Proc. Natl. Acad. Sci. USA
Vol. 76, No. 12, pp. 6582–6586, December 1979
Immunology

Differential effects of colchicine and cytochalasins on the shedding of murine B cell membrane IgM and IgD

(surface iodination/turnover/immunoglobulins/lymphocytes)

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Communicated by Edward A. Adelberg, September 10, 1979

ABSTRACT **Lactoperoxidase (EC 1.11.1.7) catalyzed cell surface radioiodination was employed to monitor the fate of murine B cell membrane (mem) IgM and IgD on radiolabeled cells in short-term culture. Both mem-IgM and mem-IgD were shed from the cell surface with biphasic kinetics. The rapid phase of mem-IgD shedding was somewhat slower (half-time = 12 hr) than that of mem-IgM shedding (half-time = 7–8 hr). The effect of temperature, colchicine, and cytochalasin on the shedding of the two membrane immunoglobulin isotypes was determined. The shedding of mem-IgD was more energy dependent than that of mem-IgM and was sensitive to colchicine but not cytochalasin. Conversely, the shedding of mem-IgM was sensitive to cytochalasin but not colchicine. The results suggest that the mechanisms of shedding of mem-IgM and mem-IgD are qualitatively distinct and may be regulated by microfilaments or microtubules, respectively.**

Serological, immunochemical, and functional studies of lymphocyte membrane immunoglobulins provide compelling evidence to support the concept that these membrane proteins serve as antigen recognition units for B lymphocytes (1, 2). The events that follow receptor–ligand interactions at the B cell surface and that lead to activation of immunocompetent cells are an area of considerable interest and speculation. An additional complexity is introduced into this problem by the finding that most mature B lymphocytes bear two isotypically distinct immunoglobulins at the cell surface. B cell membrane IgM (mem-IgM) resembles the monomeric subunit of pentameric serum IgM (3, 4) and was the first membrane immunoglobulin to be described and characterized. Subsequently, it was shown that a second class of immunoglobulin is present at the B cell surface that in mice has structural similarities to murine IgD and in humans and non-human primates shares serological identity with human IgD (5–7). Moreover, there is an indication, based on selective removal of murine membrane IgD (mem-IgD) from viable cells (8), that the nature of the signal delivered to a cell after antigen binding depends on which receptor binds the ligand. In this scheme, it is suggested that selective binding of antigen to mem-IgM leads to the inactivation of immunocompetent cells (8).

The apparent dichotomy in receptor functions of mem-IgM and mem-IgD could be due to structural differences in these molecules, association of these polypeptides with other membrane proteins, or the physiological behavior of these molecules at the cell surface. Lactoperoxidase-catalyzed cell surface radioiodination has been used to monitor the surface dynamics of lymphocyte membrane immunoglobulins (4) and surface antigens (9). Because this technique does not alter the normal turnover and shedding rates of membrane immunoglobulins (4, 10), we have utilized cell surface radioiodination to measure the membrane residence times of murine cell surface IgM and IgD. The results indicate that although both of these molecules are turned over at the cell surface, energy constraints and the requirement for intact microtubules (instead of microfilaments) suggest profound differences between mem-IgM and mem-IgD with regard to mechanisms that control their membrane residence.

MATERIALS AND METHODS

Mice. CBA/J mice were obtained from Jackson Laboratory. All animals were 8–14 weeks old when used.

Cell Preparation and Surface Radioiodination. Spleen cell suspensions were prepared and surface iodinated as detailed (9). All iodinations were performed in calcium- and magnesium-free phosphate-buffered saline at reactant concentrations of 0.7 μM lactoperoxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7; from Sigma), 50 μM H_2O_2, and 0.5 μM $Na^{125}I$. Cell viability after labeling was always greater than 96%, as determined by the exclusion of diamine (Trypan) blue dye.

Cell Culture and Drug Treatments. Radiolabeled cells were cultured at a concentration of 5×10^6 per ml in 0.5-ml volumes of RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum (GIBCO batch no. R267426), penicillin at 100 units/ml, streptomycin at 100 μg/ml, and 2 mM glutamine. Colchicine (Calbiochem), stock solution in RPMI 1640 was prepared immediately before use, and lumicolchicine was then prepared by exposure to ultraviolet irradiation for 40 min (11). Cytochalasins A and B (Calbiochem) were stored at 10 mg/ml in dimethyl sulfoxide at −20°C and diluted in RPMI 1640 immediately before use. Parallel samples of radiolabeled cells were cultured at 37°C under a 5% CO_2 atmosphere. Colchicine at concentrations of 0.01–100 μM or cytochalasin A or cytochalasin B at concentrations of 0.01–50 μl/ml was added to some of these samples at the beginning of the incubation. Incubations with 0.01–100 μM lumicolchicine and 0.1% dimethyl sulfoxide were performed in parallel to evaluate the noncytoskeletal effects of the drugs and carriers used. Cell viability was uniformly greater than 85% at 8 hr, and over 75% at 20 hr of incubation in all samples. At specified time, culture tubes were removed and centrifuged at $1500 \times g$ for 10 min. Cell-free supernatants were drawn off and dialyzed against 0.05% Triton X-100 (New England Nuclear) in Tris/NaCl buffer (50 mM

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Abbreviations: B cell, bone marrow-derived lymphocyte; mem-IgM and mem-IgD, membrane immunoglobulin M and D; NRS, normal rabbit serum; SAR, sheep antiserum to rabbit immunoglobulin; NaDodSO$_4$, sodium dodecyl sulfate.

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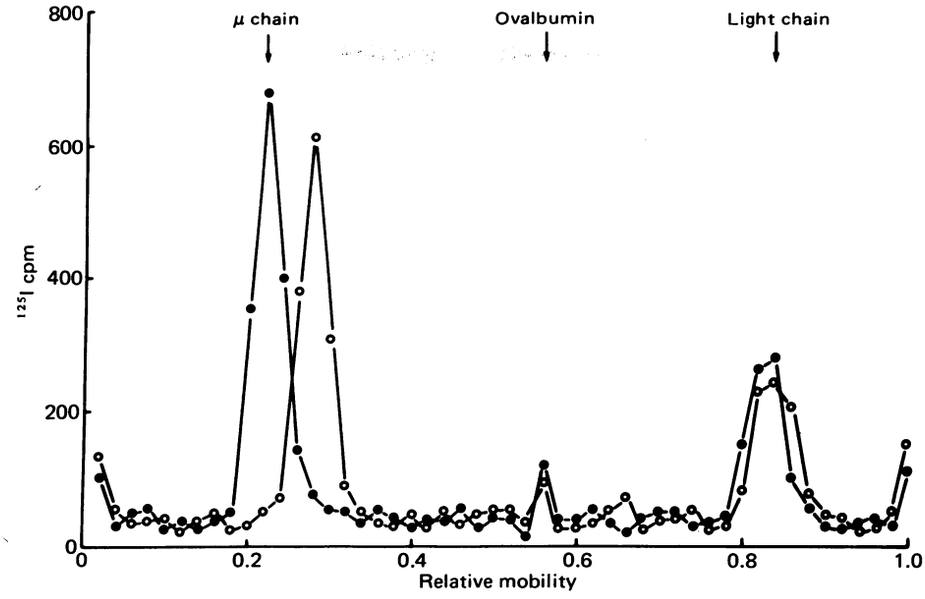

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis analysis of mem-IgM and mem-IgD isolated from CBA/J splenocyte lysates. ●, ^{125}I-Labeled cells, lysates precipitated with anti-μ serum for 1 hr at 4°C followed by SAR for 25 hr at 4°C. ○, Supernatants from the high-speed centrifugation of the anti-μ immunoprecipitates reprecipitated with anti-κ serum for 1 hr at 4°C followed by SAR for 24 hr at 4°C. Both the anti-μ and anti-κ precipitates were washed three times in 0.05% Triton X-100/Tris before preparation for electrophoresis in 10.5% acrylamide/0.25% bisacrylamide gels, with 80 1.5-mm slices per gel. Electrophoresis of NRS precipitates performed in parallel during both immunoprecipitation steps gave patterns indistinguishable from detector background (data not shown in figure).

Tris·HCl/10 mM EDTA/150 mM NaCl, pH 8.0) or against Tris·HCl buffer alone. Cell pellets containing 5 × 10^6 cells were lysed in 0.5 ml of 0.5% Triton X-100/Tris for 30 min at 4°C. Lysates, containing over 99% of the incorporated radiolabel, were separated by centrifugation at 5000 × *g* for 10 min and dialyzed against 0.5% Triton X-100/Tris. After 4–20 hr the dialyzed supernatants and cell lysates were centrifuged at 20,000 × *g* for 20 min in a Sorvall RC-5 centrifuge, and the supernatants were withdrawn for immunoprecipitation.

Antisera. Rabbit antiserum to mouse IgM (μ-chain-specific) and rabbit antiserum to mouse κ chain were purchased from Litton Bionetics Laboratories, Kensington, MD. Sheep antiserum to rabbit immunoglobulin (SAR) was the generous gift of Heinz Furthmayr, Department of Pathology, Yale University School of Medicine.

Immunoprecipitation. After high-speed centrifugation, 0.5-ml cell pellet lysates and supernatant dialysates were divided into fractions for immunoprecipitation. In each, 250 μl of sample was incubated with 5 μl of anti-μ and an equal volume with 5 μl of normal rabbit serum (NRS) for 2 hr at 4°C; then 75 μl of SAR was added to each sample, and immunoprecipitates were allowed to form for 20–24 hr at 4°C. The precipitates were centrifuged at 10,000 × *g* for 10 min, and the supernatants from the anti-μ precipitates were carefully drawn off and split in half. One half was then incubated with 5 μl of anti-κ, the other with 5 μl of NRS for 2 hr at 4°C; 75 μl of SAR was added to each of these samples, and immunoprecipitates formed over 24 hr at 4°C. All immunoprecipitates were washed with 0.05% Triton X-100/Tris buffer until maximum specificity was achieved. Background NRS radioactivity was 10–20% of specific counts for both the IgM and IgD precipitations.

Polyacrylamide Gel Electrophoresis. Indirect immunoprecipitates were dissociated with sodium dodecyl sulfate (NaDodSO$_4$)/polyacrylamide gel electrophoresis solvent buffer and 5% (vol/vol) 2-mercaptoethanol, and eluted polypeptides were resolved by discontinuous electrophoresis in polyacrylamide gels as described (9). ^{131}I-Labeled murine IgM and ovalbumin were subjected to electrophoresis with the ^{125}I-labeled membrane proteins to serve as internal markers.

RESULTS

Membrane Residence Times of mem-IgM and mem-IgD. To determine the membrane residence times of mem-IgM and mem-IgD, splenic lymphocytes were radioiodinated and cultured for 24 hr. At intervals during culture, samples of cells were removed and centrifuged, and the cell pellet was lysed with Triton X-100. Detergent lysates and culture supernatants were precipitated first with anti-μ serum to isolate mem-IgM. IgM-cleared preparations were then precipitated with anti-κ serum to isolate murine IgD. Fig. 1 shows the NaDodSO$_4$/polyacrylamide gel electrophoresis pattern obtained for reduced and alkylated radiolabeled membrane proteins bound at 0 time in detergent lysates by anti-μ or anti-κ after IgM clearing. NaDodSO$_4$/polyacrylamide gel electrophoresis patterns of anti-μ precipitates revealed immunoglobulin heavy chains that comigrated with MOPC-104E μ chain standards and light chains. Anti-κ precipitation of anti-IgM-cleared lysates revealed complete removal of mem-IgM and the presence of heavy chains of approximately 68,000 daltons and light chains. Anti-μ precipitation of IgM-cleared lysates resolved no peaks of radioactivity above detectable background. In most experiments the ratio of IgM to IgD precipitated was approximately 1.2:1.

As shown in Fig. 2*A*, loss of labeled mem-IgM from the cell surface followed biphasic kinetics, as shown previously (10). Approximately 40% of the total mem-IgM was lost rapidly from the cell surface with a half time ($t_{1/2}$) of 7–8 hr. The remaining mem-IgM was lost with a $t_{1/2}$ of approximately 48 hr. Loss of mem-IgD also followed biphasic kinetics: the $t_{1/2}$ for the rapid phase of IgD loss (25% of the total IgD) was approximately 12 hr, and the slow loss had a $t_{1/2}$ of approximately 48 hr. All of the mem-IgM lost in the first 12 hr (rapid phase) could be recovered in the culture supernatants and a slight decrease in IgM detectable in the culture fluid was observed after 12 hr of cul-

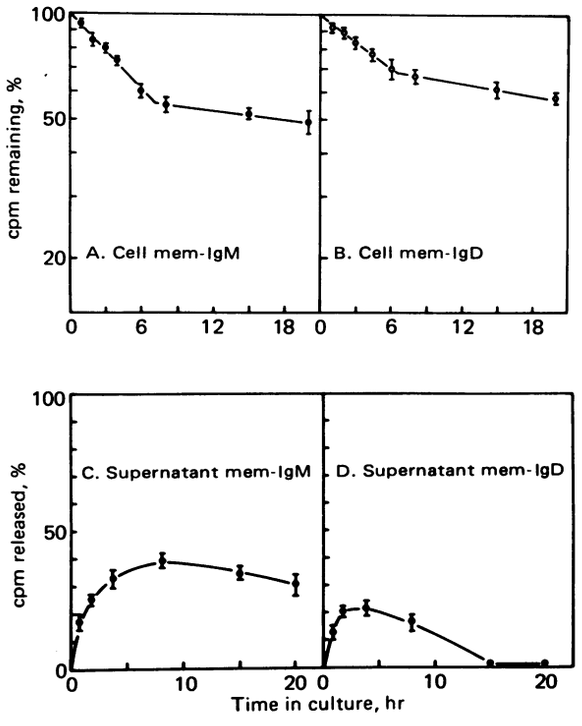

FIG. 2. Loss of mem-IgM and mem-IgD, and recovery from culture supernatants. Surface-iodinated spleen cells were cultured for 0–20 hr. Centrifuged cell pellets were withdrawn for lysis. Cell lysates and cell-free supernatants were sampled for immunoprecipitation with anti-μ serum and NRS. Supernatants from the 10,000 × g centrifugation of the anti-μ precipitates were then halved and precipitated with anti-κ serum and NRS. (A) Cell lysates precipitated with anti-μ serum; (B) supernatant of cell lysate anti-μ precipitate precipitated with anti-κ serum; (C) cell culture supernatant precipitated with anti-μ; (D) supernatants from anti-μ-precipitated culture fluids precipitated with anti-κ. Each point indicates experimental minus control cpm and represents the mean result of nine experiments; brackets show the standard error of the mean. Percentages were calculated by the formula: % cpm remaining at time x = 100 × [specific cpm (time x) − control cpm (time x)]/[specific cpm (time 0) − control cpm (time 0)]. The IgM-to-IgD ratio per cell equivalent was approximately 1.2:1.

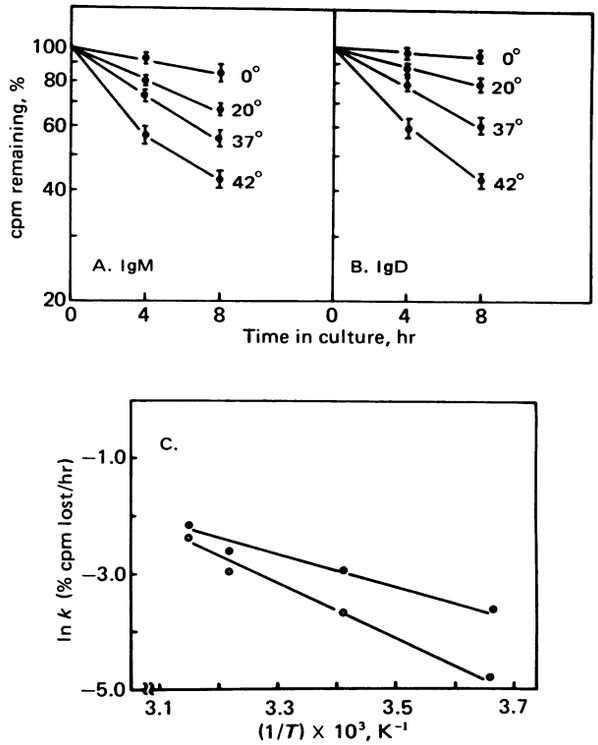

FIG. 3. Temperature dependence of mem-IgM and mem-IgD turnover. Twelve replicates of 5 × 10^6 iodinated spleen cells were cultured in parallel at 42°C, 37°C, 20°C, and 0°C. At 0, 4, and 8 hr of culture, samples were withdrawn and centrifuged, and cell pellets were lysed. Each dialyzed lysate was sampled and immunoprecipitated with anti-μ serum and NRS. Supernatants from anti-μ precipitate were analyzed for radio-labeled IgD by immunoprecipitation of samples with anti-κ serum and NRS. (A) % IgM specific cpm remaining; (B) % IgD specific cpm remaining. Each point indicates experimental minus control cpm, and represents the mean of four experiments, with brackets showing the standard error of the mean. Percentages were calculated as in Fig. 2. (C) Arrhenius plot of temperature dependence of mem-IgD (○) and mem-IgM (●) turnover. The natural logarithm of the initial turnover velocity k, expressed as % IgD and IgM specific cpm lost per hour, is plotted versus the reciprocal of the absolute temperature.

ture. In contrast to mem-IgM, 20% of the total IgD could be recovered in the supernates after 5 hr of culture but material was rapidly lost from the supernates with further culture.

Temperature Dependence of Loss of mem-Ig. The results presented above demonstrate that both mem-IgM and mem-IgD are being turned over at the cell surface, albeit with distinct membrane residence times. We next determined the temperature dependences of the loss of these cell surface proteins by culturing radiolabeled cells at various temperatures. The results, shown in Fig. 3, indicate that the losses of both mem-IgM and mem-IgD are highly temperature dependent. However, when the rate of loss is plotted as a function of the inverse of absolute temperature (Arrhenius plot), it can be seen that the slope of the line for mem-IgD release is significantly steeper than that for mem-IgM. These results suggest that the energy requirements for IgD release are far more stringent than for IgM release.

Effects of Colchicine and Cytochalasin on the Loss of mem-IgM and mem-IgD. The difference in temperature dependence of the shedding of surface labeled mem-IgM and mem-IgD suggested that the mechanisms responsible for the losses of these proteins from the cell surface may be qualitatively distinct. Microtubules and microfilaments have been implicated in the control of the lateral movement of lymphocyte surface components and cell surface topography in general (12, 13). To determine whether these cytoskeletal elements influence the shedding of membrane immunoglobulin, we next assessed whether the shedding of mem-IgD and mem-IgD was affected by colchicine and cytochalasin, drugs known to disrupt microtubule and microfilament integrity, respectively. As shown in Fig. 4 A and C, culture of radiolabeled cells with 0.01–100 μM colchicine had little or no effect on the rapid or slow loss of mem-IgM. In contrast, this drug completely inhibited the rapid and slow loss of mem-IgD in a dose-dependent fashion. Photoinactivated lumicolchicine did not inhibit the shedding of mem-IgD, indicating that the colchicine-induced inhibition of shedding is likely due to disruption of microtubule integrity (11).

Culture of labeled cells with cytochalasin A or B at 10–50 μg/ml, on the other hand, completely inhibited the shedding of mem-IgM and did not affect the shedding of mem-IgD except at 10 μg/ml, where there was a slight acceleration of shedding (Fig. 5) of this membrane immunoglobulin isotype. Titration of cytochalasin A and B indicated that cytochalasin A is a somewhat more effective inhibitor than cytochalasin B at doses lower than 10 μg/ml.

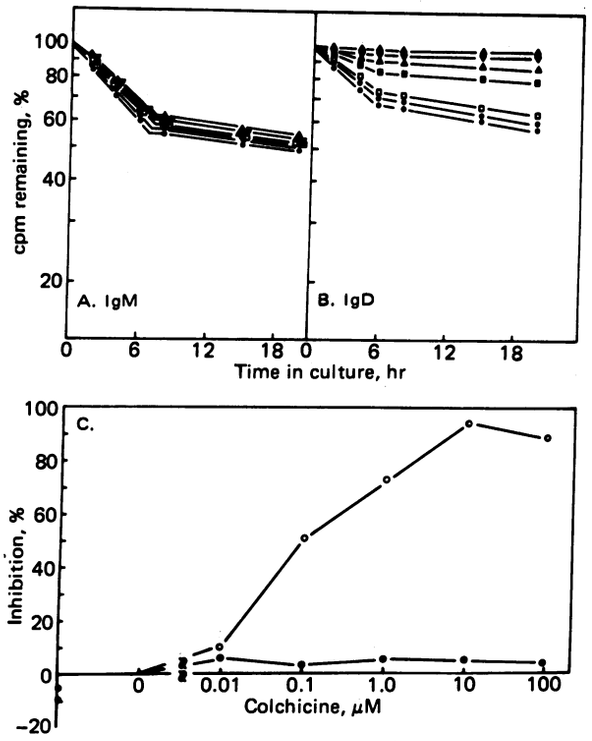

FIG. 4. Colchicine inhibits the loss of mem-IgD but not mem-IgM. Replicates of 2.5×10^6 radioiodinated spleen cells were cultured in the presence of 0–100 μM colchicine, or 100 μM photoisomerized lumicolchicine, for 0–20 hr. Cell lysates from each sample were analyzed by immunoprecipitation for mem-IgM and mem-IgD. (*A*) mem-IgM and (*B*) mem-IgD: ○, without drug; ●, 100 μM lumicolchicine; □, 0.01 μM colchicine; ■, 0.1 μM colchicine; △, 1 μM colchicine; ▼, 10 μM colchicine; ▲, 100 μM colchicine. (*C*) Colchicine dose–response of IgM (●) and IgD (○) loss compared by plotting the percent of control membrane immunoglobulin isotype loss at 6 hr that is inhibited by drug incubation: % loss inhibition at 6 hr = 100 × [specific cpm (0 hr) colchicine − specific cpm (6 hr) colchicine]/[specific cpm (0 hr) no drug − specific cpm (6 hr) no drug]. Each point indicates experimental minus control cpm and represents the mean of six experiments. Standard errors were less than ±4% for each point. The points below 0 on the ordinate are results with 100 μM lumicolchicine.

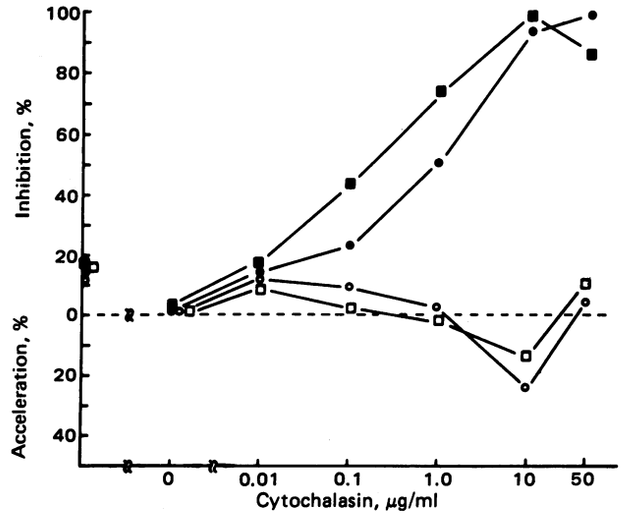

FIG. 5. Cytochalasins A and B inhibit the loss of mem-IgM but not mem-IgD. Replicates of 2.5×10^6 radioiodinated spleen cells were cultured in the presence of cytochalasin A or B at 0–50 μg/ml for 0–20 hr. Final concentration of solvent dimethyl sulfoxide in each sample was 0.1% or less. The points on the ordinate are 0.1% dimethyl sulfoxide controls. Each sample was analyzed by immunoprecipitation for the presence of radiolabeled IgM and IgD, and the inhibition of membrane immunoglobulin isotype losses by cytochalasins A and B was determined as in Fig. 4*C*. ■, Effect of cytochalasin A on mem-IgM; □, effect of cytochalasin A on mem-IgD; ●, effect of cytochalasin B on mem-IgM; ○, effect of cytochalasin B on mem-IgD. The data shown represent the means from six experiments for each drug; standard errors of the means were less than ±4% for each drug dose.

DISCUSSION

Although both mem-IgM and mem-IgD are shed from the cell surface, their release processes appear to be controlled by fundamentally distinct mechanisms.

The first suggestion that the expression and turnover of these two membrane immunoglobulin isotypes differed came from temperature-control studies (Fig. 3). At 37°C loss of IgD occurred at a somewhat (30–40%) slower initial rate than loss of IgM; however, IgD release was far more sensitive to temperature variation. As indicated by the Arrhenius plot, this reflects an approximately 2-fold higher activation enthalpy and entropy for the rate-limiting step for IgD release. Such a difference could result from either greater energetic constraints within the plasmalemma, if δ C_H domains interact more tightly with membrane lipid than those of μ chains. Alternatively, a more energy-dependent submembranous control process may mediate IgD release.

That the submembranous controls of membrane IgD and IgM release are distinct is indicated further by the disparate effects of colchicine and cytochalasin on the shedding of the two membrane immunoglobulin isotypes. Microtubules are disrupted by colchicine (14), and their assembly from tubulin subunits is extremely temperature dependent (15). The observation that colchicine (but not lumicolchicine) completely inhibited the release of mem-IgD but did not affect mem-IgM suggests that the release of IgD may be controlled by cell microtubules whereas that of surface IgM is not. Microtubules play important roles in the maintenance of the cell shape (13), secretory processes, and insertion of components into the plasma membrane (16) and could provide stability for lymphocyte membrane domains in general (12, 13). Moreover, microtubules apparently restrict the mobility of lymphocyte membrane lectin receptors (17), suggesting the possibility that some membrane components may be directly or indirectly linked to these cytoskeletal structures. On the other hand, selective inhibition of the shedding of mem-IgM was obtained with doses of cytochalasins that are known to disrupt microfilaments (18). Lower doses of cytochalasin A or B, which cause disulfide exchange (19) or inhibition of glucose transport (20), respectively, did not affect shedding. Thus, the effect of cytochalasin A or B on surface IgM release indicates that the membrane residence time of mem-IgM may be controlled by cell microfilaments. Cytochalasin also inhibits ligand-induced lateral movement of mem-IgM, suggesting involvement of microfilaments in this process also (21). However, inhibition of capping by cytochalasin is often incomplete (12). The synergistic inhibitory effect of colchicine and cytochalasin on capping (21, 22) might thus be explained by the association or control of the two immunoglobulin isotypes by different cytoskeletal elements.

Although the functional significance of membrane immunoglobulin shedding is not known, the regulation of this process by different cytoskeletal structures suggest that the membrane residence times of mem-IgM and mem-IgD are regulated by different mechanisms and implies that these molecules may be associated with distinct membrane domains or subcellular elements. This could explain in part the observation that selective binding of antigen to mem-IgM or mem-IgD may initiate

different and opposing signals to an immunocompetent B cell (8, 23). When IgD binds certain antigens, microtubule-dependent events may initiate transformation, mitosis, and differentiation. The ability of colchicine to inhibit the early premitotic events of concanavalin A-induced transformation (24) suggests a direct role for microtubules in lymphocyte activation. In contrast, selective binding of some antigens to mem-IgM may activate microfilament-dependent events that lead (in some instances) to B cell inactivation. Thus, the influence of distinct cytoskeletal elements on mem-IgD and mem-IgM may explain, in part, the observed differences in the nature of the signal transduced to the cell when antigen binds to these isotypes.

This work was supported by U.S. Public Health Service Grants AI12577, CA14216, and GM07205. S.G.E. is a predoctoral fellow of the Medical Scientist Training Program, National Institutes of Health and is supported in part by an Upjohn Graduate Fellow Research Award.

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