

THE ADHERENCE OF HUMAN Fc RECEPTOR-BEARING LYMPHOCYTES TO ANTIGEN-ANTIBODY COMPLEXES

II. Morphologic Alterations Induced by the Substrate

BY ELAINE ALEXANDER AND PIERRE HENKART

(From the Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014)

In contrast to most cell types, lymphocytes do not readily adhere either to other cells or to surfaces *in vitro*. The physiological functions of lymphocytes appear to require their circulation among the various lymphoid organs in the animal. Adherence to cells or surfaces may be required for normal immune function *in vivo* and such adhesions may be mediated by specific receptors on the cell surface. Many of the natural foreign antigens recognized by lymphocytes are microorganisms and the binding of such antigens to lymphocytes appears to be necessary to elicit an immune response. Recently, many examples of cell-cell interaction required for various immune reactions have been described (reviewed in 1, 2), and while some of these may be mediated by soluble factors (3), cell-cell adhesion may be required for others. For example, it has been shown that clusters of adhering cells are necessary for the *in vitro* generation of antibody-forming cells (4). The antigen-specific adhesion between lymphocytes and macrophages (5, 6) seems likely to be functionally important. In lymphocyte-mediated cytotoxicity of foreign cells, a specific binding of the target cell by the killer cell appears to be a necessary first step in the lytic process (reviewed in 7).

Because of their nature, these specific cell-cell surface interactions have been difficult to study. The morphologic changes induced by the binding of murine spleen cells to antigen immobilized on nylon fibers has been studied as a model system (8). The present investigation describes the morphologic consequences of the adhesion of human lymphocytes to immobilized antigen-antibody complexes. We have shown that this interaction is mediated by the lymphocyte Fc receptor and results in a strong binding of Fc-bearing cells to antibody-coated antigenic surfaces.¹ The interaction of lymphocytes with such surfaces can be considered a model for killer-target cell interaction in antibody-dependent lymphocyte-mediated cytotoxicity. During these studies, it was observed that the shape and morphology of the adherent cells was markedly altered when compared to that of normal lymphocytes. These alterations and experiments to elucidate their mechanism(s) are described in this paper.

¹ Henkart, P., and E. Alexander. The adherence of human Fc-bearing lymphocytes to immobilized antigen-antibody complexes. I. ⁵¹Cr binding assay and lymphocyte preparative technique. Manuscript submitted for publication.

Materials and Methods

Colchicine, vinblastine, and phenylmethane sulfonyl fluoride (PMSF)² were obtained from Sigma Chemical Co., St. Louis, Mo.; EDTA and ethylene bis(oxyethylenedinitrilo)tetraacetic acid (EGTA) from Fisher Scientific Co., Pittsburgh, Pa.; cytocholasin B from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.; concanavalin A (Con A) from Miles Laboratories Inc., Miles Research Div., Elkhart, Ind.; purified phytohemagglutinin (PHA) from The Wellcome Research Laboratories, Beckenham, England; and diisopropyl fluorophosphate (DFP) from Aldrich Chemical Co., Inc., Milwaukee, Wis. Colcemid was the kind gift of Dr. J. R. MacIntosh, University of Colorado, Boulder, Colo. Ricin was purified by the method of Nicolson and Blaustein (9). Wheat germ agglutinin (WGA) was purified according to LeVine et al. (10).

Human peripheral blood lymphocytes were prepared as described previously.¹ Plastic surfaces were coated with antigen-antibody complexes using a 1/100 dilution of heat-inactivated rabbit anti-DNP or anti-TNP.¹ Plastic surfaces were also coated with lectins by incubating with solutions of 1 mg/ml for 20 min at 22°C followed by thorough washing.

Light Microscopy of Lymphocytes Adherent to Antigen-Antibody-Coated Plastic. Parafilm wells were made in the bottom of tissue culture Petri plates as outlined in detail elsewhere.¹ Cell suspensions containing 40,000–200,000 cells in 20 μ l of Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS) were allowed to settle for 20 min onto wells with 7 mm diameters. When desired, nonadherent cells were removed by inverting the plates for 10 min, gently aspirating the inverted drop, and washing away the remaining nonadherent cells with 50- μ l aliquots of MEM. The wells containing 20 μ l of medium were examined directly and photographed in an inverted phase microscope at \times 290 (correcting the optics for the lens effect of the curved surface of the medium). For higher power observation with noninverted microscopes using phase and Nomarski optics, a cover glass was placed on top of the wells. Most experiments were performed at room temperature. In studies of cell motility, the temperature was maintained at 37°C using an air curtain incubator. When fixation was desired, the adherent cells were fixed at room temperature in 0.15 M sodium cacodylate, pH 7.4, containing 2.5% glutaraldehyde.

Time-lapse Cinematography. Time-lapse cinematography was performed at 37°C using Plus-X reversal film (Eastman Kodak Co., Rochester, N. Y.) with 2.25-s exposures every 5 s. The objective lens was focused on scratches placed on the plastic surface. Lymphocytes were then pipetted into the medium above this area of the Petri plate and observed as they settled onto the substrate.

Analysis of Drug and Temperature Effects on Lymphocyte Morphology. The effects of various drugs and temperature (Table II) on the morphology of adherent cells were quantitated by careful examination of 150–200 adherent lymphocytes in photomicrographs (\times 290) taken with an inverted-phase microscope. Each was graded independently on a scale of 0–2, for both its degree of flattening and its extent of elongation, and an average value of flattening and elongation computed for each condition. Adherent cells that were neither flattened nor elongated were graded as 0; cells with an intermediate degree of flattening or elongation as 1; and cells which were markedly flattened or maximally elongated with classical uropods as 2. The criteria for these scales are shown in Table I. The inhibition of flattening and elongation was calculated by comparing these average values for the drug-treated cells with the average values for the same cells on the same day in MEM in the absence of drugs. Thus each of the values for inhibition in Table II are derived from scoring 1,500–2,000 cells graded according to their morphology.

Scanning Electron Microscopy. Adherent lymphocytes were fixed for scanning electron microscopy by adding an equal volume of 3.0% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, to the media contained within the antigen-antibody complex-coated wells. After 2 h the plates were thoroughly washed and stored in buffer at 0°C. The washed lymphocytes were either dehydrated through a graded series of ethanol and prepared by critical-point drying (11) or freeze dried directly from water (12). The specimens were vacuum coated gold palladium on a rotating tilting stage and examined with an Etec Autoscan microscope (Etec Corporation, Hayward, Calif.) at 20

² Abbreviations used in this paper: BSA, bovine serum albumin; Con A, concanavalin A; DFP, diisopropyl fluorophosphate; EGTA, ethylene bis(oxyethylenedinitrilo)tetraacetic acid; FCS, fetal calf serum; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PMSF, phenylmethane sulfonyl fluoride; SEM, scanning electron microscopy; WGA, wheat germ agglutinin.

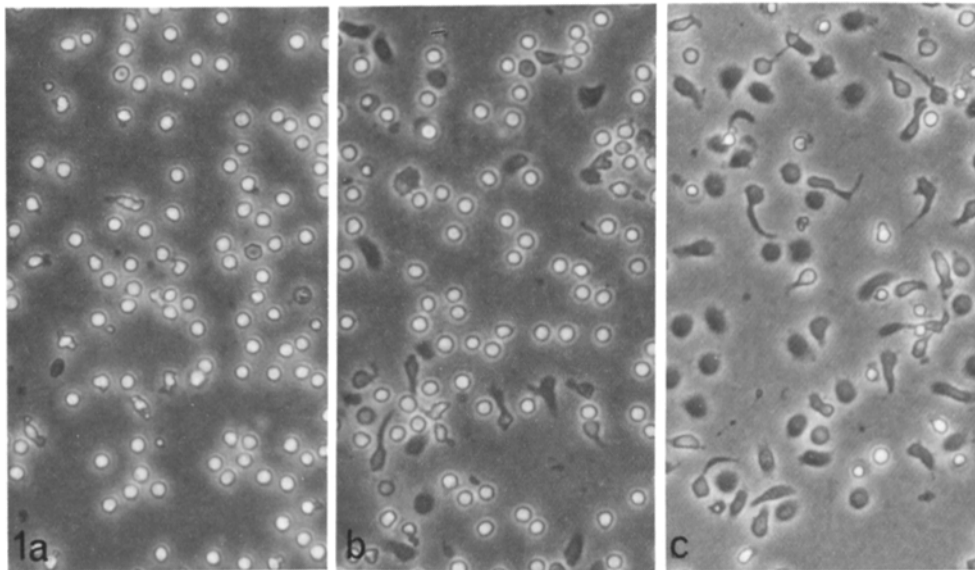


FIG. 1. Human lymphocytes settled onto modified substrates. $\times 290$. (a) Purified lymphocytes allowed to settle onto antigen-coated substrate. Virtually all of the cells remain spherical and phase bright. (b) Purified lymphocytes allowed to settle onto antigen-antibody-coated substrate. A subpopulation of cells appear flattened (phase dark) and some are elongated. (c) Adherent lymphocytes on immobilized antigen-antibody complexes. Nonadherent cells have been removed. The cell concentration of the starting population for this plate was higher than for (a) or (b) in order to provide a higher adherent cell density.

kV and 45° tilt. The cells were photographed on Polaroid 55 film. (Polaroid Corp., Cambridge, Mass.).

Results

Alterations of Lymphocyte Morphology Induced by Binding to Immobilized Antigen-Antibody Complexes. When lymphocytes in MEM were allowed to settle onto plastic surfaces coated with antigen-antibody complexes, a striking change in the appearance of a subpopulation of cells was observed with the phase microscope (Fig. 1 b). Between 5 and 30% of the purified lymphocytes from normal individuals became phase dark (flattened) and increased their area of contact with substrate, indicating cell spreading. Frequently, these flattened cells also became elongated. In contrast, when lymphocytes were allowed to settle onto uncoated plastic surfaces or onto plastic surfaces coated with antigen alone (Fig. 1 a), only 0–2% of the cells underwent a similar morphological transformation. As previously demonstrated, the cells which adhere to these immobilized antigen-antibody complexes are Fc receptor-bearing lymphocytes.¹ As shown in Fig. 1 c, most but not all of the adherent cells have become phase dark. Thus the morphologically altered subpopulation of lymphocytes bears the Fc receptor and most of these cells become flattened upon the brief contact with surfaces coated with antigen-antibody complexes.

The presence of an Fc receptor appears to be required for the induction of these morphologic changes in lymphocytes.¹ In normal individuals, the percent of

phase-dark cells is generally slightly lower than the percent which bind aggregated IgG or soluble immune complexes. F(ab')₂ antibody did not induce these shape changes, nor did the goat, sheep, or mouse antibodies previously shown to be inactive in mediating adhesion to these surfaces.¹

Soluble antigen-antibody complexes were formed in antigen excess using TNPP₁₇-bovine serum albumin (BSA) and the same antibody (rabbit anti-TNP) that was used to coat the TNP-plastic surfaces. These soluble complexes did not trigger the morphologic changes induced by the attachment of lymphocytes to immobilized complexes and the cells appeared similar to those in Fig. 1 *a*. However, some clumping of the Fc receptor-bearing cells did occur.¹

The morphological alterations accompanying lymphocyte adhesion to lectin-coated plastic surfaces were also studied. Con A-, PHA-, WGA-, and ricin-coated polystyrene surfaces bound 5–50% of human lymphocytes. In the case of Con A, this binding was inhibited by alpha-methyl-mannose, a specific saccharide inhibitor of Con A binding. Of the lymphocytes adherent to such immobilized Con A, only 9% underwent a morphological alteration (similar to that of adherent lymphocytes on immobilized antigen-antibody complexes) while the majority remained spherical. Immobilized PHA induced similar morphological alterations in 16% of the adherent cells, while the nonmitogenic lectins, WGA and ricin, induced less than 1.0% of the adherent cells to alter their shape as viewed with the phase microscope.

As previously documented,¹ the vast majority of cells adherent to antigen-antibody-coated plastic in our experiments are lymphocytes, not monocytes. In experiments using cell preparations obtained from Ficoll-Hypaque density gradients and not depleted of monocytes, the adherent population also contained a larger cell possessing a large irregularly shaped, convoluted nucleus and a characteristic broad phase-dark ruffled membrane around the cell periphery. The appearance of these cells was very similar to that previously reported for macrophages induced to spread by contact with immobilized antigen-antibody complexes (13, 14). Thus, it appeared that most of the occasional contaminating monocytes in our preparations could be recognized by phase morphology alone, and such cells have not been considered in the following discussion.

Light Microscopy of Lymphocytes Adherent to Immobilized Antigen-Antibody Complexes. Low power phase micrographs (Fig. 1 *c*) illustrate the diversity in the morphology of the lymphocytes attached to antigen-antibody-coated substrate. Further details of this variation were apparent at higher power using phase and Nomarski optics as shown below. At one extreme of the range of adherent cell morphology were the phase-bright spherical cells whose appearance resembled that of normal lymphocytes in suspension. Other cells were primarily phase bright, but possessed small phase-dark processes radiating peripherally and apparently in contact with the substrate. More common were cells which appeared to have flattened to a further degree, with phase-dark nuclei surrounded by cytoplasm bordered by more prominent phase-dark irregularities (Fig. 1 *b* and *c*). While many of these cells were spread symmetrically along the plane of the substrate, others showed a variable degree of elongation. The combined variables of flattening and elongation gave rise to the diversity of adherent cell shapes. Extremely elongated lymphocytes (Fig. 1 *b* and *c*) pos-

TABLE I
Distribution of Adherent Lymphocyte Morphology

		Percent of total adherent cells*		
Degree of flattening‡		None	Intermediate	Marked
Degree of elongation‡	None	8.3 ± 1.2	13.8 ± 1.7	9.3 ± 1.1
	Intermediate	0	24.9 ± 2.1	22.8 ± 2.0
	Uropod	0		20.9 ± 2.1

* Mean ± standard error of the mean, for a total of 13 experiments with a minimum of 200 cells scored per experiment.

‡ Adherent lymphocytes were graded both as to their degree of flattening and of elongation. The criteria used to score lymphocytes were as follows: Phase-bright cells, no flattening; phase-gray or black and white cells, intermediate flattening; and phase-dark cells, marked flattening. Likewise, cells with one axis at least twice as long as their width were classified as elongated to an intermediate degree; and cells with well-defined hand mirror shapes and tails as uropod bearing.

sessed a long, thin "tail," suggestive of the "uropod" (15) classically associated with motile lymphocytes (16, 17). Table I presents the distribution of adherent cell morphology when graded by degree of flattening and extent of elongation and relates these two parameters, respectively, to the phase appearance and shape of the adherent cells. There was considerable variation in the distribution of these forms from experiment to experiment, as indicated by the ranges. This was particularly true for different individuals, but was also noted to a lesser extent in different experiments from the same donor.

The distribution of morphological types of lymphocytes adherent to antigen-antibody-coated plastic was independent of the dose of antibody used to coat the TNP plastic. For example, even at suboptimal antibody concentrations (0.3 μ l/ml anti-TNP), those lymphocytes which did adhere had a distribution of shapes similar to those lymphocytes adhering to the TNP-plastic coated with 10 μ g/ml anti-TNP. The orientation of the elongated lymphocytes appeared to be random; this was true using the standard antigen-antibody-coated plastic, and also in cases where a gradient of immobilized antibody was created on the substrate.

Higher power phase and Nomarski optics revealed a number of remarkable features of these adherent lymphocytes. The small phase-dark irregular borders of many of these cells were suggestive of, but smaller than, the ruffled membranes seen in fibroblasts in culture (18; Fig. 2 *a* and *b*). The nuclei of many elongated cells changed from their essentially spherical shape to irregular or elongated forms (Fig. 2 *a-d*) which in some cases extended well into the more constricted region associated with the uropod. The cytoplasmic organelles tended to redistribute and concentrate in the Golgi-centriolar region or within the uropod (Fig. 2 *c* and *d*). Apparent contact between cell membranes of adjacent lymphocytes was commonly observed (Fig. 2 *a-d*), and occasionally such cells appeared to crawl over or underneath one another (Fig. 2 *b*).

Time-Lapse Cinematography. Time-lapse cinematography of cells settling onto antigen-antibody-coated substrate revealed that flattening and elongation

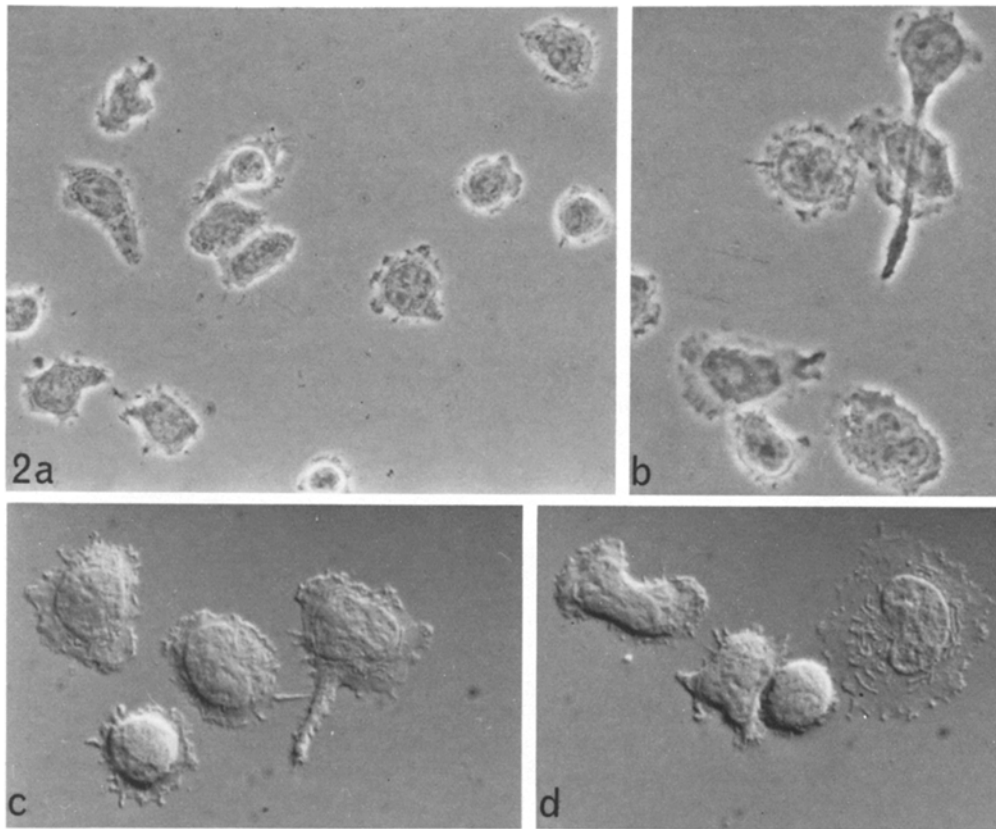


FIG. 2. Adherent Fc receptor-bearing lymphocytes. (a and b) Phase photomicrographs of adherent lymphocytes. The heterogeneity of cell shape is demonstrated by the flattened, elongated, and uropod-bearing lymphocytes. Phase-dark irregularities at the cell periphery appear to be ruffled membranes. (a), $\times 1,062$; (b), $\times 1,710$. (c and d) Nomarski photomicrographs of adherent lymphocytes. Peripheral ruffled membranes and microextensions, as well as intracellular organelles concentrated in the peri-Golgi region can be visualized. (c), $\times 2,044$; (d), $\times 2,085$.

occurred very rapidly after cell contact with substrate. With the microscope focused on the plastic substrate while the cells were settling from suspension, the time of contact with the substrate for each cell was established as it came into focus and ceased being transported by the convection currents of the medium. For those cells which eventually underwent a morphological alteration, an average interval of 80 s (43 cells measured; range 15–375 s) elapsed between contact with the substrate and the detection of the onset of clearly observable flattening.

The process of flattening was even more rapid. The lymphocytes required an average time of 42 s to flatten, (43 cells measured; range 10–100 s). The first step in this process frequently appeared to involve the formation of small phase-dark ruffled skirt-like extensions at the peripheral cell-substrate interface. Elongation and uropod formation were commonly completed within several minutes of cell contact with antigen-antibody-coated substrate.

Time-lapse cinematography, as well as sequential phase photomicrographs, showed that individual cells continued to undergo a wide range of morphological alterations after initial attachment and spreading. The degree to which individual cells were flattened and elongated varied strikingly with time. In some cases, for example, flattened cells reverted to the spherical phase-bright form characteristic of cells in suspension. In other cases, transitions between symmetrically flattened and elongated forms were observed. In general, the most phase-dark symmetrically flattened cells tended to undergo few or none of these changes with time.

Translational movement was observed frequently among adherent lymphocytes. Such movement was random (nondirectional) and temperature dependent. During given periods of observation between 30 to 50% of the adherent lymphocytes were actively motile. The remainder of the cells reversibly extended and retracted processes while remaining essentially stationary. Individual cells alternated between relatively stationary and motile phases. The rate of movement varied markedly, both between different cells, and for the same cell observed at different times. Elongated and uropod-bearing forms commonly are observed undergoing locomotion. Individual motile lymphocytes photographed at 1-min intervals over a time period of 10 min traversed an average of 4.7 $\mu\text{m}/\text{min}$ (25 cells measured; range 1.8–9.3 $\mu\text{m}/\text{min}$). This rate of locomotion is three- to eightfold lower than that reported for cultured lymphocytes (16, 17) and suggests that the firm adhesion of some cells to substrate may actually impede translational movement.

In this regard, markedly flattened lymphocytes with centrally placed, phase-dark nuclei surrounded by a concentric rim of cytoplasm were not generally observed to undergo translational movement. However, some flattened adherent cells showing little or no elongation were capable of considerable translational motion. For example, one particularly active cell was followed for 28 min at 37°C and its position was plotted at 50-s intervals. This analysis revealed that the lymphocyte moved randomly at a rate averaging 8 $\mu\text{m}/\text{min}$. This movement was accompanied by the continual extension and retraction of small pseudopodia. Higher magnification Nomarski time-lapse cinematography also revealed that the long slender microextensions observed in nonelongated flattened cells and the microspikes of uropod-bearing cells were in rapid motion. Uropod formation generally followed flattening, but occasionally one could see an elongated lymphocyte in suspension which attached directly to substrate via its uropod. Such cells were considered to be among the small percent of normal human lymphocytes which spontaneously form small uropods (15).

Scanning Electron Microscopy. The three dimensional structure of lymphocytes adherent to immobilized antigen-antibody complexes was revealed by scanning electron microscopy (SEM), as shown in Figs. 3 and 4. The diversity of shapes of adherent cells seen with phase optics was even more striking when viewed with SEM. As shown in Fig. 3 *a-f* the nonelongated adherent lymphocytes are flattened to a variable degree, and commonly resemble "fried eggs," with the central nucleus flattening less than the surrounding cytoplasm (Fig. 4 *a-c*). Such cells often possessed slender microextensions of various lengths (Figs. 3 *a-d* and 4 *a-b*) and/or ruffled membranes (Figs. 3 *b-e*, and 4 *b* and *d*),

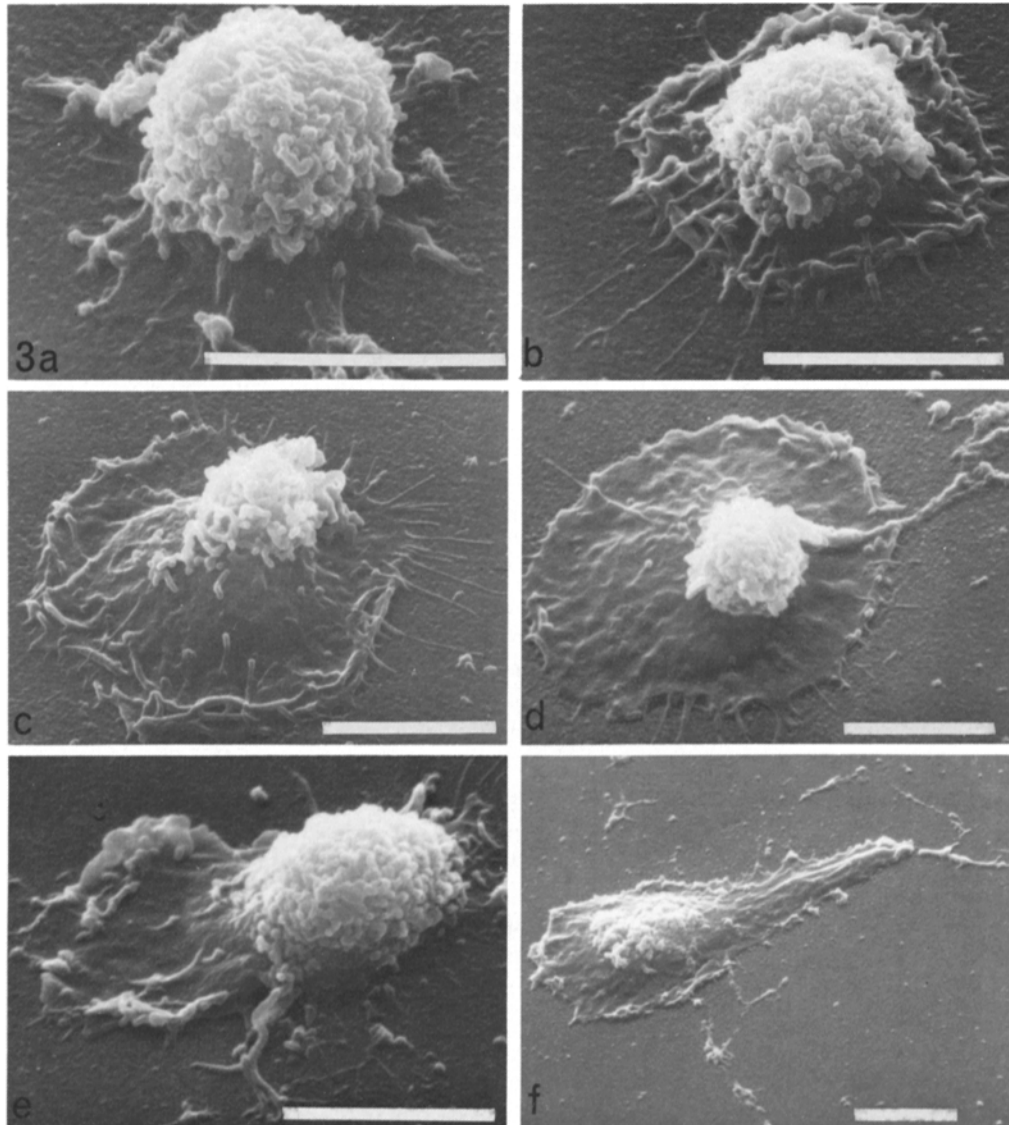


FIG. 3 *a-f* and 4 *a-f*. Scanning electron micrographs of normal Fc-bearing human lymphocytes adherent to immobilized antigen-antibody complexes. Cells in various stages of flattening and elongation give rise to a diversity of shape and surface topography. Relatively symmetrically flattened cells resemble "fried eggs" in appearance. Uropod-bearing lymphocytes and other elongated cells are illustrated. See text for description. These adherent cells can round up and detach from substrate, returning to their former spherical shape covered once again with short microvilli.¹ Bar represents 5 μm .

which were similar in appearance to those seen in fibroblasts, where they have been implicated in motility (18). The most flattened adherent cells had diameters of up to 20 μm (as opposed to an average 5- μm diameter when fixed in suspension) and extremely thin cytoplasm.

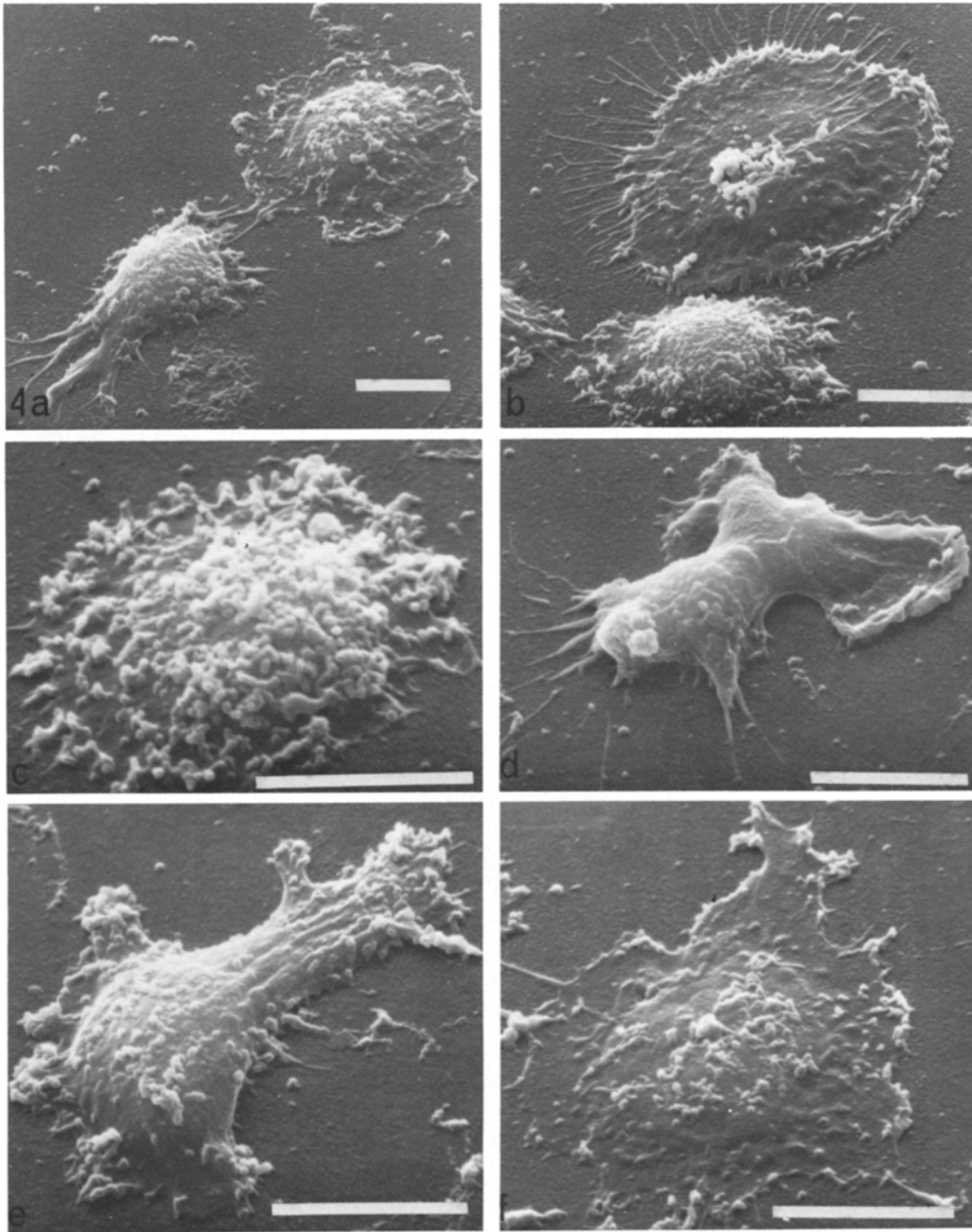


FIG. 4 a-f

The elongated adherent lymphocytes also assumed a wide range of morphologic appearances. Prominent pseudopodia bordered by ruffled membranes were seen (Fig. 4 *d-e*). The uropods were complex and of varying lengths and diameters, often terminating in long slender microextensions or microspikes (Fig. 4 *a* and *d*). Contacts between adjacent cells observed with light microscopy

were particularly well visualized by SEM (Fig. 4 *a*, *b* and *f*). SEM also revealed that the surface structure of these cells altered as a consequence of their adhesion to immobilized complexes. The uniform covering of short microvilli seen when lymphocytes are fixed in suspension (19) was largely replaced by a smoother surface topography, although some cells appeared to retain a variable number of microvilli on their upper surfaces (Figs. 3 *e* and *f*, 4 *a-f*). In general the less flattened cells had a greater number of microvilli on their surfaces (Fig. 3 *a-d*). This relationship suggests that the microvilli provide much of the increased surface membrane area needed when the lymphocytes deviate from their spherical shape. As shown previously,¹ these adherent lymphocytes can be released from this substrate by treatment with EDTA and recovered. Such cells have been seen to be spherical and uniformly covered with microvilli when fixed in suspension. Occasional cells with short foot processes were seen, suggestive of uropods which had not been fully retracted. It thus appears that these striking morphological alterations of lymphocyte shape and gross surface structure are largely reversible over a period of less than 30 min.

Effect of Temperature on Morphologic Alterations. When lymphocytes were settled onto antigen-antibody-coated plastic at 0°C, adhesion of the Fc receptor-bearing cells measured by the ⁵¹Cr-labeling technique¹ was similar to that observed when cells were settled at room temperature. When such adherent cells were fixed at 0°C and examined with the phase microscope, the shape changes characteristic of cells settled at 22 or 37°C were substantially inhibited (Fig. 5 *b*). Analysis of the morphological types of adherent cells fixed at 0°C in four experiments shows that the degree of elongation was markedly inhibited when compared to the same donor's adherent cells fixed at 22°C (Table II). No uropod-bearing cells were observed at the cold temperature. The extent of flattening was also inhibited at 0°C but this parameter showed more variability between different experiments.

The inhibition of morphologic changes by cold was only partially reversible. When adherent cells which had settled at 0°C were warmed to 37°C, the shape changes characteristic of the higher temperature appeared gradually. After 90 min of warming, such adherent populations still contained a higher percentage of spherical and nonelongated cells than those which had been settled and maintained at 37°C for the same period of time. No differences in morphology between cells settled at 22 and 37°C were observed.

Inhibition of Shape Changes by Drugs. The effect of sodium azide on the morphology of lymphocytes adherent to immobilized antigen-antibody complexes was unique among the drugs tested. While this mitochondrial oxidase inhibitor caused a marked inhibition of the formation of elongated cells, essentially no inhibition of flattening was observed (Fig. 5 *c*; Table II). The effects of

FIG. 5. Effect of temperature and drugs on lymphocyte morphological changes. Phase photomicrographs of adherent lymphocytes which were pretreated with drugs or preincubated in the cold before exposure to antigen-antibody-coated substrate. All micrographs were from the same experiment. The degree of inhibition of flattening and elongation by EDTA, cytochalasin B, and vinblastine illustrated herein was at the upper range of that observed. (a) Control, Eagle's MEM plus 10% FCS. (b) 0°C. (c) Sodium azide, 0.02%. (d) Colchicine, 10⁻⁴ M. (e) Vinblastine, 10 μg/ml. (f) Cytochalasin B, 10 μg/ml. (g) PBS, pH 7.2. (h) EDTA, 0.01 M.

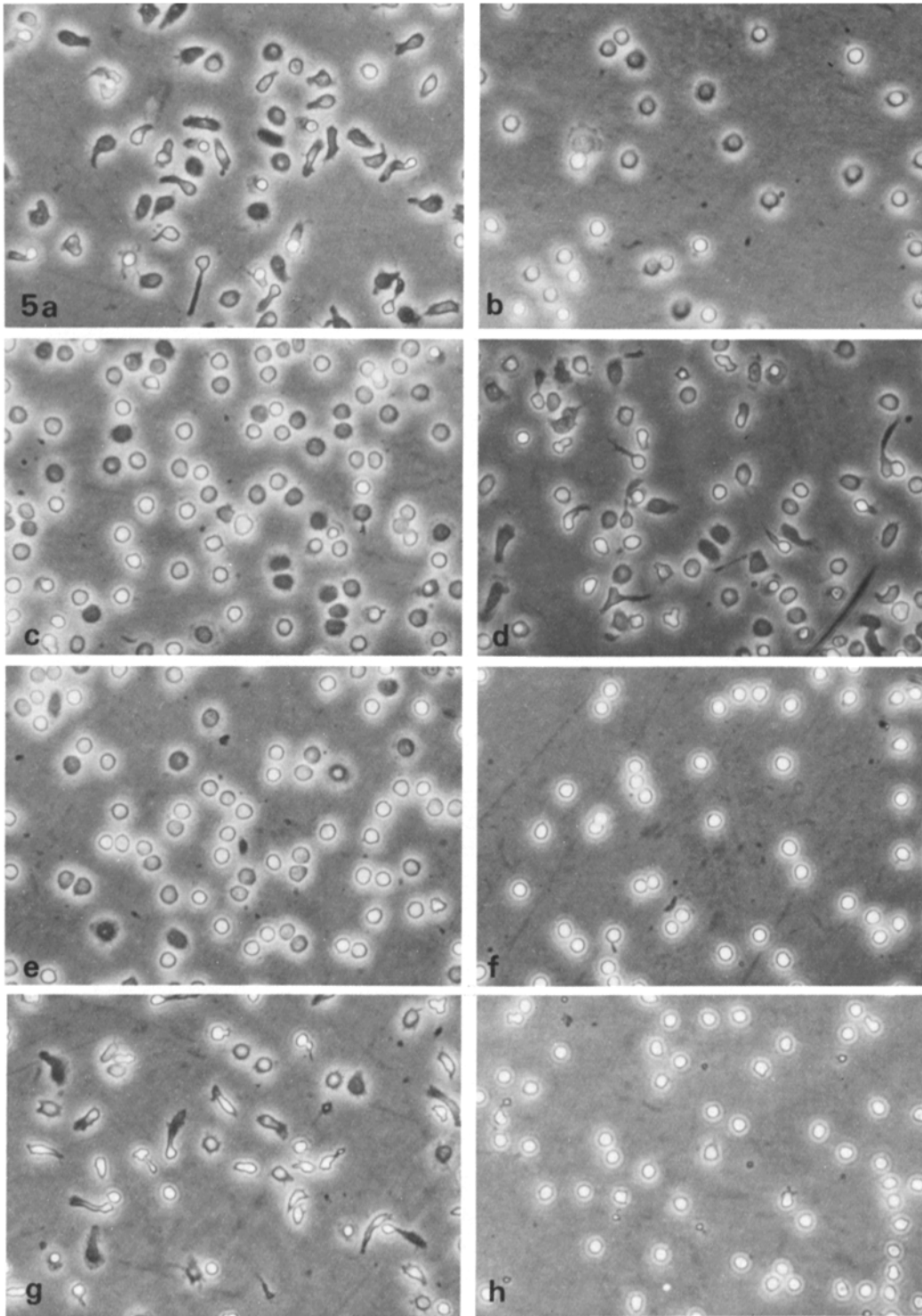


TABLE II
Inhibition of Shape Changes by Temperature and Drugs

Conditions*	No. exp.	Mean % inhibition (range)	
		Flattening	Elongation
0°C	4	39 (1-78)	88 (68-98)
NaN ₃ (0.02%)	3	0 (-19 to 10)	82 (75-87)
Colchicine (10 ⁻⁴ M)	4	10 (9-13)	26 (18-36)
Vinblastine (10 ⁻⁴ M)	4	51 (38-68)	95 (85-100)
Cytochalasin B (10 µg/ml)	5	68 (14-99)	83 (61-100)
DMSO (1%)	4	-7 (-32 to 8)	17 (-2 to 20)
PBS	5	14 (-5 to 19)	0 (-15 to 21)
EDTA (0.01 M)	4	64 (26-96)	80 (72-91)
EGTA (0.01 M)	6	58 (31-91)	64 (40-100)

* Lymphocytes were preincubated in suspension either at 0°C or with drugs for 20 min at room temperature before their being allowed to settle onto antigen-antibody-coated substrate. The extent of flattening and degree of elongation was scored as outlined in Table I. The inhibition of these parameters was calculated as outlined in the text.

this drug, like cold temperature, were only partially reversible with time. DNP (10⁻⁴ M) also strikingly inhibited elongation in these cells, but appeared to inhibit the flattening process to a small degree. The effects of this uncoupler of oxidative phosphorylation were not studied quantitatively, since it could be argued that some of its effects were mediated by competitively inhibiting the antigen-antibody interaction.

The effects of colchicine (10⁻⁴ M) and vinblastine (10⁻⁴ M) were also examined. The former demonstrated a minimal effect on the flattening process and a small but consistent inhibition of elongation (Figure 5 *d*; Table II). This inhibition was not readily apparent upon casual inspection; many uropod-bearing cells remained. This colchicine was shown to be active by effectively inhibiting mitosis of cultured murine lymphoma cells at 10⁻⁶ M. Colcemid (10⁻⁴ M) like colchicine, showed only a small inhibition of these morphological alterations. In contrast, vinblastine showed a striking inhibition of cell elongation and a substantial inhibition of flattening (Fig. 5 *e*; Table II).

Cytochalasin B (10 µg/ml) markedly inhibited both the flattening and elongation of these cells (Fig. 5 *f*; Table II). No uropod-bearing cells were detected and, in general, a substantial number of the cells remained spherical and phase bright, although still bound to the antigen-antibody-coated plastic. In the example illustrated (Fig. 5 *f*), marked inhibition of flattening occurred and all of the cells in the field are nonflattened. Dimethyl sulfoxide, the solvent for cytochalasin B, was present at 1% in these experiments, but had a negligible effect by itself (Table II). Lower concentrations (1 µg/ml) of cytochalasin B had a similar but somewhat less pronounced effect. No synergistic effects were noted using a mixture of 1 µg/ml cytochalasin B and 10⁻⁴ M colchicine. The serine esterase inhibitors DFP and PMSF (at 10⁻³ M) had no effect on the normal morphological alterations induced by immobilized antigen-antibody complexes.

Role of Divalent Cations on Lymphocyte Adherence and Morphology. Lymphocytes suspended in phosphate-buffered saline (PBS) in the ab-

sence of divalent cations or serum adhered to antigen-antibody-coated plastic and underwent morphologic changes indistinguishable from those described in complete medium (Fig. 5 *g*; Table II). However, in the presence of 0.01M EDTA or EGTA in PBS these morphologic changes were substantially inhibited (Fig. 5 *h*). Under these conditions, most of the adherent cells remained spherical (phase bright) or demonstrated only a slight degree of flattening as indicated by their light gray shading under phase optics. Cells which had attached to plastic in the presence of EDTA were readily dislodged from the plastic by shearing forces in the medium, in contrast to the firm adhesion of the adherent cells attached in medium alone.

These chelating agents reversed the morphologic changes. Treatment of adherent cells settled in complete medium with a solution of 0.01M EDTA in PBS resulted in a rounding up and eventual detachment of the flattened cells from substrate over a period of 30 min, indicating that EDTA treatment reversed the flattening and elongation which had occurred previously. In contrast to chelating agents, cations such as Mn^{+2} or La^{+3} (at 10^{-4} M) did not affect the morphological alterations induced by antigen-antibody complexes.

Discussion

The present investigation demonstrates that normal human peripheral Fc-bearing lymphocytes can be rapidly triggered to undergo dramatic changes in shape and surface structure upon brief contact with immobilized antigen-antibody complexes. Evidence to suggest that these morphological changes appear to be the result of an active response of the lymphocyte to contact with immobilized complexes, as opposed to a passive deformation of the cell, is as follows: (*a*) the inhibitory effects of drugs, chelating agents, and temperature on these morphologic changes (Table II); (*b*) the formation of pseudopodia and ruffled membranes; (*c*) the development of uropods in many cells; (*d*) the induction of translational movement; (*e*) the reversibility of the process with time; (*f*) the failure of the great majority of lymphocytes which attach to lectin-coated substrates to undergo similar morphologic changes and the inability of murine lymphocytes (whose Fc receptors are capable of binding this antibody) to respond morphologically to this stimulus.¹

The functional consequences of this active response remain unclear at present. The cell flattening and spreading seen in these lymphocytes have not, to our knowledge, been observed previously in lymphocytes. The formation of uropods is induced by lymphocyte adhesion to macrophages (20), by mitogens after a period of culture (21), by anti-immunoglobulin (22), and by target cells recognized by killer cells (23). The ability of lymphocytes to respond so rapidly to the physiologic stimulus of antigen-antibody complexes with such profound structural modifications reveals a previously unrecognized potential of normal resting lymphocytes. Such morphologic and structural changes may be of functional immunologic significance *in vivo*.

The molecular mechanisms involved in the observed shape changes appear to be complex. However, the speed with which these changes occur precludes processes which require protein synthesis from playing a role, and implies that the necessary components must be present in a readily mobilizable form. We

have dissected the morphological changes into two basic components: a flattening process and an elongation event. The latter phenomenon may be dependent on the former since many of the drugs (Table II) inhibit elongation to a greater extent than the flattening. Mitochondrial inhibitors appear to have no effect on flattening, while drastically inhibiting elongation and this finding may be interpreted as an energy requirement for this component. A role for microfilaments in this shape change is implied by the effects of cytochalasin B (24). Such microfilaments have been implicated in changes of cell shape in other systems (8, 22). The role of microtubules is less clear from the present drug experiments, since both colchicine and colcemid, generally considered to be the most specific inhibitors of microtubule polymerization (25, 26), have a minimal effect on these lymphocyte shape changes. On the other hand, vinblastine (27) and cold treatment (28), which cause microtubular disruption, both strikingly inhibit flattening and essentially abolish elongation. However, transmission electron microscopy indicates that adherent lymphocytes exposed to colchicine and colcemid retain the majority of their microtubules, while the vinblastine- and cold-treated cells are virtually devoid of such organelles (29).

A relationship between the observed shape changes and the phenomenon of "capping" of lymphocyte surface receptors is suggested by a number of observations: (a) The necessity for lattice formation in capping (30) and the parallel necessity for the antigen-antibody complexes to be immobilized for the induction of the observed shape changes; (b) the apparent capping of Fc receptors during the shape change;¹ and (c) the induction of both capping and shape changes by anti-immunoglobulin treatment of murine B lymphocytes. While capping and shape changes may share some common mechanisms, the sensitivity of the shape changes induced by binding to immobilized antigen-antibody complexes to chelating agents, vinblastine, and cytochalasin B has been variably observed with the capping phenomenon (22, 30). Likewise, whereas immune complex- and anti-immunoglobulin-induced shape changes and translational movement require lymphocyte interaction with substrate, capping does not (22).

Adhesion of other Cell Types to Substrate. The process of adhesion of Fc receptor-bearing lymphocytes to antigen-antibody-coated plastic morphologically resembles the adhesion of other cell types to substrate. Murine macrophages adhere naturally to glass or plastic, but the degree of their spreading can be enhanced by a number of agents including immobilized antigen-antibody complexes (13, 14) and various divalent cations (31). A similar phenomenon has been reported with polymorphonuclear leukocytes, where spreading and degranulation are induced by such substrates (32, 33).

The process of fibroblast adhesion and spreading onto glass substrate in many respects parallels that which we have observed for some lymphocytes settling onto antigen-antibody-coated plastic. In particular, SEM (34) shows many morphological parallels between fibroblast adhesion and the lymphocyte morphological alteration we have observed. The lymphocyte flattening process, however, occurs far more rapidly than the corresponding changes in fibroblasts (34).

The mechanism of morphological alteration triggered by lymphocyte adhesion to immobilized complexes may be in part similar to that of the spreading phenomena observed in these other cell types. In common with the lymphocyte

alteration, the spreading process by the above cells is dependent on divalent cations (14, 31) and is inhibited by cold temperature (14), metabolic inhibitors (35), cytochalasin B (14, 35), and vinblastine (35).

Lymphocyte Substrate Interaction. Early investigators (16, 17) observed motile "hand mirror" shaped lymphocytes with prominent tail-like organelles, uropods, migrating from lymph node explants in tissue culture. Uropod-bearing lymphocytes attached to substrate have been observed in unstimulated cultures (22, 36, 37, 38), and it appears that guinea pig lymphocytes from the peritoneal cavity show a higher percentage of uropod-bearing forms than those from blood and spleen (37). Lymphocyte cultures stimulated by allogeneic cells (36), PHA (39), and antigen (6) show an increased frequency of uropod-bearing lymphocytes. It has been suggested that these cells represent a subset of T lymphocytes whose uropods may be organelles mediating specific functional interactions (40).

B lymphocytes, however, also have been shown to be capable of uropod formation. Murine B lymphocytes treated with anti-immunoglobulin (22) show an increased frequency of uropod formation. These uropods, and to a lesser extent those formed by T cells (6, 40), appear less elongated and structurally less complex than those we have observed. Whether these differences are due to subpopulation, species, or method of induction is presently unresolved.

Both lymphocyte adherence and spreading on immobilized antigen-antibody complexes and the induction of uropod-bearing murine lymphocytes by treatment with anti-immunoglobulin are temperature and metabolism dependent and are inhibited by cytochalasin B, but not colchicine (22). However, the serine esterase inhibitor DFP has no effect on the behavior of lymphocytes on immobilized complexes, but does inhibit the induction of uropod-bearing forms by anti-immunoglobulin (22).

The interaction of lymphocytes with surfaces bearing ligands which bind to surface receptors has been reported in a number of cases (41, 42). The morphological appearance of DNP-sensitized murine B lymphocytes bound to nylon fibers derivatized by DNP-BSA was studied by Rutishauser et al. (8). These cells undergo a morphological alteration in response to this interaction, but the reported changes are distinctly different from those we have observed. No flattening or spreading along the surface of the derivatized nylon fibers occurs in this case. The elongation which occurs is oriented largely perpendicular to the surface. Continuous morphologic changes occur with time, but no translation along the surface is observed. The shape changes are maximal at 60 min. The drug sensitivities of these morphological alterations are similar to those we have observed in our system. Thus, while in many respects these two systems differ, interesting parallels are present and common mechanisms may exist in both processes.

Lymphocyte-Cell Interaction. The actual mechanisms of lymphocyte interaction with other lymphocytes and macrophages during the immune response is presently the subject of much study and speculation. One glimpse into such an interaction has been provided by recent studies of the adhesion of guinea pig lymphocytes to macrophages (5, 6). It has been shown that in the antigen-dependent adhesion between syngeneic lymphocytes and macrophages, many of the lymphocytes have formed uropods (5, 6) and some subsequently are stimu-

lated to divide (6). The morphology of these T lymphocytes appears similar to that which we have observed in the interaction of Fc-bearing lymphocytes with immobilized complexes.

Studies of the lymphocyte-mediated lysis of foreign cells provides the most detailed analysis of specific lymphocyte-cell adhesion. This interaction is most analogous to the lymphocyte-antigen-coated substrate adhesion since normal human lymphocytes efficiently lyse antibody-coated target cells. It is clear that cytolysis by immune T lymphocytes and by nonimmune lymphocytes of antibody-coated target cells requires intimate contact between the killer and target cells (42). These interactions have been observed morphologically for both types of killing (44, 45), and the presence of nonspherical, uropod-bearing lymphocytes adherent to the target cells had consistently been noted. Flattened forms similar to those we have observed have not been reported, but this may be in part due to the difference in geometry between cells and flat surfaces and also to the fluidity of the target cell membrane. Indeed, such flattened forms were not seen when lymphocytes were settled on antibody-coated lipid bilayers (38).

Both the killing process as a whole and the killer cell-target cell adhesion are inhibited by the same conditions — cold, mitochondrial inhibitors, and cytochalasin B (46, 47) — which we have demonstrated inhibit the morphological changes in lymphocytes adherent to antigen-antibody-coated surfaces. Recent experiments (46) demonstrate that T-cell killing proceeds by a rapid EDTA and temperature-sensitive killer-target adhesion step followed by a second step requiring 6 min during which the lethal target cell injury occurs. Both of these two rapid steps occur at rates comparable to that of the morphological changes we have described. One interpretation of the similar ionic, temperature and metabolic requirements, drug susceptibilities, and kinetics of morphological alterations and of killing is that such morphological alterations are required for the killing process. An alternative and equally plausible explanation is that both processes are in turn dependent on a common cellular process which in turn is sensitive to these conditions.

The interaction of human Fc receptor-bearing lymphocytes with immobilized antigen-antibody complexes may provide a model system for studying an interesting physiological response of the lymphocyte to a signal originating at the cell surface. While this signal is not mitogenic, it has marked metabolic,³ as well as, morphological consequences to the cell. The mechanisms by which this signal triggers this complex cellular response and the relationship of these changes to the ability of cells to lyse antibody-coated target cells will be the object of further study.

Summary

The adhesion of human Fc receptor-bearing lymphocytes to immobilized antigen-antibody complexes is accompanied by marked alterations in cell shape, resulting in flattening of greater than 90% of the adherent cells. In addition, about 65% of the adherent cells become elongated, with distinct uropods being present in about 1/3 of these cells. Scanning electron microscopy demonstrates

³ Henkart, P., C. Neels, and J. Ryan. Manuscript in preparation.

that most of the surface microvilli are lost, while ruffled membranes and long microextensions are formed during the shape change. Time-lapse cinematography shows that the major shape changes occur within a few minutes after contact with the substrate, and that the adherent cells undergo translational motility. Both flattening and elongation of the adherent cells are inhibited by low temperature, chelating agents, cytochalasin B, and vinblastine, while sodium azide selectively inhibits elongation and uropod formation. It is argued that these morphological changes result from an active response of the cell to the immobilized complexes, and that such alterations may be related mechanistically to the ability of the cells to kill antibody-coated target cells.

We wish to express our appreciation to Ms. Cindy Neels for her excellent and enthusiastic technical assistance; Dr. Anthony Breuer for time-lapse cinematography; Doctors Timothy Triche and Raul Braylan for helpful discussions and for gracious use of scanning electron microscope facilities; Dr. William Terry for his support, interest and critical discussions; and Mrs. Marilyn Schoenfelder for her professional manuscript typing.

Received for publication 23 October 1975.

Bibliography

1. Greaves, M. F., J. J. T. Owen, and M. C. Raff. 1973. Cell interactions in immune responses. T and B lymphocytes. Excerpta Medica. American Elsevier Publishing Co., Inc., New York. 149.
2. Unanue, E. R. 1972. The regulatory role of the macrophage. *Adv. Immunol.* 15:95.
3. Feldmann, M., and A. Basten. 1972. Cell interactions in the antibody response in vitro. IV. Comparison of the effects of antigen specific and allogeneic factor. *J. Exp. Med.* 136:772.
4. Mosier, D. E. 1967. A requirement for two cell types for antibody formation *in vitro*. *Science (Wash. D. C.)*. 158:1573.
5. Nielsen, M. B., H. Jensen, O. Braendstrup, and Ole Werdelin. 1974. Macrophage-lymphocyte clusters in the immune response to soluble protein antigen in vitro. *J. Exp. Med.* 140:1245.
6. Lipsky, P. E., and A. S. Rosenthal. 1975. Macrophage-lymphocyte interaction. II. Antigen-mediated physical interactions between immune guinea pig lymph node lymphocytes and syngeneic macrophages. *J. Exp. Med.* 141:138.
7. Cerottini, J. C., and K. Brunner. 1974. Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. *Adv. Immunol.* 18:67.
8. Rutishauser, U., I. Yahara, and G. M. Edelman. 1974. Morphology, motility, and surface behavior of lymphocytes bound to nylon fibers. *Proc. Natl. Acad. Sci. U. S. A.* 71:1149.
9. Nicolson, G. L., and J. Blaustein. 1972. The interaction of *Ricinus communis* agglutinin with normal and tumor cell surfaces. *Biochim. Biophys. Acta.* 266:543.
10. LeVine, D., M. J. Kaplan, and P. J. Greenaway. 1972. The purification and characterization of wheat-germ agglutinin. *Biochem. J.* 129:847.
11. Anderson, T. F. 1951. Techniques for the preservation of three dimensional structure in preparing specimens for the electron microscope. *Trans. N. Y. Acad. Sci.* 13:130.
12. Wetzel, G., G. B. Cannon, E. L. Alexander, B. W. Erickson, Jr., and E. W. Westbrook. 1974. A critical approach to the scanning electron microscopy of cells in suspension. In *Scanning Electron Microscopy*. O. Johari and I. Corvin, editors, I. I. T. Research Institute, Chicago, Ill. 581.

13. North, R. J. 1968. The uptake of particular antigens. *J. Reticuloendothelial Soc.* 5:203.
14. Rabinovitch, M., and M. J. DeStefano. 1973. Macrophage spreading *in vitro*. I. Inducers of spreading. *Exp. Cell Res.* 77:323.
15. McFarland, W., and G. P. Schechter. 1970. The lymphocyte in immunological reactions *in vitro*: ultrastructural studies. *Blood.* 35:683.
16. Lewis, W. H., and L. T. Webster. 1921. Migration of lymphocytes in plasma cultures of human and lymph nodes. *J. Exp. Med.* 33:261.
17. DeBruyn, P. P. H. 1944. The motion of the migrating cells in tissue cultures of lymph nodes. *Anat. Res.* 89:295.
18. Abercrombie, M., J. E. M. Heaysman, and S. M. Pegrum. 1970. The locomotion of fibroblasts in culture. III. Movement of particles on the dorsal surface of the leading lamella. *Exp. Cell Res.* 62:389.
19. Alexander, E. L., and B. Wetzel. 1975. Human lymphocytes — similarity of B and T cell surface morphology. *Science (Wash. D. C.)*. 188:752.
20. Rosenstreich, D. L., E. Shevach, I. Green, and A. S. Rosenthal. 1972. The uropod-bearing lymphocyte of the guinea pig. Evidence for thymic origin. *J. Exp. Med.* 135:1037.
21. Biberfeld, P. 1971. Uropod formation in phytohaemagglutinin (PHA) stimulated lymphocytes. *Exp. Cell Res.* 66:433.
22. Unanue, E., K. A. Ault, and M. J. Karnovsky. 1973. Ligand-induced movement of lymphocyte surface macromolecules. IV. Stimulation of cell motility by anti-Ig and lack of relationship to capping. *J. Exp. Med.* 139:295.
23. Biberfeld, P., G. Biberfeld, P. Perlmann, and G. Holm. 1973. Cytological observations on the cytotoxic interaction between lymphocyte and antibody-coated monolayer cells. *Cell. Immunol.* 7:60.
24. Wessells, N. K., B. S. Spooner, J. F. Ash, M. O. Bradley, M. A. Ludend, E. L. Taylor, J. T. Wrenn, and K. M. Yamada. 1971. Microfilaments in cellular and developmental processes. *Science (Wash. D. C.)*. 171:135.
25. Wilson, L., J. R. Bamberg, S. B. Mizel, L. M. Grisham, and K. M. Creswell. 1974. Interaction of drugs with microtubule proteins. *Fed. Proc.* 33:158.
26. Vasiliev, J. M., I. M. Gelfand, L. U. Domnina, O. Y. Ivanova, S. K. Komm, and L. U. Olshevskaja. 1970. Effect of colcemid on the locomotory behavior of fibroblasts. *J. Embryol. Exp. Morphol.* 24:625.
27. Bensch, K. G., and S. E. Malawista. 1969. Microtubular crystals in mammalian cells. *J. Cell Biol.* 40:95.
28. Olmsted, J. B., and G. G. Borisy. 1973. Microtubules. *Annu. Rev. Biochem.* 42:507.
29. Henkart, P., M. Henkart, and E. Alexander. 1975. Behavior and fine structure of human Fc receptor bearing lymphocytes adherent to immobilized antigen-antibody complexes. Proceedings of the 10th Leukocyte Culture Conference. Academic Press, Inc., New York. In press.
30. Cohen, H. J. 1975. Human lymphocyte surface immunoglobulin capping: normal characteristics and anomalous behavior of chronic lymphocytic leukemic lymphocytes. *J. Clin. Invest.* 55:84.
31. Rabinovitch, M., and M. J. DeStefano. 1973. Macrophage spreading *in vitro*. II. Manganese and other metals as inducers or as co-factors for induced spreading. *Exp. Cell Res.* 79:423.
32. Hawkins, D. 1971. Biopolymer membrane: a model system for the study of the neutrophilic leukocyte response to immune complexes. *J. Immunol.* 107:344.
33. Henson, P. M. 1971. The immunologic release of constituents from neutrophil leukocytes. I. The role of antibody and complement on nonphagocytosable surfaces or phagocytosable particles. *J. Immunol.* 107:1535.

34. Rajaraman, R., D. E. Rounds, S. P. S. Yen, and A. Rembaum. 1974. A scanning electron microscope study of cell adhesion and spreading in vitro. *Exp. Cell Res.* 88:327.
35. Rabinovitch, M., and M. J. DeStefano. 1974. Macrophage spreading in vitro. III. The effect of metabolic inhibitors, anesthetics and other drugs on spreading induced by subtilisin. *Exp. Cell Res.* 88:153.
36. MacFarland, W., D. H. Heilman, and J. F. Moorhead. 1967. Functional anatomy of the lymphocyte in immunological reactions in vitro. *J. Exp. Med.* 124:851.
37. Lipsky, P. E., and A. S. Rosenthal. 1973. Macrophage lymphocyte interaction. I. Characteristics of the antigen-independent binding of guinea pig thymocytes to syngeneic macrophages. *J. Exp. Med.* 138:900.
38. Henkart, P., and R. Blumenthal. 1975. Interaction of lymphocytes with lipid bilayer membranes: a model for lymphocyte-mediated lysis of target cells. *Proc. Natl. Acad. Sci.* 12:2789.
39. Biberfeld, P. 1971. Cytotoxic interaction of phytohemagglutinin-stimulated blood lymphocytes with monolayer cells: a study by light and electron microscopy. *Cell. Immunol.* 2:54.
40. Rosenthal, A. S., and D. L. Rosenstreich. 1974. The lymphocyte uropod: a specialized surface site for immunologic recognition. *Biomembranes*. Plenum Publishing Corporation, New York. 5:1.
41. Chess, L., R. P. MacDermot, and S. F. Schlossman. 1974. Immunologic functions of isolated human lymphocyte subpopulations. I. Quantitative isolation of human T and B cells and response to mitogens. *Immunology.* 113:1113.
42. Choi, T. K., D. R. Sleight, and A. Nisonoff. 1974. General method for isolation and recovery of B cells bearing specific receptors. *J. Exp. Med.* 139:761.
43. Perlmann, P., and G. Holm. 1969. Cytotoxic effects of lymphoid cells *in vitro*. *Adv. Immunol.* 11:117.
44. Able, M. E., J. C. Lee, and W. Rosenau. 1970. Lymphocyte-target cell interaction in vitro. *Am. J. Pathol.* 60:421.
45. Biberfeld, P., G. Biberfeld, P. Perlmann, and G. Holm. 1973. Cytological observations on the cytotoxic interaction between lymphocytes and antibody-coated monolayer cells. *Cell. Immunol.* 7:60.
46. Martz, E. 1975. Early steps in specific tumor cell lysis by sensitized mouse T lymphocytes. I. Resolution and characterization. *J. Immunol.* 115:261.
47. Henney, C. S., and J. E. Bubbers. 1973. Antigen-T lymphocyte interactions: inhibition by cytochalasin B. *J. Immunol.* 111:85.