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Immunospecific Labeling of Mouse Lymphocytes in the Scanning Electron Microscope

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Bone marrow-derived (B) and thymus-derived (T) Balb/c mouse lymphocytes were identified in the scanning electron microscope (SEM) by the immunospecific attachment of one of several kinds of large-molecular-weight markers distinguishable in SEM. These markers (tobacco mosaic virus, keyhole limpet hemocyanin, bushy stunt virus, and bacteriophage T4) could be modified with hapten groups and linked with anti-hapten antibody, in an indirect (sandwich) scheme, to hapten-modified anti-cell-surface antibody bound to the cell surface.

Hapten-modified antibodies to B cell antigens (goat anti-mouse-immunoglobulin) or to T cell antigens (rabbit anti-mouse brain) were employed to identify these two lymphoid cell types in unfractionated spleen, mesenteric lymph node, bone marrow, and thymus cell populations. The topography of B cells was always indistinguishable from that of T cells. No surface features were found to be unique to either cell type. In suspension, the majority of B and T cells had one or no microvilli regardless of the tissue source of the labeled cells. Cells in suspension that had microvilli (usually 10% of the total cell population) were always unlabeled. However, after cell contact with a glass surface, approximately half of both the B and T cell populations had a villous topography.

Key words: scanning electron microscope, lymphocytes

INTRODUCTION

Since the discovery that lymphocytes, the cells responsible for the immune response, can be classified into two functional types, the bone marrow-derived (B) and thymus-derived (T) lymphocytes, much effort has been expended in describing features of these cells that would serve to distinguish one type from the other. Consistent structural differences between B and T cells have not been observed by light or transmission electron microscopy. Lymphocytes are most readily classified by the presence of cell surface antigens unique to one or the other cell type, which can be identified by labeling techniques employing specific antibodies in fluorescent or electron microscopy.

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At the beginning of the work described in this paper, we hoped to find some features of lymphocyte surface topography as viewed in the scanning electron microscope (SEM)* that would be unique to either B or T cells. To this end, we have used a hapten-sandwich method (Fig. 1) to attach markers visible in SEM to antibodies specifically bound to cell surface antigens (1). Antiserum to mouse immunoglobulin (anti-MIg) binds to B cells specifically, and antiserum to mouse brain (anti-MBr) cross-reacts with T but not B cells (2). This labeling technique permits accurate identification of individual B or T cells in a mixed lymphoid population.

In general, we find the two mouse lymphoid types to be identical in their appearance in SEM. There have been a number of reports that have indicated some topographic differences between B and T cells (3–5), although the majority of published observations of lymphocytes in SEM (6–13) are consistent with our results. Preliminary reports of some of our results have been presented (1, 14).

METHODS

Antisera and Purified Antibodies

Antibodies to the haptens p-azophenyl-β-D-lactoside (anti-lac), and p-azobenzenearsonic acid (anti-ars) were isolated from pooled, high-titer antisera from rabbits hyperimmunized by standard procedures against hapten conjugates of keyhole limpet hemocyanin (KLH), prepared as previously described (15), and were purified by Sepharose affinity chromatography (16).

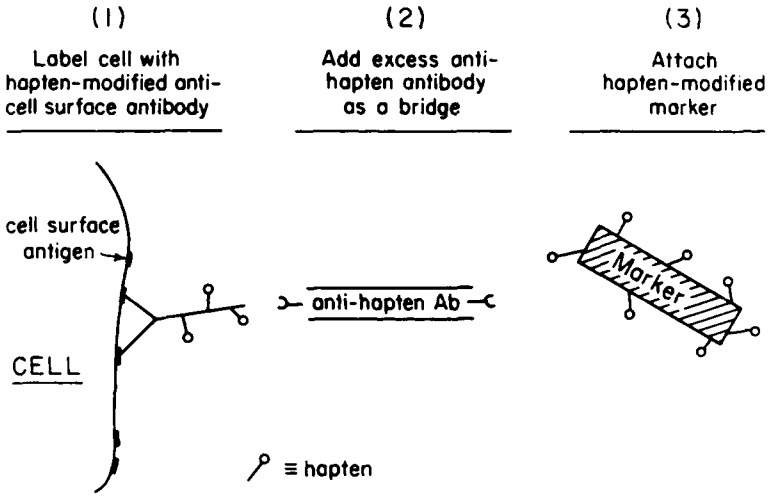

Fig. 1. Schematic representation of the hapten-sandwich labeling method. (1) Reprinted by permission.

*Abbreviations: Anti-lac-KLH, conjugate of anti-lac antibody and KLH; anti-MIg, polyvalent goat anti-mouse-immunoglobulin (B-cell-specific antibody); anti-MBr, rabbit anti-mouse-brain (T-cell-specific antibody); ars, p-azobenzenearsonic acid hapten; ars(amid)anti-MBr, anti-MBr amidinated with ars hapten; BSV, bushy stunt virus; KLH, keyhole limpet hemocyanin; lac, p-azophenyl-β-D-lactoside hapten; MV, topographic categories relating to cells bearing more than 20 microvilli (MV++), 2–20 microvilli (MV+), or fewer than 2 microvilli (MV–); SEM, scanning electron microscope; T4, bacteriophage T4; TMV, tobacco mosaic virus; VBS, veronal-buffered saline.

Polyvalent goat anti-mouse-immunoglobulin (anti-MIg) was prepared as previously described (1). In indirect (hapten-sandwich) immunofluorescent microscopy (17), this reagent labeled 44% of mouse spleen cells and less than 1% of mouse thymus cells.

Rabbit anti-mouse-brain (anti-MBr) antiserum was prepared according to Golub (2); the anti-T-cell activity of similar antisera has been extensively characterized (17). The immunoglobulin fraction was obtained by chromatography on DEAE-cellulose (1). In indirect (hapten-sandwich) immunofluorescent microscopy, this reagent labeled 54% of mouse spleen cells, 85% of mouse thymus cells, and less than 0.5% of mouse femoral bone marrow cells.

The percentage of cells labeled in SEM was usually the same as or less than (but never greater than) that obtained from corresponding cell preparations with the same reagent in fluorescent microscopy.

SEM Markers

Tobacco mosaic virus (TMV) and bushy stunt virus (BSV) were gifts from Dr. A. Knight, University of California, Berkeley. KLH was prepared as previously described (17). Bacteriophage T4 was grown under standard conditions from stocks supplied by Dr. M. Chamberlain, University of California, Berkeley.

Modification of Immunoglobulin and Markers

The azo-hapten conjugates of immunoglobulins (lac-anti-MIg and lac-anti-MBr) and of SEM markers (lac-TMV, lac-BSV, lac-T4, and ars-TMV) were prepared under standard reaction conditions (1, 17): 3 mg protein per ml and 4×10^{-4} M lac- or ars-diazonium reagent, in 0.2 M borate buffer, pH 8.5, were gently mixed overnight at 4°C. TMV, BSV, and T4 were treated for 2 hr with 0.1% glutaraldehyde before hapten modification, to prevent the disruption of these large-molecular-weight markers during hapten coupling (17). The conjugate of anti-lac and KLH (anti-lac-KLH) was prepared in a standard one-step procedure: 5 mg anti-lac was coupled to 50 mg KLH in 10 ml 0.1 M sodium phosphate buffer, pH 6.8, by the slow addition of 5% glutaraldehyde to a final concentration of 0.1%. Free aldehyde was subsequently quenched by dialysis against 0.1 M ammonium carbonate, pH 7.2, at 4° for 3 hr. All modified marker preparations were purified by gel chromatography as previously described (1).

The amidinated anti-MBr reagent (ars(amid)anti-MBr) was prepared by reacting the immunoglobulin fraction of anti-MBr antiserum at 7 mg/ml with 0.05 M o-azobenzenearsonic acid-p-OH-methylbenzimidate (produced by reaction of a 1:1 molar ratio of ars-diazonium to the benzimidate) in 0.25 M borate buffer, pH 8.5, for 18 hr at room temperature (18, 19). The resulting modified antibody was purified by gel chromatography (19).

Cell Preparations

All cell preparations were derived from unimmunized Balb/c mice (Diablo, Berkeley, CA). Spleen, thymus, and mesenteric lymph nodes were excised, washed, and teased into single-cell suspensions in veronal-buffered saline (VBS) as specified previously (1). Bone marrow cells were teased from tissue squirted from excised femurs into VBS.

Labeling of Cells for SEM

Lymphoid populations were labeled in suspension in VBS containing 0.2% bovine serum albumin (Pentex Biochemical, Kankakee, IL) and 0.1% sodium azide (1). Cells

were treated sequentially with hapten-modified anti-cell antibody, anti-hapten antibody, and hapten-modified marker particles (except for anti-lac-KLH, which was added after incubation of the cells with lac-anti-MIg). Each labeling step was followed by extensive washing of the cells. Labeled cell preparations were either fixed for SEM preparation in suspension with 1–2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 10 min to 2 days, or allowed to settle upon glass coverslips for 1 hr at room temperature and then fixed for 30 min (1). Cell preparations were then washed and dehydrated through an increasing concentration of ethanol in water, then Freon-113 in ethanol, and finally critical-point-dried from Freon-13 as previously described (1). Cells were dried either in suspension in Nuclepore chambers (1) or on glass coverslips.

Specimens were examined in a Cambridge Stereoscan Mark IIa, or a Coates and Welter Cwikscan 100-4 field emission SEM. Cells were examined for the presence of label and categorized further according to the occurrence on the observed cell face of greater than 20 microvilli (MV++), of 2–20 microvilli (MV+), or of less than 2 microvilli (MV–). A microvillus was identified by its uniform, cylindrical shape with a diameter of about 0.1 μ and 0.2 μ or greater in length. For each specimen, a minimum of 100 cells were counted, though the number was usually 200 or greater.

RESULTS

Cells Bearing MIg Antigens

Spleen, mesenteric lymph node, thymus, and bone marrow cells were labeled for MIg (Table I). Cells labeled in suspension were subsequently fixed for SEM examination either in suspension or upon a coverslip after contact with the glass for 1 hr.

Prepared in suspension at 0°, MIg-bearing cells are round, with an undulating surface characterized by fewer than 2 microvilli per cell. The example representative of these cells (Fig. 2a), a spleen cell, also bears a surface projection larger and more irregularly shaped than a microvillus. This feature was often found associated with such cells, but was never observed to display a marker for MIg. The majority of all lymphoid cells fixed in suspension, whether labeled or unlabeled (Fig. 2b), have this topography (Table I). No more than 10% of cells in these preparations had 2 or more microvilli (Fig. 2c), although this percentage varied with the type of lymphoid tissue examined and from one mouse to the next. MIg+ cells fixed in suspension from bone marrow and mesenteric lymph node (Fig. 4d) also exhibited a nonvillous topography.

In general, when lymphoid cells were allowed to contact glass for 1 hr at room temperature, an increase was noted in the incidence of cells with 2 or more microvilli in both MIg bearing (Fig. 3a) and unlabeled cell populations. Table I shows this increase for the MIg-bearing populations of spleen and bone marrow. Many unlabeled (Fig. 3b) and labeled cells (Figs. 3c, d) retained the relatively nonvillous topography associated with the majority of cells fixed in suspension (Figs. 2a, b).

The appearance of MIg+ cells was the same regardless of the marker employed. Figure 4 contains examples of B (MIg+) lymphocytes labeled with each of 3 other types of markers: KLH, BSV, and bacteriophage T4.

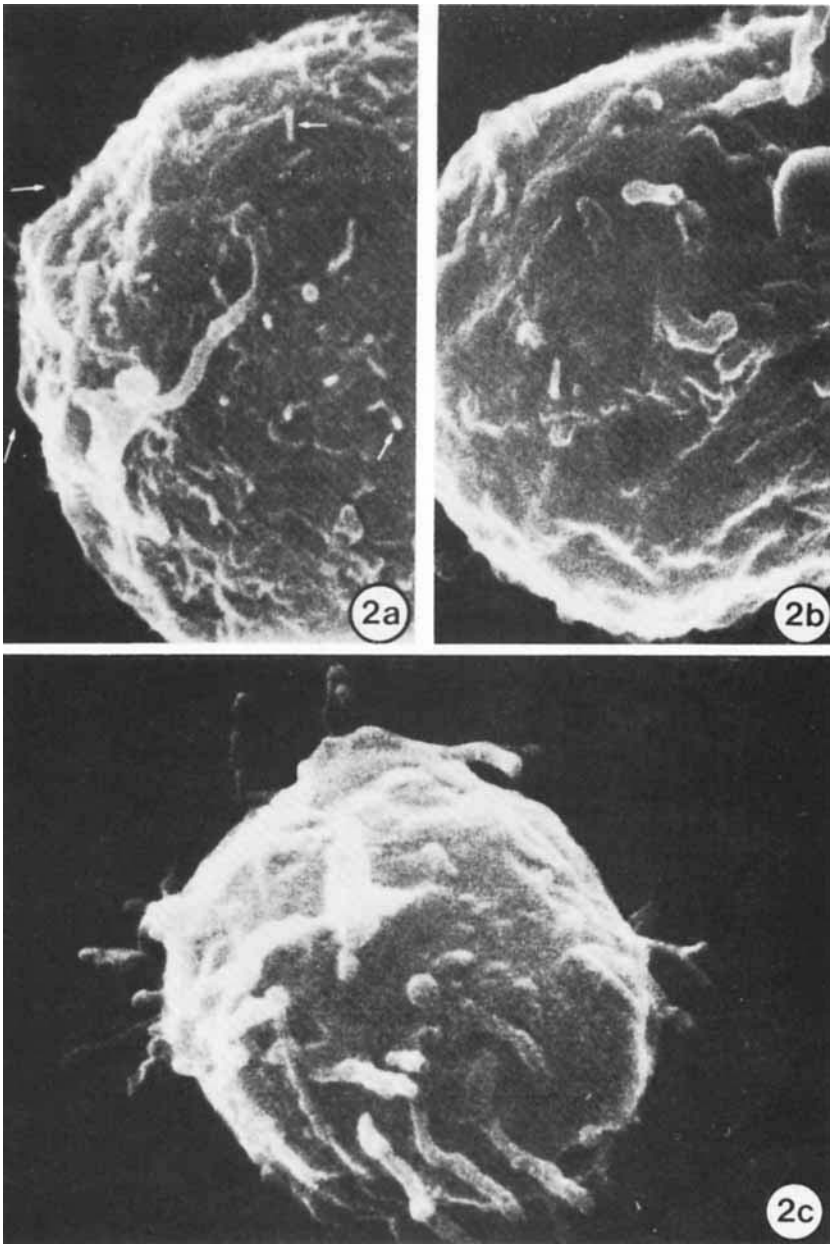

Fig. 2. SEM micrographs of spleen cells treated with lac-anti-MIg, anti-lac, and lac-TMV, and prepared for SEM in suspension (all are from one preparation, × 22,000): a) B lymphocyte with TMV attached (arrows) and devoid of microvilli (MV−); b) unlabeled cell with topography similar to B cell in Fig. 2a; c) unlabeled cell with numerous microvilli (MV+). (1) Reprinted by copyright permission of the Rockefeller University Press.

TABLE I. SEM Topography of MIg-Bearing Cells From Four Lymphoid Populations

No. of expts.	Cell source	Total MIg+	Topography[b] of: MIg+ cells MV++	MIg+ cells MV+	MIg+ cells MV−	MIg− cells MV++	MIg− cells MV+	MIg− cells MV−
	Prepared in suspension							
5	Spleen	33[a] (28–37)	< 1[a]	< 1	33 (28–37)	6[c] (3–9)		60 (54–65)
1	Lymph node	26	< 1	< 1	26	< 1	9	57
2	Bone marrow	4 (2–5)	< 1	< 1	4 (2–5)	< 1	2 (2)	86 (82–90)
	Prepared on glass							
2	Spleen	38 (28–49)	4 (3–6)	19 (19)	15 (6–24)	9 (8–10)	21 (17–25)	32 (26–37)
1	Thymus	1	< 1	< 1	1	2	41	43
2	Bone marrow	3 (2–4)	< 1	2 (2–3)	< 1	4 (4–5)	50 (47–54)	32 (31–33)

[a]Percentage of total cells counted; scatter in parentheses.
[b]Cells that bear less than 2 (MV−), 2–20 (MV+), or over 20 microvilli (MV++); scatter in parentheses.
[c]Represents the sum of (MV++) + (MV+) frequencies.

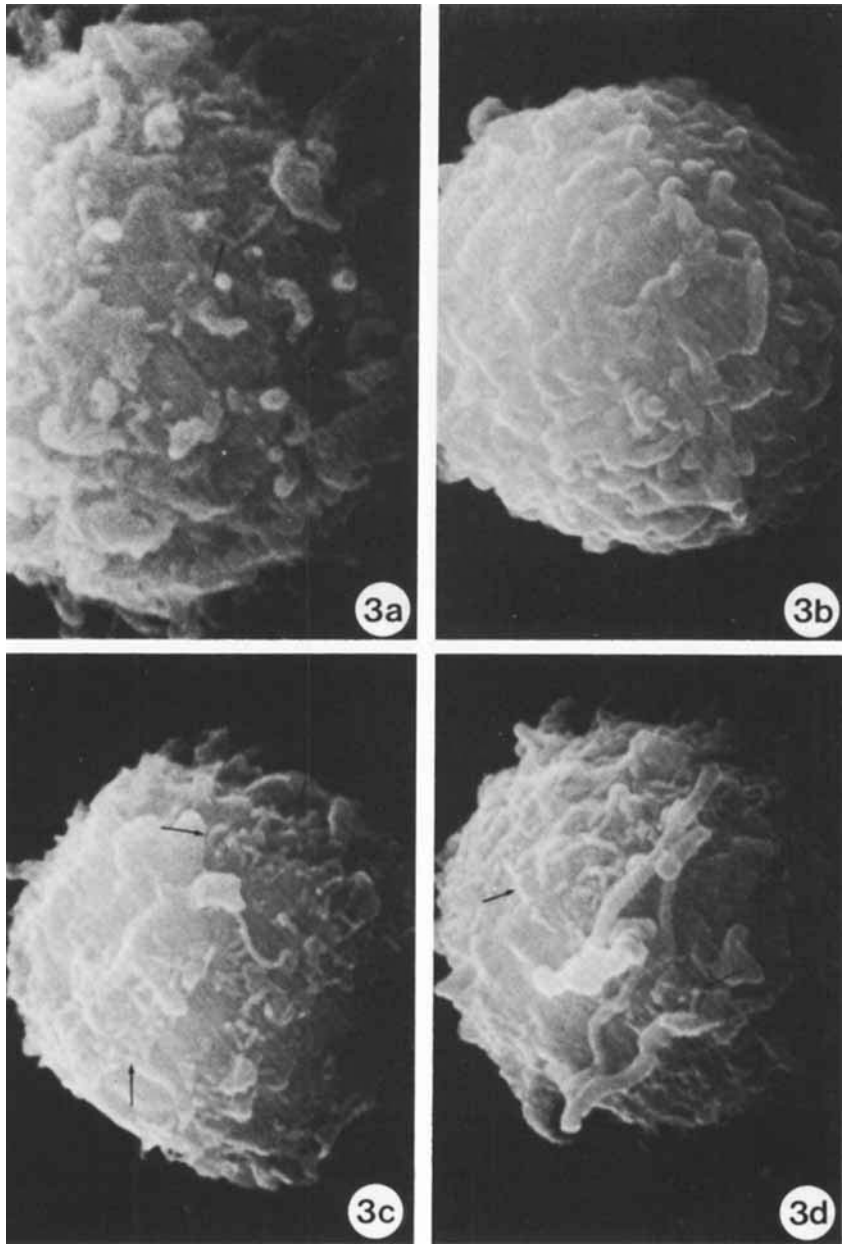

Fig. 3. SEM micrographs of spleen cells treated in suspension with lac-anti-MIg, anti-lac, and lac-TMV, and prepared for SEM on a glass coverslip (all are from one preparation; × 21,000): a) labeled B lymphocyte (TMV, arrows) with numerous microvilli (MV++); b) unlabeled spleen cell, without microvilli (MV−); c) labeled B lymphocyte (TMV, arrows), similar in topography to cell in Fig. 3b (MV−); note patched appearance of label; d) labeled B lymphocyte (TMV, arrows) with several microvilli (MV+); again note patched label. (1) Reprinted by copyright permission of the Rockefeller University Press.

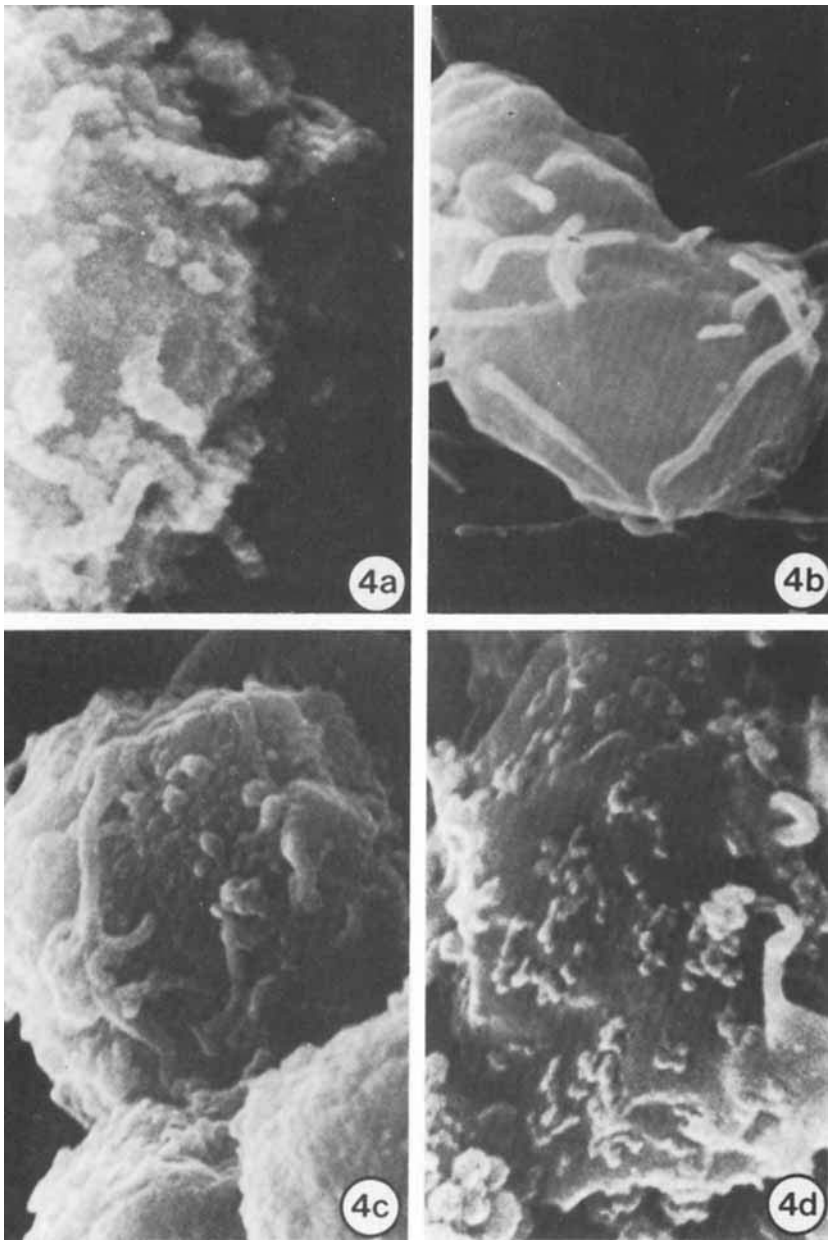

Fig. 4. Mouse cells labeled with lac-anti-MIg and three types of marker particles other than TMV: a) spleen cell labeled with anti-lac-KLH (note particularly on the microvilli) and prepared on glass (× 25,000): b) cell found adjacent to B cell in Fig. 4a, unlabeled with anti-lac-KLH (× 8,000); c) spleen cell treated further with anti-lac and lac-BSV (arrows), and prepared on a glass coverslip (× 14,000); d) lymph node cell treated further with anti-lac and lac-T4, and prepared in suspension (× 10,000).

In order to eliminate the possibility of topographical changes induced in the cells by the labeling procedure, spleen cells were labeled for MIg antigens either live or after a 5 min fixation in 1.8% formaldehyde in phosphate-buffered saline (19). By fluorescent microscopy, this fixation procedure inhibited redistribution or patching of MIg antigens without reducing the fluorescence intensity. MIg-bearing cells fixed before labeling for SEM were present in the same frequency, and were identical in their topography to live cells labeled and only then fixed in suspension.

Cells Bearing MBr Antigens

Cells representative of the four lymphoid tissues labeled for MIg were also labeled for the presence of MBr antigens (Table II). TMV was used as the marker in each experiment. Cells were prepared, as above, either in suspension at 0° or after contact upon glass for 1 hr at room temperature.

Lymphoid cells labeled for MBr antigens were almost identical in topography to cells labeled for MIg. The majority of MBr-bearing cells from spleen and bone marrow prepared in suspension at 0° were roughly spherical and devoid of microvilli, but with some irregular surface undulations (Figs. 5a, b). A small proportion of MBr+ spleen cells exhibited a few microvilli, but most cells with this topography were unlabeled (Fig. 5c). There were always numerous unlabeled cells identical in topography to cells labeled for MBr (Fig. 5d).

As described above for MIg+ cells, the frequency of MBr-bearing cells with microvilli increased upon cell preparation on glass. In such preparations there were labeled and unlabeled cells represented in each of the three topographical categories. Figure 6a shows two spleen cells, each with an intermediate number of microvilli; one cell is heavily labeled with TMV (Fig. 6b), the other is unlabeled. In the same preparation were MBr+ cells devoid of microvilli (Fig. 6c) and those with numerous microvilli (Fig. 6d).

DISCUSSION

These results indicate that all B (MIg+, MBr−) and all but a few T (MBr+, MIg−) cells prepared in suspension lack microvilli. A small proportion of cells, particularly in the peripheral lymphoid tissues (spleen and lymph node), exhibited microvilli in suspension, but this topography was not reflected in the constituent B and T cells. On the other hand, when lymphoid cell populations were allowed to make contact with a glass substrate, a higher frequency of cells with microvilli was observed in both B and T cell populations as well as in the whole lymphoid population. However, either in suspension or on glass, no unique topographic features of either cell type were observed that could serve to distinguish one from the other.

The initial reports of major topographic differences between B and T cells from humans (3, 4) and mice (4, 5) remain to be confirmed. In these publications, the topography of a lymphocyte type was determined indirectly, either by correlating the frequency of cells of a given SEM topography with the frequency of a given cell type in normal lymphoid populations or in populations enriched for B or T cells (3–5), or by examining cultured human lymphoblastic cell lines bearing specific receptors found on normal B or T cells (3). There is no evidence that such correlations are valid.

TABLE II. SEM Topography of MBr-Bearing Cells From Four Lymphoid Populations

No. of expts.	Cell source	Total MBr+	Topography[b] of: MBr+ cells MV++	MBr+ cells MV+	MBr+ cells MV−	MBr− cells MV++	MBr− cells MV+	MBr− cells MV−
	Prepared in suspension							
3	Spleen	19[a] (9–24)	< 1[a]	4 (0–12)	15 (9–24)	< 1	9 (5–13)	71 (56–86)
2	Bone marrow	2 (0–5)	< 1	<1 (0–1)	2 (0–4)	< 1	6 (2–10)	88 (85–91)
	Prepared on glass							
1	Spleen	62	9	37	16	17	15	6
1	Lymph node	50	25[c]		25	39[c]		11
1	Thymus	72	< 1	31	41	1	6	7
2	Bone marrow	< 1 (0–1)	< 1	< 1 (0–0.5)	< 1 (0–0.5)	55[c] (50–60)		38 (27–50)

[a]Percentage of total cells counted; scatter in parentheses.
[b]Cells that bear less than 2 (MV−), 2–20 (MV+), or over 20 microvilli (MV++); scatter in parentheses.
[c]Represents the sum of (MV++) + (MV+) frequencies.

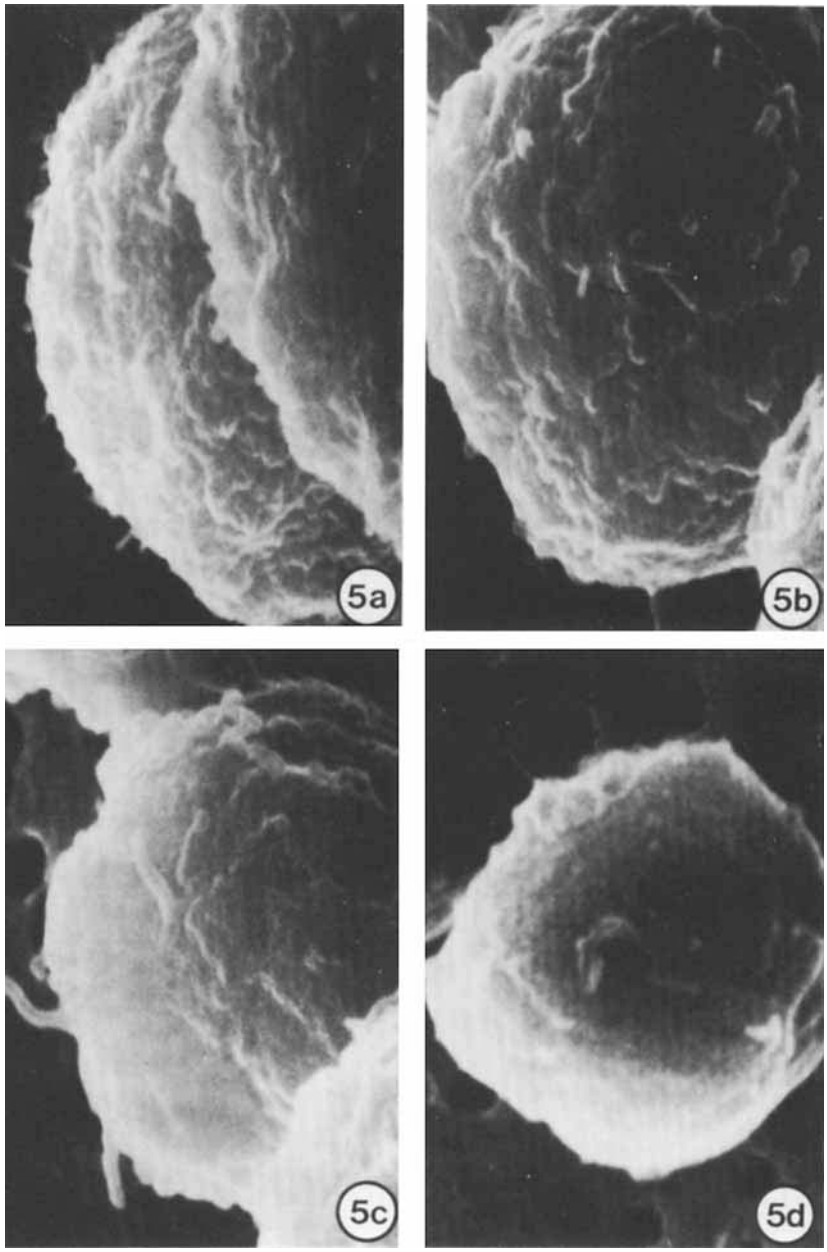

Fig. 5. SEM micrographs of spleen cells treated with lac-anti-MBr, anti-lac, and lac-TMV, and prepared for SEM in suspension (all are from one preparation): a) labeled T lymphocyte in background (TMV is seen well on cell periphery) without microvilli (MV−, × 22,000); b) labeled T lymphocyte (TMV, arrows) without microvilli (MV−, × 22,000); c) unlabeled cell with a few microvilli (MV+, × 22,000); d) unlabeled cell similar in topography to cells in Figs. 5a, b (MV−, × 18,000).

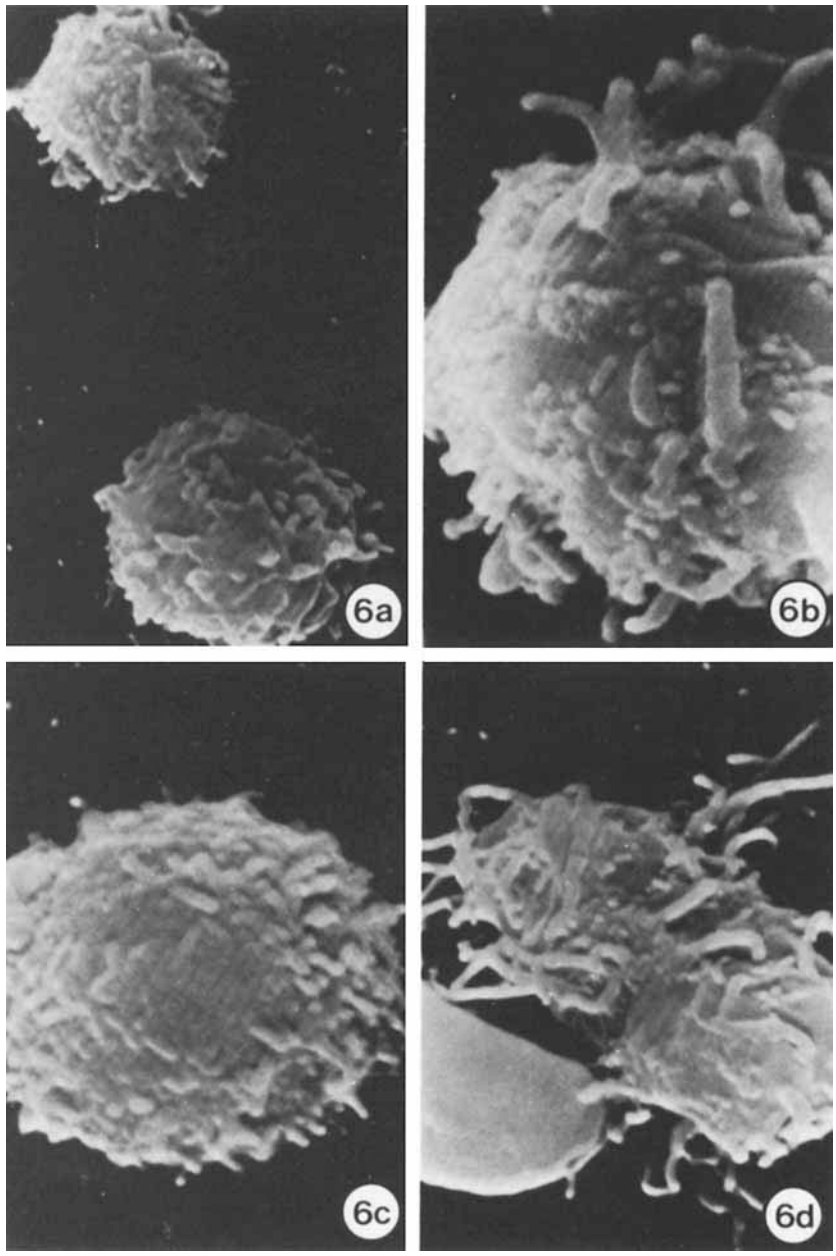

Fig. 6. SEM micrographs of spleen cells treated with ars(amid)anti-MBr, anti-ars, and ars-TMV; cells were prepared for SEM on glass (all are from one preparation): a) two cells, one labeled (upper cell), the other unlabeled; both have several microvilli (MV+, × 6,000); b) higher magnification of labeled cell in Fig. 6a; note patched appearance of label on cell surface (× 12,000); c) labeled T lymphocyte (TMV particles cover the cell surface) without microvilli (MV−, × 12,000); d) labeled T lymphocyte (TMV are primarily in the center of the cell surface) with numerous microvilli (MV++); note the unlabeled erythrocyte (× 7,000).

Only in subsequent reports from these investigators (20, 21) have conclusions been based upon the identification of lymphoid cells by specific markers in SEM. With such methods, more heterogeneity of topography was observed in B and T populations than had been found earlier (3–5). Obviously, the hope that lymphocyte types could be accurately discriminated on the sole basis of topography was too simplistic a notion.

Since the conditions under which cells are prepared appear to affect the appearance of cell populations in SEM (8, 22–24), we have examined lymphoid populations that have been exposed to a minimum of manipulation. It is likely that the early observations of Polliack et al. (3–5) were affected by the procedures used to enrich for a given cell type (24) or by the SEM specimen-preparation method (8). Antibody-mediated labeling of cells in SEM permits the identification of B and T lymphocytes within the original, unfractionated lymphoid population and can supply a less distorted view of cell topography, especially when employed after light fixation of the cells to be labeled (12, 19).

The labeling process caused no detectable alteration in the topography of labeled live B lymphocytes in suspension (19). Because of its bivalent nature, antibody can cross-link and redistribute cell surface antigens, as has been observed for the patching, capping, endocytosis and shedding of Ig integral to the plasma membranes of B cells (25). Topographic changes may accompany each of these antibody-induced phenomena. However, under the conditions used in this work for labeling cells (specifically, in the presence of 0.1% sodium azide), only patching of membrane antigens could occur (see Figs. 3c and 6b), which did not result in any detectable change in the appearance of the cells.

The method of dehydrating fixed cell suspensions in Nuclepore filter chambers (1) may induce artifacts in cell topography (19). Cells can be fixed in suspension and then allowed to attach to polylysine-treated coverslips (26) for SEM preparation. Our preliminary experiments with this method indicate that mouse spleen contains three times as many villous cells as were observed in cell suspensions prepared on Nuclepore filters. It has not yet been established whether polylysine-coated glass selectively retains villous cells. In any case, we have found that spleen cells labeled for MIg and prepared in such a manner are still 90% nonvillous in topography.

No determination was made in this study of how many cells of the original population placed into a filter chamber or onto a coverslip were recovered in the final SEM specimen. This important factor has been assessed and published in only one instance (27). Although the frequencies of lymphoid cells labeled in SEM reflect the frequencies labeled in fluorescence (19), there may be a selective loss of cells with a certain topography in filter chambers or from glass surfaces. In fact, the selective adherence of cells to a substrate is the basis of some techniques of separating cell types from lymphoid populations (28).

The alterations in topography of lymphoid cells in contact with glass are a striking consequence of the extracellular environment. The percentage of cells bearing microvilli in cell preparations allowed to settle upon glass coverslips was always greater than in cell populations fixed in suspension (Tables I, II). Similar changes in lymphoid cell shape upon attachment to glass (29) and to nylon wool (24) have been observed in SEM.

Microvilli may be one means by which both B and T lymphocytes make contact with cells and surfaces that they encounter. A recent SEM study of whole spleen and lymph node tissue (13) suggests that lymphocytes residing within a tissue lack microvilli,

but that the circulating lymphoid cells are villous to facilitate their contact with and movement across the endothelial barrier between the bloodstream and the lymphatic stroma. This hypothesis is supported by an analysis of repulsion barriers to cell adhesion, where it has been calculated that a plasma membrane projection of the diameter of a microvillus on a cell approaching another cell or surface of like charge (glass) would be most likely to make the initial contact (30). There are numerous additional instances where lymphocytes are seen to make contact with other cells by means of their microvilli (6, 20, 23, 31).

NOTE ADDED IN PROOF

The method of preparing prefixed cells for SEM on polylysine-treated coverslips discussed above has been described and elegantly quantitated by Sanders, et al. (32) and has been used to describe the topographic similarities between human B and T cells (33).

ACKNOWLEDGMENTS

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