

SYNTHESIS, INTRACELLULAR DISTRIBUTION, AND SECRETION  
OF IMMUNOGLOBULIN AND H-2 ANTIGEN IN  
MURINE SPLENOCYTES\*

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Despite considerable interest in the genetic control and biochemistry of histocompatibility antigens (1), virtually nothing is known of their biosynthesis, intracellular transport, insertion into the plasma membrane, and subsequent fate. It has been suggested that histocompatibility (H-2)<sup>1</sup> alloantigens are primarily confined to the plasma membrane (2, 3). H-2 alloantigens have been described in subcellular fractions such as the microsomes (4-6); however, the extent of contamination of these fractions by plasma membrane (7) was not adequately assessed.

We have previously studied the biosynthesis and intracellular fate of immunoglobulin (Ig) in plasma cells and lymphocytes using two approaches: (a) labeling of Ig with precursors, subcellular fractionation, disruption of fractions in detergent, and isolation of radioactive Ig by means of specific immunoprecipitation (8); (b) enzymatic radioiodination of cell-surface Ig followed by cell lysis and immunoprecipitation of surface molecules of high specific activity (9) and determination of release of cell-surface Ig from cultured radiolabeled cells (10).

In the present studies, these approaches have been applied to a study of H-2 alloantigen and Ig in splenocytes from normal A/J mice. The results suggest that both macromolecules remain membrane bound from synthesis until exteriorization. In contrast to Ig, however, H-2 alloantigens are not secreted by these cells and are not shed from the cell surface.

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<sup>1</sup> *Abbreviations used in this paper:* H-2, histocompatibility antigen 2; Ig, immunoglobulin; MEM, minimal essential medium; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

### Materials and Methods

**Cell Cultures.**—Adult female A/J mice (Jackson Laboratory, Bar Harbor, Maine) were killed by cervical dislocation and spleen cell suspensions prepared as previously described (11). Cell counts and viability were determined in 0.05% trypan-blue phosphate-buffered saline (PBS). The cells were suspended in Eagle's minimal essential medium ([MEM] Grand Island Biological Co. Grand Island, N. Y.) lacking leucine and containing 10% fetal calf serum (Grand Island Biological Co.). They were labeled with 70–75  $\mu\text{Ci}[^3\text{H}]\text{leucine/ml}$  ( $\text{L-}[4\text{-}^3\text{H}]\text{-leucine[N]}$  30.8 Ci/mM; New England Nuclear, Boston, Mass.) at a concentration of  $14.5 \times 10^7$  cells/ml. Eight aliquots each containing  $17.4 \times 10^8$  cells were incubated from 0 to 4 h at 37°C in a CO<sub>2</sub> incubator. Unlabeled leucine was added to a concentration 100-fold that of labeled leucine to one sample after 2 h of incubation. Effectiveness of the "chase" was estimated by determining acid-precipitable counts in small aliquots of the cell suspension at 30-min intervals. The radioactivity in these aliquots either remained stable for 2 h of chase or increased 25% in individual experiments.

**Radioiodination.**— $1\text{--}3 \times 10^8$  A/J spleen cells were radioiodinated using lactoperoxidase (9), washed, and suspended at  $10^7$ /ml in MEM containing fetal calf serum. The culture was divided into five aliquots and incubated in a CO<sub>2</sub> incubator; samples were taken at time intervals up to 24 h and the cells and medium separated by centrifugation at 1500 g for 15 min. The medium was recentrifuged at 10,000 g for 30 min and adjusted with Nonidet P-40 ([NP-40] Shell Chemical Corp., New York) to 0.5%. Cells were washed, lysed in NP-40 (11), and both medium and lysate dialyzed overnight against PBS at 4°C.

**Cell Fractionation.**—After incubation, the cells were centrifuged, the supernatant (secretion) was decanted, the cells were washed three times in cold PBS, resuspended in 2.5 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris) Cl, pH 7.5 (20°C) 0.025 M KCl-0.005 M MgCl<sub>2</sub>-0.25 M sucrose, and were disrupted with 200 strokes of a tight-fitting Dounce homogenizer (Kontes Glass Co., Vineland, N. J.). Unbroken cells, debris, and nuclei were sedimented at 1,500 g for 20 min and three-fourths of the postnuclear supernatant fluid was centrifuged for 2 h at 100,000 g in the type 40 rotor of the Spinco Model L ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) to pellet the microsomes. Postmicrosomal supernatant (cell sap) was decanted and microsomal pellets were resuspended in 1.0 ml of Tris buffer by Dounce homogenization. Postnuclear supernatant, microsomes, and cell sap were adjusted with NP-40 to 0.5% to release all membrane-bound radioactivity. All samples, including secretions, were dialyzed against cold PBS overnight, centrifuged at 10,000 g for 10 min, and small aliquots were precipitated with 5% trichloroacetic acid (TCA) (12) and counted (12). Fractionation of iodinated splenocytes indicated that the microsomal fraction contained 16% and the cell sap 4% of the acid-precipitable material from the cell surface; the remaining 80% pelleted with the nuclei.

**Immunoprecipitations.**—These were performed using a "sandwich" procedure (11): The binding antiserum, produced in rabbits, contained specificities to mouse  $\mu$ ,  $\gamma$ ,  $\kappa$ , and  $\lambda$  chains or to bacteriophage  $\phi\chi$  174 (control). Complexes were precipitated with an excess of goat antirabbit Ig. The supernatants of the anti-Ig precipitates were divided into two aliquots and treated with antiserum B6/TL anti-A strain leukemia ASL1 (=  $H\text{-}2^b$  anti- $H\text{-}2^a$  = anti- $H\text{-}2.4$ , 11, 23, 25) (13) or a control serum (normal A/J or BALB/c serum or anti- $H\text{-}2^b$ ). After 30 min at 37°C, excess goat antimouse Ig was added and the samples were incubated at 37°C and 4°C (11). The precipitates were washed two to three times in cold PBS, dissolved, and counted (11, 12). Values obtained from the control precipitations were subtracted from the values obtained for specific precipitation in calculating the amount of radioactive Ig and H-2 antigen.

In several experiments precipitates were reduced and alkylated (11), dialyzed (11) and electrophoresed on 5% acrylamide gels in sodium dodecyl sulfate (SDS), or electrophoresed without reduction and alkylation on agarose-2.5% acrylamide gels (10).  $\mu$ ,  $\gamma$ , and L chains

or 19S IgM, 8S IgM, and 7S IgG served as markers on companion gels. All gels were fractionated and counted as previously described (11, 12).

#### RESULTS

*Kinetics of Incorporation.*—As seen in Fig. 1, after 3 h of labeling, the incorporation of [<sup>3</sup>H]leucine into acid-precipitable protein and Ig in the postnuclear supernatant (lysate) slowed markedly suggesting that the rate of synthesis had approached that of degradation and/or secretion. In contrast, incorporation

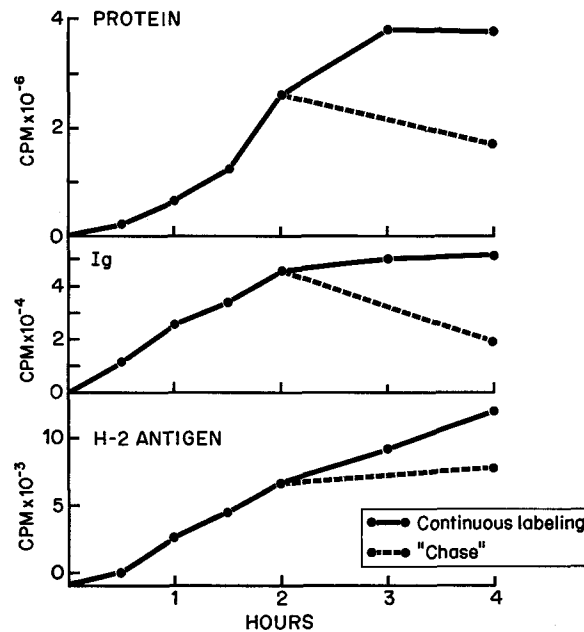


FIG. 1. Kinetics of incorporation of [<sup>3</sup>H]leucine into total protein (*upper panel*), Ig (*middle panel*), and H-2 antigen (*lower panel*) in normal A/J splenocytes. After indicated periods of incubation, aliquots of the cell suspension were used to prepare the postnuclear supernatant from which Ig and H-2 were immunologically precipitated.

into H-2 alloantigen continued at a steady rate. To determine whether [<sup>3</sup>H]leucine in the medium had been depleted and had thereby caused the decreased rate of incorporation into protein, unlabeled spleen cells were incubated with the supernatant of the 4 h sample. The new spleen cells incorporated [<sup>3</sup>H]leucine into protein for 1 h at the same rate as in the initial experiment. After a chase with cold amino acids (from 2 to 4 h) intracellular protein and Ig declined and the majority of the chased radiolabeled protein, including Ig, was recovered in the secretions. Intracellular H-2 antigen increased very slightly and was not detected in the incubation medium. The chase with unlabeled leucine that was

performed routinely in the presence of 10% fetal calf serum (which was necessary for optimal cell survival) varied in effectiveness in individual experiments (in all cases, it was at least 75% effective). Additional experiments were performed, therefore, in which  $10^{-4}$  M cycloheximide was used rather than unlabeled leucine as a chase. In addition, a reduced concentration of cells ( $10^7$ /ml) and isotope (25  $\mu$ Ci of [ $^3$ H]leucine/ml) were used. The results indicate, that in contrast to the preceding experiments, incorporation into protein continued at a linear rate for the 4 h of culture. After chase (which was > 99% effective) total protein declined 15% and Ig 50% during the 2 h of chase. The results with H-2 antigen were essentially the same as before, i.e., there was *no* change in the amount of radioactive H-2 antigen associated with the cells during the period of chase and *no* H-2 antigen was detected in the incubation medium at any time. These results suggest that H-2 alloantigen is not secreted or rapidly degraded by splenocytes, whereas Ig (and other unidentified proteins) is rapidly secreted.

*Intracellular Distribution of Total Protein, Ig, and H-2.*—Ig is primarily confined to microsomes in both plasma cells and a lymphocytic cell line (8). In order to determine the intracellular distribution of Ig and H-2 antigens in splenocytes, cells were labeled with [ $^3$ H]leucine and after 4 h, the cells were homogenized and the postnuclear supernatant was fractionated into microsomes and cell sap. Table I summarizes the results of four individual experiments. As can be seen, an average of 29% of labeled protein was in the microsome fraction, whereas 68% was found in the cell sap. In contrast, an average of 76% of radioactive Ig and 75% of H-2 antigen was associated with microsomes.

The distribution of Ig and H-2 antigen between microsomes and cell sap was also studied as a function of time of labeling and chase. Fig. 2 illustrates the results of one experiment and Table II summarizes data from four experiments. As can be seen in the table, there are fluctuations in the averaged data (probably because varying amounts of microsomes are lost in the nuclear pellet) but certain patterns emerge. The percentage of acid-precipitable radioactivity in the microsomes that is incorporated into Ig appears to decline (5.2–3.6%),

TABLE I  
*Distribution of Incorporated Radioactivity in Subcellular Fractions of A/J Spleen Cells Labeled with [ $^3$ H]Leucine\**

Fraction	Acid-precipitable protein	Ig	H-2 antigen
Microsomes	29 $\pm$ 2	76 $\pm$ 10	75 $\pm$ 13
Cell sap	68 $\pm$ 6	24 $\pm$ 3	16 $\pm$ 1
Percent of total recovered	97 $\pm$ 4	100 $\pm$ 7	91 $\pm$ 12

\* Percentages based on 100% for the postnuclear supernatant. Average values of four experiments (H-2 antigen was assayed for in the cell sap in only two experiments). The radioactivity in the supernatant after 4 h was as follows: acid-precipitable protein,  $4\text{--}11 \times 10^6$  cpm; Ig,  $6\text{--}12 \times 10^4$  cpm; and H-2 antigen,  $1\text{--}2 \times 10^4$  cpm.

whereas the percentage incorporated into H-2 antigen increased during the 4 h of labeling (0.32–0.50%). As expected, only a very small proportion of the total radioactive protein in the cell sap was Ig (0.30–0.55%) or H-2 antigen (0.03–0.06%). Further, there was no increase of Ig in the cell sap accompanying

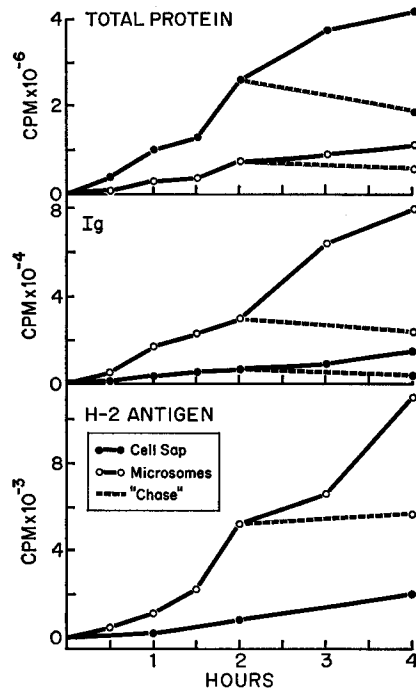


FIG. 2. Incorporation of [ $^3\text{H}$ ]leucine into total protein, Ig, and H-2 antigen in microsomes and cell sap of A/J splenocytes. Cells were labeled and at intervals thereafter microsomes and cell sap were separated, fractions were treated with NP-40, and radioactivity in acid precipitates and immunoprecipitates was determined.

TABLE II  
*Percentage of Acid-Precipitable Radioactivity That Was Incorporated into Ig and H-2 Antigens in the Subcellular Fractions*

Duration of labeling	Microsomes		Cell sap	
	Ig	H-2	Ig	H-2
<i>min</i>				
30	5.2	0.32	0.55	—
60	4.7	0.21	0.35	0.04
90	4.0	0.38	0.54	—
120	3.0	0.40	0.47	0.03
180	3.6	0.44	0.35	—
240	3.6	0.50	0.30	0.06
120 + 120 "chase"	2.3	0.51	0.26	—

— = not determined

the loss of I $\gamma$  from the microsomes, suggesting that there is no transit of I $\gamma$  into the cell sap before secretion.

The specificity of the immunoprecipitation was investigated by analyzing reduced and alkylated precipitates by SDS-acrylamide gel electrophoresis. Fig. 3 shows the results obtained using a 4 h sample of one experiment for I $\gamma$

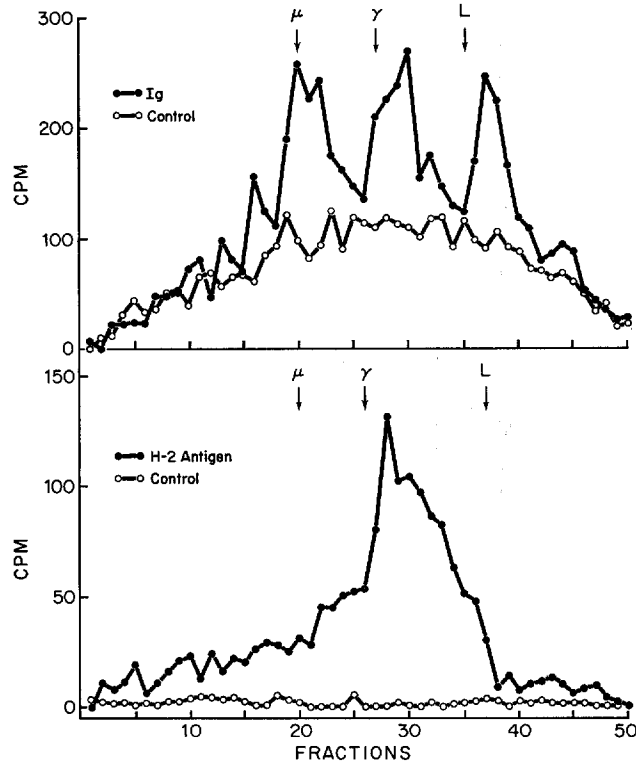


FIG. 3. Acrylamide gel electrophoresis of I $\gamma$  and H-2 antigen from microsomes of [ $^3$ H]-leucine-labeled cells. Normal A/J splenocytes were labeled for 4 h, the microsomal fraction was treated with NP-40, specific precipitation of I $\gamma$  and H-2 performed, and the precipitates were dissolved and reduced and alkylated. Markers for  $\mu$ ,  $\gamma$ , and L chains were electrophoresed on companion gels.

and H-2.  $\mu$ ,  $\gamma$ , and L chains were present in the anti-I $\gamma$  precipitate, whereas the anti-H-2 precipitate showed a major peak with molecular weight of approximately 45,000 daltons (14), consistent with reports by others using a variety of techniques (1, 15). This finding confirms that the anti-H-2 serum used was specific for H-2 determinants. The slightly elevated background of the specific compared with the control precipitate may be due to other membrane proteins that are still bound to the H-2 molecule.

Although these results indicate that the vast majority of I $\gamma$  enters the micro-

somes and does not traverse the cell sap, it is not clear whether the Ig found in the cell sap is released from microsomes ruptured during the fractionation procedure or whether a small portion of the Ig is normally synthesized in the cell sap. The following experiment was performed to determine if ruptured microsomes could contribute to Ig in the microsomal supernatant: Cells were incubated with [ $^3\text{H}$ ]leucine for 4 h and microsomes containing  $2.2 \times 10^5$  acid-precipitable cpm were obtained by the usual fractionation procedure. The labeled microsomes were then added to unlabeled cells and the cell fractionation procedure was repeated. Approximately 20% of recovered acid-precipitable radioactivity appeared in the microsomal supernatant. Since the labeled microsomes were being fractionated a second time, it is impossible to interpret the quantitative significance of this leakage since it could be argued that the microsomes were more fragile from the previous homogenization. Nevertheless, the experiment is consistent with the possibility that Ig and H-2 alloantigens in the microsomal supernatant are derived from disrupted microsomes.

*Secretion.*—After incubation of normal mouse spleen cells with [ $^3\text{H}$ ]leucine, radioactive Ig but not H-2 was found in the cell supernatant (secretion). After an initial lag period, Ig appeared and by 2 h was secreted at a constant rate. By 4 h, the secreted Ig represented 20% of the total radioactive protein in the medium. Fig. 4 shows that the anti-Ig precipitate of a secretion consisted of 19S IgM and 7S IgG, with small amounts of 8S IgM and “free” chains. The ratio of 19S IgM to IgG varied in individual experiments presumably depending on the immunologic status of the animals. A reduced and alkylated precipitate from another secretion revealed  $\mu$ ,  $\gamma$ , and L chains.

The above experiments failed to reveal secretion of H-2 antigen during 4 h of incubation. Occasionally, at 4 h a trace amount of H-2 was detected (0.03–0.10% of acid-precipitable protein) in the medium; this could have been derived from a few labeled and lysed cells. The chase of H-2 indicates no detectable degradation of H-2 antigen during the 2 h.

*Release of H-2 Antigen and Ig from the Cell Surface.*—Previous experiments had indicated that after 3 h in culture, surface radioiodinated splenocytes release approximately 30% of their cell-surface Ig, but none of their H-2 antigen (10). These experiments were repeated but with a 24 h period of incubation.

As seen in Fig. 5, for the first 6 h in culture, cells released greater than 40% of their cell-surface Ig, 30% of their protein, and less than 2% of their H-2 alloantigen. During this time, there was no detectable loss in cell viability. When viability declined (10–24 h) to approximately 50%, there was only a slight increase in loss of Ig from the cells, and the loss of H-2 antigen approached 8%. These results confirm the selectivity of loss of cell-surface proteins and suggest that this loss is not due to cell death.

#### DISCUSSION

These studies indicate that the majority of both Ig molecules and H-2 antigens in splenocytes are membrane bound during their intracellular life.

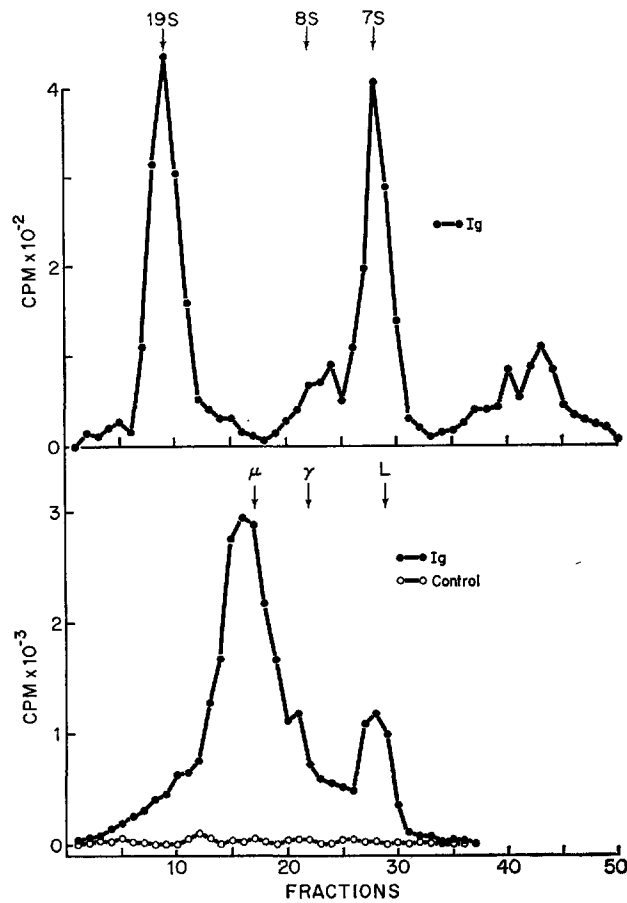


FIG. 4. Acrylamide gel electrophoresis of [ $^3\text{H}$ ]leucine labeled Ig secreted by mouse splenocytes. Dissolved precipitates were electrophoresed on SDS agarose acrylamide gels (*above*) or reduced and alkylated and electrophoresed on SDS acrylamide gels (*below*) with appropriate markers. The precipitates used were from two different experiments in which the ratio of IgM/IgG varied.

This concept implies but does not prove synthesis on membrane-bound polyribosomes, entrance into the cisterni of the rough endoplasmic reticulum, and transport within smooth vesicles until exteriorized. The results with regard to Ig are entirely consistent with previous more detailed studies of Ig synthesis in a human lymphocytic cell line (Daudi) that appears to be a neoplastic B cell line (12).

There were striking differences, however, between Ig and H-2 alloantigens with respect to the kinetics of labeling with [ $^3\text{H}$ ]leucine: (*a*) During continuous labeling, incorporation into radioactive Ig slowed markedly at 2–3 h; incorpora-



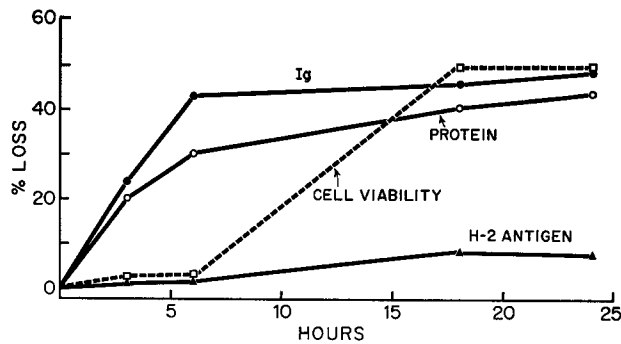


FIG. 5. Loss of acid-precipitable protein, Ig, and H-2 antigen from the surface of radioiodinated A/J splenocytes in culture. Ig and H-2 antigen were determined by immunoprecipitation, total protein by acid precipitation, and viability by trypan blue exclusion.

tion into H-2 alloantigens did not. (b) After 2 h of chase with cold amino acids or cycloheximide, there was a substantial decline in radioactive Ig but *not* in H-2 alloantigen. (c) Radioactive Ig was recovered in the incubation medium; H-2 alloantigen was not readily detected in the incubation medium. We interpret these differences as follows: Ig is rapidly secreted from splenocytes with an intracellular half-life of approximately 1.5 h (Fig. 1). Since the cells are presumably in a steady state in which synthesis and secretion are balanced, uniform labeling is virtually achieved in 2–3 h resulting in a near plateau of radioactive intracellular Ig. In contrast, turnover of H-2 alloantigens occurs at a slower rate, primarily because they are not secreted; hence, H-2 alloantigens do not achieve uniform labeling in the same period of time.

Another striking difference between Ig and H-2 alloantigens is the release of cell-surface Ig but not H-2 antigens from surface radioiodinated cells. The present findings extend earlier observations (10) and suggest that cell death is not responsible for these findings. Not only did release occur without evidence of cell death, but release slowed as cells began to die 6–24 h after cultivation. Since all nucleated cells have H-2 alloantigens on their surfaces (2, 3), the same cells that do *not* release surface H-2 alloantigens are releasing Ig.

Regardless of whether the release of Ig is a physiologic event or an *in vitro* artifact of iodination and/or cultivation, the question arises as to the mechanism responsible for release of one cell-surface protein and not another. The first point to be made is that Ig as well as many other cell-surface proteins are released on a fragment of plasma membrane (10). We suggest as a working hypothesis that Ig and these other cell-surface proteins represent peripheral proteins in the fluid mosaic model of Singer and Nicholson (16), whereas H-2 alloantigens represent integral ones. This idea would be consistent with our observations that suggest that Ig is fully exteriorized (11, 17). Thus, the forces of interaction between H-2 alloantigens and surrounding lipid and protein molecules would account for the great stability of the H-2 membrane complex.

The mechanisms underlying release of selected fragments of plasma membrane are obscure. Release could occur from fixed non-H-2 containing areas of the surface or at surface sites from which H-2 antigens have migrated before clasmotosis. In either event, the possibility that membranous vesicles are pinched off is attractive when one considers the elongated microvilli that characterize the surface of small lymphocytes (18) (Wortis et al., personal communication).

Our findings, with regard to cell-surface H-2 alloantigens, do not imply lack of their turnover on the cell surface. In fact, the observation that H-2 antigens can be readily labeled with radioactive precursors in a "resting" cell population implies turnover. Cepellini et al. (personal communication) and Schwarz and Nathenson (19) have shown that lymphocytes regenerate cell-surface histocompatibility alloantigens in approximately 6 h after their removal by proteolysis. Their findings are not incompatible with those reported here. We have suggested that H-2 alloantigens could be molecules in the walls of post-Golgi vesicles that shuttle to and from the plasma membrane transporting Ig (and other proteins) to the plasma membrane for exteriorization (10, 17). This concept implies that H-2 antigens alternately become exteriorized and interiorized and that degradation (and synthesis) could occur at a slow rate.

#### SUMMARY

A/J spleen cells were labeled with [<sup>3</sup>H]leucine and at intervals thereafter were homogenized and separated into microsomes and cell sap. Ig and H-2 antigens were assayed in the cell fractions and cell supernatants using immunoprecipitation. In addition, cells labeled by enzymatic radioiodination were incubated to determine the rates of release of Ig and H-2 antigens from the surface. The results indicate that the majority of Ig and H-2 antigens remain membrane bound throughout their intracellular life. In contrast to Ig, H-2 antigens are neither secreted nor shed from the cell surface. It is suggested that Ig is a peripheral protein of the cell membrane, whereas H-2 antigens are integral ones. The release of Ig on a fragment of plasma membrane could occur at fixed cell surface areas that contain no H-2 antigens or from which they have migrated before release.

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