LYMPHOCYTE MEMBRANE DYNAMICS

METABOLIC RELEASE OF CELL SURFACE PROTEINS*

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The proliferation and differentiation of lymphoid cells can be stimulated by specific antigens, antigen-antibody complexes, plant mitogens and hormones. The initial event in the response to these diverse agents is believed to be the binding of stimulator molecules to receptors located on the cell surface (1, 2). However, the mechanism by which the binding of receptor to its ligand triggers cell differentiation and division is unknown. Prerequisite to the understanding of the cellular events occurring subsequent to surface receptor-stimulator interactions is knowledge of the nature of such receptor molecules and the manner in which they are associated with the cell membrane.

We have approached the problem of identification of cell surface receptors by developing a method based on the lactoperoxidase-catalyzed radioiodination of proteins (3), which allows covalent labeling of proteins present on the surface of lymphoid cells (4). Electron microscopic evidence indicated that only the outer surface of the cell is labeled by this method. In addition, sufficient radioactivity is incorporated into the surface to allow fractionation of iodinated proteins by a variety of techniques. This method has facilitated the isolation of immunoglobulin from the surface of mouse spleen cells (5) and human and mouse thymus cells.[1]

Since cell viability is not adversely affected by this gentle method of iodination, physiological studies of accessible surface proteins may be carried out using living lymphocytes. The experiments reported herein demonstrate that many proteins associated with the cell surface exist in a dynamic state. We

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[1] Marchalonis, J. J., J. L. Atwell, and R. E. Cone. Isolation of surface immunoglobulin from lymphocytes from human and murine thymus. *Nature (London)*. In press.

provide evidence that normal and neoplastic lymphoid cells release certain surface proteins at a rapid rate through a process requiring cellular respiration and protein synthesis.

Materials and Methods

Lactoperoxidase (LP)[2].—The enzyme was prepared and standardized as previously described (3). Stock solutions of enzyme containing 60 μg of LP/ml were prepared in phosphate-buffered saline (PBS), pH 7.3, which was 0.05 M with respect to phosphate and 0.15 M with respect to NaCl.

Hydrogen Peroxide.—A stock solution of 10 M H_2O_2 (analytical grade) was obtained from Ajax Chemicals Ltd., Melbourne, Australia. Dilutions of the stock solution were made in PBS. Opened bottles of 10 M H_2O_2 were discarded after 3 wk.

Radioactive Iodide.—Carrier-free iodide-^{125}I as NaI was obtained from the Radiochemical Centre, Amersham, England, at a concentration of 90–160 mCi/ml. Carrier-free iodide-^{131}I was supplied by the Australian Atomic Energy Commission, Lucas Heights, N. S. W., at a concentration of 500 mCi/ml.

Cells.—Male and female CBA/H/Wehi and (CBA × C57BL)F_1 mice, weighing approximately 20–25 g each, were used as a source of normal lymphoid cells. Cells of a murine lymphoma, S1AT.4, a thymidine-resistant clone derived from a mouse lymphoma cell line (6), and myeloma (MOPC460) maintained in vitro were supplied by Dr. A. W. Harris, Immunogenetics Laboratory, Walter and Eliza Hall Institute. Thymus-derived and bone marrow–derived thoracic duct lymphocytes were kindly supplied by Dr. J. Sprent, Experimental Pathology Unit, Walter and Eliza Hall Institute. Thymus-derived thoracic duct lymphocytes were obtained by thoracic duct cannulation. The preparation of these cells is described elsewhere (7). Bone marrow–derived thoracic duct lymphocytes were obtained by thoracic duct cannulation of mice with congenital thymic aplasia (noninbred mice carrying *nu/nu* locus). 100% of these cells lack reactivity to thymus-specific anti-θ antiserum (J. Sprent, unpublished results). Sheep erythrocytes were stored in modified Alsever's solution and were washed three times with PBS, pH 7.3, before use.

Antimetabolites.—Iodoacetamide (British Drug Houses, Poole, England) and sodium azide (Ajax Chemicals Ltd., Melbourne, Australia) were dissolved in tissue culture medium to appropriate concentrations. Puromycin (2 mM in PBS) and antimycin A (10 mM in absolute ethanol) were obtained from Sigma Chemical Co., St. Louis, Mo.

Antisera.—Rabbit antiserum to sheep red blood cells (SRBC) was obtained from Dr. M. Feldmann, Cellular Immunology Unit, Walter and Eliza Hall Institute of Medical Research. Rabbit antiserum to mouse γ-globulin (MGG) was obtained from Cappel Laboratories, Downingtown, Pa. Purified rabbit and mouse γ-globulin was prepared as previously described (8). Immunoelectrophoresis and cellulose acetate electrophoresis of these preparations showed no extraneous protein contamination. Purified γG-immunoglobulin from normal rabbit serum (NRG), rabbit anti-sheep red blood cell serum (RASG), and rabbit anti-mouse γ-globulin (RAMIgG) were iodinated with iodide-^{131}I or iodide-^{125}I according to the method of Ada et al. (9). We thank Mr. J. Pye for performing the iodinations.

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[2] *Abbreviations used in paper:* EBSS, Eisen's balanced salt solution; FCS, fetal calf serum; HEPES, *N*-2-hydroxyethyl-*N*-2-ethane sulfonic acid; LP, lactoperoxidase; MEM, Eagle's minimal essential medium; MGG, mouse gamma globulin; NRG, normal rabbit serum; PBS, phosphate-buffered saline; RAMIgG, rabbit anti-mouse γ-globulin; RASG, rabbit anti-sheep red blood cell serum; R_f, distance migrated relative to that of a methyl green dye marker (rate of flow); SRBC, sheep red blood cells.

Cell Preparations.—Mice were killed by cervical dislocation, spleens and thymuses removed, and teased on a stainless steel mesh into chilled Eisen's balanced salt solution (EBSS). Cell debris and clumps were removed by settling the cell suspensions over 1 ml of fetal calf serum (FCS, Commonwealth Serum Laboratories, Melbourne, Australia) for 5 min at 4°C. After one wash with EBSS, dead cells and red blood cells were removed by Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) gradient centrifugation as previously described (4). Cells prepared in this fashion were 93–98% viable as judged by eosin dye exclusion. Tumor cells and thoracic duct lymphocytes were washed four times in chilled EBSS and resuspended in PBS, pH 7.3, for iodination. The cells were 98–99% viable as judged by the exclusion of eosin dye.

Lactoperoxidase-Catalyzed Iodination.—External surface proteins of the above cells were iodinated according to the method previously described (4). For studies of the kinetics of release of cell surface proteins (where high specific activity was not critical), a solution of iodide-^{125}I or iodide-^{131}I diluted in K-^{127}I carrier to give a specific activity of 1 and 0.5 mCi/ml, respectively, was used. Carrier-free iodide-^{125}I was used in experiments involving fractionations of iodinated proteins. Radioactivity was assessed by counting in a Packard autogamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) equipped with a deep-well sodium iodide crystal detector. Cells iodinated by this procedure incorporate approximately 10–15% radioactive iodide. Viability of the cells, as judged by eosin dye exclusion, is not altered by the iodination procedure.

Binding of RAMIgG to Spleen Cells.—Normal spleen cells or spleen cells iodinated with iodide-^{131}I were washed twice in EBSS + 5% (v/v) FCS and then were centrifuged through an FCS gradient, 100, 50, and 25% FCS (v/v) in EBSS. The cells were resuspended to 5×10^6/ml in EBSS and incubated for 30 min at 4°C with 50 μl of RAMIgG-^{125}I (15.5 μg/0.5 ml) or NRG-^{125}I (15.6 μg/0.5 ml). The cells were washed once with EBSS and 5% FCS and twice in FCS gradients.

Coupling of Mouse γ-Globulin to Sheep Erythrocytes.—This was carried out by means of a modification of the method of Gold and Fudenberg (10). Freshly prepared chromic chloride (1 mg/ml in normal saline) catalyzed the coupling of mouse globulin to SRBC. One ml of mouse globulin (4 mg/ml) was added to 0.5 ml of packed SRBC followed by the addition of 0.5 ml of chromic chloride. The SRBC–mouse globulin mixture was incubated for 4 min at room temperature and washed three times with PBS + 1% (v/v) FCS. Experiments using ^{125}I-labeled mouse γG-globulin showed that this was an effective coupling method and that the bound protein did not elute appreciably upon incubation at 37°C.

Binding of Rabbit Antisera to SRBC.—One-tenth ml of RAMIgG-^{131}I or RASG-^{131}I was added to 0.1 ml of 1% SRBC-MGG or SRBC in PBS + 1% FCS, respectively. The mixtures were incubated for 30 min at 37°C and 30 min at 4°C followed by two washes with PBS + 1% FCS. These conditions were previously determined to give specific uptake of RAMIgG by SRBC–MGG–coated cells and RASG by SRBC.

Incubation of Lymphoid Cells.—For studies of the release of surface proteins, cells were suspended to a concentration of 5×10^6–1×10^7 cells/ml in Eagle's minimal essential medium (MEM) supplemented with nonessential amino acids (F-15, Grand Island Biological Co., Grand Island, N. Y.) and FCS (5% v/v) and buffered with 20 mM *N*-2-hydroxyethyl-*N*-2-ethane sulfonic acid (HEPES, Calbiochem, Los Angeles, Calif.). The cells were incubated in 12-ml polystyrene centrifuge tubes at 37°C in a water bath or on a roller drum in a 37°C warm room.

Disc Electrophoresis in Polyacrylamide Gel.—This procedure was carried out under dissociating conditions according to procedures described by Parish and Marchalonis (11). The gels were sliced into 35–40 fractions using a Canalco slicer (Canalco Inc., Rockville, Md.). Slices were counted in plastic tubes using the Packard autogamma spectrometer. Mobilities are expressed as distance migrated relative to that of a methyl green dye marker (R_f).

RESULTS

Release of Radioiodinated Surface Proteins.—To determine whether accessible surface proteins labeled by lactoperoxidase-catalyzed iodination might be released by the cell during in vitro incubation, lymphocytes obtained from normal (unimmunized) mice were iodinated with iodide-^{125}I or -^{131}I diluted in K-^{127}I carrier. After the labeling procedure the cells were washed twice, suspended in tissue culture medium, and incubated at 37°C. Aliquots

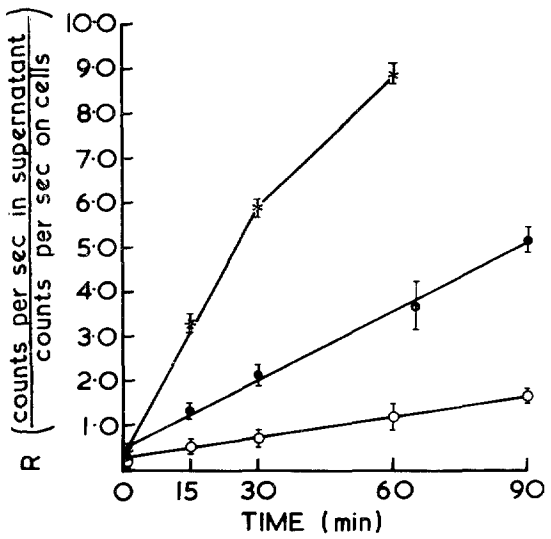

FIG. 1. Kinetics of release of radioiodinated surface proteins from lymphocytes. Release of surface proteins from thymus cells (✳–✳) or spleen cells (●–●) labeled with iodide-^{125}I; (○–○), release of RAMIgG-^{125}I from spleen cells. Each point represents the arithmetic mean of the results of five to eight individual experiments, the vertical bar indicating the standard error of the arithmetic mean.

were removed at intervals, centrifuged at 1500 rpm for 10 min at 4°C, and the amount of radioactivity was determined in both supernatant and cell pellet. As may be seen in Fig. 1, radioactive material was released by spleen cells and thymus cells labeled with iodide-^{125}I when incubated in vitro at 37°C. To minimize errors in sampling, the release of radioactive material as a function of time of incubation is represented as the ratio (R) of radioactivity in the supernatant to radioactivity remaining on the cells. Under the present conditions, this index of the amount of material released by the cells increased linearly with time. In addition, labeled material was lost at least three times more quickly by thymus lymphocytes than by spleen cells obtained from the same animal. The lactoperoxidase-catalyzed iodination of proteins labels

many types of surface proteins with accessible tyrosine residues (4). To determine whether the release of an individual protein from the cell surface could be followed, spleen cells were labeled with RAMIgG-^{125}I. After the labeling procedure the cells were washed twice in FCS gradients and incubated as described above. As may be seen in Fig. 1, the rate of release of RAMIgG-^{125}I from spleen cells was one-third that of the rate of release of material from spleen cells labeled with radioactive iodide. This result was obtained whether these rates were determined using spleen cells labeled with iodide-^{125}I or RAMIgG-^{125}I only or cells doubly labeled with both iodide-^{131}I and RAMIgG-^{125}I. The RAMIgG was specifically bound to the cell surface. In these experiments spleen cells bound at least 20 times more RAMIgG-^{125}I than NRG-^{125}I under conditions which would inhibit pinocytosis (12). In addition, more than 90% of the NRG-^{125}I which did bind to the cells was lost during the first 15 min of incubation. The release of RAMIgG-^{125}I might have been because of the dissociation of antigen-antibody complexes. To test this possibility, RAMIgG-^{131}I was specifically bound to SRBC coated with mouse γG-globulin and the cells were incubated as described above. Sheep erythrocytes conjugated with rabbit anti-SRBC IgG-^{131}I were incubated in similar fashion. In both cases less than 25% of the radioactivity was recovered in the supernatant after as much as 24 hr incubation at 37°C. Thus, the release of RAMIgG-^{125}I which was bound to spleen cells does not appear to be the result of the dissociation of antigen-antibody complexes. The possibility that RAMIgG is released bound to cell surface immunoglobulin is under investigation.

Metabolic Requirements for Release.—During the period in which the release of surface label was followed there was no change in cell numbers or viability as determined by eosin dye exclusion, indicating that cell death was not causing the release of labeled material. To determine whether the release of labeled material was an active process, cells were first incubated in serum-free medium in the presence of agents which suppress energy-yielding processes or protein synthesis. After treatment with inhibitors the cells were washed twice, iodinated and incubated in HEPES medium containing the inhibitor. Controls were treated in the same fashion with the exception that the inhibitor was excluded from the medium. Pretreatment of the cells with inhibitors affected neither the viability of the cells nor the extent to which the cells could be labeled. The amount of labeled material released by the cells after incubation was however affected, as may be seen in Table I. When iodinated spleen cells were incubated at 4°C or were treated with metabolic inhibitors, the release of labeled material was markedly suppressed. These results indicate that the release of labeled materials is an active metabolic process and that protein synthesis is required. In addition, the cells lost radioactive material when serum was excluded from

the incubation medium, indicating that the process was not because of stimulation of the cell membrane by serum components (12).

Fractionation of Released Surface Proteins.—Since we have previously shown that iodide incorporated into lymphoid cells by the lactoperoxidase technique is covalently bound to surface proteins (4), the released protein observed here could be representative of all surface proteins or of some specific fractions

TABLE I

Effect of Antimetabolites and Temperature on Release of Cell Surface Proteins from Mouse Spleen Cells

Treatment of cells before iodination	Incubation conditions after iodination	Per cent release of surface proteins relative to control after 2 hr incubation
None	HEMF* at 37°C	100
None	HEM‡ at 37°C	100
None	HEMF at 4°C	23
1×10^{-1} M azide for 1 hr at 37°C	HEMF + 1×10^{-1} M azide at 37°C	60
1×10^{-2} M iodoacetamide for 1 hr at 37°C	HEMF + 1×10^{-2} M iodoacetamide at 37°C	22
5×10^{-5} M antimycin A, for 1 hr at 37°C	HEMF at 37°C	21
4×10^{-5} M puromycin for 2 hr at 37°C	HEMF + 4×10^{-5} M puromycin at 37°C	70
4×10^{-5} M puromycin for 4 hr at 37°C	HEMF + 4×10^{-5} M puromycin at 37°C	40

Cells were incubated in HEM ± inhibitor at a concentration of 5×10^6–1×10^7 cells/ml. After incubation the cells were washed twice, iodinated, and resuspended in HEMF ± inhibitor for further incubation. Data represent the pooled results of 24 individual experiment. S/C ratios for untreated cells varied from three to four in these experiments.

* HEMF, HEPES-buffered (MEM) + 5% FCS.

‡ HEM, HEPES-buffered MEM.

which may be preferentially released. To determine the nature of labeled material released, ^{125}I-labeled iodide cells from two tumor cell lines were incubated for 1–2 hr in a small volume of tissue culture medium. After incubation the cells were centrifuged at 4°C and the supernatant removed. Urea and mercaptoethanol were added to the supernatant to a final concentration of 9 M and 0.2 M, respectively. The supernatants were then subjected to polyacrylamide disc electrophoresis. Figs. 2 *a* and *b* compare supernatants and urea-soluble surface proteins obtained from iodinated cells. The electrophoresis conditions used were those previously established (11) to resolve immunoglobulin chains and fragments in a molecular weight range from 10,000 to 100,000. As was found

previously (4), the cell lines shared some components but were quantitatively and qualitatively different in others. The material released by the cells during incubation was similar to components removed from the cell surface when iodinated cells were disrupted in urea immediately after iodination. However, in all instances, some components were not released by the cell during the period of incubation. This is particularly evident in the myeloma cell line (Fig. 2 *b*). A component with an R_f of approximately 0.8 was not released to any appreciable effect after 2 hr incubation at 37°C. Similar findings were

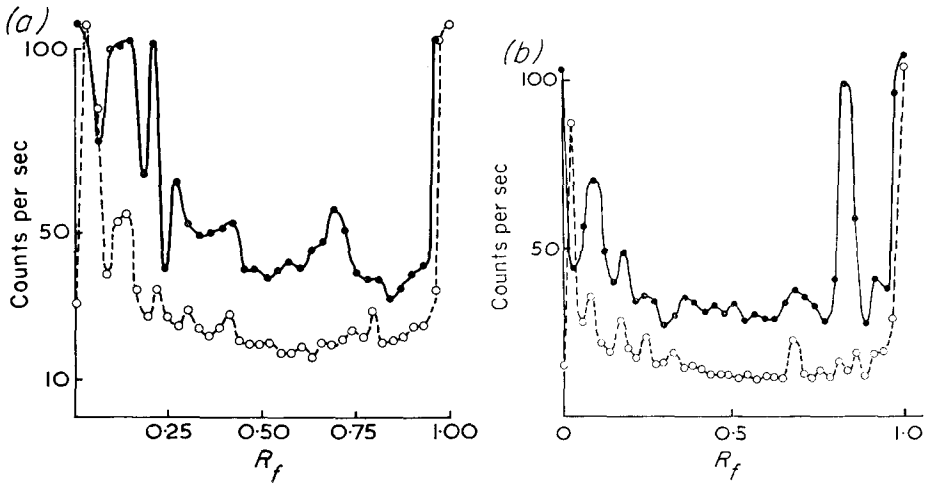

FIG. 2. Comparison by disc electrophoresis in 5% acrylamide containing 9 M urea, acetic acid of urea-soluble surface proteins of cells, and supernatants from lymphoma S1AT.4 and myeloma MOPC460. (2 *a*) Lymphoma S1AT.4; (2 *b*) Myeloma MOPC460. (●–●), urea-soluble surface proteins obtained from cells; (○–○), surface proteins present in supernatant after 2 hr incubation.

obtained when supernatant obtained from ^{125}I-labeled spleen cells, thymus cells, thymus-derived, and bone marrow–derived thoracic duct lymphocytes were compared with iodinated surface proteins extracted from the cells.

The latter results suggested that although most surface proteins iodinated by the lactoperoxidase-catalyzed reaction are released by the cells, they may be released at different rates. To test this possibility, thymus-derived and bone marrow–derived lymphocytes were iodinated with iodide-^{125}I. After iodination 1×10^7 cells of each type were disrupted in 9 M urea + 0.2 M mercaptoethanol while another 1×10^7 cells were incubated for 1 hr at 37°C and then disrupted in urea. The urea-soluble proteins obtained at each time point were then frac-

tionated by polyacrylamide gel electrophoresis. As may be seen in Figs. 3 *a* and *b*, both thymus-derived and bone marrow-derived lymphocytes lost approximately twice as much material in components characterized by R_f values of 0.5 or greater than in components with R_f values <0.5. Although the present

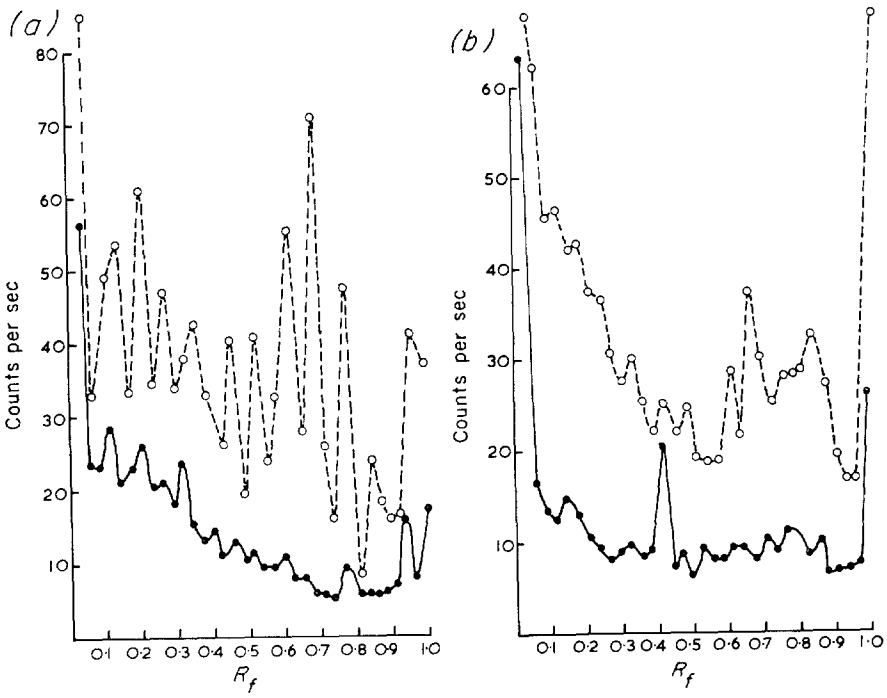

FIG. 3. Comparison by disc electrophoresis in 8% acrylamide containing 9 M urea, acetic acid of urea-soluble surface proteins obtained from thymus-derived and bone marrow-derived lymphocytes immediately after iodination and after 1 hr incubation at 37°C. (3 *a*) Bone marrow-derived cells; (3 *b*) Thymus-derived cells. (○– –○), urea-soluble surface proteins immediately after iodination. (●– –●), urea-soluble surface proteins after 1 hr incubation at 37°C.

electrophoretic technique does not entirely exclude the effect of charge, it is likely that the latter proteins have significantly higher molecular weights than those of the rapidly released molecules. In addition, individual components are lost to different degrees. This is particularly evident in a component present on the surface of thymus-derived cells having an R_f of 0.4–0.43.

DISCUSSION

The observation that normal and neoplastic lymphoid cells release cell surface constituents is in accordance with findings for a variety of other cell types

(13–15) and suggests that this phenomenon may be characteristic of many types of mammalian cells. The suppression of the release of cell surface proteins by low temperature or the addition of inhibitors of cell respiration such as antimycin A or iodoacetate indicates that this process requires active cellular metabolism. This view is supported further by the observation that sheep erythrocytes did not release surface proteins when incubated under similar conditions. It is unlikely that the proteins released by the cells represent a population of molecules which were absorbed to the surface because (*a*) all the cells were extensively washed before and after iodination and (*b*) spleen or thymus cells which were preincubated for 2–4 hr before iodination label to the same extent and release surface proteins at the same rate as cells which have not been preincubated. Furthermore, components identical to those released by labeled cells have been detected in supernatants obtained from serum-free medium in which unlabeled normal or neoplastic cells have been incubated.[3] This observation indicates that the process is not induced by the incorporation of radioactive iodide into the cell surface and suggests that the release of cell surface proteins may represent rapid turnover of these components. The ability of puromycin to inhibit membrane turnover (16) and the release of cell surface components supports this contention. However, most turnover rates for plasma membranes that have been reported previously (16–20) were considerably slower than the half-life for labeled surface protein reported herein. The difference might be because of differences in cell types and in the method of analysis. Most studies of membrane turnover have involved incorporation of radioactive amino acids into the membrane and subsequent isolation of purified membrane fractions. This mode of analysis might result in the loss of components which are loosely bound to the membrane (21) and which are synthesized and released at a rapid rate. It has been shown that various proteins within the membrane turn over at different rates (20, 22). In agreement with the latter study, the results shown in Figs. 2 and 3 demonstrate that cell surface proteins are released at different rates. Thus, the difference in release kinetics observed for thymus cells and spleen or bone marrow–derived cells may be because of differences in the types or amounts of accessible proteins present at the cell surface. The elution kinetics shown in Fig. 1 for spleen cells and thymus cells would then represent a composite for the rates of release of various labeled proteins, the rate of the fastest released component predominating. Preliminary experiments involving gel filtration on Sephadex G-25 indicate that thymus cells possess three to five times more of a rapidly released surface component than do spleen or bone marrow–derived cells. This material is released at a rate consistent with the half-life determined for the cell coat (23), a glycoprotein matrix, external to the plasma membrane (15,

[3] Marchalonis, J. J. Unpublished results.

24). The cell coat has been thought to play a critical role in the migration patterns of circulating lymphocytes and in the ability of cells to aggregate (15, 24). The presence, in large quantity, of material on the surface of thymus cells which has properties similar to that of the cell coat suggests that this material may be important in the interactions which occur between thymus-derived and bone marrow–derived cells during the initiation of the immune response (25, 26).

The fact that RAMIgG bound specifically to spleen lymphocytes is released at an appreciable rate is consistent with the findings of Wilson et al.[4] who have employed a variety of antisera and cell types. Their observed rate of metabolic release of RAMIgG correlated well with ours. These observations strengthen the conclusion that different surface proteins are released at individual rates. Preliminary evidence obtained by gel filtration[5] suggests that the RAMIgG is released as a high molecular weight complex, presumably bound to its receptor globulin. We have taken advantage of the finding that surface immunoglobulin may be released from the cell and have isolated immunoglobulin from the surface of normal human and mouse thymus cells.[1] The immunoglobulin molecule consists of μ-type heavy chains and light chains and has a molecular weight of 200,000.

The effects of metabolic inhibitors taken in conjunction with the linear rather than expotential nature of release kinetics indicates that the phenomenon is not because of enzymatic degradation of surface constituents. The linear kinetics suggest further that these proteins may be "queued" within the cell for insertion into the membrane. As a component is inserted, another is released. Deviations from linearity in arithmetic graphs of counts *versus* time can be explained by the presence of components which are released at different rates. Components are exhaustively depleted in order of decreasing rate of release. The time-dependent suppression of the release of surface proteins by puromycin supports this hypothesis. Thus, if a "pool" of surface components were present within the cell, the release of such components from the surface might be expected to continue until the pool was depleted.

The release of cell surface proteins by lymphoid cells indicates that a dynamic state exists at the interface between the cell and its environment. If such molecules function as receptors their rapid turnover may play an important regulatory role in the initiation of responses to antigens, hormones and other environmental stimuli. Thus, the binding of specific antigen to membrane-bound immunoglobulin might inhibit the release of this molecule. Inhibition of the release of receptors might cause conformational changes in

[4] Wilson, J. D., G. J. V. Nossal, and H. Lewis. Metabolic characteristics of lymphocyte surface immunoglobulin. Manuscript submitted for publication.

[5] Atwell, J. L., and H. Lewis. Unpublished observations.

the membrane, resulting in the triggering of division and differentiation of the cell. In addition, the uptake of free receptors or receptor-stimulator complexes by other cells could be a mechanism of communication between distant cells.

SUMMARY

Cell surface proteins of normal and neoplastic lymphocytes were labeled with iodide-^{125}I by lactoperoxidase-catalyzed iodination. Incubation of ^{125}I-labeled iodide cells in vitro resulted in the release of iodinated surface proteins at a rapid rate which was dependent on cellular respiration and protein synthesis.

Comparisons by disc electrophoresis showed a marked similarity between urea-soluble surface proteins extracted from iodinated cells and iodinated material released by the cells during in vitro incubation.

The rate of release of cell surface proteins from thymus cells was three times faster than that of spleen cells or bone marrow–derived thoracic duct lymphocytes. In addition, different proteins were released at different rates as evidenced by the rate of release of ^{125}I of rabbit anti-mouse immunoglobulin specifically bound to mouse spleen cells and comparisons by disc electrophoresis of urea-soluble iodinated surface proteins extracted from cells before and after incubation.

The results suggest that a dynamic state exists at the cell surface. The possible role of the release of cell surface proteins in cell regulation and communication is discussed.

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