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Anchorage and lymphocyte function. Spreading-capacity distinguishes common thymocytes and peripheral T lymphocytes

P. OTTESKOG* & K.-G. SUNDQVIST† *Department of Clinical Immunology*† and Department of Periodontology*, Karolinska Institutet, Huddinge Hospital, Sweden*

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Summary. Contact of T-enriched human blood lymphocytes with an adhesive surface in the presence of Concanavalin A (Con A) almost immediately induced a sequence of motile changes in virtually all cells. The initial event in this spreading process was the formation of filopodia distinct from the microvilli of lymphocytes in suspension. The filopodia were accompanied by lamellipodia, ruffles and flattening of the nucleus. *Contact with a nonadhesive substratum in the presence of Con A did not trigger this sequence of changes.* Cytochalasin B and D or low temperature inhibited the contact-induced changes. With the exception of a small number of cells (5–15%), T-enriched lymphocytes that were allowed to settle in the absence of Con A showed a radius of action (area occupied by the cells/translational movement per hr) of $39\mu m^2/<1\mu m$. The small 'motile' population showed a radius of action of 74 $\mu m^2/8$ μm. The Con-A-mediated spreading-process yielded a radius of action of the lymphocytes of 117 $\mu m^2/6$ μm. This augmented radius of action markedly facilitated cell–cell interaction in a high frequency of the cells and appeared to be a prerequisite for such interactions at 'low' cell density. Thymocytes reactive with OKT 6 antibodies or belonging to the 'high-density' fraction of cells attached to a Con-A-coated surface to the same extent as peripheral OKT 3 positive lymphocytes, but did not exhibit the morphological changes characteristic of a spreading-process. In contrast, OKT 6 negative thymocytes or thymocytes with a relatively low density showed spreading indistinguishable from that of OKT 3 positive peripheral lymphocytes.

These results characterize the spreading-process in human T lymphocytes and demonstrate its functional importance for interactions with the environment. Spreading-capacity appears to reflect the stage of maturation of T cells.

Correspondence: Dr Karl-Gösta Sundqvist, Department of Clinical Immunology, Huddinge University Hospital, S-141 86 Huddinge, Sweden.

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INTRODUCTION

The interaction of lymphocytes with surfaces of other cells and non-cellular structures is a fundamental function necessary for the induction of immunity, homing and the expression of cell-mediated effector functions. Very little is known about the ways in which lymphocytes explore the surface of other cells or penetrate into tissues. Unlike other leukocytes, which readily adhere to serum-coated glass or plastic surfaces, lymphocytes show low adhesiveness and little or no locomotion when allowed to settle. It is known that stimulation by mitogens, allogeneic cells or exposure to anti-immunoglobulin (Ig) antibody or serum albumin, or even incubation in the absence of mitogen for a

few days, enhance lymphocyte motility (Schreiner & Unanue, 1976; Wilkinson, Parrot, Russell & Sless, 1977; O'Neill & Parrot, 1977; Chang, Celis, Eisen & Solomon, 1979). However, the mechanism of these effects is unknown and the number of motile cells is limited; e.g. 10–20% of human lymphocytes showed a motile shape during phytohaemagglutinin stimulation (Biberfeld, 1971), and the increase in motile forms caused by anti-Ig over the 'spontaneous motility' was approximately 15%. In order to analyse the interaction between lymphocytes and their environment we have studied the influence of contact with a solid surface on the behaviour of this cell (Sundqvist, Otteskog, Wanger, Thorstensson & Utter, 1980; Sundqvist & Wanger, 1980; Sundqvist & Wanger, 1981). These experiments showed that appropriate lectin-mediated contact with adhesive surfaces triggers a spreading-process in the vast majority of all peripheral lymphocytes (Sundqvist *et al.*, 1980) and indicated that anchorage to a surface did not impair lymphocyte function (Sundqvist & Wanger, 1980; Sundqvist & Wanger, 1981). Spreading is probably not essentially different from locomotion (Grinnel, 1978; Bessis & de Boisfleury, 1976). The present study is an attempt to characterize in detail the initial contact-induced motile response of primate T lymphocytes and possibly relate it to their stage of maturation. Therefore we have utilized thymocytes and peripheral T lymphocytes from human and monkey and compared their behaviour when allowed to settle on Concanavalin-A-(Con A) coated substratum. Thymus contains functionally immature cortex thymocytes and relatively mature medullary cells (Blomgren & Andersson, 1970; Papiernik, Laroche & Bach, 1977; Goust & Perry, 1981; Waldman, 1978). To distinguish different subsets of thymocytes/T lymphocytes, monoclonal antibodies (Reinhertz & Schlossman, 1981) and cell separation methods (Goust & Perry, 1981) have been used.

MATERIALS AND METHODS

Cells

Lymphocytes were obtained from peripheral blood of healthy human donors. The mononuclear cells were isolated by sedimentation in gelatin followed by iron powder treatment (Natvig, Perlman & Wigzell, 1976). Thymic cells, from children undergoing cardiac surgery for cardiovascular malformations or from Macaca cynomolgus monkeys, were obtained by mincing the tissues. Adherent cells were removed by incubation in tissue culture flasks in RPMI medium with 10% foetal calf serum (FCS). T-lymphocyte enriched cells were obtained by using sheep erythrocyte rosette sedimentation (E-rosetting) (Natvig *et al.*, 1976). These cells comprising 87–96% sheep erythrocyte rosette-forming cells were used in the experiments performed to study spreading. Erythrocytes were lysed by addition of 0·83% NH_4Cl. The cells were cultivated ($1{\cdot}5 \times 10^6$/culture) in RPMI 1640 medium with 10% FCS in Falcon 1008 Petri dishes. The number of spontaneously adherent cells and cells reactive with OKM 1 antibodies (ORTHO) was generally less than 1%.

Separation of thymocytes on Percoll gradient

Thymocytes were separated on a discontinuous gradient of Percoll as previously described (Goust & Perry, 1981). The cells were layered on top of a gradient of 90%, 80%, 70%, 60%, 50% and 40% Percoll and centrifuged for 25 min at 600*g* at room temperature. In a separate tube, density marker beads (Pharmacia, Uppsala, Sweden) were added and centrifuged simultaneously. Each layer was harvested from the top of the gradient and washed twice before study of spreading capacity.

Spreading

In order to induce spreading, T-lymphocyte enriched cells or thymocytes were allowed to settle on glass cover slips in RPMI medium containing 10% FCS and Con A 100, 50 and 25 μg/ml at 37°. Alternatively the glass cover slips were incubated with Con A 100 μg/ml for 60 min at 37° and subsequently washed in phosphate-buffered saline (PBS). The cells were then induced to spread on the Con-A-coated surface in medium without Con A. In some experiments cytochalasin B or D (Sigma) was present during the induction of spreading. Poly-Hema substratum was prepared as previously described (Sundqvist *et al.*, 1980).

Immunofluorescence (IF)

One million cells were reacted with 50μl of monoclonal mouse anti-human T-cell antibodies (OKT3, OKT6 and OKM1 from Ortho Pharmaceuticals, Raritan, NJ) for 30 min at 4°. The cells were then induced to spread in the presence of Con A for 60 min at 37° and fixed in 4% paraformaldehyde for 10 min at 4°. Thereafter the cells were reacted with fluorescein isothiocyanate (FITC) conjugated sheep anti-mouse immunoglobulin (SBL, 111 610. F/P molar ratio 3·5,

protein concentration 4·8 mg/ml) diluted 1/10. The morphology and IF staining of the cells were examined simultaneously in a Leitz Orthoplan fluorescence microscope with interference contrast equipment and Ploemopak containing filters for FITC. Photographic recordings were made on Kodak Tri-X and Echtachrome 400 films.

Scanning Electron Microscopy (SEM)

One million T-enriched cells suspended in 0·3 ml of RPMI medium were fixed by addition of an excess of 2·5% glutaraldehyde (in PBS). These cells were referred to as lymphocytes in suspension. After incubation in glutaraldehyde for at least 60 min at room temperature, the cells were attached to glass cover slips preincubated with an aqueous solution of poly-l-lysine (Sigma Chemicals) at a concentration of 1 mg/ml. Cells incubated on glass in the absence and presence of Con A were fixed *in situ* after removal of the medium by addition of an excess of 2·5% glutaraldehyde. The fixed lymphocytes were rinsed in distilled water and dehydrated in a series of acetone dilutions followed by incubation in absolute acetone and critical point drying. The preparations were covered with gold (70Å) using a Polaron gold sputter and then examined in a Philips S501 scanning electron microscope (grid voltage 15 kV).

All figures of cell diameters and length of microvilli presented in 'Results' are based on data obtained by SEM and therefore generally lower than those obtained from living cells (for discussion see Newell, 1980).

Live-cell microscopy

The behaviour of lymphocytes on substratrum was observed continuously in microscopes with phase contrast equipment or interference contrast optics according to Normarski. The cells were allowed to settle in petri dishes or falcon bottles placed in a room with adjustable temperature which was kept at 37°. Photographic exposures were made every second minute. The films were analysed by projection on a screen. The tracks of individual lymphocytes were depicted with the advancing lamellipodium as reference point. The total length of an isolated lymphocyte path was determined as the mean of three independent measurements. The area occupied by the lymphocytes settled on substratum was calculated from interference contrast pictures. The weight of the area on the photographs corresponding to individual lymphocytes was determined and related to the weight of a standard area.

Terminology

Short cylindrical and fingerlike extensions of the lymphocyte surface of uniform diameter and length 0·2 to 1 μm have been termed microvilli. Broad-based extensions with a length exceeding 1 μm were termed filopodia. Flattened lamellar projections were termed lamellipodia. Broad-based undulating lamellar extensions have been termed ruffles.

RESULTS

Morphology of thymocytes and peripheral T lymphocytes in suspension and after contact with a glass surface

Thymocytes from both human and monkey, when fixed in suspension, consisted of one major morphology type comprising 76–90% of the cells obtained from six separate individuals. These cells were almost devoid of microvilli and had a mean diameter of 4·2 μm (3·7–4·5) (Fig. 1a). The rest of the cells (10–24%) carried microvilli in a number similar to or slightly lower than that of blood lymphocytes (Fig. 1b). The mean diameter of T-enriched blood lymphocytes fixed in suspension was 4·6 μm (4–5·1) and 84–97% of these cells (variation in seven individuals) carried microvilli with a length of 0·1–0·7 μm. The rest of the cells (5–16%) carried few or no microvilli but showed ruffles or ridges.

Both thymocytes and T-enriched blood lymphocytes that were allowed to settle on a glass surface for 60 min were generally round with a surface morphology indistinguishable from that of cells in suspension. However, approximately 10% of the peripheral T cells showed a non-spherical shape. Examination of the surface of peripheral T cells in close contact with the substratum revealed villus-like projections attached to the substratum with a length of 1–2 μm in 63–89% of the cells obtained from separate individuals (Fig. 1c). A fraction of the settled peripheral T cells (25–65%) exhibited more prominent cylindrical projections with a length of 1–5 μm. Thymocytes settled on the glass surface generally did not exhibit any substratum-attached projections.

Lymphocyte-morphology in relation to substratum-adhesiveness and Con A concentration

Human T-enriched blood lymphocytes were allowed

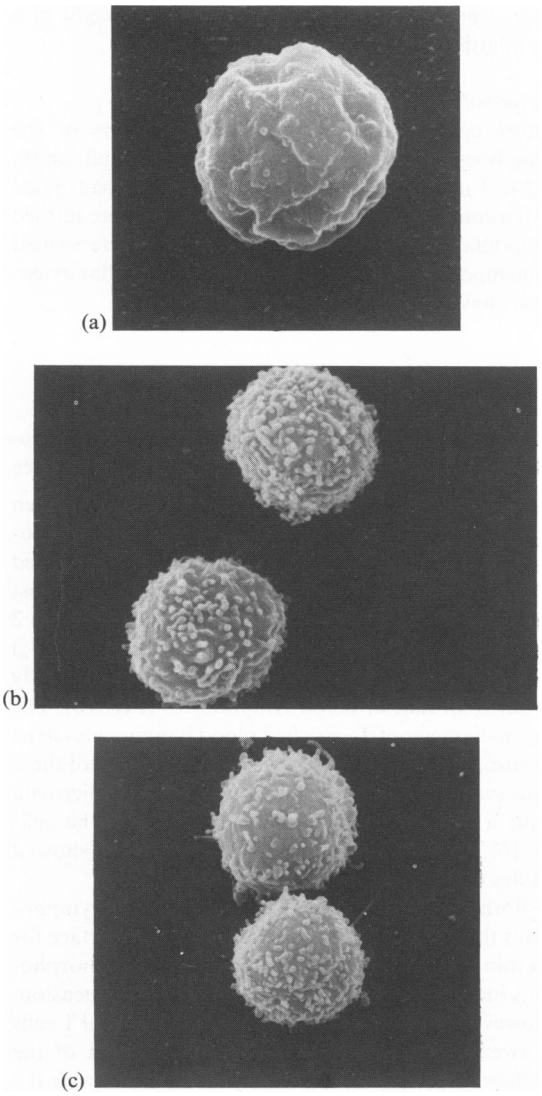

Figure 1. Morphology of human thymocytes (a, magnification × 16,500) and T-enriched blood lymphocytes (b, magnification × 9000) in suspension. (c) shows T-enriched blood lymphocytes allowed to settle on a glass surface in the absence of Con A for 60 min.

to settle on glass, tissue culture plastic and poly-Hema in the presence of Con A 100, 50 and 25 μg/ml. On the poly-Hema surface the cells were essentially round at all Con A concentrations, indistinguishable from lymphocytes allowed to settle in the absence of Con A (Fig. 1c).

Lymphocytes settled on glass and plastic showed different degrees of cytoplasmic spreading, dependent on the concentration of Con A used. At Con A 100 μg/ml, 55–92% of the T-enriched cells from seven different individuals exhibited pronounced spreading (Fig. 2a). At Con A 50 μg/ml, 34–65% of the cells showed lamelar cytoplasmic spreading, and at Con A 25 μg/ml the corresponding figures were 10–35%. This indicated that the higher the Con A concentration the more pronounced was the spreading. Human or monkey thymocytes that were allowed to settle on glass in the presence of Con A 100, 50 and 25 μg/ml, attached to the substratum to the same extent as peripheral T cells (see next section), but, with the exception of a minor cell population (< 10%), showed virtually no spreading (Fig. 2b). Separation of the thymocytes on a discontinuous gradient of Percoll yielded four cell layers (i–iv) corresponding to the level of different density marker beads centrifuged simultaneously. Cells from the first two layers (low-density cells) showed a relatively high degree of Con-A-induced spreading (75% spread cells). The number of spread cells in the third and fourth layer representing high density thymocytes was below 5%.

T-enriched blood lymphocytes and human thymocytes were identified in relation to interaction with substratum by a combination of immunofluorescence with monoclonal anti-T-cell antibodies and interference contrast microscopy (Fig. 3). The spread blood lymphocytes (Fig. 3a) generally reacted with the monoclonal anti-pan T-cell antibody OKT3 (Fig. 3b). When the thymocytes, attached to a Con-A-coated surface were examined, the spread cells (Fig. 3c), comprising less than 10% of all lymphocytes, generally were T6 negative (Fig. 3d). Of the other unspread cells, 75–100% expressed T6 antigen (Figs 3c, 3d).

The presence of cytochalasin B 10 μg/ml or cytochalasin D 1 μg/ml prevented the Con-A-induced attachment to substratum and subsequent spreading of human T-enriched lymphocytes. When the cells were allowed to settle on substratum in the presence of Con A at 4° the number of adherent cells was virtually the same as at 37°. The number of fully spread cells however was reduced from values above 90% to below 10%.

Sequence of morphological changes in thymocytes and peripheral T lymphocytes allowed to settle on substratum

Thus, the induction of cytoplasmic spreading in T lymphocytes required that Con A was present and that

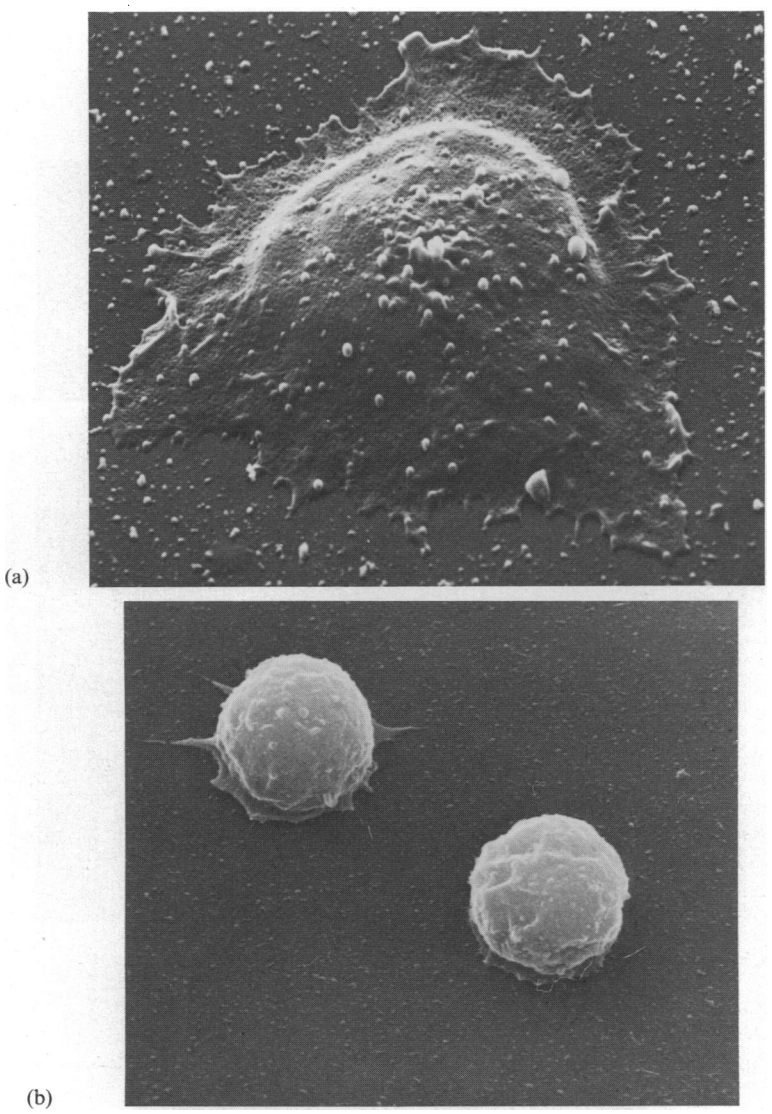

Figure 2. Morphology of T-enriched human blood lymphocytes (a, magnification ×9000) and common thymocytes (b, magnification ×8000) allowed to settle on a surface in the presence of Con A 100 μg/ml for 60 min.

the cells were in contact with an adhesive surface. In order to study the kinetics of spreading-changes, thymocytes and peripheral T lymphocytes were allowed to settle on a Con-A-coated surface. The cells were fixed before and after 2, 5, 15 and 60 min. The number of lymphocytes that attached under the conditions used were 85–100% for thymocytes and 90–100% for peripheral T lymphocytes. The number of attached cells reached a maximum within 15 min. The peripheral lymphocytes exhibited four morphology types:

(i) spherical non-spread cells indistinguishable from cells in suspension and 4·3 μm (3·9–6·7) in diameter (Fig. 4a);

(ii) spherical villous cells with substratum-attached filopodia 1–5 μm in length (Fig. 4b);

(iii) lymphocytes showing lamellar cytoplasmic spreading from the cell base and absence of microvilli

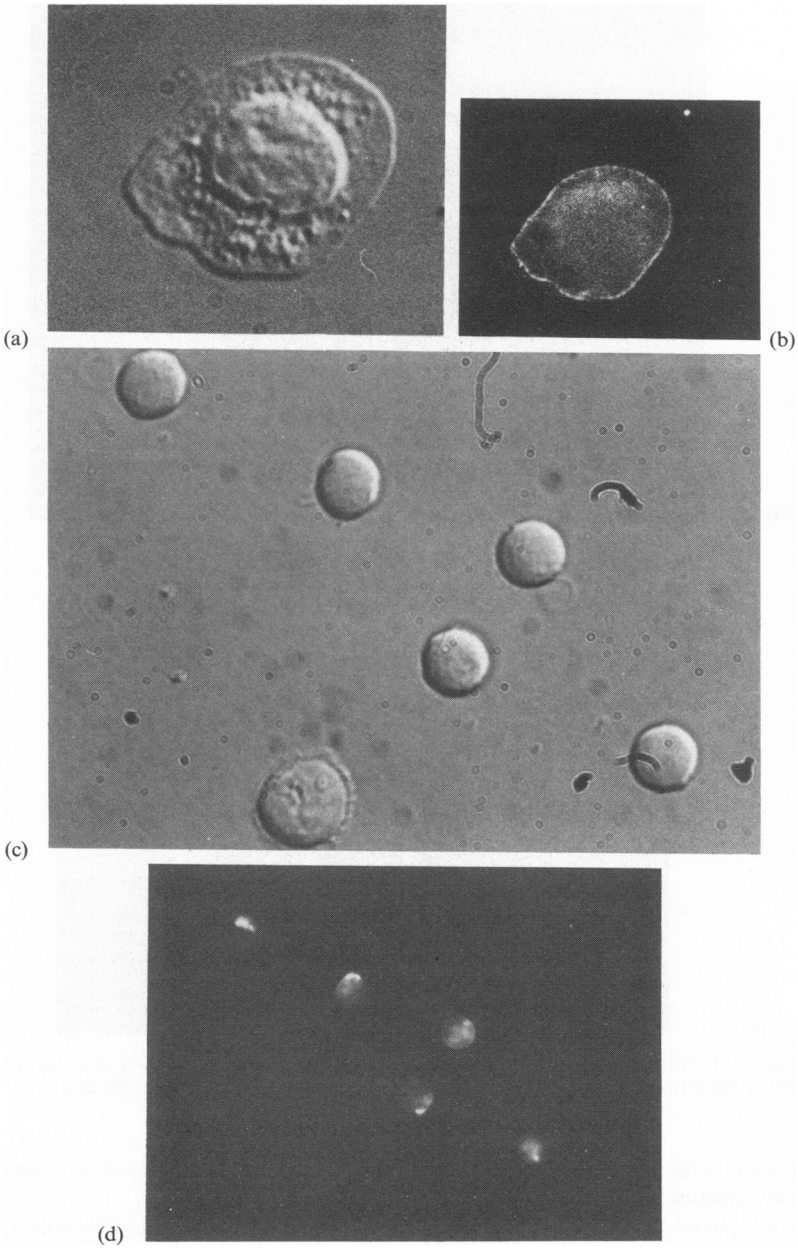

Figure 3. Identification of human peripheral lymphocytes (a, magnification × 3000; b, magnification × 1200) and thymocytes (c, magnification × 3000; d, magnification × 1200) in relation to interaction with a Con A coated glass surface. (a) and (c) show cell morphology as revealed by interference contrast microscopy. (b) and (d) show the reactivity of the cells with OKT 3 (b) and OKT 6 (d). It appears from (d) that the spread thymocyte in (c) is T6-negative and that the unspread thymocytes are T6-positive.

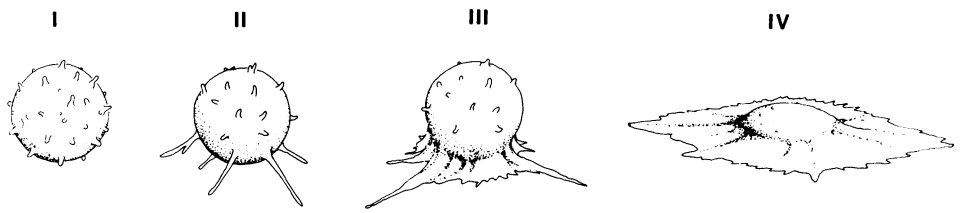

Figure 4a. Sequence of morphological changes during spreading of human T-enriched blood lymphocytes induced by Con A 100 μg/ml: I, suspension morphology (represents Fig, 1b); II, initial contact and formation of filopodia (represents Fig. 4b); III, lamellar cytoplasmic spreading (represents Fig. 4c); IV, flattening of the central cell mass (represents Fig. 2a).

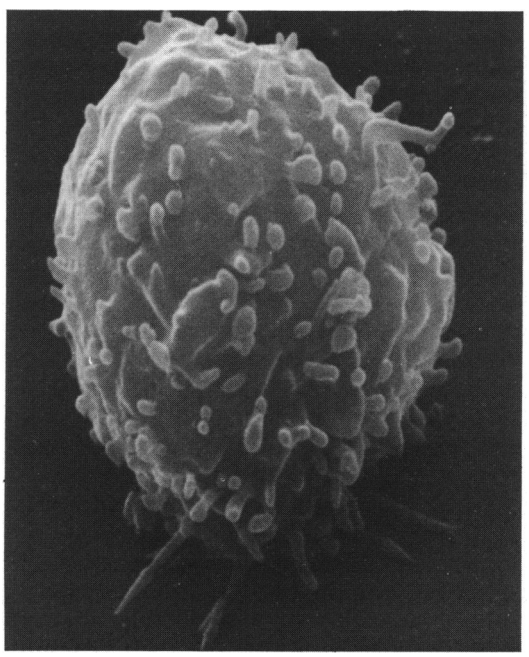

Figure 4b. Filopodia-formation—initial stage of spreading. Magnification × 19,000.

in the flattened areas. The peripheral lamellae of these cells often showed ruffles (Fig. 4c);

(iv) lymphocytes showing lamellar cytoplasmic spreading and nuclear flattening (Fig. 2a).

These differences in cell morphology were related to the length of interaction between lymphocytes and substratum (Fig. 5). Accordingly there was a time-dependent transition from type i to type ii, iii and iv, indicating that the morphological variations i–iv represent different stages of the spreading process.

The substrate-attached thymocytes from both species exhibited time-dependent changes of morphology similar to those of peripheral T cells (Figs 4a, 4b; Fig. 5). However, the number of thymocytes that showed an alteration of cell morphology was considerably lower than that of the peripheral T cells (Fig. 6). Thus, in different individuals less than 10% of the thymocytes showed spreading similar to peripheral T cells. Most attached cells (73–91% in different individuals) remained spherical throughout the incubation period, showed no filopodia, lamellipodia or nuclear flattening, and lacked microvilli.

Live-cell microscopy

Table 1 summarizes some results obtained by means of observation of living cells. It appears that conditions that promoted spreading as described in the previous sections also markedly augmented cell motility compared with that of 'non-spread' cultures. All parameters of motility, i.e. number of motile cells, radius of action (area occupied by the cells/length of translational movement) and most significantly the tendency to cell–cell interaction, were higher under 'spreading-conditions'. The experiments clearly showed that the cells developed contacts as a consequence of the spreading/motile activity (see Table 1 and Fig. 7). We have the impression that lymphocytes allowed to settle in the absence of Con A attempt to initiate a motile response but are unable to continue and/or maintain it. Thus, as mentioned above, these cells exhibit microvilli and to some extent filopodia in areas of substrate-contact, but with the exception of a small 'population' do not develop lamellipodia.

DISCUSSION

Thus, the Con-A-mediated contact of T lymphocytes with an adhesive surface initiated a sequence of dissociable membrane events including filopodia-formation, formation of lamellipodia and flattening of

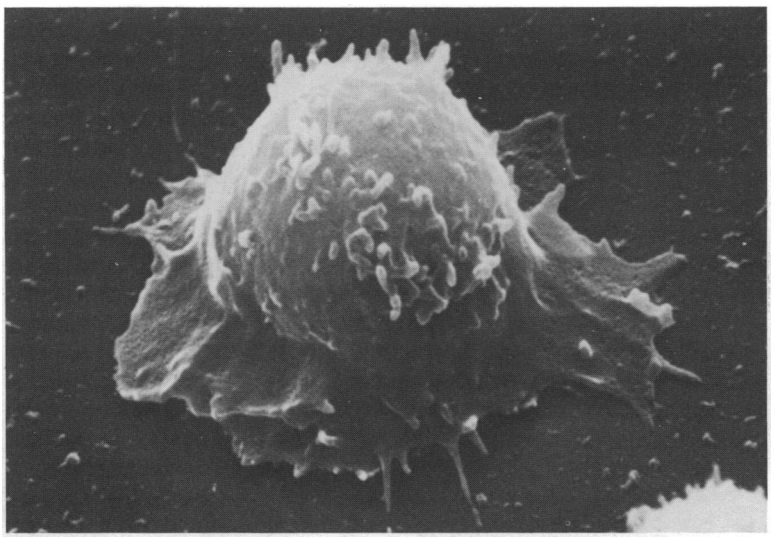

Figure 4c. Lamellar cytoplasmic spreading. Magnification × 14,000.

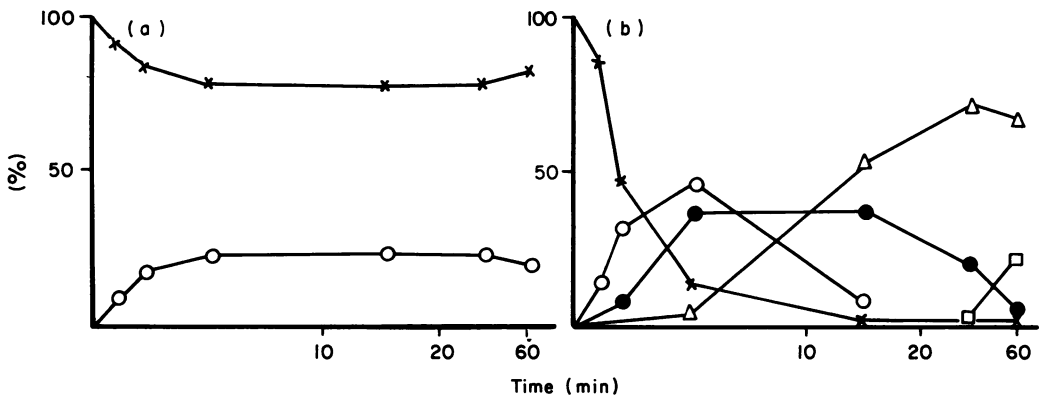

Figure 5. Kinetics of cell shape changes on uncoated (a) and Con-A-coated (b) surfaces respectively of T-enriched blood lymphocytes. (×) lymphocytes in suspension (type I in Fig. 4a); (○) lymphocytes with filopodia (type II in Fig. 4a); (●) lamellar cytoplasmic spreading (type III described in Fig. 4a); (△) flattening of the central cell mass (type IV); (□) type IV cells with prominent peripheral projections (not shown in picture).

the central cell mass. This was an active adhesion process which markedly increased the radius of action of the cells. The data further showed that this adhesion/spreading-process distinguishes thymocytes and peripheral T cells. The sequence of events characterizing the spreading seemed to be the same for all Con A concentrations. The degree of spreading, however, was dependent on the concentrations of Con A used, being most pronounced at high concentrations. The active extension of filopodia and lamellipodia, which characterizes the spreading of lymphocytes, is considered to be required for cell migration in general (Grinnel, 1978). A relatively small portion of T-lymphocytes has previously been noted to move 'spontaneously' (Schreiner & Unanue, 1976), but the present findings indicated a direct relationship between a stimulus and the motile response of the cells.

Thymus controls the development of T lymphocytes (Pahwa, Ikehara, Pahwa & Good, 1979; Bevan, 1981; Stutman, 1978). Thymic epithelium and thymic hor-

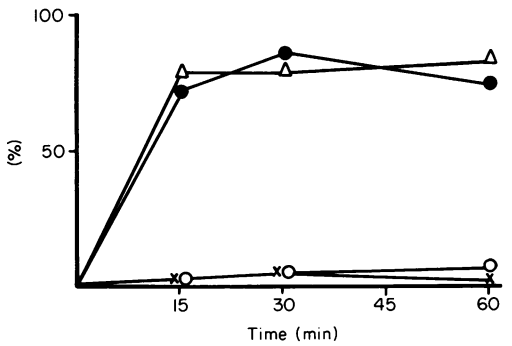

Figure 6. Spreading in relation to the length of interaction between lymphocytes and substratum in the presence of Con A 100 μg/ml. (×) Human thymocytes; (○) monkey thymocytes; (●) human T-enriched blood lymphocytes; (△) monkey blood lymphocytes.

mones probably govern the differentiation of functionally immature cortex thymocytes from relatively mature medullary cells (Bach & Carnaud, 1976; Levine & Claman, 1970). The capacity of T lymphocytes to interact in the immune response is probably imposed by the thymic microenvironment (Zinkernagel, 1978; Waldman, 1978). Common T6-antigen-bearing thymocytes, 'high-density' thymocytes and peripheral T cells defined by the T3-antigen attached to the same extent to a Con-A-coated surface, but only peripheral T cells exhibited contact-induced morphological changes characteristic of a spreading-process. The data also indicated that the mature OKT 6 negative thymocytes and low-density thymocytes underwent contact-induced changes. The difference in spreading-capacity between thymocytes and peripheral T lymphocytes was 'qualitative' rather than 'quantitative'. This suggests that the capacity to spread on a Con-A-coated surface correlates with immunocompetence. One possible explanation for the lack of spreading in thymocytes is that induction of this process requires a membrane receptor or some cytoskeletal component absent in these cells. In addition, however, there are two related explanations for the difference in spreading capacity between thymocytes and peripheral T cells. Accordingly, thymic lymphocytes have markedly less microvilli than peripheral T cells (Newell, 1980; Fig. 1). Thymocytes also have a high nuclear to cytoplasmic ratio. It has been proposed that microvilli represent reserve surface membrane necessary for the spreading of non-lymphoid cells (Erickson & Trinkaus, 1976). It follows from this hypothesis that, because thymocytes lack microvilli, they would be unable to spread, which is consistent with the present observations. That thymocytes have relatively little cytoplasm may imply that their cytoskeletal machinery is too weak to generate the force required for formation of filopodia, lamellipodia and for cell flattening, particularly of the nucleus.

The capacity of spreading in mature T lymphocytes is probably functionally important. Thus, the formation of filopodia and lamellipodia and the motile activity associated with these structures, taken together with the increase in area occupied by the cells caused by the flattening, increased the radius of action of the cells considerably. This enhanced interaction with the environment was evident by the fact that spreading appeared to be a prerequisite for cell–cell interaction at the relatively low cell density used (Table 1). In the light of this it seems conceivable that the lack of spreading-capacity in common thymocytes may reflect and possibly contribute to their functional immaturity. The present results therefore prompt further studies of spreading in relation to lymphocyte differentiation and function.

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Table 1. Lymphocyte movement in relation to substratum-contact*

Experimental conditions	No. of cells examined†	Motile cells (%)†	Cell–cell interactions†‡		Radius of action ($\mu m^2/\mu m$)§			
			After 15 min (%)	After 60 min (%)	No. of cells measured	All cells	Motile forms	Non-motile forms
Con-A-coated surface	214	90	1	12	20	117(97–132)/6(1–21)	117(97–132)/6(1–21)	not determined
Uncoated surface	208	14	< 1	< 1	20	43(34–102)/2(0–26)	74(63–102)/8(3–26)	39(34–45)/ < 1(0–2)

* Lymphocytes were allowed to settle and observed continuously during a 60 min period. Cell density 10^5 cells/cm^2.

† Determined from phase-contrast pictures.

‡ The figures show per cent cells in contact 15 and 60 min after initiation of the experiment.

§ Denotes the area occupied by the cells/the length of translational movement. Figures before parenthesis denote mean values and figures within parenthesis show extreme values.

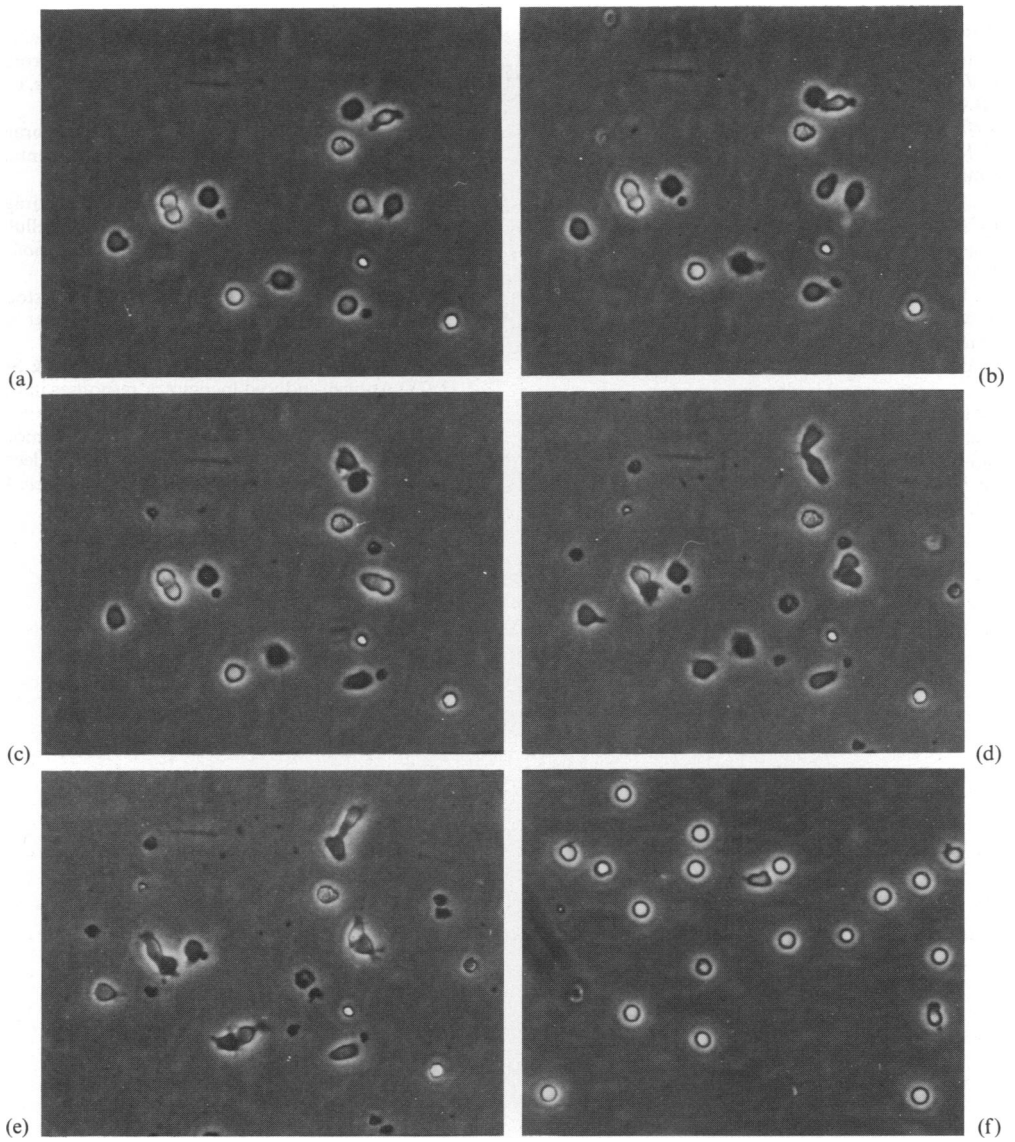

Figure 7. Phase contrast pictures illustrating the formation of contact between T-enriched lymphocytes settled on substratum in the presence of Con A (a–e). The photographs were taken (a) 10, (b) 15, (c) 20, (d) 25 and (e) 35 min after initiation of the experiment. (f) shows the same lymphocytes after 35 min in the absence of Con A. Spread cells are dark and spherical ones are phase bright.

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