

## AN ELECTROPHORETIC DIFFERENCE BETWEEN SURFACE AND SECRETED IGM OF MURINE SPLENOCYTES\*

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Murine splenocytes synthesize and secrete pentameric (19S) immunoglobulin M (IgM) (1). Murine splenocytes also have IgM on their surface, but in a monomeric (8S) form (2, 3). Cell surface Ig is rapidly released (shed) by radioiodinated cells in culture (4, 5). Shed Ig differs from secreted Ig in that it is monomeric; it appears to be attached to a fragment of plasma membrane; and it accounts for only a small portion of extracellular Ig (4).

The presence of Ig in two compartments of lymphoid cells (the secretory pathway and the cell surface) provides an opportunity to investigate the nature of membrane association of proteins. As an initial step in the comparison of cell surface Ig with secretory pathway Ig, an electrophoretic study of their constituent chains using sodium dodecyl sulfate (SDS)-polyacrylamide gels was initiated. The results of this study are reported here.

### Materials and Methods

*Preparation and Incubation of Iodinated Splenocytes.*—The procedures for isolation and iodination of spleen cells from 6–10-wk old BALB/c mice were as previously described (2, 4). Briefly, cells were teased from spleens previously perfused with 0.01 M sodium phosphate, 0.15 M NaCl (PBS), pH 7.2, into PBS. They were filtered, washed several times with PBS, and refiltered. The suspension was iodinated at room temperature at a concentration of  $0.5\text{--}0.8 \times 10^8$  cells/ml with 100  $\mu\text{g}$  lactoperoxidase/ml, 1 mCi [ $^{125}\text{I}$ ]NaI/ml (Amersham/Searle Corp., Arlington Heights, Ill.), and 0.44 mM  $\text{H}_2\text{O}_2$ . Iodination was terminated by dilution with cold PBS. After several washings with PBS, cells were either lysed in 0.5% Nonidet P-40 (Shell Chemical Corp., New York)-PBS and the nuclei removed by centrifugation or were incubated in Eagle's minimal essential medium (MEM) as previously described (3).

*Incubation of Cells with [ $^3\text{H}$ ]Tyrosine.*—Cells were teased into Eagle's MEM lacking tyrosine, but with added nonessential amino acids and 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.), washed once, and incubated at  $1 \times 10^7$  cells/ml with 20  $\mu\text{Ci}$  L-[ $^3\text{H}$ ]tyrosine/ml (New England Nuclear, Boston, Mass.). After incubation, cells were separated from the medium, washed, and lysed as previously described (2, 4). The incubation medium was dialyzed and concentrated as previously described (4).

*Immunoprecipitation and Gel Electrophoresis.*—Ig was immunologically precipitated from lysates and secretion using mouse gamma globulin (Miles Laboratories, Kankakee, Ill.) and goat antimouse Ig serum (2). Washed immunoprecipitates were solubilized, reduced, alkylated, and electrophoresed on 5% acrylamide, 0.5 M urea, 0.1% SDS gels (22). Gels were fractionated

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and counted in Beckman Cocktail D in a Beckman LS350 liquid scintillation spectrometer. There was less than 0.01% spillover of  $^3\text{H}$  counts per minute (cpm) into the channel used for  $^{125}\text{I}$  radioactivity.  $^3\text{H}$  radioactivity was counted with either 2.3 or 37% overlap of  $^{125}\text{I}$  cpm. The cpm reported here were corrected for this overlap.

#### RESULTS

Reduced and alkylated specific anti-Ig precipitates from [ $^{125}\text{I}$ ]surface-labeled murine splenocytes were coelectrophoresed with reduced and alkylated specific precipitates from cell supernatants of murine splenocytes labeled for from 2 to 4 h with [ $^3\text{H}$ ]tyrosine. A gel pattern typical of five separate experiments is shown in Fig. 1. No difference in width or mobility of the peaks of light (L) chains of

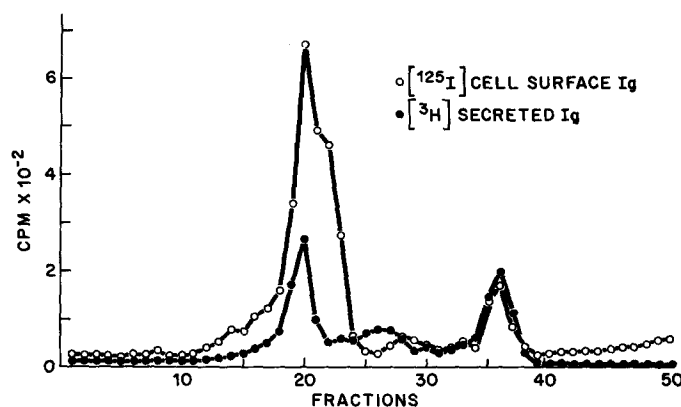


FIG. 1. SDS-polyacrylamide gel coelectrophoresis of reduced and alkylated [ $^{125}\text{I}$ ]splenocyte surface Ig and [ $^3\text{H}$ ]tyrosine-labeled Ig secreted by splenocytes.

cell surface and secreted Ig was detected. We had considered the possibility that plasma membrane L chain is identical with precursor L chain (6, 7). Precursor L chain might have an affinity for membranes, since cleavage of precursor L chain to mature L chain occurs in association with microsomes (7). The possible identity of plasma membrane and precursor L chain is now eliminated since the resolution of the gel system was more than sufficient to detect the 15–20 amino acid difference (6, 7) between precursor and mature L chains.

The peak of cell surface heavy chain ( $\mu$ ) was different from that of  $\mu$ -chain in secreted Ig. In contrast to secreted  $\mu$ -chain, the cell surface  $\mu$ -chain peak was wider and asymmetrical, exhibiting, in this experiment, a shoulder on the fast side. Attempts to further resolve this peak by extending the duration of electrophoresis or using smaller gel fractions were unsuccessful suggesting that the peak does not consist of only two species of  $\mu$ -chain but a spectrum of species with differing mobilities.

Cell surface  $\mu$ -chains were also compared in four experiments with intracellular  $\mu$ -chains biosynthetically labeled for 1 h with [ $^3\text{H}$ ]tyrosine. One such experiment is shown in Fig. 2. The surface  $\mu$ -chain peak was slightly broader than the

intracellular species. The peak of intracellular  $\mu$ -chain, however, occurred in the same fractions as the cell surface peak. Thus, the extent of electrophoretic heterogeneity of  $\mu$ -chain from the cell surface is not found either intracellularly or extracellularly. Again, no difference in mobility or peak width between [ $^3\text{H}$ ]L chain and [ $^{125}\text{I}$ ]L chain was detected.

The possibility that iodination of Ig could cause changes in mobility of  $\mu$ -chains was considered. A dialyzed supernatant from spleen cells cultivated for 4 h in the presence of [ $^3\text{H}$ ]tyrosine was reacted with NaI,  $\text{H}_2\text{O}_2$ , and lactoperoxidase under conditions that resulted in substantial conversion of tyrosyl to iodotyrosyl residues (detected as a 29% increase in  $A_{290}$  at pH 7.2) (8). When the Ig was precipitated from this reaction mixture, reduced, alkylated, and electrophoresed, no significant alteration of  $\mu$ - or L chain mobility or peak width when compared with the noniodinated control was noted. A lysate of cells that had been labeled for

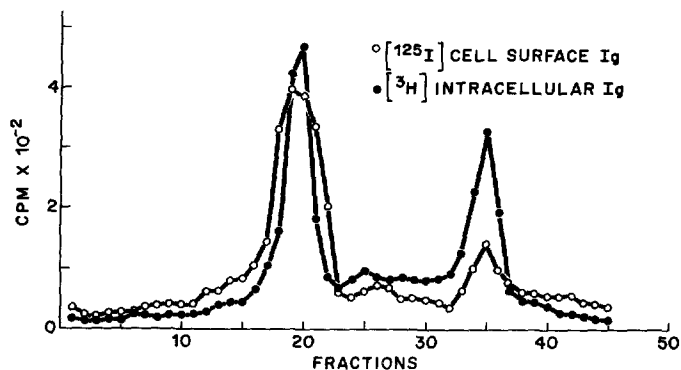


FIG. 2. SDS-polyacrylamide gel coelectrophoresis of reduced and alkylated [ $^{125}\text{I}$ ]surface Ig and [ $^3\text{H}$ ]tyrosine-labeled Ig from lysates of splenocytes.

60 min with [ $^3\text{H}$ ]tyrosine was similarly iodinated. A 27% increase in  $A_{290}$  at pH 7.2 occurred. Such treatment resulted in no significant change in mobility or peak width of  $\mu$ - or L chains.

Since cell surface iodination is performed under conditions of hydrogen peroxide excess (2) and since lactoperoxidase can catalyze the oxidative dimerization of tyrosyl residues (9), the possibility that such oxidation was responsible for the observed cell surface  $\mu$ -chain profile was tested. A dialyzed incubation medium from spleen cells incubated for 4 h with [ $^3\text{H}$ ]tyrosine was reacted with 100  $\mu\text{g}$  of lactoperoxidase and 0.9  $\mu\text{mol}$   $\text{H}_2\text{O}_2$  (2.0 ml vol) for 30 min at room temperature. Ig was then immunoprecipitated, reduced, alkylated, and electrophoresed. Such oxidation did not cause any change in mobility or peak width of [ $^3\text{H}$ ] $\mu$ -chains when compared with a control sample incubated without enzyme and  $\text{H}_2\text{O}_2$ . Thus, the observed heterogeneity is not an artifact due to iodination or oxidation.

Previous results from this laboratory (4) have shown that approximately 50% of the cell surface Ig is shed very rapidly. The possibility was considered that one species of cell surface  $\mu$ -chain was preferentially shed over others. Consequently, radioiodinated spleen cells were incubated for 4 h after which the cells were separated from the incubation medium. Ig was precipitated from the cell lysate and from the medium, reduced, alkylated, and electrophoresed. The electrophoretic profile of Ig chains retained on the cells during incubation closely

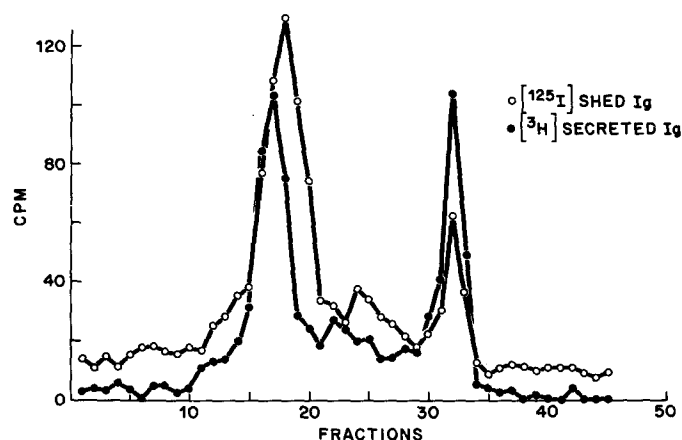


FIG. 3. SDS-polyacrylamide gel coelectrophoresis of reduced and alkylated  $[^{125}\text{I}]\text{Ig}$  shed from radioiodinated splenocytes and  $[^3\text{H}]\text{tyrosine-labeled Ig}$  secreted by splenocytes.

resembled the profile shown in Fig. 1. The profile of shed immunoprecipitable material is shown in Fig. 3 (one of three experiments). The shed  $\mu$ -chain peak was significantly broader than the  $\mu$ -chain profile of  $[^3\text{H}]\text{tyrosine-labeled}$  secreted Ig, while no difference could be detected in the L chain profiles. Thus all  $\mu$ -chain species were shed to some extent. The observation that both slow and fast radioiodinated species of  $\mu$ -chain can be recovered in approximately equivalent amounts after incubation indicates that one is not a precursor of the other. This is consistent with the view that cell surface Ig is not a major precursor to secreted Ig (4, 10).

#### DISCUSSION

The present results demonstrate that splenocyte surface  $\mu$ -chain is heterogeneous. The heterogeneity is not due to the following artifactual causes: iodination; lactoperoxidase-catalyzed oxidation; degradation during cell lysis; and preparation for electrophoresis. The latter can be concluded from Fig. 2 in which internal  $\mu$ -chain did not exhibit the heterogeneity of the surface species. In this experiment,  $[^3\text{H}]\text{tyrosine-labeled}$  cells and  $^{125}\text{I}$ -labeled cells were carried through the same procedures. In addition, the observed  $\mu$ -chain heterogeneity is not due to the presence of other heavy chain classes ( $\gamma$  or  $\alpha$ ), since previous experiments from this laboratory (2) have shown that virtually all splenocyte surface H chain in young BALB/c mice is  $\mu$ .

The biological basis for the heterogeneity of  $\mu$ -chains on the cell surface is unknown. Our studies have not established whether different types of  $\mu$ -chain are present on the same cell. Since the spleen cell population is heterogeneous, it is possible that this heterogeneity is reflected in cell surface Ig. Three possibilities can be excluded, however, by the present results. First, the heterogeneity is not due to sequence heterogeneity arising from different antibody specificities,

since such heterogeneity should also be reflected in Ig secreted by splenocytes. Second, one type of  $\mu$ -chain does not seem to be the biosynthetic precursor of another since all are recovered in approximately equal amounts after incubation of iodinated cells. Third, since all types of  $\mu$ -chain were shed (Fig. 3), the presence on the surface of sheddable and nonsheddable subpopulations of  $\mu$ -chain with differing mobility is also ruled out.

The chemical basis for the heterogeneity of  $\mu$ -chain on the cell surface could be differences in either of the two known constituents of Ig, carbohydrate and protein, or to a variation in a third hitherto undescribed component, e.g. covalently attached lipid (11). The role of carbohydrate in the heterogeneity of cell surface  $\mu$ -chain is supported by two observations. First, the presence of carbohydrate in proteins in general (12, 13) and in Ig in particular (14) reduces mobility during SDS-polyacrylamide gel electrophoresis. Indeed, differing carbohydrate content may be responsible for the slower mobility of extracellular compared with internal [ $^3\text{H}$ ] $\mu$ -chain (Figs. 1 and 2; and unpublished observations), since the terminal sugars of Ig, galactose, fucose, and sialic acid are added near the time of secretion (15–18). Second, only cell surface  $\mu$ -chains are heterogeneous. L chains are not. This is consistent with localization of carbohydrate on  $\mu$ -chains and its absence on L chains (19).

One of us has suggested (20) that during intracellular transport Ig is bound to the interior of Golgi vesicles in order to facilitate final glycosylation. We now suggest that acquisition of the entire complement of Ig carbohydrate results in release of Ig into the interior of the Golgi vesicle and subsequent secretion upon exteriorization of that vesicle. Once the Golgi vesicle is exteriorized, addition of sugars ceases and any incompletely glycosylated Ig remains associated with the plasma membrane. In this manner  $\mu$ -chains containing little carbohydrate (fast moving) and  $\mu$ -chains only a few sugar residues short of completion (those coelectrophoresing with secreted  $\mu$ -chains) appear on the outside of the plasma membrane. Thus, in summary, we propose that the heterogeneity of cell surface  $\mu$ -chain is due to variably incomplete glycosylation and that this incomplete glycosylation is responsible for membrane association of cell surface IgM.

#### SUMMARY

$\mu$ -chains on the surface of murine splenocytes are more heterogeneous on SDS-polyacrylamide gel electrophoresis than both secreted and intracellular  $\mu$ -chains labeled with [ $^3\text{H}$ ]tyrosine. No difference in heterogeneity among cell surface, secreted, or intracellular L chains was detected. The possible role of carbohydrate in  $\mu$ -chain heterogeneity is discussed.

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