell surface Ig.

In interpreting the above experiments, the fundamental problem of cell

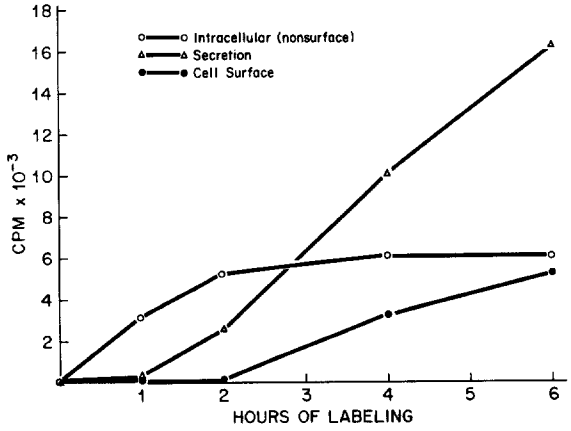

FIG. 7. Kinetics of labeling with [^{3}H]tyrosine of intracellular (nonsurface), surface, and secreted Ig in splenocytes. Cells were treated as described in Fig. 1. Cell surface Ig was subtracted from total cell-associated Ig in order to determine intracellular (nonsurface) Ig.

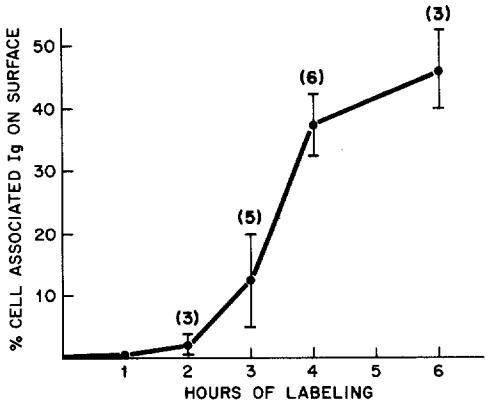

FIG. 8. Kinetics of labeling with [^{3}H]tyrosine of cell surface Ig. The points are averages of a number of experiments; the number is indicated in parentheses; bars represent the ranges of values.

heterogeneity must be emphasized. Thus, the cell population studied consists predominantly of small or medium sized lymphocytes (80%), a moderate percentage of lymphoblasts (5–15%), and a small proportion of plasma cells (1–5%) (35, 36). However, a plasma cell is over 100-fold more active in Ig synthesis than a small lymphocyte,[2] hence the biosynthetic contributions of plasma cells are major ones. Thus, the presence of even 1% of plasma cells in the suspension of splenocytes could account for one-half of the Ig synthesized by the total population. From studies of murine myelomas (32, 37–41) it is known that intracellular Ig of plasma cells is uniformly labeled in 1–2 h, and thus in the splenocyte suspension, the plasma cells probably account for the initial rapid appearance of radioactivity in intracellular Ig and its rapid rise for a period of 2 h. The slower rise between 2 and 6 h is probably due to the increase in specific activity of lymphocytic Ig which is turned over slowly.

In contrast, plasma cells probably make little contribution to total cell surface Ig because of their small numbers. Thus, the specific activity of cell surface Ig increases throughout the duration of the experiment presumably because of the slow turnover of lymphocyte surface Ig. The sigmoid nature of the curve for the proportion of Ig on the cell surface (Fig. 8) and the relatively rapid rise of that phase of the curve after appearance of cell surfae Ig argue for a true latent period.

Turnover of Cell Surface Ig. In order to further study the turnover of cell surface Ig, pulse-chase experiments were performed. If the interpretation is correct that rapidly labeled Ig (0–2 h) predominantly represents plasma cell Ig, then the rapidly labeled pool should be chased into the secretory compartment without a noticeable effect on the cell surface. In contrast, the slowly labeled Ig (which accounts for the rise in total intracellular Ig between 2–6 h) should be in lymphocytes and destined to appear on their surface. Thus, cells were labeled for 90 min with [^{3}H]tyrosine and then were chased with excess unlabeled amino acid. The chase was over 94% effective.

A representative experiment is shown in Fig. 9. As can be seen, there was a rapid decrease in intracellular Ig for the first 2 h of chase, accompaned by a concomitant rise in secreted Ig. In contrast, when surface Ig appeared (1 h after chase) it did not rise over the next hour of chase, while the intracellular Ig was falling. These results support the above interpretation.

It is also to be noted that radiolabeled cell surface Ig presumably on lymphocytes was not noticeably decreased during the last 5 h of chase. This finding suggests that only a small proportion of surface Ig had been labeled during the 1.5 h of labeling and that turnover is slow. Hence, in a given period of time, labeling can occur but not a detectable chase.

Fig. 10 summarizes five experiments and indicates the percentage of cell-associated Ig on the surface as a function of time of chase. This curve rose

[2] E. S. Vitetta, and J. W. Uhr, unpublished observations.

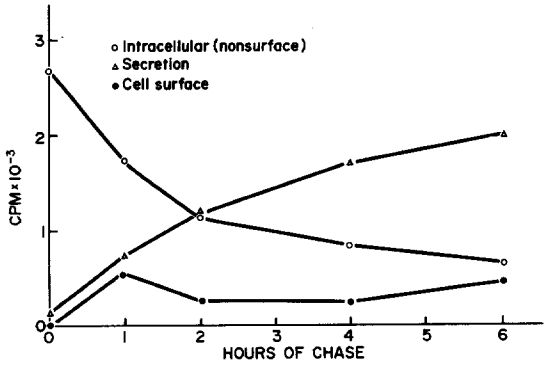

FIG. 9. Kinetics of chase of intracellular-(nonsurface) secreted and cell surface Ig. Cells were labeled for 90 min with [^{3}H]tyrosine and then were washed and incubated in complete medium.

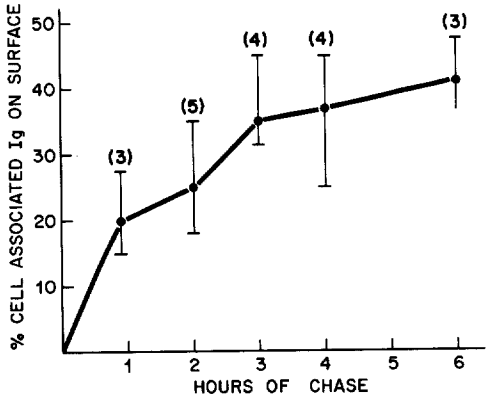

FIG. 10. Kinetics of chase of cell surface Ig. See Figs. 8 and 9.

because the cell-associated pool decreased due to secretion. After 6 h of chase, almost one-half of the remaining Ig, the majority of which is presumably in lymphocytes, was on the surface. If an effective chase could be continued for a long period, virtually all cell-associated radioactive Ig should appear on the cell surface.

DISCUSSION

The present studies describe a new method for the labeling of cell surface Ig. The method consists of the treatment of lymphoid cells with heterologous anti-Ig in order to aggregate cell surface Ig. The washed cells are then treated with exogenous Ig in order to block free anti-Ig-binding sites. The cells are then lysed in nonionic detergent and large antigen-antibody complexes are pelleted by high speed centrifugation. The soluble antigen-antibody complexes remaining in the supernate are then immunoprecipitated with antibody to the

heterologous Ig. The Ig remaining in the supernate (not complexed to heterologous anti-Ig) is compared to the Ig in control cells (not treated with anti-Ig). The difference between these values is considered to be Ig that was present on the cell surface and which was removed from the supernate by the aforementioned maneuvers.

The validity of this method has been firmly established: (*a*) All exogenous reagents added to the cells were trace iodinated with ^{125}I and their fate determined in individual experiments. Thus, it has been ascertained that virtually all RAMIg and exogenous MIg which remain bound to the cells after washing are pelleted in the high speed centrifugation. (*b*) The small number of soluble complexes which remain were shown to be quantitatively immunoprecipitated by antibody to the heterologous Ig. (*c*) Admixture experiments proved that there are no free anti-MIg-binding sites that could bind intracellular Ig (after cell lysis) or secreted Ig (during the treatment period). (*d*) Optimal conditions for aggregation of cell surface Ig, such as temperature of incubation and concentration of reagents were determined. (*e*) No lysis of cells occurred during this procedure which could inadvertently alter the value of cell surface Ig determined.[3]

Melchers and Andersson (39) have independently used anti-MIg as a means for determining the amount of cell surface Ig. The major differences between the approach described by these authors and our own are that they did not block free RAMIg sites with exogenous Ig and they performed the procedure at 37°C. Our studies suggest that under these circumstances, there would be free anti-Ig-binding sites which could bind intracellular Ig released after cell lysis.

One of the major questions to be addressed in our study is the site of synthesis of cell surface Ig. Two major possibilities were considered: (*a*) Cell surface Ig might be synthesized in the RER and transported to the Golgi complex as Ig destined to be secreted. (*b*) Cell surface Ig could be made on polyribosomes attached to the plasma membrane. We had previously suggested the secretory pathway hypothesis based on pulse-chase studies of a neoplastic line of B lymphocytes (Daudi cells) (3, 4) but the results of these studies although consistent with the hypothesis were incomplete. The new method presented in this paper allows a more effective approach to this question. Thus, the secretory pathway hypothesis predicts a series of transport steps resulting in a considerable latent period between synthesis of Ig and its appearance on the cell surface analogous to the lag before secretion. The plasma membrane

[3] Thus, as a function of time of incubation, there could be an increased sensitivity of cells to lysis by treatment with RAMIg. This would result in a decrease in radioactivity in both total protein and Ig but would not affect these parameters in the control cells. Hence, the amount of cell surface Ig detected by aggregation would be higher than the true value. This possibility was excluded by the finding that radioactivity in total protein was not decreased in cells incubated for up to 6 h and then treated with RAMIg (see Fig. 4).

hypothesis suggests little or no lag between synthesis and exteriorization. Continuous labeling experiments showed that intracellular Ig appeared within minutes after labeling spleen cells and rose rapidly for a period of 2 h whereas cell surface Ig was not detected until 1.5–2 h after labeling. At this time, the amount of radioactive cell surface Ig rose rapidly for the next 4 h. The presence of a long latent period and a rapid increase in cell surface Ig after its appearance argue for a true latent period and against the possibility that cell surface Ig is labeled from the beginning at a slow rate and only reaches the threshold of detection after 1–2 h. It should be noted, however, that during the first 2 h of labeling, the vast majority of intracellular Ig is in plasma cells which thereby diminishes the sensitivity of detection of lymphocyte cell surface Ig (which is derived by a subtraction value of total cellular Ig in treated cells from that in control cells). Notwithstanding this cautionary note, we believe the simplest interpretation of the data is that cell surface Ig is synthesized in the RER and transported via the Golgi complex to the cell surface.

Further analysis of the kinetics of labeling and "pulse-chase" experiements revealed additional findings: (*a*) Total intracellular Ig is labeled rapidly for 2 h and then much more slowly. (*b*) Chase of intracellular (nonsurface) Ig is also rapid for 2 h at which time over one-half of the Ig has been secreted. (*c*) Chase of cell surface Ig for 6 h does not change the absolute amount of radioactive Ig on the cell surface but the percentage of cell-associated Ig that is on the surface is greater than 40. Melchers and Andersson have reported essentially similar findings (39).

These results together with other biosynthetic and electron microscopic observations suggest the following concept of the cellular and subcellular events underlying exteriorization of Ig in a splenocyte population. There is a small percentage of plasma cells in spleens from unimmunized animals (approximately 2%) (35, 36); each plasma cell has a large intracellular pool of Ig which is turning over rapidly; the majority of Ig molecules secreted by these cells do not have a cell surface phase (35). In contrast, small lymphocytes represent the majority of cells in normal spleen (36); each has a small intracellular pool of Ig which is turning over slowly (35) and the vast majority of Ig molecules have a cell surface phase (35). There is little or no secretion but shedding of Ig occurs in vitro (1, 42–44). The small number of plasma cells have as much intracellular Ig as all the lymphocytes. Activated lymphocytes are probably intermediary cells in terms of their biosynthetic and secretory functions.

The difference in the turnover of cell surface Ig in experiments utilizing ^{3}H labeling and those using enzymatic iodination with ^{125}I deserve comment. The latter technique usually revealed a biphasic loss of cell surface Ig: half-life of 6–8 h for the first 3–4 h of culture, followed by a very long half-life measured in days thereafter (1). The initial rapid loss of iodinated cell surface Ig can be accounted for by the shedding of Ig into the incubation media (1). Shedding could be due to effects of the radioiodination procedure and thus not occur with ^{3}H labeling. Our tentative interpretation is that rapid shedding of cell surface proteins is due to pinching off of microvilli on B lymphocytes (45–47) and may not be physiological; the physiologic rate of turnover of cell surface Ig appears to be much slower. Clearly, more studies are necessary to resolve this issue.

These studies describe for the first time the presence of carbohydrate in cell

surface Ig. It was demonstrated that [^{3}H]fucose, -galactose, and -glucosamine were all incorporated into cell surface Ig. It was also possible to calculate what percentage of cell-associated Ig was on the cell surface with each of the three sugars as well as with tyrosine. The proportions should be related to the time of addition of the particular sugar; that is, the closer to the time of exteriorization that the sugar is added, the higher is the proportion of cell surface Ig to total cellular Ig. The results indicate that 81% of [^{3}H]fucose-labeled Ig that is cell associated is on the surface; the other values are 57% for galactose, 47% for glucosamine, and 41% for leucine. This sequence is reminiscent of the sequence of addition of carbohydrate moieties to Ig in plasma cells as determined by the proportion of cellular to secreted radioactive Ig as a function of duration of labeling, subcellular fractionation, and electron microscopy (32, 48–51). The present results, therefore, further support the concept that cell surface Ig is synthesized and transported like secretory Ig. The results along with past studies suggest that glucosamine is added onto nascent chains and that additional sugars (glucosamine, galactose, and fucose) are incorporated in the Golgi complex.

The antibody-aggregation method was also used to extend earlier observations. Thus, it was found that 8S IgM is the major cell surface Ig on murine splenocytes (5–7). This finding excludes the possibility that depolymerization of 19S IgM is induced by enzymatic radioiodination. The absence of cell surface 19S IgM together with the demonstration of its presence in cell lysates indicates that polymerization occurs elsewhere in the cell. We suggest that such polymerization probably occurs in post-Golgi vesicles after glycosylation is complete.

The concept that the IgM molecule is bound to the plasma membrane in the lymphocyte by its Fc fragment (5) received further support from two findings. (*a*) Rabbit antibody that was monospecific for μ-chain was far less effective in binding cell surface Ig on intact cells than rabbit antibody specific for κ-chains. (*b*) The only free chains detected on the surface of lymphoid cells by this new method were μ-chains. These findings suggest that the binding site for the cell surface is on the μ-chain of the IgM monomer.

SUMMARY

A new method for the detection of cell surface immunoglobulin labeled with isotopic precursors is described. The method consists of the aggregation of surface Ig on cells with specific antibody (heterologous) and the subsequent removal of antigen-antibody complexes by the combination of high speed centrifugation and immunoprecipitation of remaining soluble complexes using antibody to the heterologous Ig. Using this method, the kinetics of appearance of cell surface Ig and its turnover were studied in murine splenocytes. The results suggest that cell surface Ig is synthesized and transported in the same manner as secretory Ig rather than being synthesized on the plasma membrane. The turnover of intracellular and cell surface Ig in lymphocytes is slow.

In contrast, intracellular Ig in plasma cells is rapidly secreted and usually without a cell surface phase. Cell surface Ig was shown to be radiolabeled with [^{3}H]glucosamine, -galactose, and -fucose. The proportion of cell surface to intracellular (nonsurface) Ig labeled with these precursors suggests the same sequence of addition of sugars to Ig destined to be on the surface of lymphocytes as with Ig which will be secreted by plasma cells. Results with this new method also confirm earlier conclusions based on experiments using cell surface iodination: 8S IgM is the predominant Ig on the surface of murine splenocytes and the molecule appears to be attached by its μ-chains.

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