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Crawling movements of lymphocytes on and beneath fibroblasts in culture

(activated lymphocytes/cell motility/T-cell leukemias/surface antigens)

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ABSTRACT **Some lymphocytes become highly motile upon immunological stimulation *in vivo* or *in vitro*. When introduced into a culture of 3T3 or L cells and followed by live-cell microscopy, some of these lymphocytes were observed to crawl on top of, along the edges of, and preferentially beneath the attached fibroblasts. The crawling could be as rapid as 20 μm/min, easily detectable without a time-lapse device. The striking ability of crawling lymphocytes to penetrate beneath attached 3T3 cells provided a quantitative means to compare the crawling activity of different lymphocyte populations under various conditions. Crawling was diminished by inhibitors of energy metabolism, by agents that disrupt the cytoskeleton, and by absence of Mg^{2+} and Ca^{2+}, but not of Ca^{2+} alone. Crawling lymphocytes were virtually absent in normal thymus and spleen cells. They increased greatly in 5-day mixed lymphocyte cultures and in peritoneal exudate lymphocytes taken after mice had been immunized with allogeneic tumor cells. T cells accounted for most of the crawlers. Of two T-cell leukemias tested, $R1^+$ cells were crawlers whereas EL-4 cells were not. The *H-2* haplotype of the 3T3 fibroblasts (i.e., whether syngeneic or allogeneic) had no apparent effect on lymphocyte crawling activity. The crawling may relate to the exploration of cell surface antigens by lymphocytes (immune surveillance), to the mode of action of cytotoxic T cells, to the migration of lymphocytes across blood vessel walls, or to the penetration of lymphocytes into "solid" masses of normal tissue or tumor cells.**

The interactions of lymphocytes with other cells are important in the induction and regulation of immune responses and in the expression of immune functions, such as the destruction of target cells bearing abnormal surface antigens. Very little is known about the ways in which lymphocytes explore the surface of other cells to hunt for appropriate surface antigens, or penetrate into a "solid" mass of cells, or cross endothelial or mesothelial boundaries to make contact with extravascular cells. The present paper describes an approach to the analysis of these exploratory and migratory activities. In particular, it describes the crawling behavior of lymphocytes on and beneath attached fibroblasts in culture and describes conditions for comparing the crawling activity of lymphocyte populations that have been activated in various ways.

MATERIALS AND METHODS

Mice and Cells. In the following list of mouse strains, tumors, and cell lines, the haplotypes of *H-2*, the major histocompatibility complex of the mouse, are given in parentheses. Mice of the BALB/c AnN (*d*) and of the congenic BALB.B (*b*), and BALB.K (*k*) strains were produced at the Massachusetts Institute of Technology. 3T3 fibroblasts derived from these mice were developed by Hale *et al.* (1). A line of L cells [transformed fibroblasts from the C3H/HeJ (*k*) strain] was obtained from H. Green. The T-cell lymphomas, $R1^+$ from C58/J (*k*) and EL-4 from C57BL/6 (*b*) mice, were obtained from M. Bevan and H. Winn, respectively. LPC-1, a myeloma of BALB/c AnN, was adapted to grow in culture (2). L cells, $R1^+$ and EL-4, and LPC-1 were grown in minimal essential medium (low Ca^{2+}), RPMI-1640, and L-15 (GIBCO), respectively, each supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, Rockville, MD).

Lymphocyte Stimulation. Spleen cells were cleared of erythrocytes with NH_4Cl and resuspended in culture medium (1, 2). In mixed lymphocyte cultures, 7×10^6 "responder" spleen cells were incubated with 3×10^6 γ-irradiated (1000 rad; 1 rad = 1.00×10^{-2} J/kg) allogeneic "stimulator" cells in 2 ml of medium for 4–6 days (1, 2). Lymphocytes at 1.0×10^7 cells per ml were also stimulated with concanavalin A (Worthington) at 2 μg/ml or with lipopolysaccharides (Difco) at 10 μg/ml for 2 days to generate predominantly T- and B-cell blasts, respectively. BALB/c AnN mice were immunized by an initial ("priming") intraperitoneal injection of 2×10^7 EL-4 cells and a second ("booster") intraperitoneal injection 4 weeks later of 2×10^7 cells, either EL-4 cells or BALB.B spleen cells (both have the $H\text{-}2^b$ haplotype).

Separation of B and T Cells. To obtain B lymphocytes, T cells were eliminated by treating spleen cells or cells recovered from mixed lymphocyte cultures or peritoneal exudates with anti-Thy-1 antibodies and complement (normal rabbit serum). The anti-Thy-1 was a 1:2000 dilution of ascites fluid from mice bearing a hybridoma that produces monoclonal anti-Thy 1.2 (3); dead cells were removed on a density gradient. To obtain T lymphocytes, the same mixed cell populations were passed through a nylon wool column to remove B cells and macrophages (4). Peritoneal exudate cells were incubated in a petri dish at 37°C for 1 hr to allow macrophages to adhere to the plate; only the nonadherent cells (lymphocytes) were tested.

Live-Cell Microscopy. Chambers for visualizing cells by microscopy were constructed essentially as described (5). L or 3T3 cells (2×10^5) in 5 ml of Dulbecco's modified Eagle's medium (GIBCO)/10% serum were plated on a 75 × 25 mm glass slide (Fisher) in a 100-mm petri dish (Falcon). After 4 hr (37°C in 10% CO_2/90% air) the cells were covered by a 22 × 22 mm glass cover slip (Corning) supported by bits of cover slip (0.1-mm thick) at each corner to form a chamber. Lymphocytes at 1.0×10^6 cells/ml in the same medium were introduced into the chamber, which was then sealed along its edge with nontoxic wax. While the chamber was observed under a Zeiss microscope (Model 2432, equipped with a camera), it was maintained at 37°C with an Air Curtain incubator (Sage, Cambridge, MA). Cells remained viable and active for more than 12 hr, but observations were generally completed in 3–4 hr. Photographs were taken with Kodak PX panchromatic film (Eastman Kodak) at ×160 or ×400 magnifications.

Assay for Lymphocytes That Crawl underneath 3T3 Cells. 3T3 cells (1.5×10^5) in 5 ml of medium were plated on a 60-mm petri dish, and after 4 hr 2.0×10^6 lymphocytes, whose crawling

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activity was to be tested, were added. After 2 hr at 37°C, the dish was agitated and nonadherent cells were removed by aspiration. The dish was then washed three times with 5 ml of 3.7% formaldehyde in phosphate-buffered saline (0.15 M NaCl/0.01 M K phosphate, pH 7.4); 5 ml of the same solution was kept in the dish for 30 min at room temperature and then replaced by phosphate-buffered saline (no formaldehyde) for storage at 4°C until the cells were counted. Lymphocytes that had crawled beneath 3T3 cells appeared under the phase-contrast microscope as dark forms ("shadows") that could be readily distinguished from the bright refractile lymphocytes on top of 3T3 cells (which could adhere without necessarily having crawling capability); only the lymphocytes beneath 200 3T3 cells were counted (under ×160 magnification in 10–15 randomly chosen fields) to measure crawling activity.

Scanning Electron Microscopy. The foregoing procedure for counting lymphocytes that had crawled beneath fibroblasts was modified by plating the cells in a petri dish that contained about 12 × 12 mm pieces of petri dish plastic. After 2 hr the pieces of plastic were removed and the cells on them were fixed *in situ* with 4% glutaldehyde in RPMI-1640 (room temperature, 30 min) before being processed further for scanning electron microscopy (6).

RESULTS AND DISCUSSION

Lymphocyte Movement and Crawling on and beneath Fibroblasts. Previous studies on the lytic activity of cytotoxic T cells by microcinematography suggested that these cells were motile (7–9). The present studies focused on the motile activity of various lymphocytes on individual fibroblasts.

At 37°C, under the phase-contrast microscope, some of the lymphocytes that were stimulated *in vivo* or *in vitro* were observed (without a time-lapse device) to change shape continuously and to move about. When the live-cell chambers contained spread L cells, some of the motile lymphocytes crawled on top of or along the edges of these fibroblasts (Fig. 1); it was especially striking that when a lymphocyte came to the edge, it crawled beneath more often than above the fibroblasts (Figs. 1*B*, 2 and 3). The proportion of cells that changed shape, moved, and crawled on fibroblasts varied with the source of the lymphocytes and with their treatment (see below). The crawling could be as rapid as 20 μm/min, or about 100 times faster than migrating 3T3 cells (10), and was often a repetitious back and forth movement (Fig. 3*A*). In crawling underneath fibroblasts, the lymphocytes flattened their bodies substantially and could be seen through the thin cytoplasm of spread L cells or 3T3 cells as "dark shadows" (Fig. 2). This behavior of lymphocytes is reminiscent of the preference of motile transformed fibroblasts to underlap, rather than overlap, other cells upon contact (11).

The contact area between lymphocytes and fibroblasts generally involved a large part of the lymphocyte surface (Fig. 1), and some lymphocytes encircled the thin cytoplasmic extensions of fibroblasts (Fig. 1*C*). Occasionally, lymphocytes maintained physical contact with fibroblasts by means of very fine processes (as thin as 0.1–0.5 μm in diameter). These linkages broke when the culture was agitated. It was only when cytotoxic T cells were included in the lymphocyte population that the crawled-upon fibroblasts occasionally underwent lysis (Fig. 3*B*) (see below).

Scoring of Lymphocytes underneath 3T3 Cells under Various Conditions. Lymphocytes may stick to fibroblasts without being able to crawl, but only those with crawling activity can find their way underneath spread 3T3 cells. Therefore, to compare the crawling activity of various lymphocyte populations under different conditions, we counted only the lymphocytes beneath 3T3 cells.

Crawling was inhibited almost completely by decreasing the incubation temperature to 20–25°C, or partially by treating cells with 10 mM NaN_3. The presence of cytochalasin B or colchicine, both of which disrupt cytoskeleton, abolished the lymphocytes' shape-changing activity, motility, and crawling on fibroblasts, which may account for the ability of these agents to block cytotoxic T-cell lysis of target cells (12, 13). (3T3 cells gradually retracted their spread cytoplasm in the presence of cytochalasin B at 10 μg/ml or 50 μM colchicine.) Because the presence of Mg^{2+} is essential for the formation of cytotoxic T cell–target cell "conjugates" and the lytic process also requires the presence of Ca^{2+} (14, 15), we examined the effects of removing these ions. As shown in Table 1, crawling was partially

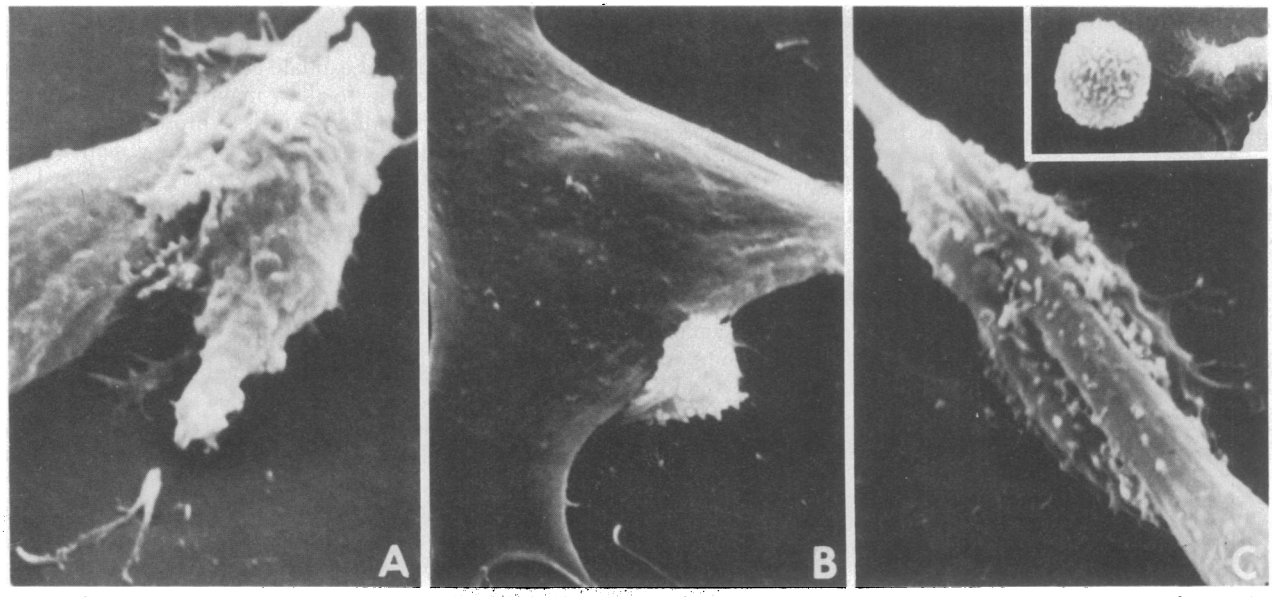

FIG. 1. Contact between lymphocytes and fibroblasts. Lymphocytes were recovered from a 5-day mixed lymphocyte culture [BALB/c AnN ($H\text{-}2^d$) spleen cells incubated with γ-irradiated BALB.K ($H\text{-}2^k$) spleen cells] and were plated with L cells ($H\text{-}2^k$). The cells were prepared for scanning electron microscopy. Note lymphocytes clasping the processes of spindle-shaped L cells in *A* and *C* and a lymphocyte half underneath a spread L cell in *B*. (*A*, ×7200; *B*, ×4300; *C*, ×5700.) The *inset* shows a round, presumably inactive, lymphocyte. (×2900.)

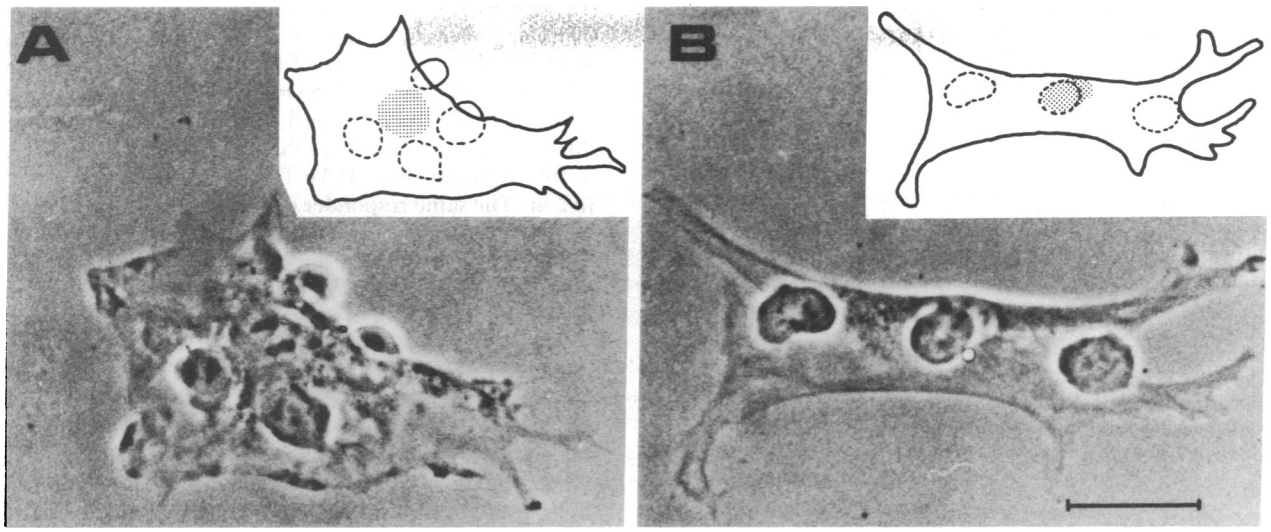

FIG. 2. Lymphocyte crawling beneath fibroblasts. Lymphocytes were prepared as in Fig. 1 and the fibroblasts were 3T3 cells derived from BALB.K mice. The diagrams show cell contours; broken lines indicate lymphocytes underneath 3T3 cells; shaded areas indicate nuclei of 3T3 cells. (*A* and *B*, ×720.) The bar measures 25 μm.

inhibited by removing Ca^{2+} and Mg^{2+}, but not by removing Ca^{2+} alone.

Crawling Activity in Various Lymphocyte Populations. Lymphocytes with crawling activity were rare in thymus and spleen of nonimmune mice. However, the frequency was greatly increased among cells recovered after 5 days in mixed lymphocyte culture, and it was especially high in the peritoneal exudate lymphocytes taken from mice that had been immunized with allogeneic tumor cells (Table 2). It is noteworthy that the intensity of crawling activity in peritoneal exudate lymphocytes was correlated with the intensity of the antigeneic stimulation *in vivo*: when the cells injected as the "booster" stimulus were the same as those injected in the "priming" stimulus, the resulting crawling activity was maximal; when the booster cells shared only some alloantigens with the priming cells, the resulting activity was less, though still greatly elevated

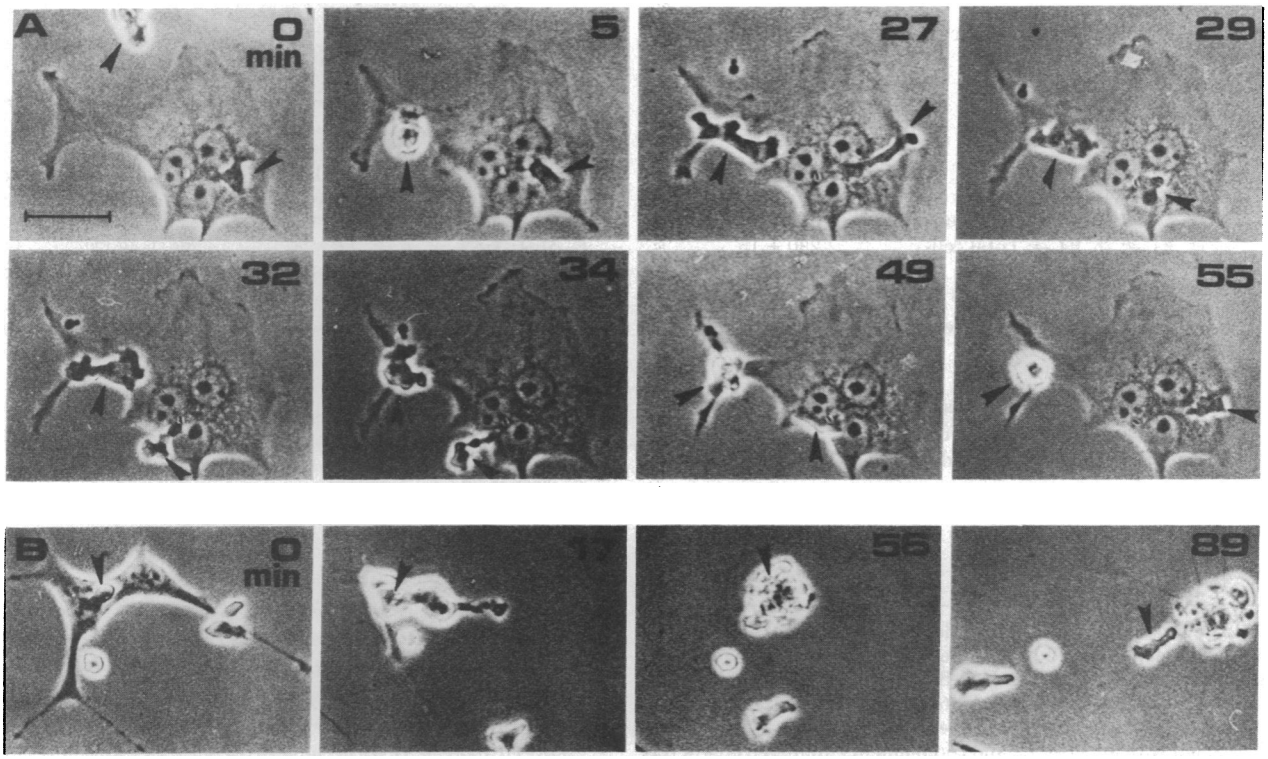

FIG. 3. (*A*) Repetitious crawling of lymphocytes beneath fibroblasts. Two lymphocytes (arrows) crawl beneath a trinuclear L cell. The lymphocyte at upper-left approached the L cell (0–5 min), slipped beneath it (27–34 min), and then emerged but remained in contact with the L cell (49–55 min). The lymphocyte at lower-right emerged from beneath the L cell just before the photographic sequence was initiated, and then the lymphocyte repetitiously underlapped (5, 29, 49 min) and emerged from (27, 32, 55 min) the L cell. (*B*) Lysis of an L cell by lymphocytes. One lymphocyte (arrow) is underneath the L cell and another encircles a process of the L cell. Cell lysis began before 17 min and was complete before 56 min. Lymphocytes in *A* and *B* were prepared as in Fig. 1. The photographic sequences took place about 30–50 min after the cells were plated. (×480). The bar measures 25 μm.

Table 1. Effects of various conditions and agents on the crawling activity of lymphocytes*

Treatment	Activity†
None	99 ± 6
Room temperature	2.8 ± 1.5
NaN_3 (10 mM)	46 ± 4
Cytochalasin B (10 μg/ml)	0 ± 0
Colchicine (50 μM)	0 ± 0
EGTA (2.0 mM)‡	96 ± 2
EDTA (3.2 mM)‡	40 ± 4

* Lymphocytes and 3T3 fibroblasts were as described in the legend of Fig. 2.

† The number of lymphocytes found beneath 200 attached 3T3 cells in an assay in which 2×10^6 lymphocytes were cultured for 2 hr with 1.5×10^5 fibroblasts. Values are mean ± SD of four determinations, two from each of duplicate preparations.

‡ [Ethylenebis(oxyethylenenitrilo)] tetraacetic acid (EGTA) depletes Ca^{2+} in the medium; EDTA depletes both Ca^{2+} and Mg^{2+}.

(Table 2). The frequency of crawlers increased only slightly when normal spleen cells were treated with concanavalin A or lipopolysaccharides for 2 days.

T cells accounted for most of the crawlers in the mixed lymphocyte cultures. It is probable that all cytotoxic T cells are crawlers, but because of the abundance of crawlers in the antigenically stimulated peritoneal exudate lymphocytes and in mixed lymphocyte culture, it is probable that not all crawlers are cytotoxic T cells. When cytotoxic T lymphocytes in the cells recovered from mixed lymphocyte culture were specifically directed against products of the 3T3 cells' H-2 complex, about 5–10% of the attached 3T3 cells were observed to undergo lysis in 2 hr. The same responder cells lysed about 30% of the same 3T3 cells (at the same responder to target cell ratio) in a standard test tube assay, based on the release of ^{51}Cr from lysed target cells (unpublished data). Only a small proportion of the crawled-upon 3T3 cells underwent lysis.

Table 2 also shows that the cells ($R1^+$) of a T lymphoma from the C58/J strain were crawlers, whereas the cells (EL-4) of a T lymphoma from the C57BL/6 strain were not. It would be interesting to determine if crawling and noncrawling T-cell leukemias arise from T cells at different maturation stages. It would also be of interest to determine the correlation (if any) between the crawling activity and surface differentiation antigens of lymphocytes (16) from diverse lymphomas. The cells of LPC-1, a myeloma from the BALB/c AnN strain, were noncrawlers.

Table 2. Crawling activity of various lymphocytes*

Cells	Activity
A. Nonimmunized mice	
Spleen T cells	2.5 ± 0.6
Spleen B cells	2.0 ± 0.8
Thymocytes	2.3 ± 1.0
B. 5-day mixed lymphocyte culture	
Total cells	82 ± 6
T cells	118 ± 13
B cells†	6.3 ± 1.5
Control spleen cells‡	29 ± 4
C. Alloimmunized mice§	
1°, EL-4; 2°, none: Total cells	9.1 ± 1.2
1°, EL-4; 2°, EL-4: Total cells	230 ± 15
T cells	236 ± 12
B cells†	15 ± 2.5
1°, EL-4; 2°, BALB.B spleen cells: Total cells	82 ± 5
D. Mitogen-stimulated culture	
Concanavalin A-treated T cells¶	8.8 ± 1.6
Lipopolysaccharide-treated B cells¶	25 ± 3
E. From cultured tumor cell lines	
$R1^+$ (T-cell lymphoma)	51 ± 3
EL-4 (T-cell lymphoma)	0 ± 0
LPC-1 (myeloma)	0 ± 0

* BALB/c AnN mice provided the spleen cells in part A, B, and D, and the peritoneal exudate lymphocytes in part C. In part B, the stimulator cells were γ-irradiated BALB.K spleen cells. The stimulation in parts C and D is described in *Materials and Methods*. For activity measurements, see legend of Table 1.

† Treatment with anti-thy 1.2 and complement eliminated 88% of the cells from 5-day mixed lymphocyte cultures and 93% of peritoneal exudate cells from the alloimmunized mice.

‡ Five days in culture without stimulator cells.

§ BALB/c AnN mice were injected first (1°) with EL-4 ($H\text{-}2^b$) cells and then (2°) with nothing or with EL-4 or BALB.B ($H\text{-}2^b$) spleen cells.

¶ T and B cells from the spleen were separated before stimulation.

Fibroblast *H-2* Haplotype and Lymphocyte Crawling Activity. Because T cells generally seem able to recognize products of the major histocompatibility complex on the surface of other cells (1, 10, 17, 18), we determined whether the crawling activity of lymphocytes depends upon the *H-2* haplotype of the fibroblasts. Table 3 shows that the active lymphocytes crawled equally well on 3T3 cells with diverse *H-2* haplotypes, whether these fibroblasts carried "self" or "nonself" H-2 antigens, or the particular allogeneic H-2 antigens that stimulated the responder lymphocytes in mixed lymphocyte culture. That the lymphocyte–fibroblast contact is probably

Table 3. Crawling activity is not apparently affected by fibroblast *H-2* haplotype

Lymphocytes*	H-2 haplotype of BALB 3T3 cells	H-2 relationship between 3T3 cells and lymphocytes†	Activity‡
1. Anti-$H\text{-}2^k$	*k*	allo (target)	43 ± 4
	d	syn	45 ± 6
2. Anti-$H\text{-}2^d$	*d*	allo (target)	80 ± 7
	k	syn	48 ± 5
3. Anti-$H\text{-}2^k$	*k*	allo (target)	33 ± 4
	d	allo	38 ± 3
4. Anti-$H\text{-}2^d$	*d*	allo (target)	53 ± 2
	k	allo	45 ± 2
5. Con A blasts ($H\text{-}2^k$)	*k*	syn	14 ± 3
	d	allo	12 ± 2
6. Anti-$H\text{-}2^k$	*k*		47 ± 3
	k§		44 ± 5

* Generated in mixed lymphocyte cultures with spleen cells from mice of congenic strains: 1 and 6, BALB/c anti-BALB.K; 2, BALB.K anti-BALB/c; 3, BALB.B anti-BALB/c; 4, BALB.B anti-BALB.K. In 5, the lymphocytes were blasts produced by treatment of BALB.K spleen cells with concanavalin A (Con A).

† Allo, allogeneic; syn, syngeneic.

‡ For activity measurements, see legend of Table 1.

§ Anti-$H\text{-}2^k$ antiserum was present in the assay. The antiserum was generated in BALB.B mice that received more than 10 biweekly intraperitoneal injections of BALB.K spleen cells.

not predominantly related to H-2 antigens is also suggested by the finding that the presence of antiserum against the H-2 antigens of 3T3 cells did not affect the ability of the lymphocytes to crawl beneath these 3T3 cells.

Because crawling activity appears to be indifferent to *H-2* haplotypes, it could lead to nonspecific adherence to attached cells, and this could interfere with efforts to purify or enrich for cytotoxic T cells by specific adsorption on fibroblast monolayers (19), unless the adsorption is carried out under conditions (such as low temperature) in which crawling activity is negligible.

Possible Physiological Functions. It is easy to imagine that crawling activity might be essential for several lymphocyte functions *in vivo*. For instance, it could be essential for (*i*) the lytic activity of cytotoxic T cells, (*ii*) exploration of cell surface for patches of appropriate antigens (i.e., nonspecific crawling precedes a possible specific reaction), (*iii*) migration across vascular endothelial and pleural and peritoneal mesothelial layers, and (*iv*) penetration into "solid" masses of cells, such as tumors.

Some of these possibilities are testable. For example, crawlers (such as $R1^+$ leukemic cells, see Table 2) and noncrawlers may be labeled with different radioisotopes, and their migration across blood vessels and into strategic sites (such as spleen or transplanted growing tumors) or solid tissues (such as muscle) may be followed and compared. Preliminary observations have shown that crawling lymphocytes can penetrate confluent monolayers of fibroblasts or endothelial cells in culture (unpublished results).

Note Added in Proof. This work was presented, in part, at a meeting of the American Association of Immunologists, April 1979, Dallas, Texas (20).

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