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Two Distinct Mechanisms Involved in the Infiltration of Lymphocytes into Tumors

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We have analyzed the mechanism controlling the infiltration of lymphocytes into tumor tissues. W3/25 (+) (helper/inducer phenotype) T cells obtained from tumor tissues of T-9 sensitized rats produced soluble factors. We demonstrated that the soluble factors were responsible for the infiltration of T lymphocytes into tumor tissues by using a modified Boyden chamber technique. We established a system in which we stained filters of the Boyden chamber by an immunoperoxidase technique, thus directly determining the phenotype of cells that had actually migrated into the filters in response to the soluble factors. Upon fractionation of soluble factors produced by W3/25 (+) T cells, four peaks of lymphocyte migration factor (LMF) activity were detected. Peaks B and C exhibited strong LMF activity and specifically attracted R1-10B5 (+) (suppressor/killer phenotype) T cells. Thus, the infiltration of R1-10B5 (+) T cells into tumor tissues was partly explained by LMF produced by tumor-infiltrating W3/25 (+) T cells. The expression of a putative receptor for LMF by lymphocytes may also influence the degree of lymphocyte infiltration into tumors.

Key words: Chemotactic factor — Boyden chamber — Immunoperoxidase staining

We previously demonstrated that tumor-infiltrating T cells played an important role in the regulation of inoculated syngeneic tumor cell growth.[1–6] Histopathological examination demonstrated that tumor tissues were infiltrated by neutrophils and macrophages at 6 to 12 hr after tumor injection. The infiltration of W3/25 (+) (helper/inducer phenotype) T cells into tumor tissues was found at 4 days after tumor inoculation and subsequently W3/25 (+) T cells were replaced by R1-10B5 (+) (suppressor/killer phenotype) T cells at 6 days after tumor injection.[7] However the mechanism that controls the infiltration of lymphocytes into tumors is not well understood. We found that tumor-infiltrating neutrophils produce soluble factor(s) that attract W3/25 (+) T cells.[7] A modified Boyden chamber has been used for the detection of soluble factors that attract lymphocytes.[7,8] Although we used purified W3/25 (+) T cells, cells that migrated into the filter of the Boyden chamber amounted to approximately 0.6% of applied cells.[7] Therefore, we could not formally rule out the possibility that some contaminating cells migrated in response to soluble factors rather than W3/25 (+) T cells. In order to determine if soluble factors regulate the infiltration of T cells into tumor tissues, it is essential to establish a system in which we can directly determine the cell types of cells migrating into the filter. With this in mind, we have used various fixation methods and tested if a certain fixation is suitable for the subsequent immunoperoxidase staining of lymphoid cells by using various monoclonal antibodies against rat lymphoid cells. We report here that 1% paraformaldehyde fixation for 20 min at room temperature is suitable for the subsequent immunoperoxidase staining of cells in the filter. Since tumor-infiltrating R1-10B5 (+) T cells were responsible for the eradication of tumor cells and W3/25 (+) T cells preceded the appearance of R1-10B5 (+) T cells,[3,7] we tested if W3/25 (+) T cells produce soluble factors that attract R1-10B5 (+) T cells. Furthermore we analyzed the mechanisms that regulate the sensitivity of lymphocytes to soluble factors.

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MATERIALS AND METHODS

Animals Inbred 6- to 8-week-old female Fischer and Lewis rats were purchased from Shizuoka Animal Center (Hamamatsu).

Reagents and Antibodies The monoclonal antibodies (MoAb), R1-3B3, R1-10B5, W3/25 and R2-1A6 that detect rat lymphocyte antigens comparable to mouse Lyt-1, Lyt-2,3,[9] L3T4[10] and rat neutrophil-macrophage specific antigen,[11] respectively, were used in this study. A murine MoAb specific for rat B cells, RLN-9D3 was also used in this study.[12] A fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse immunoglobulin (Ig) was purchased from Meloy Laboratories, Springfield, VA and was absorbed with normal rat serum coupled to Sepharose-2B as described previously.[13]

Sensitization of Fischer Rats with T-9 Cells Fischer rats were sensitized with T-9 cells as described previously.[3] Briefly, T-9 cells were injected subcutaneously into the dorsal area of syngeneic Fischer rats. Ten days after the inoculation of T-9 cells, the tumor mass was surgically removed and 1×10^7 mitomycin C-treated T-9 cells were injected subcutaneously once a week, 6 times.

Cell Fractionation The method used for cell fractionation was the same as described previously.[7] Briefly, spleen cells and tumor-infiltrating cells were fractionated into B cell- and T cell-enriched fractions by using a nylon wool column. The T cell-enriched fraction was further fractionated into W3/25 (+) T cells or R1-10B5 (+) T cells by a panning method using R1-10B5 and W3/25 MoAb. Peritoneal exudate cells (PEC) elicited by intraperitoneal injection of proteose pepton were collected by washing the peritoneal cavity with phosphate-buffered saline, pH 7.2 (PBS).[11] PEC were then fractionated into neutrophil- and macrophage-enriched fractions by density gradient centrifugation with Percoll. The purity of cells in each fraction was determined by an indirect immunofluorescence technique using various MoAbs and was analyzed by using a fluorescence-activated cell sorter as described.[14] The purity of T cell, B cell, W3/25 (+) T cell, R1-10B5 (+) T cell, macrophage, and neutrophil fractions was 93%, 86%, 96%, 95%, 96% and 96%, respectively.

Production of Lymphocyte Migration Factor Ten million T-9 cells were injected into the right footpads of T-9-sensitized rats 1 week after the completion of sensitization. Tumor tissues were surgically removed at 6 hr after tumor inoculation. Tumor-infiltrating neutrophils were purified as described previously[7] and were cultured for 24 hr at a cell density of 5×10^6/ml. Culture supernatant (CS) was recovered and used as LMF-a. LMF-a was fractionated on a Mono Q column as described previously.[7] Peak A of LMF-a, which attracted lymphocytes as well as macrophages and neutrophils, and peak D of LMF-a, specifically chemotactic for W3/25 (+) T cells, were obtained as described previously[7] and used in this study. Tumor tissues were also surgically removed at 4 days after tumor injection. Tumor-infiltrating cells were fractionated into W3/25 (+) T cells. These W3/25 (+) T cells were cultured for 24 hr at a cell density of 1×10^6/ml. CS was recovered and used as LMF-4d. LMF-4d was dialyzed against 20m*M* Tris HCl buffer, pH 8.0, and applied to a Mono Q column. The gradient was generated using 20m*M* Tris HCl buffer, pH 8.0, with 1*M* NaCl at a flow rate of 0.5 ml/min. Each fraction was tested for LMF activity.

Detection of Lymphocyte Migration Factor Activity Lymphocyte migration activity was assessed by a modified Boyden chamber technique.[8] Spleen cells were obtained from Fischer rats. Lymphocytes to be used as responding cells were obtained from spleens of Fischer rats by Ficoll-Hypaque gradient centrifugation. The purity of lymphocytes was more than 96% as judged from the morphology and by esterase staining. In some experiments, various purified subsets of spleen cells were used as responding cells. To assess the cell migration activity, 1×10^5 cells in 0.2 ml of RPMI-1640 medium were placed in the upper one of two chemotactic chambers separated by a 5 μm cellulose nitrate filter (Sartorius, Gottingen, West Germany) to prevent entry of the cells into the lower compartment where 200 μl of RPMI-1640 medium alone (negative control) or RPMI-1640 containing an experimental sample was placed. After a 3 hr incubation at 37°, the filter was removed, fixed with 10% formalin in PBS for 20 min at 4° and stained with hematoxylin. Cell movement was quantitated by counting the total number of cells that had migrated more than 40 μm from the upper surface of the filter in five randomly selected microscope high-power fields (HPF; 10×40). Migrating cell number was determined by subtracting the background cell number (the medium was used as a control sample) from the counted cell number in the presence of a sample.

Immunoperoxidase Staining In order to clarify what type(s) of cells migrate into a chemotactic filter in response to LMF-a or LMF-4d, filters were stained by an immunoperoxidase technique. Chemotactic filters were fixed with various fixatives for various times at various temperatures. After being washed, filters were incubated with various MoAbs for 1 hr at room temperature and then washed three times with PBS. Filters were then reacted with biotinylated goat anti-mouse Ig for 30 min. After washing, the filters were reacted with

avidin-peroxidase conjugate.[15] The enzyme reaction was developed with 0.05% 3,3′-5,5′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05*M* Tris HCl buffer, pH 7.6.

Cell Activation Spleen cells of Lewis rats were cultured in complete medium at a cell density of 5×10^6/ml in the presence of 4 μg/ml concanavalin A (Con A). After 3 hr, cells were recovered, washed, resuspended in complete medium at a cell density of 3×10^6/ml and cultured for 45 hr. The supernatant was recovered and used as Con A-activated cell supernatant (CAS). Five million popliteal lymph node cells obtained from normal Fischer rats were incubated with 0.5 μg/ml Con A, 0.5n*M* 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or 30% CAS for 1 hr at 37°. Cells were also cultured for 48 hr at 37° in the presence of 25 μg/ml lipopolysaccharide (LPS) or 0.5m*M* periodate.

Results

Effects of Various Fixatives on Filters and on Reactivity of Cells to Antibodies Spleen cells, lymphocytes, purified R1-10B5 (+) T cells, macrophages neutrophils, or B cells were incubated against peak A of LMF-a and purified W3/25 (+) T cells were incubated against peak D of LMF-a. After 3 hr of incubation, filters were removed and fixed with various reagents. Filters were then stained by an immunoperoxidase technique using R1-3B3, R1-10B5, W3/25, RLN-9D3 and R2-1A6 MoAb. As shown in Table I, the nitrocellulose filters were totally destroyed by acetone and methanol fixation at 4° for 5 min. After the fixation of filters by ethanol, cells that had migrated into filters were clearly stained by R1-3B3, R1-10B5 and R2-1A6 MoAb, whereas these migrating cells were not stained by W3/25 MoAb in spite of the use of purified W3/25 (+) T cells in the LMF assay. With PLP fixation, the reactivity of migrating cells to various MoAbs was well preserved. However, the reactivity to W3/25 MoAb was occasionally destroyed by PLP fixation. With glutaraldehyde fixation, the reactivity of migrating cells to MoAbs tested was completely lost. In contrast, the reactivity of migrating cells to various MoAbs was well preserved after the fixation of filters with 1% paraformaldehyde in PBS. Next, we fixed filters by 1% paraformaldehyde at various temperatures for various periods of time. We

Table I. Effects of Various Fixatives on Filters and on Reactivity of Cells to Antibodies[a]

Fixative		Filter	LMF assay: Responder cells	LMF assay: Factor[b]	Reactivity of cells to antibodies
Acetone	100%	destroyed	Spleen cells	peak A	ND[c]
Methanol	50%	destroyed			
	70%	destroyed	Spleen cells	peak A	ND[c]
	95%	destroyed			
Ethanol	20%	well preserved	R1-10B5 (+) T cells	peak A	good
	40%	well preserved	W3/25 (+) T cells	peak D	lost
	50%	well preserved	B cells	peak A	good
			macrophages and neutrophils	peak A	good
Paraformaldehyde	1%	well preserved	R1-10B5 (+) T cells	peak A	good
			W3/25 (+) T cells	peak D	good
			B cells	peak A	good
			macrophages and neutrophils	prak A	good
PLP		well preserved	R1-10B5 (+) T cells	peak A	good
			W3/25 (+) T cells	peak D	lost
			B cells	peak A	good
			macrophages and neutrophils	peak A	good
Glutaraldehyde	5%	well preserved	lymphocytes	peak A	lost
			macrophages and neutrophils	peak A	lost

a) Filters were fixed with various fixatives at 4° for 5 min, then stained by the immunoperoxidase technique using R1-3B3, R1-10B5, W3/25, RLN-9D3, or R2-1A6 MoAb.
b) Peaks A and D of LMF-a were used in this study.
c) ND; not determined.

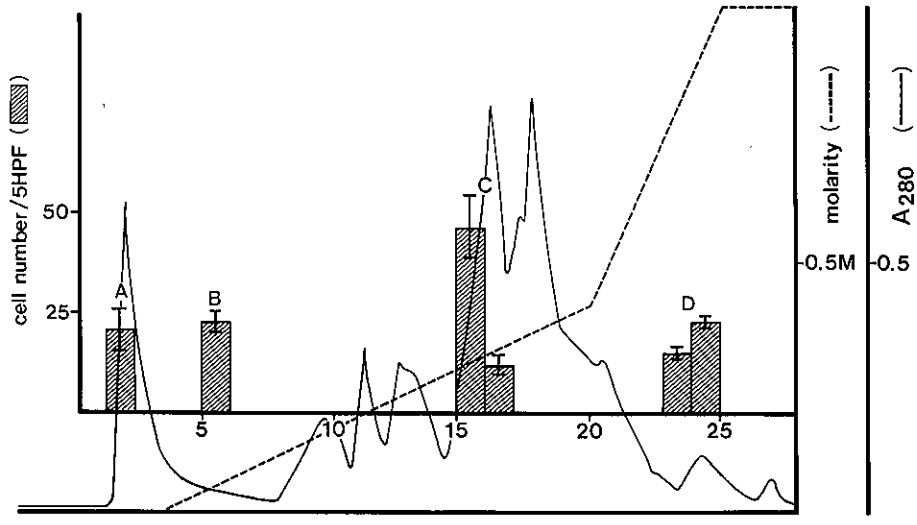

Fig. 1. Representative elution profile of LMF activity on a Mono Q anion exchange column. LMF-4d was obtained as described in "Materials and Methods," and applied to a Mono Q column. The elution profile of LMF activity is based on 3 experiments. Values represent migrating cell number per 5 HPF±SEM. Protein concentration is shown as absorbance at 280 mm.

Table II. Sensitivity of Various Lymphoid Cells to Each Peak of LMF-4d[a]

Cells used in the LMF assay[b]	Peak A		Peak B		Peak C		Peak D	
	×1	×2	×1	×2	×1	×2	×1	×2
T cells	10±2	10±3	20±2	20±3	38±4	36±4	18±2	4±1
R1-10B5 (+) T cells	9±3	9±2	21±3	20±4	36±3	36±4	18±4	4±2
W3/25 (+) T cells	3±2	3±1	3±4	3±2	4±3	3±2	16±3	4±3
B cells	4±1	3±2	4±3	4±2	4±1	4±2	16±4	3±2
Macrophages	14±2	13±2	5±4	4±3	5±2	4±2	4±3	4±2
Neutrophils	15±3	12±4	4±3	3±2	4±3	4±2	4±3	4±2

a) LMF-4d was fractionated on a Mono Q column, and each peak (undiluted or 1:2 diluted) was used in the LMF assay. Each sample was tested in triplicate. Data are from 3 experiments and values represent the mean ±SEM.

b) Cells used in this assay were prepared as described in "Materials and Methods."

found that 1% paraformaldehyde at room temperature for 20 min is best for the preservation of reactivity of the migrating cells to MoAbs.

Fractionation of LMF-4d on a Mono Q Anion Exchange Column LMF-4d was applied to a Mono Q column. Each fraction was tested for LMF activity using spleen cells as responding cells. Four peaks of LMF activity were detected (Fig. 1). The strongest LMF activity was eluted at 0.3*M* NaCl (peak C). A weak LMF activity was detected as an unbound fraction (peak A). Two other peaks, B and D, were eluted at 0.06*M* and 0.9*M* NaCl, respectively. Each peak eluted from the Mono Q column was tested for LMF activity against various purified lymphoid cells. As shown in Table II, peak B as well as peak C attracted only R1-10B5 (+) T cells. Peak A attracted T cells as well as macrophages and neutrophils, whereas peak D attracted T cells and B cells.

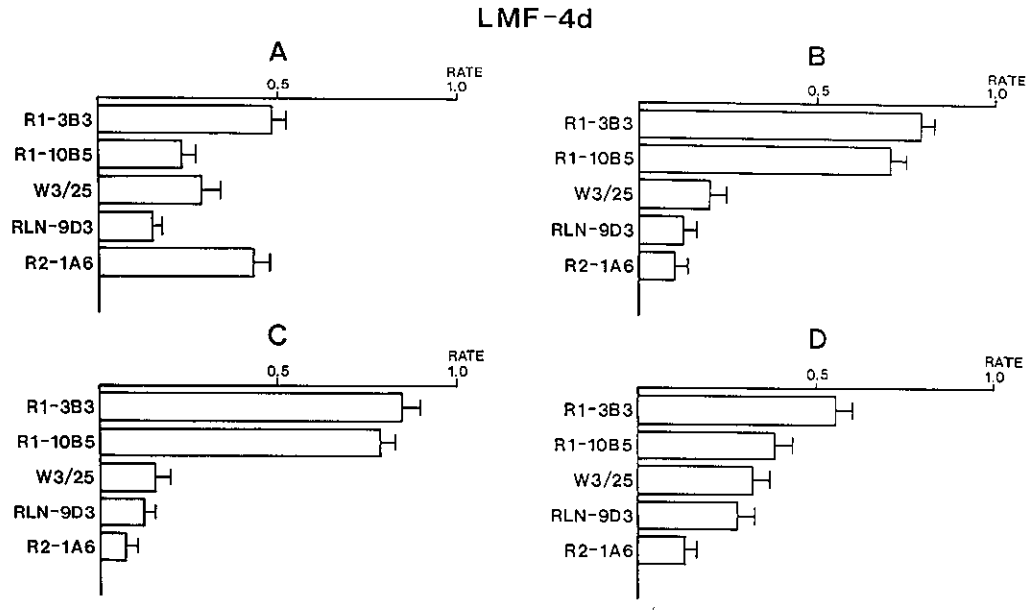

Fig. 2. Differential sensitivity of various lymphoid cells to each peak of LMF-4d. Spleen cells were incubated against various peaks of LMF-4d (A, peak A; B, peak B; C, peak C; D, peak D) by using a modified Boyden chamber. Filters were stained with various MoAbs. Values represent immunoperoxidase-positive migrating cell number/total migrating cell number per 5 HPF±SEM.

Identification of a Cell Subset by Immunoperoxidase Staining In the next experiment, each peak eluted from a Mono Q column was tested for LMF activity against normal spleen cells by using a modified Boyden chamber. After a 3 hr incubation, filters were fixed with 1% paraformaldehyde in 0.1*M* phosphate buffer, pH 7.4, for 20 min at room temperature. After being washed with PBS, filters were stained by immunoperoxidase staining. As shown in Fig. 2, cells which migrated in response to peaks B and C were stained by R1-3B3 and R1-10B5 MoAbs, indicating that migrating cells were R1-10B5 (+) T cells. Cells which migrated in response to peak A were stained by R1-3B3 as well as R2-1A6 MoAb, indicating that macrophages, neutrophils, and T cells migrated in response to peak A. Peak D did not attract R2-1A6 (+) cells, whereas T cells and B cells migrated in response to peak D. Representative results are shown in Fig. 3. Spleen cells migrating into filters in response to peak C of LMF-4d were stained by R1-3B3 (A) and R1-10B5 MoAb (B), but not by W3/25 MoAb (C). Spleen cells migrating into filters in response to peak A of LMF-4d were stained by R1-3B3 (D) and R2-1A6 MoAb (E), but not by RLN-9D3 MoAb (F).

Modulation of the Lymphocyte Sensitivity to LMF Since our previous data demonstrated that various lymphocytes exhibited different sensitivities to LMF-a and LMF-4d,[7] the potential mechanisms for the differential sensitivity of lymphocytes to LMF were also analyzed. As shown in Table III, popliteal lymph node cells obtained from normal Fischer rats did not migrate in response to LMF-4d or LMF-a. Upon incubation of lymph node cells with periodate, Con A, or CAS, lymph node cells gained the ability to migrate in response to LMF-4d, whereas periodate and Con A-activated lymph node cells failed to migrate in response to LMF-a. In contrast, lymph node cells failed to migrate in response to LMF after LPS or TPA treatment.

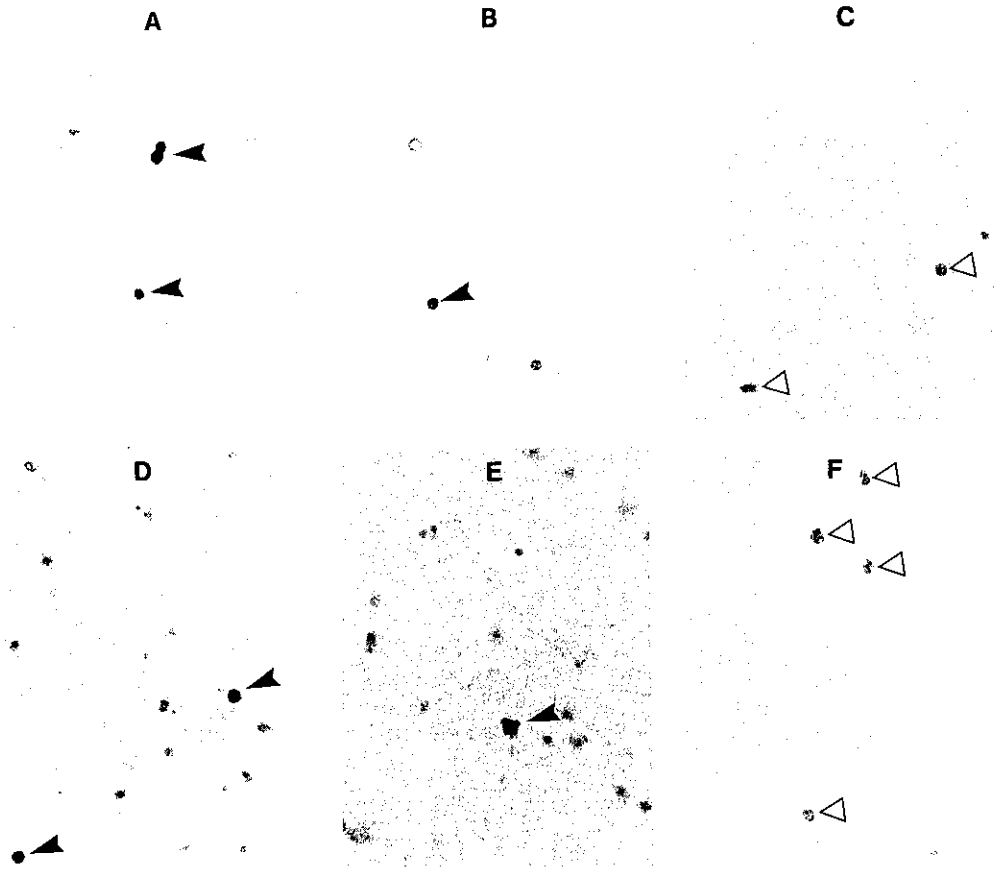

Fig. 3. Immunoperoxidase staining of cells that infiltrated into filters. Spleen cells that migrated into filters in response to peak C of LMF-4d were stained by R1-3B3 (A) and R1-10B5 (B), but not by W3/25 (C). In contrast, spleen cells that migrated into filters in response to peak A of LMF-4d were stained by R1-3B3 (D) as well as R2-1A6 (E), but not by RLN-9D3 (F). Positive staining is indicated by ◀. Negative staining is indicated by ◁.

Discussion

The modified Boyden chamber analysis has been used for the detection of soluble factors that attract lymphocytes. Although we have used a purified population of cells as responding cells in the LMF assay, the purity was only approximately 90%. In addition, cells that migrated into the filter of the Boyden chamber amounted to 0.6% of the applied cells at most.[7] By the Boyden chamber technique, one could therefore not define which type(s) of cells actually migrated into filters in response to LMF. It is possible that some contaminating cells migrated into filters. We established a system that permitted us to directly identify and quantitate cells that migrated into filters. As shown in Fig. 2 and Table II, the results obtained by the conventional Boyden chamber technique and by direct visualization of cells by the immunoperoxidase technique are essentially the same. Therefore, peaks B and C indeed attracted R1-10B5 (+) T cells. Thus, infiltration of R1-10B5 (+) T cells into tumor tissues may be partly explained by the production of a chemotactic factor.

Table III. Modulation of Sensitivity of Lymphocytes to LMF

Treatment[b]	Peak C of LMF-4d	Peak D of LMF-a
None	5±3[c]	4±1
Periodate	31±2	3±1
LPS	4±2	2±1
CAS	37±4	15±2
Con A	47±6	4±2
TPA	4±1	ND[d]

a) LMF-a and LMF-4d were fractionated on a Mono Q column, and peak D of LMF-a and peak C of LMF-4d were used in the LMF assay. Popliteal lymph node cells were obtained from normal Fischer rats and were used as responding cells.
b) Popliteal lymph node cells were treated as described in "Materials and Methods."
c) Each sample was tested in triplicate, Data are based on 3 experiments and values represent the mean±SEM.
d) ND; not determined.

However, more importantly, binding of chemotactic factors to their receptors is presumably the first step in the mechanisms of chemotactic action.[16] In this regard, it should be noted that lymphoid cells exhibited different sensitivities to LMF-a and LMF-4d.[7] Regional lymph node cells obtained from T-9-sensitized rats 1 day after T-9 injection exhibited sensitivity to LMF-a, but not to LMF-4d, whereas regional lymph node cells obtained from T-9 sensitized rats 6 days after T-9 injection showed sensitivity to LMF-4d, but not to LMF-a. Therefore, it is conceivable that the infiltration of lymphocytes into tumors is also regulated by a mechanism controlling the expression of chemotactic receptors (or sensitivity to chemotactic factors). Upon activation of popliteal lymph node cells by Con A or periodate, lymph node cells gained the ability to respond to LMF-4d, but not to LMF-a. As reported previously,[7,17] spleen cells obtained from normal Fischer rats migrate in response to LMF-a as well as LMF-4d. Activation of cells by TPA or the combination of TPA and Ca^{2+} ionophore abolished the ability of spleen cells to respond to LMF-4d. Therefore, it seems likely that sensitivity of lymphocytes to LMF is not a constitutive property of lymphocytes. Rather, the sensitivity of lymphocytes to LMF may be up- or down-regulated by local microenvironmental stimuli in the course of immune reactions.

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