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Differences in the Surface Proteins of Mouse B and T Cells

(iodination/lymphocytes/mitogens/polyacrylamide gels/antilymphocyte serum)

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ABSTRACT **We have selectively labeled the surface of mouse thymus-dependent (T) and thymus-independent (B) lymphocytes by means of lactoperoxidase-catalyzed iodination. Examination of the labeled proteins by electrophoresis in the presence of sodium dodecylsulfate followed by autoradiography revealed differences in the cell surface proteins of B and T cells. Proteins from labeled thymocytes, T cell lymphomas, and both normal and activated peripheral T cells give a broad band of radioactivity corresponding to at least two protein components with apparent molecular weights of 170,000 to 190,000. This band is absent from autoradiographs of iodinated B cell proteins, which instead have another band corresponding to a protein with an apparent molecular weight of about 220,000. We have shown that these T and B cell marker proteins are synthesized by the cell and are not serum components selectively bound to the cell surface. We have also established that these proteins are the major iodinated species precipitated from Nonidet P-40 cell lysates by rabbit anti-mouse lymphocyte serum, suggesting that they are the major antigens recognized by rabbit antibodies to mouse lymphocytes.**

The physiological role of the immune system is to discriminate between self and nonself. In mammals and birds this task is shared by two classes of lymphocytes: T cells, which differentiate in the thymus, and B cells, which are generated independently of the thymus. After antigenic stimulation, T cells give rise to the effector cells of cell-mediated immune reactions such as delayed-type hypersensitivity and graft rejection as well as cells which cooperate with B cells in the antibody response. The only established function of stimulated B cells is to synthesize and secrete antibody.

Serologically detectable cell surface differences between T and B cells have been exploited to identify each class of lymphocyte and to study their physiological roles. Antigen recognition, the characteristic migratory properties of both B and T cells, and the effector reactions of T-cell-mediated immunity are all believed to be associated with the lymphocyte plasma membrane. As a consequence, further detailed studies of the lymphocyte cell surface are warranted not only to provide additional means of discriminating between T and B cells, but as an approach directed towards understanding their different immunological roles.

We have selectively labeled the cell surface of mouse T and B cells by lactoperoxidase-catalyzed iodination and examined the labeled proteins by gel electrophoresis in the presence of sodium dodecylsulfate. The resolution of our analysis has been enhanced by using a high pH discontinuous buffer system, polyacrylamide gel gradients, and by identifying the iodinated proteins by autoradiography. We have labeled relatively pure populations of T and B cells activated by selective stimulation of normal spleen cells with mitogens or allogeneic cells, cells from the lymphoid organs of normal and congenitally athymic mice, as well as a variety of lymphoid cell lines. The use of these cell populations in combination with the high resolution of our analytical procedures has allowed us to identify hitherto unrecognized structural differences between T and B cells.

Abbreviations: Con A, concanavalin A; NP-40, Nonidet P-40; LPS, lipopolysaccharide.

MATERIALS AND METHODS

Normal Lymphoid Cells. Cell suspensions from the spleens, lymph nodes, and thymuses of BALB/c mice and spleens and lymph nodes of mice with the congenital athymic character (*nu/nu*) backcrossed onto a BALB/c background (1) were prepared by teasing with forceps in balanced salt solution (2). Red cells were removed from cell suspensions by selective lysis with Tris-buffered ammonium chloride (3).

Lymphoid Cell Lines. Tumor lines of B or T cell origin were maintained as suspension cultures in Dulbecco's modified Eagle's medium supplemented with either 10% fetal calf serum or 10% horse serum. Cells from exponentially growing cultures were used in all iodination experiments.

Preparation of Activated T and B Cells. Concanavalin A (Con A) twice crystallized was from Miles-Yeda, Ltd., and used without further purification. Lipopolysaccharide (LPS) from *Salmonella typhosa* 0901 (Difco) was boiled 60 min at 1 mg/ml in 0.1 M sodium phosphate buffer pH 8.0 before use (1). Pea lectin was purified by affinity chromatography (4).

Spleen cell suspensions from $B_6D_2F_1$ mice (2- to 6-months-old) were prepared by teasing spleens in balanced salt solution with forceps. In most experiments red cells were removed by selective lysis with Tris-buffered ammonium chloride. Cells were then cultured (2×10^6 cells per ml) with either Con A (2 μg/ml), pea lectin (5 μg/ml), or LPS (10 μg/ml) in Falcon tissue culture dishes containing RPM1 1640 medium supplemented with 2 mM glutamine, 5% fetal calf serum, antibiotics, and 50 μM 2-mercaptoethanol. Cultures were incubated at 37° for 2–3 days in a water-saturated atmosphere of 10% CO_2–7% O_2–83% N_2.

Stimulation of C57BL/6 spleen cells (histocompatibility type H-2b) with mitomycin-C-treated BALB/c spleen cells (H-2d) was carried out as described (5). The culture period was 4–5 days.

Lactoperoxidase-Catalyzed Iodination of Cells. Before iodination, damaged cells were removed from cell suspensions by

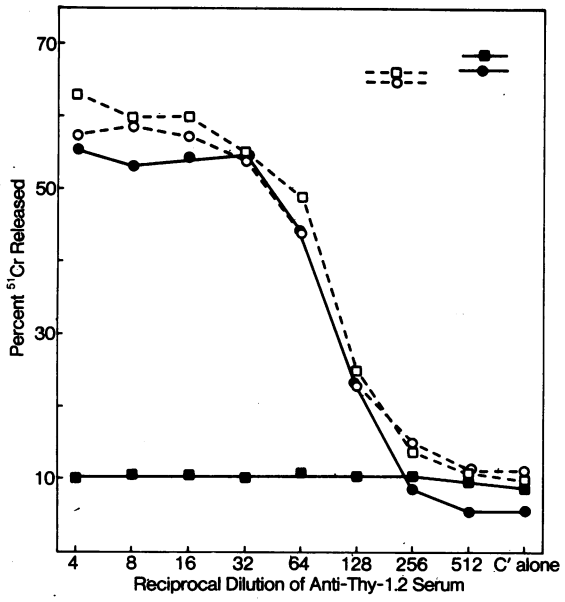

FIG. 1. Cytolysis of mouse spleen cells by anti-Thy-1.2 serum and complement after selective stimulation by mitogens or allogeneic cells. Viable cells were isolated from stimulated spleen cell cultures. After labeling with $Na^{51}CrO_4$ (29), samples of each cell suspension [2.5×10^5 cells in 0.1 ml of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffered medium containing 0.1% bovine serum albumin] were incubated with various dilutions of anti-Thy-1.2 serum for 20 min at 37°. Cells were then washed and incubated for 45 min at 37° with complement (0.1 ml of a 1:10 dilution of guinea pig serum absorbed with mouse spleen cells). The ^{51}Cr released by the cells was measured and expressed as a percentage of the total ^{51}Cr label originally present in the cells. Shown are the titration curves of ^{51}Cr release induced by different dilutions of anti-Thy-1.2 serum and complement from cells stimulated with either Con A (□), pea lectin (○), LPS (■), or allogeneic cells (●). Also shown in the top right of the figure is the percent ^{51}Cr release induced by rabbit anti-mouse lymphocyte serum from samples of the same cell populations.

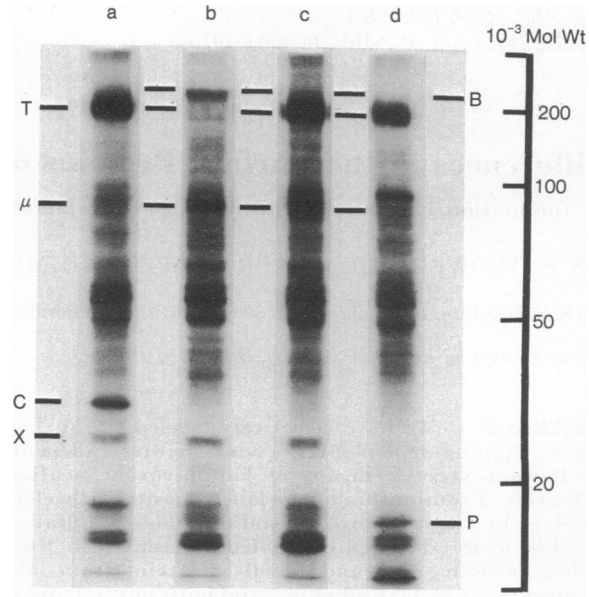

FIG. 2. Iodination of activated T and B cells. Autoradiographs of iodinated proteins from spleen cells stimulated with: (a) Con A, (b) LPS, (c) allogeneic cells, (d) pea lectin. See *text* for explanation of symbols.

density fractionation in isotonic unbuffered salt solution (pH 5.1) containing bovine serum albumin (ρ 1.094) (6). Cell suspensions prepared in this manner were more than 95% viable as judged by dye exclusion. After three washes with 0.15 M NaCl–0.01 M sodium phosphate buffer (pH 7.2), cells were resuspended in the same buffer containing 5 mM glucose. Lactoperoxidase-catalyzed iodination was performed following the procedure described by Hubbard and Cohn (7) in which the oxidation of glucose by glucose oxidase generates a continuous low concentration of hydrogen peroxide. Cell suspensions (10^7 cells per ml) were incubated for 10 min at room temperature with 250 μCi/ml of carrier-free $Na^{125}I$ (New England Nuclear Corp.) in the presence of 20 μg/ml of lactoperoxidase (Calbiochem) and 0.1 units/ml of glucose oxidase (Calbiochem). The rate of incorporation of ^{125}I into trichloroacetic-acid-insoluble material was linear for 5 min and the reaction was complete after 10 min. The omission of either enzyme from the incubation medium reduced the incorporation of ^{125}I into trichloroacetic-acid-insoluble material by more than 95%. An experimental control in which lactoperoxidase was omitted from the iodination mixture was always performed.

After iodination the cells were washed at 4° with 0.15 M NaCl–0.01 M phosphate buffer, dissolved in electrophoresis sample buffer (10^7 cells per ml) containing 2% (w/v) sodium dodecylsulfate and 2% (v/v) 2-mercaptoethanol, and boiled for at least 2 min.

Gel Electrophoresis and Autoradiography. Polyacrylamide slab gel electrophoresis in the presence of sodium dodecylsulfate was performed with a high pH discontinuous buffer system (8). The resolving gel (10 cm long) consisted of a linear 7.5–15% polyacrylamide gel gradient prepared by pumping the polymerization mixture from a conventional, two-chamber, gradient generator into the gel template at a flow rate of 2 ml/min. To facilitate the formation of the acrylamide gradient, 20% (w/v) sucrose was included in the 15% acrylamide mixture. To prevent premature polymerization of the acrylamide, low concentrations of ammonium persulfate, 0.035% (w/v), and *N,N,N',N'*-tetramethylenediamine, 0.025% (v/v), were used to catalyze the reaction.

Samples of labeled proteins equivalent to 3 to 5×10^5 cells were applied to the gels in 20–50 μl of sample buffer. After electrophoresis (12 mA constant current for approximately 4 hr) the gels were fixed, stained with Coomassie blue (9) and dried onto filter paper, and autoradiography was carried out with either Kodak RP14 or Kodak NS54T x-ray film.

Antisera. Anti-Thy-1.2 serum was prepared essentially as described (10). The serum was heat-inactivated at 56° for 10 min and absorbed with AKR/J spleen cells (1×10^8 cells per ml). Rabbit antiserum to mouse lymphocytes was prepared exactly as described by Levy and Medawar (11). Goat antiserum to rabbit immunoglobulin was prepared as previously described (12).

RESULTS

Activated T and B Cells. Activated T and B cells were prepared by selective stimulation of normal spleen cells by mitogens or allogeneic cells. Con A, pea lectin, or allogeneic cells selectively stimulated the proliferation of T cells; LPS

selectively stimulated B cells. The proportion of T cells in the activated cell populations was estimated by ^{51}Cr release as a measure of cell lysis induced by anti-Thy-1.2 serum and complement (Fig. 1). From the data shown, if one assumes that the ^{51}Cr release induced by antilymphocyte serum represents total cell lysis, it can be calculated that 90% of Con-A- or pea-lectin-stimulated spleen cells and 80% of spleen cells stimulated by allogeneic cells which survive culture are T cells, whereas less than 2% of the viable LPS-stimulated cells are T cells.

The profiles of the proteins from activated T and B cells labeled by lactoperoxidase-catalyzed iodination are shown in Fig. 2. Activated T cells, independent of the nature of the activating stimulus, yield a broad band of radioactivity, which in other experiments was resolved into at least two components representing iodinated species with apparent molecular weights ranging from 170,000 to 190,000. This band is absent from the autoradiograph of activated B cell proteins, which have instead a narrow band corresponding to a protein with an apparent molecular weight of about 220,000. These initial observations suggested that these iodinated proteins may represent marker proteins for T and B cells, and data which we present subsequently support this view.

In addition to the T and B cell marker proteins, other differences between the autoradiographs of the labeled proteins from the different cell populations can be seen. Pea-lectin-stimulated cells have a unique band (P) corresponding to a protein with an apparent molecular weight of 14,000. Since the intensity of this band was greatly reduced on autoradiographs of labeled proteins from cells incubated at 37° for 90 min with 50 mM methyl α-D-mannoside before they were iodinated, it is probably the labeled β subunit of pea lectin (4) bound to the cell surface. Similarly the iodinated species (C) that had an apparent molecular weight of 27,000 and was associated only with Con-A-stimulated cells is the subunit of surface-associated Con A. The iodinated species that can be identified as the μ chain of surface-bound IgM by its electrophoretic mobility and by immunoprecipitation with rabbit anti-mouse immunoglobulin serum is much more intense on autoradiographs of labeled proteins from populations of activated B cells than activated T cells. A number of bands present on autoradiographs of labeled proteins from T cells activated by Con A or allogeneic cells (for example, band X) are of much lower intensity on the autoradiograph of labeled proteins from pea-lectin-activated cells. The significance of these last differences remains to be determined.

Normal Lymphoid Tissues. Cells from the lymphoid tissues of normal and athymic mice were examined for the presence of the T and B cell marker proteins. Fig. 3 shows that the T cell marker protein was detected on autoradiographs of labeled proteins from thymocytes, spleen cells, and lymph node cells of normal mice but is virtually absent from the autoradiographs of labeled proteins from spleen and lymph node cells of athymic mice. The B cell marker protein was not detected on the autoradiograph of labeled proteins from thymocytes but can be identified on those of labeled proteins from spleen and lymph node cells of both normal and athymic mice. The tissue distribution and the relative intensity of the radioactive bands corresponding to the marker proteins support the view that the T cell marker protein is present on peripheral T lymphocytes and the B cell marker protein is present on peripheral B lymphocytes. However, cell fractionation studies will be required to establish that the marker proteins are present on all peripheral lymphocytes of the appropriate origin.

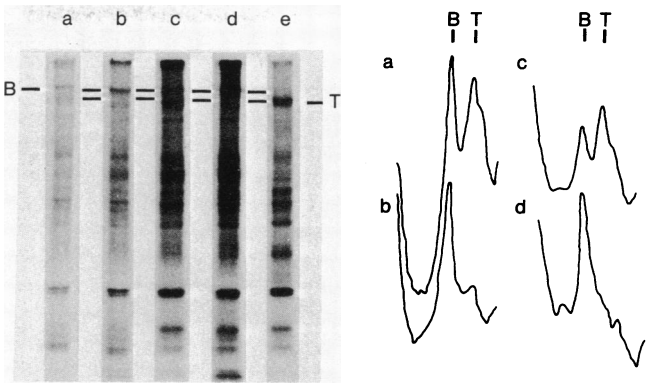

FIG. 3. Iodination of cells from normal lymphoid tissues. On the left are autoradiographs of iodinated proteins from: (a) BALB/c spleen cells, (b) (*nu/nu*) spleen cells, (c) BALB/c lymph node cells, (d) (*nu/nu*) lymph node cells, (e) BALB/c thymocytes. On the right are densitometer tracings showing the relative intensity of the T and B cell marker proteins on the autoradiographs. (a), BALB/c spleen cells; (b), (*nu/nu*) spleen cells; (c), BALB/c lymph node cells; (d) (*nu/nu*) lymph node cells.

Lymphoid Cell Lines. Tumor lines of T or B cell origin were also examined for the presence of the T and B cell marker proteins. The T cell marker protein was present on each of four lymphomas expressing the Thy-1 antigen, S49 (13), EL4 (14), WEHI 22 (15), and BW5147 (14) and also on Thy-1 negative variants of S49 and BW5147 (16), and C1498, a myeloid leukemia (14).* In contrast, neither the T nor B cell marker proteins could be detected on S194/2, a myeloma which synthesizes and secretes IgA (13); PU5-1.8, a Thy-1 negative cell line reported to express surface immunoglobulin (17); and RAW 112 and RAW 8, two lymphosarcoma cell lines of unknown cellular origin induced by Abelson leukemia virus (W. Raschke, P. Ralph, J. Watson, M. Sklar, and H. Coon, unpublished results). A selection of these results is shown in Fig. 4.

Biosynthetic Labeling Experiments. We wished to establish whether the T and B cell surface marker proteins were synthesized by the cells or whether they were serum components selectively bound to the cell surface. Examination of iodinated fetal calf serum failed to reveal serum components with electrophoretic mobilities similar to either marker protein, nor could the marker proteins be detected on autoradiographs of proteins from unlabeled cells preincubated with iodinated fetal calf serum. Examination of the proteins from activated T and B cells biosynthetically labeled with a mixture of [^{14}C]aminoacids revealed labeled proteins with electrophoretic mobilities characteristic of both marker proteins. However, the pattern was so complex that it seemed probable that other cell proteins not labeled by lactoperoxidase-

* Although originally classified as a myeloid leukemia, the C1498 cell line derived in this laboratory from a transplantable C1498 tumor obtained from Jackson Laboratories expresses Thy-1 and by this criterion consistent with the presence of the T cell marker protein, is a T lymphoma.

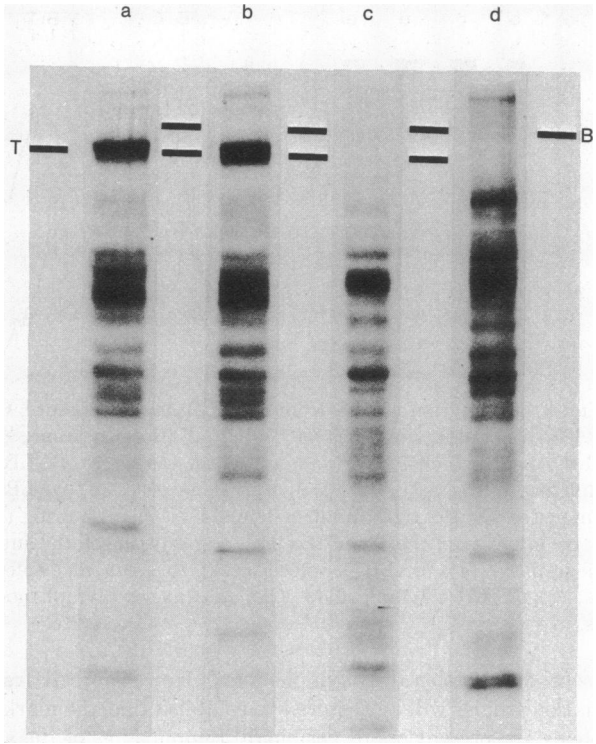

FIG. 4. Iodination of lymphoid cell lines. Autoradiographs of iodinated proteins from: (a), BW5147 Thy-1.1 positive T lymphoma; (b), BW5147 Thy-1.1 negative T lymphoma; (c) S194/2 myeloma (d), RAW 112 Abelson virus lymphoma.

catalyzed-iodination but labeled biosynthetically coelectrophoresed with the T and B cell marker proteins and interfered with their identification. Examination of the culture supernatants of [^{14}C]aminoacid-labeled cells failed to reveal selective shedding or secretion of either the T or B cell marker proteins.

A means of substantially reducing the complexity of the autoradiographs of biosynthetically labeled proteins became apparent when we observed that the T and B cell marker proteins were the major iodinated proteins recognized by rabbit anti-mouse lymphocyte serum. As shown in Fig. 5a and b, the marker proteins were the major iodinated species that could be precipitated by rabbit anti-mouse lymphocyte serum from the supernatants of cells labeled with ^{125}I and lysed with the neutral detergent NP-40. Three different preparations of antisera gave similar results, precipitating 3–4% of the trichloroacetic-acid-precipitable radioactivity from NP-40 lysates of activated T cells and 1–2% of the trichloroacetic-acid-precipitable radioactivity from NP-40 lysates of activated B cells. Normal rabbit serum precipitated only 0.2–0.3% of the radioactivity of lysates of either cells. NP-40 lysates of activated B and T cells labeled with [^{14}C]amino acids were prepared and immunoprecipitation carried out with rabbit anti-mouse lymphocyte serum. Examination of the precipitates by gel electrophoresis and autoradiography showed that the B cell marker protein was present on autoradiographs of ^{14}C-labeled proteins from activated B cells and that the T cell marker protein could be detected on autoradiographs of ^{14}C-labeled proteins of activated T cells, formally demonstrating that the marker proteins were synthesized by the cells (Fig. 5c and d).

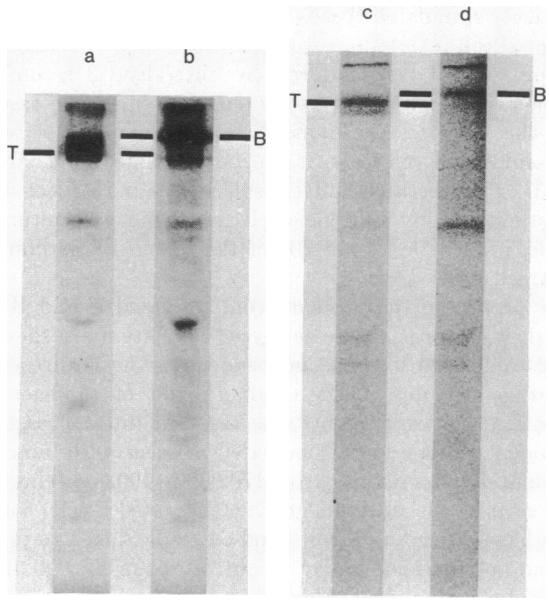

FIG. 5. Immunoprecipitation of the T and B cell marker proteins with rabbit anti-mouse lymphocyte serum. Viable activated T and B cells were either iodinated or biosynthetically labeled with 10 μCi of [^{14}C]aminoacids (5×10^6 cells per ml) for 4 hr in RPM1 1640 containing 10% of the usual concentration of amino acids and supplemented with 1% Sephadex-G-50-filtered fetal calf serum (30). After washing, the cells were dissolved (10^7 cells in 0.3 ml) in 1% NP-40 in 0.15 M NaCl–0.01 M sodium phosphate buffer (pH 7.2) by incubation on ice for 30 min. After removal of nuclei by low-speed centrifugation the lysate was centrifuged at 16,000 $\times$ g for 30 min. To a sample (10–20 μl) of the NP-40 lysate supernatant were added 0.2 ml 0.1% NP-40 in 0.15-M NaCl–0.01 M phosphate buffer (pH 7.2) and 3–6 μl of either rabbit anti-mouse lymphocyte serum or normal rabbit serum, and the mixture was incubated for 20 min at room temperature. Goat anti-rabbit immunoglobulin serum (0.2 ml) was then added and the mixture was incubated for 45 min at room temperature followed by at least 4 hr of incubation in the cold. After extensive washing the precipitates were examined by electrophoresis and autoradiography. Shown are the autoradiographs of the immunoprecipitates of iodinated (a) activated T cells (Con-A-stimulated), (b) activated B cells (LPS-stimulated), or [^{14}C]aminoacid-labeled (c) activated T cells (Con-A-stimulated), and (d) activated B cells (LPS-stimulated). The immunoprecipitates from [^{14}C]aminoacid-labeled cells were analyzed on a 9% acrylamide gel. The T and B cell markers were identified on both gels by comparison with the mobility of the T and B cell marker proteins in the mixture of total iodinated cell proteins.

DISCUSSION

The main purpose of the experiments we have reported is to demonstrate that we can identify T and B cell marker proteins by analysis of the labeled proteins from cells iodinated by the lactoperoxidase technique. This technique selectively labels surface components of undamaged cells, and we have confirmed that the marker proteins are associated with the lymphocyte plasma membrane by subcellular fractionation of homogenates of labeled cells with sucrose gradient centrifugation (R. Hamilton, M. Hamilton, M. J. Bevan, and I. S. Trowbridge, unpublished results). The T cell marker protein was associated with T cells at all stages of maturation examined (thymocytes, normal, and activated peripheral T cells) and with lymphomas identified as T cells by their ex-

pression of Thy-1. In contrast the B cell marker protein was found on normal and activated B cells but was not detected on a myeloma nor on a putative B lymphoma. The tumor cell lines of B cell origin that we examined are products of abnormal differentiation and it is possible that their cell surface differs from that of normal cells. Alternatively the B cell marker protein may only be found on B cells at certain stages of maturation.

The T and B cell marker proteins could be precipitated from NP-40 cell lysates by rabbit anti-mouse lymphocyte serum. Using this procedure to reduce the complexity of the autoradiographs of the biosynthetically labeled proteins from activated T and B cells, we established that these proteins are synthesized by the cells and are not serum components selectively bound to the cell surface. It is probable that the T and B cell marker proteins are the major antigens recognized by rabbit anti-mouse lymphocyte antibodies; however, it is possible that there are other major cell surface antigens that are not labeled by lactoperoxidase-catalyzed-iodination and consequently are not detected by autoradiography. In addition it is not possible to formally exclude that the T and B cell marker proteins are noncovalently associated with other membrane components present in the NP-40 cell lysates and that it is these molecules that carry the antigenic determinants recognized by rabbit anti-mouse lymphocyte serum.

Several cell surface components of mouse lymphocytes—immunoglobulin (18), H-2 (19), Thy-1 (20, 21), thymus leukemia antigen (22, 23) and Ia antigen (24, 25)—have been isolated by serological techniques and partially characterized. Neither the B nor the T cell marker protein described in these studies appears to be identical with any of these previously identified cell surface components.

An important problem which remains is to determine the number of T and B cell marker proteins on the cell surface. Because the selectivity of lactoperoxidase-catalyzed-iodination, which determines the efficiency of labeling of individual membrane proteins, is unknown, we cannot estimate with confidence the number of T and B marker proteins on each cell from our present data. Since these proteins are probably recognized by rabbit anti-mouse lymphocyte antibodies, it may be possible to prepare, by absorption, monospecific antisera directed against the marker proteins. We could then use the double antibody assays described by Williams (26–28) to obtain quantitative data.

Finally, defined differences similar to those we have described between mouse T and B cells may exist between the two classes of lymphocytes in other species. It is clear that if such differences between T and B cells are identified in man, then their characterization may lead to the development of highly specific antisera against either T or B cells which may be of clinical value.

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