THE UROPOD-BEARING LYMPHOCYTE OF THE GUINEA PIG

EVIDENCE FOR THYMIC ORIGIN

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Lymphocytes bearing the highly characteristic foot appendage, or uropod, have been described in small numbers in several different lymphoid populations from a variety of sources and species (1–4). Although lymphocytes with uropods remain a minor component of the total lymphoid population, their frequency can apparently be increased or "stimulated" in vitro by the mixed lymphocyte reaction (2) or by the mitogen, phytohemagglutinin (3, 4). Thus the possibility is raised that the uropod is a marker for "activated" lymphocytes. Alternatively the uropod may represent a region of membrane structural specificity limited to a distinct subset of lymphocytes.

Lymphocytes may be classified in a variety of ways. Lymphoid cells are divided into at least two functional subpopulations based upon their sites of differentiation. Thus the thymus-derived or "T" lymphocytes[1] appear to be responsible for cell-mediated immunity, and the bone marrow–derived or "B" lymphocytes are the apparent precursors of antibody-forming cells. These two populations of cells can be distinguished by the presence of easily detectable membrane immunoglobulin on the B lymphocyte (5), and by the presence of surface differentiation antigens on the T lymphocyte (6). T lymphocytes may be further subdivided by their ability to respond to antigen in vitro, and in the immunized guinea pig, there is clearly a hierarchy of antigen reactivity among different lymphoid subpopulations. Thus, peritoneal exudate lymphocytes are much more reactive to a variety of soluble protein antigens than those derived from draining lymph nodes (7), and thymocytes respond poorly to these antigens, if at all (8).

In order to study the relationship between immunocompetence and uropod formation, lymphocytes from the thymus, draining lymph node, and peritoneal exudate of immunized guinea pigs were obtained and the number of uropod-bearing lymphocytes determined with both light and electron microscope techniques immediately after isolation and before any in vitro antigenic

[1] *Abbreviations used in this paper:* B lymphocytes, bone marrow–derived lymphocytes; BSA, bovine serum albumin; FCS, fetal calf serum; MEM, Eagle's minimal essential medium; PELS, peritoneal exudate lymphocytes; PPD, purified protein derivative; TdR-^{3}H, tritiated thymidine; T lymphocytes, thymus-derived lymphocytes.

stimulation. Antigen reactivity was then subsequently determined in each population using the in vitro lymphocyte proliferation assay. Finally, to determine if uropods were found on B cells, T cells, or on both types of lymphocytes, lymphocyte populations containing both B and T cells were studied using an anti-immunoglobulin reagent as a marker for B cells and were simultaneously observed for uropod formation.

We have found that in the lymphoid populations studied there exists a general correlation between the ability to respond to antigen in vitro and uropod formation. Furthermore, in the guinea pig, all those lymphocytes that are spontaneously forming uropods do not bear easily detectable surface membrane immunoglobulin and thus appear to be thymus-dependent or T cells.

Materials and Methods

Strain 2 female guinea pigs were immunized in the four footpads with complete Freund's adjuvant containing 2 mg/ml of *Mycobacterium tuberculosis* H37Ra. 2–4 wk later, 25 ml of sterile mineral oil (Marcol 52, Humble Oil & Refining, Co., Houston, Tex.) was injected intraperitoneally, and 3 days later thymus, draining lymph nodes, and the peritoneal exudate were harvested. Peritoneal exudate lymphocytes were separated from contaminating neutrophiles and macrophages by using an adherence column of nylon fiber (Fenwal Labs., Morton Grove, Ill.) combined with fine glass beads (type 100–5005, Minnesota Mining & Manufacturing Co., St. Paul, Minn.), as previously described (10). The resulting cell population contained more than 95% lymphocytes with a viability of greater than 98% as determined by trypan blue exclusion. Thymus and lymph nodes were trimmed of fat, and single cell suspensions were prepared by teasing with needle and forceps. When indicated in the protocol, thymus and lymph node cells were also purified by passage over columns of nylon and glass beads. Techniques of immunization, cell preparation, and column purification have been described in detail elsewhere (7, 9, 10). After isolation, the cell populations were suspended in Eagle's minimal essential media (MEM, Spinner modification) with 10% fetal calf serum (FCS) and incubated at 37°C for 30 min. Living cell suspensions were examined using Nomarski interference contrast optics on a Zeiss microscope (Carl Zeiss, Oberkochen, West Germany), and uropod-bearing lymphocytes were quantitated. Since uropod formation is temperature dependent, all preparations were allowed to equilibrate to 37°C for 15 min before counting, and during the period of microscope examination, the cells were maintained at 37°C with a Sage air-curtain incubator (Sage Equipment Co., Div. of Orion Research Inc., Cambridge, Mass.). For electron microscope studies, cell suspensions were incubated at either 4° or 37°C for 30 min, fixed in 1% glutaraldehyde for 30 min, processed, and embedded in Maraglas (Polysciences, Inc. Warrington, Pa.) for examination in a Philips 300 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.).

For simultaneous evaluation of immunoglobulin-bearing cells and the presence or absence of uropods, whole unfractionated lymph node cells, which contain both B and T cells (9), were incubated in serum-free media, at 4°C for 30 min with a fluoresceinated rabbit anti-guinea pig Fab antiserum (obtained from Dr. Victor Nussenzweig, New York University School of Medicine) (23). This procedure reproducibly stains approximately 30% of lymph node cells with a characteristic "speckled" pattern (25). The cell suspensions were washed twice, resuspended in media with 10% FCS, allowed to equilibrate to 37°C for 15 min, and then maintained at 37°C with the air-curtain incubator. Using a Leitz Ortholux microscope (E. Leitz, Inc., Rockleigh, N. J.), uropod-bearing lymphocytes were first identified with tungsten illumination, then the ultraviolet light was switched on and the presence or absence of fluorescence of these uropod-bearing lymphocytes was determined.

To determine the degree of antigen-mediated in vitro lymphocyte proliferation in different lymphoid populations, cells from different sources were suspended in Spinner's MEM containing 10% strain 2 guinea pig serum plus added glutamine, sodium pyruvate, nonessential amino acids, and penicillin (7). Triplicate tubes of 3×10^6 cells/tube were cultured for 72 hr, with or without 10 μg/ml purified protein derivative (PPD) (Connaught Medical Research Labs, Willowdale, Ontario, Canada), and then were pulsed with 3.0 μCi of tritiated thymidine (TdR-^{3}H, 6.7 Ci/mM, New England Nuclear Corp., Boston, Mass.) for 4 hr and trichloroacetic acid–precipitable radioactivity was determined, as previously described (7). Stimulation is expressed as the ratio of total incorporated counts in antigen-stimulated cultures compared with total incorporated counts in control cultures.

RESULTS

Morphological Observations of Uropod-Bearing Lymphocytes of the Guinea Pig: Ultrastructural Features.—A thin section profile of a typical uropod-bearing lymphocyte is shown in Fig. 1. The uropod is most easily recognized and clearly identified by electron microscopy. Its most characteristic features are the numerous terminal finger-like projections, the microspikes (11). The uropod itself contains occasional mitochondria and more typically, numerous vacuoles of various sizes and a well-developed system of microtubules and microfilaments. Its location on the cell is usually opposite the nuclear hof, and the cytoplasmic region with which it is immediately contiguous typically contains a multitude of cell organelles, including mitochondria, microtubules, a centriole, numerous vacuoles, and the Golgi apparatus. In contrast, pseudopods, which are the means by which the cell moves, generally contain few cell organelles. The small pseudopod-like structure seen in Fig. 1 contains only cytoplasm and numerous free ribosomes. The uropod ultrastructure described here in freshly isolated guinea pig lymphocytes is similar to that described previously in human lymphocytes stimulated by phytohemagglutinin (4) and by the mixed lymphocyte reaction in humans (11, 12).

Light Microscope Observations using the Nomarski Interference Optical System.—Immediately after transfer to the slides, uropod formation in the cell suspensions was minimal. However, after 15 min under the warm stage, the lymphocytes were actively motile and forming uropods. Once equilibrated, (10–15 min) the percentage of uropod-bearing lymphocytes in individual populations remained stable for up to 180 min.

Uropod formation was a rapid and dynamic process. Lymphocytes were observed to extend or retract their uropods, in as little as 60 sec. Many uropod-forming lymphocytes were also actively motile and had a highly irregular shape which was accentuated by pseudopod formation (Fig. 2 *c*). Nevertheless, some lymphocytes formed uropods although they did not appear to be actively motile, did not have pseudopods, and had the round configuration of resting cells.

The advantage of the Nomarski system over conventional phase-contrast microscopy for observations of living cells is that it clearly delineates both

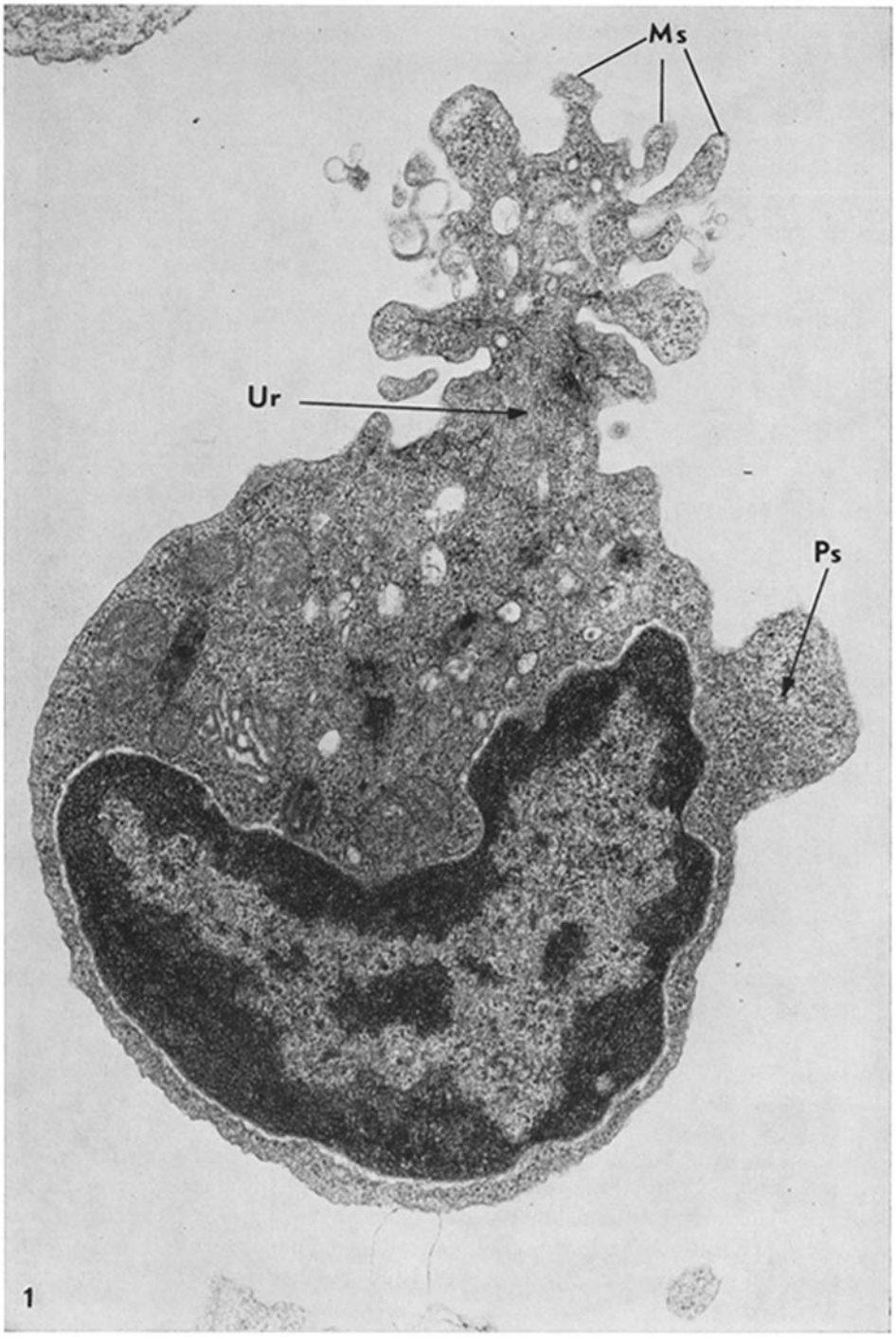

FIG. 1. Electron micrograph of a peritoneal exudate lymphocyte after equilibration at 37°C for 30 min. The cell uropod (*Ur*) is characterized by terminal microspikes (*Ms*) and content of vesicles. The small pseudopod (*Ps*) contains only ribosomes. × 28,000.

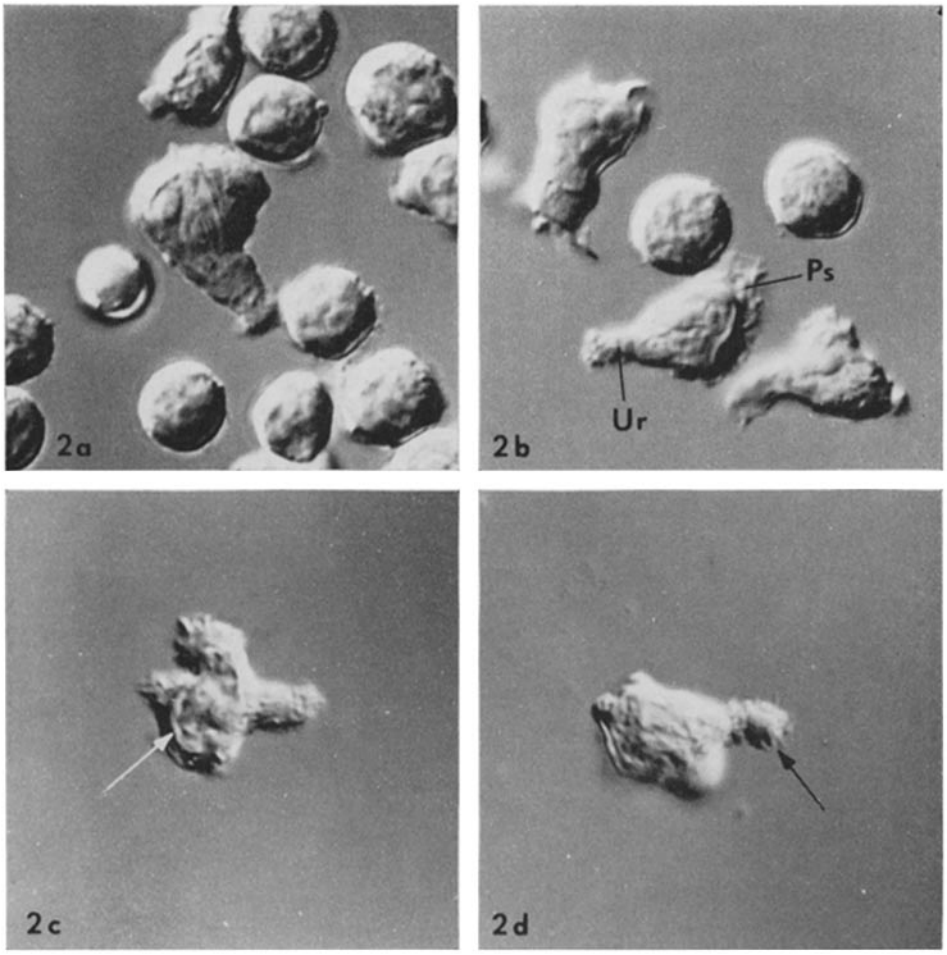

FIG. 2. Lymphocytes derived from either peritoneal exudate or lymph node, fixed after equilibration at 37°C for 30 min, and photographed using Nomarski interference contrast optics. × 1650. (*a*) Lymph node cell suspension with a large uropod-bearing lymphocyte in the center. (*b*) Peritoneal exudate lymphocytes. Note the fine-veil like pseudopod (*Ps*) and the thicker uropod (*Ur*). (*c*) Uropod-bearing lymphocyte with a typical highly irregular shape. The *arrow* points to the clearly visible nuclear rim. (*d*) Uropod-bearing lymphocyte illustrating the terminal microspikes (*arrow*).

surface structures as well as intracellular elements with good resolution. However, photographic resolution is poor because of the extremely shallow depth of focus, cell motion, and the greatly diminished transmitted light inherent in this optical system. To abolish the variable of cell motility, cells were fixed in 1% glutaraldehyde before photographing.

Several uropod-bearing lymphocytes as photographed using Nomarski optics are illustrated in Fig. 2. Terminal microspikes appear here as either small bumps or fine filaments covering the end of the uropod (Fig. 2 *d*). The uropod itself has a thickness equal to that of the cell body. In contrast, the cell pseudopod presents a thin, more veil-like appearance (Fig. 2 *b*). The outline of the nuclear rim is also visible by this technique, and the small nuclear indentation that is characteristic of many of these cells can also be seen (Fig. 2 *c*). Monocytes are distinguishable from lymphocytes by their large size, numerous long surface microvilli, and a characteristic heavily cratered surface that we have not observed with any other cell type. The intracellular granules and multilobed nuclei of neutrophiles make this cell type also easy to identify.

Quantitation of Uropod-Bearing Lymphocytes in Thymus, Lymph Node, and Peritoneal Exudate Populations.—After incubation at 37°C for 30 min, cell suspensions were mounted on warm cover slips and slides and allowed to equilibrate for 15 min on the warm stage before counting. Thus, the maximum time in culture after isolation and before counting was less than 120 min. Each cell was focused on individually in order to check for uropods that were out of the plane of focus, however, the total time of observation of each cell was generally less than 30 sec, and no attempt was made to observe uropod-negative lymphocytes for longer periods to see if they would become motile and form uropods. At least 300 cells were examined per slide.

Peritoneal exudate lymphocytes clearly contain the highest percentage of uropods (Table I). In counting lymph node cells, only viable cells were counted since up to 30% of these cells may be dead or damaged (7). Nevertheless, there was significantly less uropod formation in lymph node cells than in peritoneal exudate lymphocytes (PELS). Thymus cells had the fewest uropod-bearing lymphocytes.

Since PELS are prepared by a column adherence procedure, both the thymus and lymph node cell preparations were also column purified and the percentage of uropods determined. Column purification of lymph node cells more than doubles the percentage of uropod-bearing lymphocytes (Table I). However, similar column treatment of thymus cells has no effect at all on uropod formation suggesting that the increase in frequency of uropod-bearing cells seen with lymph node cells is the result of the removal of a nonuropod-bearing cell (see below) and is not the result of nonspecific artifacts introduced by the column-purification procedure.

In our observations on these living cell preparations, we have not observed uropod formation by monocytes or macrophages confirming the work of other investigators (13). Monocytes exhibit extensive pseudopod formation, especially as the cells begin to adhere to the glass, but these pseudopods can be differentiated from uropods by the absence of microspikes and their thin veil-like appearance. Polymorphonuclear leukocytes, on the other hand, have been occasionally seen to form what appear to be uropod-like structures. However,

neutrophiles are a significant contaminant only in the PEL preparation and can be identified by their typical granules and nuclear structure.

Comparison of PPD-Induced Lymphocyte Proliferation of Thymus, Lymph Node, and Peritoneal Exudate Lymphocytes.—In order to determine if there was any correlation between the percentage of uropod-bearing lymphocytes in a given population, and its subsequent ability to respond to antigen in vitro, the

TABLE I

Quantitation of Uropod-Bearing Lymphocytes from Guinea Pig Lymph Node, Peritoneal Exudate, and Thymus

Column purification	% Uropod-bearing lymphocytes*		
	Thymus	Lymph node	PEL
No	3.2 (1–6)	7.3 (3–12)	—
Yes	3.8 (2–6)	15.2 (7–24)	36.7 (15–57)

* Uropod counts were performed on living cells maintained at 37°C and observed using Nomarski interference contrast optics. In each experiment, at least 300 cells were examined from each population. Results are the geometric means of at least four experiments, expressed as the mean and the range.

TABLE II

Comparative In Vitro Lymphocyte Proliferation of Thymus, Lymph Node, and Peritoneal Exudate Lymphocytes Induced by PPD

	Stimulation ratio (S/C)*		
	Thymus	Lymph node	PEL
Immune‡	1.8 ± 0.4	4.7 ± 1.3	36.1 ± 6.3
Nonimmune	1.3 ± 0.4	1.1 ± 0.2§	1.0 ± 0.5

* S/C is the ratio of total incorporated counts of Tdr-^{3}H in antigen-stimulated cultures to the total incorporated counts in control cultures. Results are expressed as the arithmetic mean ± the standard error of the mean of three experiments.

‡ Immune refers to immunization history (complete Freund's adjuvant) of guinea pigs from which cells were derived. Nonimmune animals had no prior immunization.

§ Lymph node cells from nonimmunized animals were derived from the normally large anterior cervical lymph nodes.

relative antigen reactivity of these populations was tested using the antigen-induced lymphocyte proliferation assay.

In confirmation of our previous studies with other antigens, in vitro lymphocyte proliferation induced by PPD is clearly highest in PELS. Lymph node cells exhibit a small but significant proliferative response, but the response of thymus cells to PPD is not significantly greater than that of control cultures (Table II). This preparation of PPD produces no nonspecific stimulation, as is shown by the absence of stimulation when these cell populations are derived from normal

nonimmunized animals, or from animals given only incomplete adjuvant and saline.

Simultaneous Observations of Cells Bearing Immunoglobulin and Cells Forming Uropods.—Adherence columns have been found to preferentially remove B cells (9, 10, 14–16). Therefore, the finding of an increased percentage of uropod-bearing cells after passage of lymph node lymphocytes over an adherence

TABLE III

*Surface Immunoglobulin on Uropod-Bearing Lymph Node Lymphocytes**

Experiment number	No. of uropod-positive lymphocytes with surface immunoglobulin present*
1	0/18
2	0/20
3	1/25

* Number of cells with surface immunoglobulin over the total number of uropod-bearing lymphocytes counted. The percentage of uropod-positive lymphocytes in each experiment ranged from 8 to 11%, and the percentage of surface immunoglobulin positive cells ranged from 25 to 30%.

TABLE IV

Lack of Uropod Formation by a B Cell–Enriched Lymph Node Lymphocyte Population

	Original lymph node lymphocytes*	B cell-enriched lymph node lymphocytes‡
Total number of cells positive for surface immunoglobulin	30/112	59/66
Uropod-positive cells with positive surface immunoglobulin	0/17	0/21

* Number of cells positive or negative over total number of cells counted. Results are the arithmetic mean of two separate experiments.

‡ B cell–enriched population was prepared by killing T cells with a heterologous antithymus-derived lymphocyte antiserum, then removing dead cells over a BSA gradient (18). The resulting population had a viability of greater than 90% by dye exclusion.

column suggested that the uropod was a structure found mainly on T cells. The direct simultaneous evaluation of lymph node cell populations for those cells bearing easily detectable immunoglobulin and those cells forming uropods demonstrated in three separate experiments that cells which formed uropods did not have immunoglobulin on their surface (Table III). In another series of experiments the percentage of B cells in the lymph node cell population was enriched by killing the majority of T cells with a heterologous antithymus-derived cell antiserum (17) and then removing dead cells by centrifugation over a bovine serum albumin (BSA) gradient (18). Although approximately 90% of the cells in these enriched populations were positive for surface membrane immunoglobulin, all of the uropod-forming cells were negative for surface

fluorescence (Table IV). It was sometimes noted, that in those cell preparations that had been over BSA gradients, a tiny speck of fluorescence was present at the end of the uropod. The appearance of this fluorescence was entirely different from the speckled fluorescence or the rare cap-type of fluorescence noted over B lymphocytes. Thus, it appears that in a mixed population of B and T cells, all the lymphocytes that are spontaneously forming uropods are T cells by the criteria of absence of easily detectable surface membrane immunoglobulin.

DISCUSSION

Previous studies pertaining to the lymphocyte uropod have emphasized stimulation of uropod formation in vitro by a variety of techniques, while noting the relatively low frequency of lymphocytes with uropods in unstimulated populations. In this study, we have shown that the rate of spontaneous uropod formation is very high in some guinea pig lymphoid subpopulations before any in vitro stimulation. Furthermore, we have also demonstrated that lymphocyte populations differ greatly in their percentage of uropod-forming cells. Thus, at any given time an average of 36% of PELS bear uropods, while only 3.8% of thymocytes have these structures. Also, we have found that there is a general correlation between the frequency of uropod formation and the ability of a given population to respond in vitro to antigen.

Peritoneal exudate lymphocytes, which have been found to have both the highest percentage of uropod-bearing cells and the greatest response to antigen, have been shown to possess several unusual features. It has been found that these cells are a highly selected population of lymphocytes that have been generated in response to a new antigenic challenge and preferentially sequestered in the inflammatory exudate (19, 20). It is very possible then, that the uropod-bearing lymphocyte of the guinea pig, both in the peritoneal exudate and in the lymph node, is that cell that has been recently activated by antigen in vivo.

The low percentage of uropod-bearing cells observed in the thymus may be due to a general unresponsiveness of thymus cells to antigenic stimuli (8), or to the fact that there is little antigenic stimulation in vivo of the thymus since antigens do not easily reach this organ when administered at distant sites (21). Alternatively, uropod formation may be a property restricted to differentiated or mature lymphocytes, and the low frequency of uropod formation of thymocytes may be related to their immaturity. Finally, it appears that uropod formation is not merely the general response of lymphocytes that are rapidly dividing, since the mitotic index in the thymus is very high although the frequency of uropod formation is low (22).

Our findings indicate that only thymus-derived or T lymphocytes spontaneously form uropods. First, PELS are greater than 98% pure T cells by the criteria of absent surface immunoglobulin, the absence of antibody-producing

cells, and lack of antigen-binding cells, and by the same criteria, 90% of column-purified lymph node lymphocytes are also T cells (9, 10). Both these cell populations are rich in uropod-bearing cells. Second, we have found that passage of lymph node lymphocytes over an adherence column, which removes B cells, significantly increases the percentage of uropod-bearing cells. If both B and T cells were uropod forming, no such increase in frequency after column purification would have been anticipated. Finally, since easily detectable surface membrane immunoglobulin is the hallmark of the B cell, the simultaneously made observation that no uropod-bearing cell had surface immunoglobulin supports the conclusion that in the guinea pig, only T cells spontaneously form uropods.

Recently, Taylor and his coworkers have shown that in the mouse, reaction of "B" cells with a specific anti-immunoglobulin reagent resulted in redistribution of the surface immunoglobulin so that it was concentrated over one pole of the cell forming a fluorescent "cap" (24). Furthermore, they noted that after several hours at 37°C, surface immunoglobulin could no longer be detected on cells that had previously borne this marker. However, we do not feel that the uropod-bearing lymphocytes that we have observed in the guinea pig are merely B cells that have lost their surface immunoglobulin due to treatment with the anti-Fab reagent. In our system, less than 5% of B cells have an immunoglobulin cap, and even after incubation at 37°C for periods as long as 3 hr, we have not detected an increased frequency of cells with caps (25). Furthermore, it is not likely that B cell uropod formation was inhibited by the presence of the fluoresceinated anti-Fab antibody on the cell surface. Work with amebae has shown that membrane motility and uropod formation proceed normally in the presence of large amounts of fluoresceinated antibody on the cell membrane (26). In addition, in lymphocytes that have been stained with a fluoresceinated anti-histocompatibility antibody which binds to both T and B cells, uropod formation is normal (Rosenstreich, D., and E. Shevach, unpublished observation).

The uropod-bearing lymphocyte and the uropod structure itself have been found to possess several properties of possible significance in immunological reactions. It has been shown that the uropod is the structure most intimately involved in lymphocyte-to-lymphocyte and lymphocyte-to-macrophage interactions (2, 11–13). Cytotoxicity mediated by immune lymphocytes has also been found to involve direct contact between a lymphocyte and target cell, often in the region of the uropod (3). Since thymus-dependent lymphocytes appear to lack easily detectable surface antigen receptors, it may be that another mechanism of interaction with antigen exists in these cells. Indeed Biberfeld demonstrated that antigen molecules such as ferritin are taken up by the lymphocyte uropod (4). It is therefore possible that antigen binding and recognition by T cells is mediated through the uptake and concentration of antigen in their uropod region. Further characterization of the nature of the

uropod-bearing lymphocyte may help to elucidate the mechanisms of cellular cooperation and the relationship between cell structure and function in immunological reactions.

SUMMARY

In this study, the frequency of uropod formation and the type of lymphocyte bearing the uropod was investigated in various guinea pig lymphocyte populations. Without prior in vitro stimulation, almost 40% of peritoneal exudate lymphocytes (PELS) form uropods, while thymocytes and lymph node cells form far fewer. Subsequent stimulation in vitro with purified protein derivative demonstrated that there is an association between antigen reactivity and frequency of uropod formation in these populations. The ultrastructure of these uropods is identical to that described for human lymphocytes stimulated with phytohemagglutinin. In the populations studied, all the lymphocytes forming uropods lack easily detectable surface membrane immunoglobulin and are therefore most likely thymus-derived or T lymphocytes.

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