CELL SURFACE IMMUNOGLOBULIN

V. Release from Murine Splenic Lymphocytes*

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We have previously described a method utilizing enzymatic radioiodination of lymphoid cells to isolate and characterize cell surface immunoglobulin (Ig)[1] (1) and alloantigens (2). Using this approach, it was demonstrated that Ig on murine splenic lymphocytes is predominantly monomeric IgM (3) and is found on bone marrow-derived lymphocytes (4). When radioiodinated splenic lymphocytes were incubated in vitro, cell surface Ig was released into the incubation medium (5).

In the present experiments, the kinetics of release of cell surface Ig has been examined and the relationship of cell surface Ig to total intracellular Ig studied. It has been shown that small lymphocytes actively synthesize and secrete Ig, a portion of which has a transient phase on the cell surface. Radioiodinated surface Ig is released in the form of a complex containing Ig noncovalently bound to a fragment of plasma membrane. Based on the above findings, we have extended a previous model for intracellular transport and secretion of Ig.

Materials and Methods

Preparation of Iodinated Spleen Cells.—Splenic lymphocytes were prepared and iodinated as previously described (3). Cells were labeled at a concentration of 5×10^7-1×10^8 cells/ml with 50 μg (0.1 ml) of lactoperoxidase (Calbiochem, Los Angeles, Calif.), 1–2 mCi ^{125}I (1.0 ml), and 0.03% H_2O_2 (0.025 ml). The iodination was terminated by the addition of 20–30 vol of cold 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.3 (PBS). The enzyme was removed by one to two washes with the same buffer. In several experiments, iodinated cells were fractionated into small and large lymphocytes on albumin gradients as previously described (3, 6). In experiments in which radioiodinated cells were cultured, cells were washed only once before iodination and once afterwards.

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[1] *Abbreviations used in this paper:* B cells, bone marrow-derived lymphocytes; Ig, immunoglobulin; NP40, Nonidet P-40; PBS, phosphate-buffered saline; RER, rough endoplasmic reticulum; SDS, sodium dodecyl sulfate; T cells, thymus-derived lymphocytes.

Cell Culture.—1–2 × 10^8 iodinated or unlabeled cells were suspended at a concentration of 1 × 10^7/ml in Eagle's minimal essential medium containing nonessential amino acids (Grand Island Biological Co., Grand Island, N. Y.) and 10% fetal calf serum. In experiments in which cells were labeled with tyrosine-^{3}H, the medium lacked both tyrosine and fetal calf serum for the first 60 min of labeling. The medium was then supplemented with 10% fetal calf serum. Cell viability was monitored by determining trypan blue exclusion.

After the incubation period, the cell suspension was centrifuged at 1000 *g* for 15 min and the cell pellets washed and lysed as previously described (3). The incubation medium (secretion) was again centrifuged at 2000 *g* for 15 min and dialyzed for 12–16 hr at 4°C against PBS. In some experiments Nonidet P-40 (NP40, Shell Chemical Co., New York) was added to the secretions to a final concentration of 0.5% before dialysis. Dialyzed secretions were concentrated to 2–5 ml by pervaporation at 25°C, dialyzed for 2 hr at 25°C against PBS, and centrifuged at 10,000 *g* for 30 min. Small samples of lysates and secretions were precipitated in trichloroacetic acid (TCA) as previously described (3).

Isolation of Radioactive Ig.—The labeled intracellular (^{3}H), cell surface (^{125}I), or secreted (^{3}H or ^{125}I) Ig was immunologically precipitated from the lysates and secretions using a "sandwich" precipitate as previously described (3). For control precipitations, a hyperimmune antiserum to an unrelated antigen was substituted for the binding antiserum. Washed precipitates were solubilized in 8 M urea and 1% sodium dodecyl sulfate (SDS) at pH 8.4, dialyzed against 2% SDS-0.1 M phosphate buffer, pH 7.0, and electrophoresed on agarose-2.5% acrylamide gels adapted from Dingman and Peacock (7) for SDS disc gels. Material was electrophoresed for 45 min on 12.5-cm gels at 15 ma per tube. 2% SDS was included in both running buffer and gels. 19S serum IgM, 8S IgM from MOPC-104E lysates, and purified mouse IgG served as markers on gels run simultaneously. Reduced and alkylated samples (3) were electrophoresed on 5% SDS acrylamide gels (8) with μ, γ, and L chain markers (from reduced and alkylated IgG and IgM) run on parallel gels. All gels were fractionated and counted as described previously (3, 9). Double-labeled (^{125}I and ^{3}H) gel samples were counted in Beckman cocktail D (3) in a Beckman LS-250 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Chloroform-Methanol Extraction of Radiolabeled Cells.—(See reference 10.) 5 × 10^7 radioiodinated cells were suspended in 1.0 ml of PBS and mixed with 20 ml of 2:1 chloroform:methanol to form a monophasic system. After incubation at 25°C for 24 hr, 4.0 ml of water was added, the mixture vortexed, and the preparation centrifuged at 4000 *g* for 30 min. The water and chloroform layers were separated and reextracted twice with chloroform and water, respectively. The resultant extracts were counted and small samples of the chloroform extract were chromatographed as described below.

n-Butanol Extraction of Radioiodinated Cells, Secretions, and Specific Precipitates.—(See reference 11.) 1 × 10^8 radioiodinated cells, 2-3-ml secretions, or 1.0 ml samples of suspended specific precipitates were mixed with 1.0 ml of *n*-butanol and incubated for 15 min at 4°C. The water and butanol layers were then separated by centrifugation at 15,000 rpm for 15 min in the SW 56 rotor of the Spinco Model L-2 ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) and reextracted twice with butanol and water, respectively. Total and acid-precipitable radioactivity were determined.

Chromatography of Chloroform-Soluble Extracts from Radioiodinated Cells.—(See reference 12.) Chromatography was performed by Dr. P. Elsbach, Department of Medicine, New York University. Material was chromatographed on silica gel H plates (Brinkmann Institute, Westbury, N. Y.). Two solvent systems were used: (*a*) chloroform (100):methanol (56):acetic acid (20):water (10) and (*b*) petroleum ether (80):ethyl ether (20):water (1). The silica gel was scraped from the plates in five parallel fractions corresponding to marker lipoprotein regions and counted.

Density Gradients for Separation of Lipoproteins.—(See reference 13.) 4 ml of medium from

radioiodinated or tyrosine-labeled cells (either NP40 treated or untreated) were overlaid with 8 ml of density 1.006 buffer containing 1.42 g of Na_2HPO_4, 7.27 g of NaCl, and 0.1 g of Na_2 ethylenediaminetetraacetate per liter of water. Samples were centrifuged in the No. 40 rotor of the Spinco Model L ultracentrifuge for 20 hr at 40,000 rpm. Tubes were sliced (13) and the 5 ml top fraction, 7 ml bottom fraction, and pellet were removed with a Pasteur pipette. The bottom fraction and the pellet were adjusted with KBr to density 1.063 (13) and these two fractions were recentrifuged, sliced, and sampled as described above. The entire procedure was repeated with the bottom and pellet fractions from the second gradient after adjusting the densities to 1.21 (13). All fractions were then adjusted with 5% NP40 to 0.5% and total, acid-precipitable, and immunoprecipitable radioactivity were determined.

RESULTS

Effect of Cycloheximide In Vivo on Cell Surface Proteins.—To determine whether there is turnover[2] of cell surface protein in splenic lymphocytes, 20-gram BALB/c mice were injected intraperitoneally with 3.0 mg of cycloheximide (Upjohn Co., Kalamazoo, Mich.). At intervals after injection, animals were killed, the spleens were removed, cell suspensions were prepared, and 5×10^7 lymphocytes were iodinated. Control animals received injections of saline or cycloheximide immediately before splenectomy.

TABLE I

Effect of Cycloheximide In Vivo on Cell Surface Protein and Ig of Mouse Splenic Lymphocytes

Time after injection of cycloheximide	No. of lymphocytes per spleen $\times 10^7$*	Acid-precipitable radioactivity‡ (TCA)	Immunoprecipitable radioactivity (Ig)	% loss of	
				TCA§	Ig§
hr		*cpm*	*cpm*		
0	4.5	3,149,300	160,980	0	0
1	3.6	670,300	31,671	63	81
2	3.6	439,000	20,338	74	88
4	3.7	886,700	40,326	53	68
6	3.8	934,200	54,646	50	63
24	5.0	1,157,600	57,646	39	63
Uninjected	4.8	1,872,200	108,811	—	—

* Three spleens taken at each time point.

‡ 5×10^7 cells labeled at each time point.

§ Based on values obtained from uninjected animals.

As seen in Table I, there was a 30% loss of lymphocytes from the spleen within 1 hr after injection with a return to normal by 24 hr. Lymphocytes were >95% viable at all time points. When equal numbers of control and experimental cells were iodinated, there was a decrease of iodinatable total cell surface protein and surface Ig, which reached a maximum of 74 and 88%, respectively, at 2 hr after injection. During the next 22 hr there was a gradual but incomplete recovery.

[2] The term, turnover, is used to mean rate of loss from the compartment in question (in this instance the cell surface) without implication as to the cause of turnover, i.e., transit or degradation or both.

There are several possible explanations for these findings: (*a*) rapid turnover of cell surface Ig, (*b*) migration of Ig-bearing lymphocytes from the spleen, or (*c*) marked decrease in the efficiency of the iodination in the presence of cycloheximide. This last possibility was not supported by the data obtained from control animals injected with cycloheximide immediately before splenectomy in which the high concentration of cycloheximide appears to have increased the extent of surface labeling.

Effect of Cycloheximide In Vitro on Cell Surface Proteins.—To eliminate the possibility of a direct effect of cycloheximide on the iodination reaction and migration of cells from the spleen, experiments with cycloheximide were performed in vitro.

Washed splenic lymphocytes were incubated with 4×10^{-4} M cycloheximide for 5 hr. The effectiveness of the cycloheximide was demonstrated by the

TABLE II

Effect of Treatment with Cycloheximide for 5 hr In Vitro on Cell Surface Protein and Ig of Mouse Splenic Lymphocytes

Experiment	% loss in radioactivity	
	Acid precipitable	Immunoprecipitable
1	28	63
2	32	50
3	25	55
4	40	59
5	37	52
6	29	51
Average	32	55

failure of these cells to incorporate several ^{3}H-labeled amino acids into protein during the period of culture. Cell viability was unaffected (95%). Control cultures received cycloheximide immediately before sampling. After centrifugation and washing, cells were radiolabeled under identical conditions and acid-precipitable and immunoprecipitable radioactivity determined. As seen in Table II, an average of 32% of total protein and 55% of Ig that could be radioiodinated was lost in 5 hr.

The rate of turnover was investigated. In these experiments, samples were removed from two master cultures, one containing cycloheximide. Cycloheximide was then added to the control sample, both samples of cells were washed and iodinated, and acid-precipitable and immunoprecipitable radioactivity was determined. Fig 1 indicates that after a lag period of 1–3 hr, surface protein that can be iodinated disappears with a half-life of greater than 8 hr, and Ig with a half-life of 6–8 hr. The lag period is probably due to the presence of an intracellular pool of completed Ig and other protein molecules that are destined to be transported to the surface.

Release of Cell Surface Proteins into the Incubation Medium.—To determine whether turnover of cell surface Ig is due to release from the cell, radioiodinated lymphocytes were incubated in vitro for 2–8 hr and samples of cells and secre-

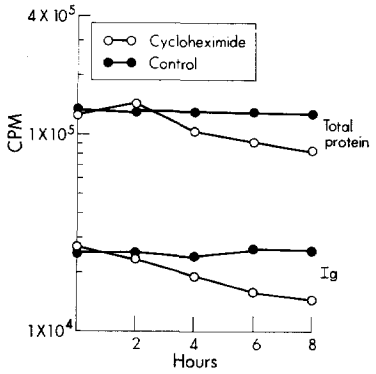

FIG. 1. Loss of cell surface protein and Ig from lymphocytes incubated in vitro with 4×10^{-4} M cycloheximide. Samples of control and cycloheximide-treated cells were iodinated and cell lysates specifically precipitated.

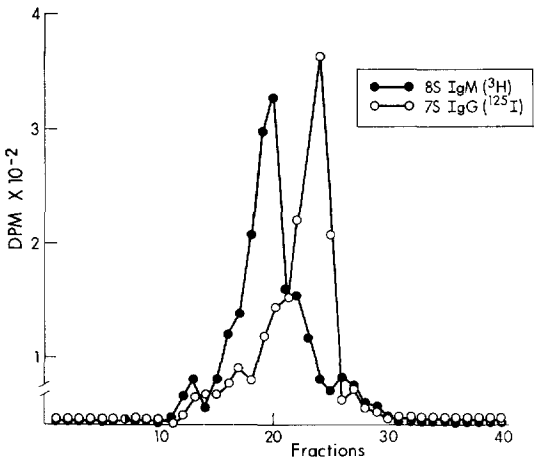

FIG. 2. Electrophoresis on SDS-agarose acrylamide gel of admixed tyrosine-^{3}H-labeled 8S IgM and ^{125}I-labeled 7S IgG. 8S IgM was isolated from lysates of MOPC-104E myeloma cells and 7S IgG was purified on diethylaminoethyl-Sephadex (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) from mouse IgG (Pentex Biochemical, Kankakee, Ill.).

tions (incubation medium treated with NP40) were examined at intervals. During this period of time, there was no change in cell viability which remained over 95%.

Large amounts of radiolabeled proteins were released into the incubation medium. 2–4% of the acid-precipitable radioactivity of secretions could be

specifically precipitated with anti-Ig. This radiolabeled Ig accounted for all of the Ig lost from the cell surface. The precipitates were analyzed on agarose-acrylamide gels in which 19S IgM, monomeric IgM, and IgG can be resolved.

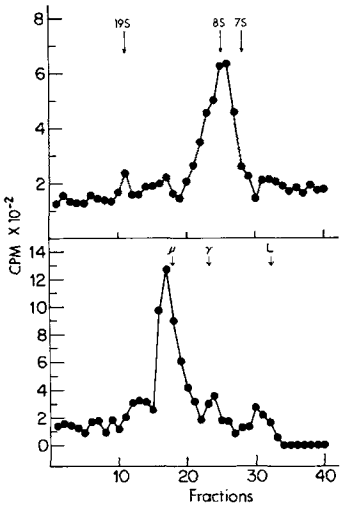

FIG. 3. Radioiodinated cell surface Ig released from splenic lymphocytes during incubation. The incubation medium was treated with NP40 before immunoprecipitation. Dissolved precipitates were electrophoresed on SDS-agarose acrylamide gels (above) or reduced and alkylated and electrophoresed on SDS acrylamide gels (below). Appropriate molecular markers were electrophoresed simultaneously on other gels.

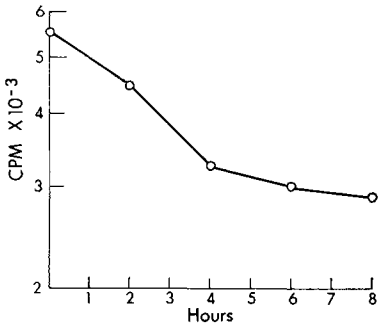

FIG. 4. Kinetics of release of radioiodinated cell surface Ig from small lymphocytes incubated in vitro.

The separation of ^{3}H-labeled IgM monomer (8S) and ^{125}I-labeled IgG is shown in Fig. 2. As seen in Fig. 3, the cell surface Ig released into the medium was identified as predominantly 8S IgM with minute amounts of IgG and possibly 19S IgM. There was no enrichment of Ig in the medium relative to the cells, i.e.,

2–4% of the radioactivity of released or cell-associated protein was Ig. The kinetics of release of Ig appeared biphasic (Fig. 4) with a half-life (calculated from the initial slope) of 5–7 hr. This half-life is similar to the turnover deduced from the in vitro cycloheximide experiments (Fig. 1) suggesting that release may account entirely for turnover.

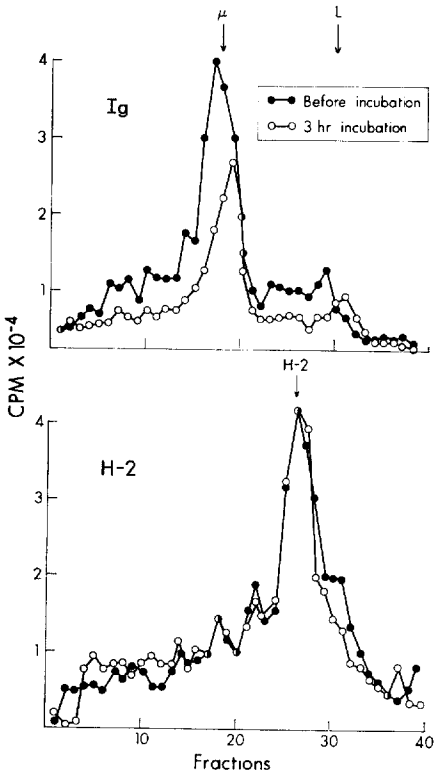

FIG. 5. Loss of radioiodinated surface Ig and *H-2*^{a} from A/J murine splenic lymphocytes incubated in vitro for 3 hr. Samples of lysates were prepared before and after incubation and the Ig precipitated by a "sandwich" technique (using rabbit anti-mouse Ig and goat anti-rabbit Ig). Supernatants of the precipitates were then treated with anti-*H-2* and the complexes precipitated with goat anti-mouse Ig. Both anti-Ig and anti-*H-2* precipitates were dissolved, reduced, and alkylated and electrophoresed on SDS-acrylamide gels.

Is the Release of Cell Surface Ig due to Cell Death?—This possibility was of particular concern since a large proportion of cell surface radiolabeled proteins released into the medium during incubation is not Ig.

Cells were labeled with ^{51}Cr and iodinated with cold NaI, incubated, and studied by both trypan blue exclusion and release of ^{51}Cr. The results indicated no significant cell lysis during the first 8 hr of culture. To investigate the ques-

tion further, another cell surface antigen (*H-2*) was examined. Previous experiments had demonstrated that this antigen could be readily radiolabeled on the cell surface and specifically precipitated from the cell lysate (2). Therefore, in these experiments, splenocytes from A/J mice were radiolabeled and incubated in vitro. Cell lysates and secretions were prepared before and after 3 hr of incubation. After specific precipitation of Ig, supernatants of the precipitates were treated with anti $H\text{-}2^a$ (a gift from Dr. E. A. Boyse, Sloan-Kettering Institute for Cancer Research). Complexes were then precipitated with an excess of goat anti-mouse Ig. Thus, using the same sample of cells, release of

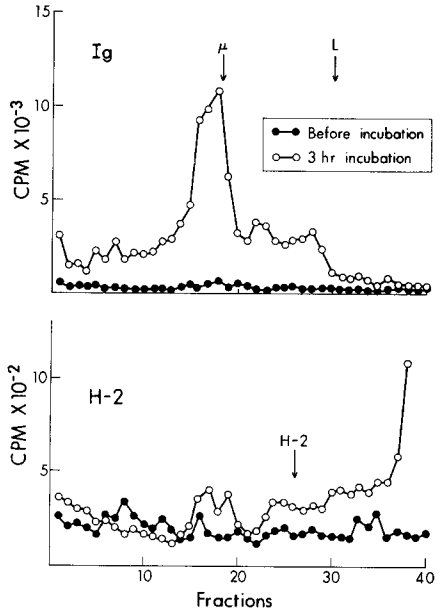

FIG. 6. Radioiodinated cell surface Ig and $H\text{-}2^a$ recovered from the incubation medium of A/J splenic lymphocytes after 3 hr of culture. See Fig. 5.

both radiolabeled Ig and *H-2* from the cells and appearance in the incubation medium were determined.

As can be seen in Figs. 5 and 6, approximately 35% of the cell surface Ig was lost from the cells and recovered in the secretions. In contrast, *H-2* antigen was not lost from these cells during the 3 hr of incubation. These data indicate that there is selectivity in the release of cell surface proteins and argue strongly against cytolysis.

Synthesis and Secretion of Tyrosine-^{3}H-Labeled Ig.—If surface Ig, which is primarily 8S IgM, is turned over and released rapidly, labeling with a precursor should reveal synthesis and secretion of such molecules. Small lymphocytes obtained from the bottom fraction of an albumin gradient incorporated tyrosine-

^{3}H into 8S IgM and 7S IgG. After 2 hr in culture, 10–20% of the intracellular Ig was secreted into the medium as 8S IgM, IgG, and a small amount of 19S IgM (Fig. 7, upper panel). Addition of NP40 to secretions did not affect the results shown in the figure. After reduction and alkylation of the specific precipitate, characteristic μ, γ, and L chain peaks were observed (Fig. 7, lower panel).

Strictly analogous results were obtained with the fraction which contained a larger proportion of large lymphocytes and plasma cells (10–15%), which

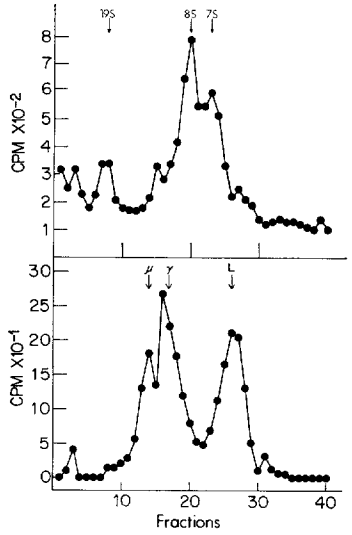

FIG. 7. Tyrosine-^{3}H-labeled Ig secreted by small lymphocytes. Dissolved precipitate were electrophoresed on SDS-agarose acrylamide gels (above) or reduced and alkylated and electrophoresed on SDS-acrylamide gels (below) with appropriate markers.

suggests that synthesis of Ig by the small lymphocyte fraction is not caused by contamination with plasma cells.

The above results confirm the prediction of synthesis and secretion of 8S IgM but also reveal synthesis and secretion of IgG, a class of Ig that was not well represented on the cell surface after enzymatic radioiodination.

Is Cell Surface Protein Released by Both T and B Splenocytes?—Since released cell surface Ig is from bone marrow-derived (B) lymphocytes (4), the released non-Ig proteins (96–98% of acid-precipitable radioactivity) could also be derived from B cells, thymus-derived (T) lymphocytes, or both. To approach the question of whether T cells release proteins, surface radiolabeled thymocytes were cultured in vitro for 3 hr. Although cell viability remained over 98% during the incubation, in three separate experiments 15–30% of the acid-precipitable radioactivity was recovered in the secretions. None of this radio-

activity was specifically precipitable with antiserum to Ig determinants, confirming earlier results (4). These results of release of surface radiolabeled proteins from thymocytes are in agreement with the results of Cone et al. (14). It is therefore probable that both T and B cells release non-Ig surface proteins.

The finding that thymocytes are actively releasing surface proteins suggests that this mechanism could account for accumulation of lymphokines in the incubation medium when T cells are stimulated by an antigen or mitogen (15).

Effect of Temperature and Pharmacologic Agents on Release of Cell Surface Ig.— If the release of cell surface proteins is an active process, this release might be inhibited by metabolic poisons (sodium azide and dinitrophenol), inhibitors of protein synthesis (cycloheximide), or agents which interfere with microfilament-

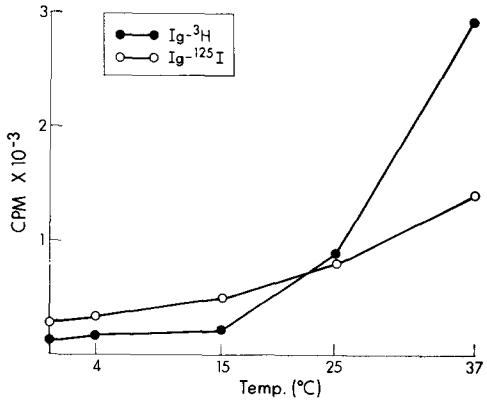

FIG. 8. Release of radioiodinated cell surface Ig or secretion of tyrosine-^{3}H-labeled Ig from splenic lymphocytes at different temperatures. Cells were incubated for 180 min after either radioiodination or labeling for 60 min with tyrosine-^{3}H. Samples of the medium were then treated with NP40 and specifically precipitated.

associated events such as pinocytosis and "cap" formation (cytochalasin) (16–19). Therefore, samples of radiolabeled cells were incubated for 3 hr with concentrations of such agents that did not affect cell viability (10^{-4}–10^{-5} M). Control tubes received equal concentrations of drugs at the end of the incubation period. None of these drugs inhibited release of cell surface Ig. Since the failure to inhibit release of Ig was unexpected under the above conditions, "conventional" secretion was examined using splenocytes which had been prelabeled for 60 min with tyrosine-^{3}H. These drugs also failed to inhibit secretion of tyrosine-^{3}H-labeled Ig by lymphocytes suggesting that release of surface Ig and secretion of intracellularly labeled Ig do not require protein synthesis or microfilament activity and are not inhibited by moderate concentrations of antimetabolites. It is possible, however, that the drug concentrations used in our experiments were insufficient to completely inhibit glycolysis and oxidative phosphorylation.

The results with cytochalasin are in agreement with results using internally labeled murine myeloma cells (20). Our results differ from those of Cone et al. (14), who observed inhibition of release of surface proteins by lymphocytes after treatment with puromycin and antimetabolites. Since these authors added puromycin before radiolabeling, their results may be caused by the effect of this drug on synthesis rather than on transport and secretion. With respect to metabolic inhibitors, the concentrations employed by Cone et al. (14) consistently killed the cells in our experiments.

The effect of temperature on release was also examined. Samples of ^{125}I- or tyrosine-^{3}H-labeled cells were incubated for 3 hr at 4°, 15°, 25°, and 37°C. A representative experiment is shown in Fig. 8. As can be seen, both secretion of

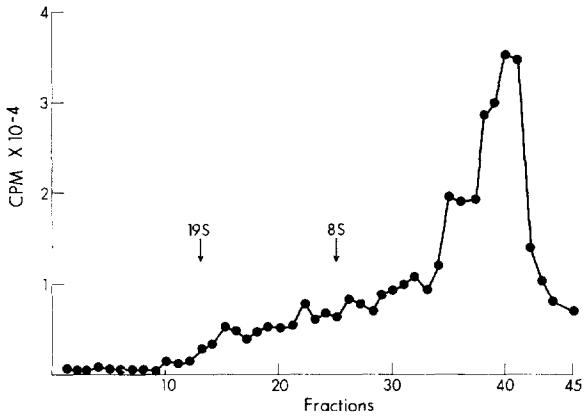

FIG. 9. Radioiodinated material released from BALB/c splenic lymphocytes after 3 hr in culture. A sample of the incubation medium was treated with 1% SDS-8 M urea and electrophoresed on an agarose-acrylamide gel with appropriate markers.

tyrosine-^{3}H-labeled Ig and release of ^{125}I-labeled cell surface Ig are temperature dependent but the slopes are different.

Characterization of the Released Material.—The following observations suggest that the release of radiolabeled surface Ig may follow a different pathway from the secretion of the majority of intracellularly labeled Ig. This release may represent shedding of plasma membrane with attached Ig.

(*a*) Only 2–4% of the radioactivity released by radioiodinated cells could be precipitated with antiserum to Ig, provided that the incubation medium was treated with detergent. This finding suggests that many other radiolabeled molecules are released from the cell surface. In addition, when samples of secretion were electrophoresed directly on the gels without prior immunoprecipitation, most of the radioactivity was associated with non-Ig molecules (Fig. 9).

(*b*) When the medium was not treated with detergent before immunopre-

cipitation, additional radioactive material (ranging from 10 to 20% of the total acid-precipitable radioactivity) was precipitated with antiserum to Ig. Moreover, if this precipitate was then washed in NP40, the additional radioactive material was released and electrophoresis of the treated precipitate revealed radioactive IgM monomer only (Fig. 10).

(*c*) Specific precipitation of secretions from tyrosine-^{3}H-labeled cells after "mock" iodination (with ^{127}I) gave the same pattern of electrophoresis whether or not the medium was treated with NP40. Such tyrosine-^{3}H-labeled secretions contained approximately equal amounts of radioactivity in IgG and IgM (Fig. 7). In contrast to ^{125}I-labeled secretions, these secretions contained 70–80%

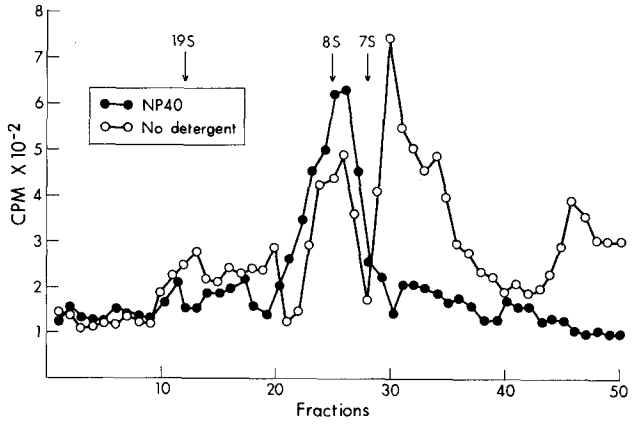

FIG. 10. Effect of detergent treatment on the electrophoresis pattern of released cell surface Ig. The incubation medium was either precipitated directly or pretreated with NP40 before precipitation. Precipitates were dissolved in 2% SDS and electrophoresed on SDS-agarose acrylamide gels. Appropriate markers were electrophoresed simultaneously.

of the total acid-precipitable radioactivity in Ig representing enrichment relative to the cells (5–10%).

In order to study the possibility that Ig was released on a fragment of plasma membrane, additional experiments were performed.

1. *Are membrane lipids released with Ig?:* In order to determine whether cell surface lipids were labeled by the lactoperoxidase method, washed, radiolabeled cells were extracted three times with a chloroform:methanol:water mixture (10). 3–4% of the radioactivity was present in the chloroform layer after three extractions. This material was then chromatographed on silica gel H with a chloroform:methanol:acetic acid:H_2O (100:36:20:10) solvent system. The radioactivity migrated with the neutral lipids.

Since lipids on the cell membrane can be iodinated by the lactoperoxidase method, the next experiments were designed to examine whether such lipids were released into the medium. Therefore, secretions from radiolabeled cells

were extracted twice with butanol and water (11). 1.4 times as much radioactivity was recovered in the butanol layer as in the water layer. This radioactivity was not acid precipitable, in contrast to that in the water layer, indicating that labeled lipids are released from iodinated cells.

The next experiments were designed to determine whether the labeled lipids were released bound to Ig. Therefore, washed precipitates from ^{125}I-labeled secretions were treated with either 0.5% NP40, *n*-butanol, 0.5 M urea, or PBS. As seen in Table III, the untreated precipitate contained 17% of the total acid-precipitable radioactivity, as compared with 2% in a control precipitate. This indicates that the radioactivity in the specific precipitate is attached to Ig and not trapped nonspecifically in the immune complex. When the specific precipitate was extracted, all of the non-Ig radioactivity could be removed with detergent (Table III, Fig. 10). Butanol and urea released smaller amounts of the non-Ig radioactivity (Table III). Tyrosine-^{3}H-labeled Ig in the secretions was unaffected by the extraction procedures used, indicating it contained no additional noncovalently bound material. It could be concluded that the cell surface Ig is released in a complex containing membrane protein and lipid, whereas all detectable tyrosine-^{3}H-labeled Ig is secreted without membrane.

TABLE III

Extraction of Immunoprecipitates from Secretions of Radiolabeled Cells

Isotope used to label Ig	Acid-precipitable radioactivity	Radioactivity in		% of specific precipitate extracted with			
		Specific precipitate	Control precipitate	0.5% NP40	*n* butanol	0.5 M urea	PBS
	cpm	*cpm*	*cpm*				
^{125}I	179,500	30,051	3513	70	9	12	1
^{3}H	274,030	168,210	0	1	2	1	1

2. *Isolation of membrane-Ig secretory complex:* Attempts to pellet the secretory complex by high speed centrifugation were unsuccessful. Therefore, it was postulated that the secretory complex might consist of small fragments of plasma membrane which could be floated in density gradients. Samples of secretion from radioiodinated cells were therefore centrifuged in density gradients ranging from density 1.006 to 1.2. Other samples were centrifuged in a similar manner after detergent treatment of the secretion.

As seen in Table IV, the majority of the immunoprecipitable radioactivity from the untreated secretions appeared in the high-density lipoprotein region (1.063–1.21). In contrast, all of the radiolabeled Ig in the detergent-treated secretions was recovered as higher density protein (>1.21). In similar experiments, secretions from mock iodinated (^{127}I) or untreated tyrosine-^{3}H-labeled cells were fractionated in this density gradient system. As seen in Table IV virtually none of the tyrosine-^{3}H-labeled Ig appeared to be of density 1.063–1.21. Since (*a*) the cells had been prelabeled with tyrosine-^{3}H for 3 hr so that

some labeled Ig is presumed to have reached the surface and (*b*) the mock iodination did not alter the results, it was concluded that only a very small percentage of Ig secreted by the cells had a surface phase.

DISCUSSION

There are four major findings to emerge from the present studies: (*a*) small lymphocytes secrete 8S IgM and IgG, (*b*) Ig on the surface of small lymphocytes (presumably antigen-specific receptor) is rapidly turned over and released from the cell surface, (*c*) cell surface Ig is released as a secretory complex consisting of Ig bound noncovalently to lipid and other plasma membrane proteins, and

TABLE IV

Analysis in Density Gradients of ^{3}H- and ^{125}I-Labeled Ig in Secretions of Splenic Lymphocytes

Isotope used§	Treatment of secretions with NP40	Radioactivity in Ig	% distribution in density gradients* Density‡			
			<1.006	1.006–1.063	1.063–1.21	>1.21
		cpm				
^{125}I	+	93,584	0	0	0	100
	−	33,548	0	1	70	30
^{3}H	+	87,890	—	—	0.7	99
	−	88,231	—	—	0.8	99
^{3}H + "mock" iodination (^{127}I)	+	35,570	—	—	1.2	99
	−	49,709	—	—	1.0	99

* Specific − control precipitate.

‡ <1.006 = very low density lipoprotein; 1.006–1.063 = low density; 1.063–1.21 = high density; >1.21 = protein.

§ Approximately 50% of the total ^{125}I radioactivity and 10% of the ^{3}H radioactivity were lost in the pellet fractions. The percentages described here are based on the soluble material only.

(*d*) *H-2* alloantigen on the cell surface is not released under similar conditions of incubation.

Active Secretion by Small Lymphocytes.—Small lymphocytes from unstimulated animals are characterized morphologically by a paucity of rough endoplasmic reticulum (RER) and, in some instances, polyribosomes (21), and functionally by an absence of DNA synthesis in the majority of cells (22) and low rates of protein and RNA synthesis (23, 24). These cells, therefore, do not resemble typical secretory cells. Nevertheless, it is well recognized that small lymphocytes can secrete antibodies (25). The present studies indicate that a population from normal spleen consisting of virtually all small lymphocytes incorporates tyrosine-^{3}H into 7S IgG and 8S IgM molecules which are actively secreted. Since a population of spleen cells enriched moderately in large lymphocytes and plasma cells did not produce greater quantities of these proteins, the results obtained with the population of small lymphocytes cannot be attributed to a minor contamination with plasma cells and/or large lymphocytes.

Prior studies revealed that almost all radioiodinated surface Ig on small lymphocytes is 8S IgM (3). The results mentioned above with tyrosine-^{3}H labeling of the same cell population suggests that very few IgG molecules have a cell surface phase. These results complement immunofluorescent studies of plasma cells by Pernis et al. (26), who found that cell surface Ig on IgG-producing plasma cells is IgM.

The question arises as to whether secretory Ig in these cells follows a similar pathway to that described in plasma cells. Biosynthetic studies of an established human lymphoblastic cell line which contains little RER indicate that Ig is formed on polyribosomes attached to the ER and that intracellular transport occurs within microsomes (27). By analogy, it is probable that the small number of membrane-bound polyribosomes in small lymphocytes are the ones concerned with synthesis of Ig. We suggest, therefore, that the major cytoplasmic differences, morphologically and functionally, between lymphocytes and plasma cells are quantitative.

Release of Cell Surface Ig by Small Lymphocytes.—When surface radiolabeled small lymphocytes are incubated in vitro, there is a rapid release of cell surface Ig and other proteins into the medium. The possibility was considered that this release might be due to cytolysis. Considerable evidence was accumulated that argues against this view: (*a*) studies of iodinated cells incubated for 8 hr using exclusion of trypan blue or release of ^{51}Cr gave no evidence of cell death; (*b*) surface radiolabeled *H-2* antigens were not released during a period of incubation in which approximately 35% of the surface-labeled Ig was released, indicating a selectivity in release of cell surface proteins; (*c*) cycloheximide experiments performed both in vitro and in vivo showed a rapid turnover of cell surface protein, including Ig; (*d*) labeling of small lymphocytes with tyrosine-^{3}H revealed secretion of 8S IgM, which is the major form of cell surface Ig. Taken together, these findings strongly suggest that some cell surface proteins, including Ig, are released by viable lymphoid cells as part of their normal physiology. Cone et al. (14), using an experimental approach remarkably similar to ours, have also shown release of cell surface proteins from lymphocytes and thymocytes. However, they did not demonstrate labeled Ig in the medium. Our results are consistent with the observations of Pernis et al. (28) that trypsin-treated lymphocytes regain their cell surface Ig, as judged by immunofluorescent studies, in 8–12 hr.

The mechanism of release is not known. Our studies indicate that protein synthesis is not required and that release can occur in the presence of antimetabolites or cytochalasin B at concentrations of 10^{-4}–10^{-5} M. However, since release is temperature dependent, energy may be required. The combination of antimetabolite "insensitivity" and temperature dependence has also been observed in the mixing of cell surface antigens in newly formed cell heterokaryons (29).

The Secretory Complex.—When cell surface Ig that is released into the incuba-

tion medium is precipitated without detergent treatment of the medium, a considerable quantity of radioactivity is in non-Ig proteins. This radioactivity can be removed before precipitation by treatment of the medium with NP40, or after precipitation by detergent washing of the immunoprecipitate. It can be less effectively released from the immunoprecipitate by washing with butanol. These observations suggest that Ig is noncovalently bound to other radiolabeled plasma membrane constituents. It was shown that lipids as well as proteins on the plasma membrane can be radiolabeled by the lactoperoxidase method, and some of these lipids are released into the medium. Taken together, these observations suggest that Ig is released as a secretory complex containing a small fragment of plasma membrane. Further evidence for this interpretation was obtained by density gradient centrifugation of radioiodinated cell surface Ig that had been released into the medium. Such Ig appeared in the high-density lipoprotein fraction (density 1.063–1.21) rather than the protein fraction (density > 1.21). Prior treatment of such material with detergent resulted in the disappearance of Ig in fractions of lower density and total recovery of Ig in the higher density protein fraction of the gradient.

The possibility was considered that the released complex could be an artifact of the iodination reaction, particularly since experiments with tyrosine-^{3}H labeling failed to reveal radioactive Ig of lipoprotein density in the medium. However, iodination with ^{127}I of tyrosine-^{3}H-labeled cells also did not reveal secretion of membrane-associated Ig. These findings suggest that cell surface Ig that is released is a minute percentage of total secreted Ig, and that the released Ig which had a surface phase thereby escaped detection. Our working hypothesis therefore is that the shedding of membranes containing Ig is not an artifact. There is an impressive similarity between this shedding and the extrusion from cells of certain viruses which "bud" from the plasma membrane and incorporate membrane antigens into the capsid of the virion (30–33).

The Selectivity of Release.—Of particular interest was the finding that another cell surface protein, the *H-2* alloantigen, was not released during a period of in vitro incubation in which about ⅓ of cell surface Ig was released. The specialization of the plasma membrane has been emphasized in recent topographical studies (34, 35). The present findings extend this concept in a dynamic sense and suggest that there may be specialized surface areas in which proteins are released and others in which release, at least at the same rate, does not occur. This concept is consistent with the studies of Aoki and Takahashi (36) in which removal of segments of the plasma membrane by virions budding from the cell surface of infected cells appears to be selective. These portions of the surface in which budding occurs may be specialized regions of the plasma membrane, characteristically lacking *H-2* and certain other cell surface antigens (37).

Secretion: a Model.—The present findings and past studies of biosynthesis suggest that our previous model of transport and secretion of Ig can be extended. The new facts and/or assumptions which must be accommodated include: (*a*)

the selectivity of release cited above, (*b*) findings which suggest that only a small proportion of secreted Ig has a surface phase, (*c*) release of surface radiolabeled Ig associated with membrane.

Fig. 11 A illustrates a model in which the cell surface is the last step in an ordered transport sequence: (*a*) intracellular transport of Ig occurs only within vesicles, (*b*) in the post-Golgi vesicle, a small proportion of Ig remains attached to the membrane, whereas the vast majority of Ig is free within the vesicle; the proportion of bound to free Ig is higher for IgM than for IgG, (*c*) the vesicle fuses with the plasma membrane, (*d*) reverse pinocytosis exteriorizes the Ig; the majority is immediately released "free," (including virtually all of the IgG), (*e*) the cell surface Ig (the attached IgM) is released as a function of

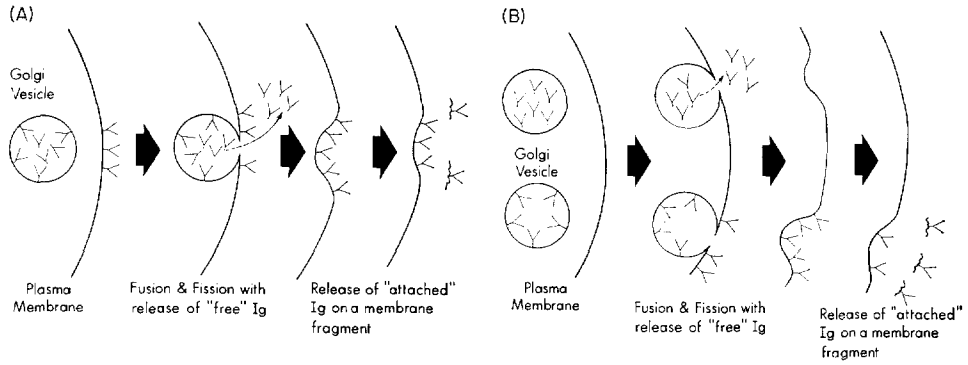

FIG. 11. Intracellular transport and secretion of "free" and surface Ig in the same (A) or separate (B) post-Golgi vesicles.

time and perhaps as a fragment of plasma membrane. It is uncertain whether shedding is analogous to the type of clasmacytosis postulated to occur in plasma cells by Avrameas and Leduc (38). Retrieval of plasma membrane may occur through pinocytosis and "shuttle" of empty vesicles as has been postulated for secretory cells in which there is a duct system (39).

An alternative model (Fig. 11 B) postulates two types of post-Golgi vesicles: one containing only free Ig and one containing only attached Ig. The former is responsible for conventional secretion of Ig and the latter for cell surface Ig. The two pathways could be regulated independently, a possibility suggested by Lerner et al. (40).

Common to both models is the postulation that attachment of protein to Golgi vesicles followed by reverse pinocytosis is a major pathway for transport of proteins to the cell surface.

The selectivity of release (*H-2* alloantigens *versus* Ig) can also be explained in two ways. If *H-2* alloantigens are more widely distributed on the surface than Ig, *H-2* antigens could be a structural part of the transport vesicles, in

which instance only a small proportion is lost (Fig. 12, upper half). On the other hand, the Ig-containing vesicles may lack *H-2* protein in their membranes and merger may be with a non-*H-2* bearing region of the surface (Fig. 12, lower half). This would result in no loss of *H-2* antigen.

A Dual Role for Antigen-Specific Receptors.—The significance of secretion of Ig with or without a surface phase by small lymphocytes is not known. Since Ig molecules with a surface phase are presumed to be antigen-specific receptors, their secretion may signify their eventual interaction with specific antigen. We postulate that 8S IgM secreted by small lymphocytes is "natural" antibody (41) whose function would be to bring antigen to an immunogenic compartment such as the surface of macrophages within lymphoid organs (42). It would

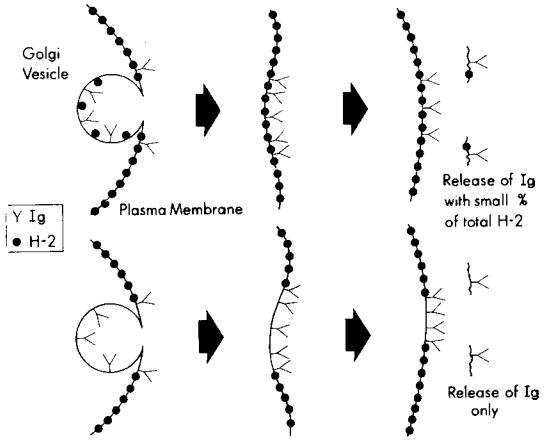

FIG. 12. Release of surface Ig with little (upper half) or no (lower half) loss of surface *H-2* antigen.

therefore be predicted that 8S IgM would be cytophilic for macrophages and that antigens for which a particular animal has no specificities would not be effectively localized. This view of the small lymphocyte as an active secretory cell implies clonal commitment with regard to synthesis of Ig (43, 44). Thus, the B lymphocyte, by actively secreting its particular antibody into a serum pool, is advertising its potential. Indeed, preliminary studies with germfree mice show release of cell surface 8S IgM similar to that observed in nonaxenic mice, suggesting that release is not dependent upon antigenic stimulation (E. Vitetta, I. Grundke, and J. W. Uhr, unpublished results). The results of antigenic stimulation can therefore be viewed entirely as an amplification process mediated via multiplication of relevant clones and differentiation of lymphocytes to plasma cells.

SUMMARY

Turnover and release of cell surface Ig and secretion of total intracellular Ig has been studied in small lymphocytes from normal mouse spleen. The major

findings to emerge are: (*a*) small lymphocytes secrete 8S IgM and IgG. A small portion of the 8S IgM, but virtually none of the IgG appears to have a cell surface phase. (*b*) Cell surface IgM is actively turned over with a half-life of 6–8 hr, and turnover can be accounted for by release into the incubation medium. Release is temperature dependent. (*c*) Released cell surface Ig is noncovalently bound to a fragment of plasma membrane. (*d*) *H-2* antigens are not released during short-term incubation. Based on the above findings, we propose a model for the transport and release of both cell surface and conventionally secreted Ig.

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