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Functional Analysis of Mononuclear Cells Infiltrating into Tumors: Establishment of a New System to Obtain Functionally Active Tumor-infiltrating Cells

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We have demonstrated that tumor-infiltrating T cells play a crucial role in tumor growth. In order to investigate the precise function of tumor-infiltrating cells, we avoided the use of any enzyme for separation of tumor-infiltrating cells from the solid tumor mass and established a new system in which we could easily obtain functionally active tumor-infiltrating cells in large amounts from the peritoneal cavity of T-9 gliosarcoma-sensitized rats. In this system, viable T-9 cells were intraperitoneally administered and peritoneal exudate cells were recovered. Importantly, cells obtained from the intraperitoneal cavity are phenotypically and functionally indistinguishable from those obtained from subcutaneous tumor masses. Furthermore, injection of purified lymphocyte migration factor-b resulted in the accumulation of CD8 (+) cytotoxic T cells in the peritoneal cavity. Therefore, this system also provides a means by which the *in vivo* function of chemotactic factor can be tested.

Key words: Tumor-infiltrating lymphocyte — Chemotactic factor

We previously emphasized the functional importance of tumor-infiltrating cells in the growth regulation of inoculated syngeneic tumor cells.[1-3] However, the precise mechanisms by which tumor-infiltrating cells are attracted to tumor sites are not well understood. In recent publications, we clearly demonstrated the presence of two distinct chemotactic factors responsible for the infiltration of T cells into tumor tissues.[4-6] Tumor-infiltrating neutrophils isolated from T-9 gliosarcoma-sensitized rats produced lymphocyte migration factor-a (LMF-a)[2] that is specifically chemotactic for helper/inducer T cells.[4] On the other hand, tumor-infiltrating helper/inducer T cells isolated from T-9 gliosarcoma-sensitized rats produced LMF-b that is selectively chemotactic for cytotoxic/suppressor T cells.[6] If chemotactic factors are actually involved in the accumulation of T cells in tumor tissues, it is essential to determine if they can attract functionally active cytotoxic T cells *in vivo*. We previously separated tumor-infiltrating cells by treating the tumor mass with collagenase and DNase.[1] In order to determine the activity of tumor-infiltrating cells, it is important to establish a system in which we can easily obtain a large amount of tumor-infiltrating cells. We report here the establishment of a new system in which tumor-infiltrating cells can easily be obtained, and which can be used to test the *in vivo* activity of LMF.

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[2] Abbreviations used in this paper: MoAb, monoclonal antibody; LMF, lymphocyte migration factor; CS, culture supernatant; PBS, phosphate-buffered saline, pH 7.2; PEC, peritoneal exudate cells; PEL, peritoneal exudate lymphocytes; CTL, cytotoxic T lymphocytes; CGF, cytotoxic cell-generating factor.

MATERIALS AND METHODS

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

Reagents and antibodies The monoclonal antibodies (MoAb) R1-3B3 and R1-10B5 detect rat T cells and cytotoxic/suppressor T cells, respectively.[7] R2-1A6 detects rat neutrophil- and macrophage-specific antigen.[8] RTH-7[9] and RLN-9D3,[5] specific for rat helper/inducer T cells and B cells, respectively, were also used in this study. 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.[10]

Separation of tumor-infiltrating cells Ten million viable T-9 cells were inoculated into the footpads of T-9 gliosarcoma-sensitized rats. The method used for the sensitization of rats with T-9 cells was described elsewhere.[1] Tumor tissues were surgically removed at the indicated times. Tissues were minced into small pieces with scissors and pipetted vigorously for 10 min. Then tumor tissues were digested with 0.025% collagenase and 0.02% DNase at 37°C for 30 min to disperse cells. The cell suspension was centrifuged on a Ficoll-Conray density gradient to obtain mononuclear cells. In some experiments, five million T-9 cells were injected into the peritoneal cavity of T-9-sensitized as well as normal rats. Peritoneal exudate cells (PEC) were recovered at 2, 4, and 6 days after T-9 injection. For the analysis of cell subsets using FACStar, the live gate was set to eliminate the T-9 cell population on the basis of large cell size as

compared to other hematopoietic cells. Preliminary experiments demonstrated that viable T-9 cells were less than 2% of PEC at 2 and 4 days after T-9 injection, and less than 1% at 6 days after T-9 injection in T-9-sensitized rats. In normal rats, 10% of PEC was T-9 cells at 4 and 6 days and less than 5% of PEC was T-9 cells due to the formation of an intraperitoneal tumor mass. For the separation of lymphocytes from PEC, discontinuous density gradient centrifugation with Percoll was used.[5] As judged from the morphology and expression of CD5, more than 90% of PEC was T lymphocytes and the contamination with T-9 cells was less than 0.5%.

Production of lymphocyte migration factor The method used for the production of LMF was the same as described previously.[4] Viable T-9 cells were inoculated into footpads of T-9 gliosarcoma-sensitized rats. After 12 h, tumor-infiltrating neutrophils were separated from tumor tissues and were cultured for 24 h at a cell density of 5×10^5/ml as described previously.[1] Culture supernatant (CS) was recovered and used as LMF-a. After 4 days, tumor-infiltrating CD4 (+) T cells were separated from tumor tissues and cultured for 24 h at a cell density of 5×10^5/ml. CS was recovered and used as LMF-b.[5]

Detection of lymphocyte migration activity Lymphocyte migration activity was assessed by a modified Boyden chamber technique.[4] Splenic lymphocytes used as responding cells were obtained from spleen cells of Fischer rats by Ficoll-Hypaque gradient centrifugation. The purity of lymphocytes was more than 96% as judged from the morphology and esterase staining. To assess the cell migration, 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 the 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 h incubation at 37°C, the filter was removed, and fixed with 1% paraformaldehyde for 5 min. Filters were then incubated with various MoAbs for 1 h at room temperature and washed with PBS three times. Filters were reacted with biotinylated goat anti-mouse Ig for 30 min. After washing, the filters were reacted with avidin-peroxidase conjugate. The enzyme reaction was developed as described previously.[11] Nuclei were counterstained 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 cell number counted in the presence of experimental samples.

Fractionation of LMF Peak D of LMF-a and peak C of LMF-b (specifically chemotactic for CD 4(+) or CD 8 (+) T cells, respectively) were obtained by the method described previously.[4,6] Briefly, CS was concentrated by 80% (v/v) saturated ammonium sulfate precipitation at 4°C for 2 h. The supernatant was clarified by centrifugation at 12,000g at 4°C for 1 h. The precipitates were redissolved in 20 mM Tris-HCl buffer, pH 7.5, with 100 mM NaCl. The samples were concentrated in about 1/40th of the original volume and dialyzed against 20 mM Tris-HCl buffer, pH 8.0. The samples were then chromatographed by using a fast protein liquid chromatography system (Pharmacia) with a Mono Q anion exchange column. The gradient was generated using 20 mM Tris-HCl buffer, pH 8.0, with 1 M NaCl. Peak D of LMF-a, which is specifically chemotactic for CD4 (+) T cells,[4] peak C of LMF-b which is specifically chemotactic for CD8 (+) T cells and peak B of LMF-b which is specifically chemokinetic for CD8 (+) T cells[6] were used in this study.

^{51}Cr release assay K562 cells or T-9 gliosarcoma cells (1×10^7) were suspended in 1 ml of RPMI-1640. [^{51}Cr]Sodium chromate (100 μCi) (Amersham) was added, and the cells were incubated at 37°C for 90 min in 5% CO_2. The cells were washed extensively and then suspended at 1×10^5 cells/ml in RPMI-1640. One hundred microliters of target cells (T-9 cells) and 100 μl of effector cells at various E:T ratios were added to each well of 96-well, round-bottomed microtiter plates. The plates were centrifuged at 25g for 10 min and then incubated for 16 h at 37°C in 5% CO_2. After incubation, the plates were centrifuged at 400g for 10 min and the supernatants were removed. The isotope release was determined with the use of an LKB Multigamma 1260 gamma counter. The control consisted of target cells incubated with 100 μl of RPMI-1640 (spontaneous release). The mean value of triplicate cultures was used for statistical evaluation. The percent specific lysis was calculated as described previously.[1]

Cytofluorographic analysis Tumor-infiltrating cells obtained from the intraperitoneal cavity were divided into portions containing 1 to 2×10^6 cells, and were sedimented for staining. Mouse MoAb, R1-3B3, R1-10B5, RTH-7, R2-1A6, and RLN-9D3 were added to the cells in 10 μl of appropriate dilutions of the MoAb, and the mixtures were incubated for 45 min at 4°C. The cells were washed three times and then were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig for an additional 30 min at 4°C. For the detection of surface Ig on these cells, the cells were stained with FITC-conjugated rabbit anti-rat Ig antibody. Cells were washed twice and were diluted appropriately for examination in the fluorescence-activated cell sorter (FACStar; Becton-Dickinson, Sunnyvale, CA).

The data were expressed as a histogram in which the intensity of fluorescence was plotted on the abscissa against the relative number of cells on the ordinate. The analysis of each sample was carried out with 20,000 cells.

RESULTS

The phenotype of PEC in response to T-9 injection Viable T-9 cells were injected into the peritoneal cavity of normal and T-9 gliosarcoma-sensitized rats. We previously reported that the accumulation of lymphocytes was observed after injection of T-9 cells into footpads of T-9 sensitized rats. However, the separation of tumor-infiltrating cells from tumor tissues required enzyme treatment of tumor tissues. Therefore, we searched for a better system for obtaining tumor-infiltrating cells. As shown in Table I, the number of peritoneal exudate cells in T-9 sensitized rats is far greater than that obtained in normal rats. Less than one million cells were recovered from normal rats at 6 days after T-9 injection, whereas 25 times more cells were recovered in T-9 sensitized rats. The difference in total cell number may be attributed to the difference in T cell number. Thirty-three times more T cells were recovered in T-9-sensitized rats as compared to the number obtained from normal rats. The percentage of neutrophils and macrophages in peritoneal exudate cells decreased from approximately 57% at 2 days to 14% at 6 days in T-9-sensitized rats, whereas 54% at 2 days and 37% at 6 days of PEC cells were neutrophils and macrophages in normal rats. In the next experiments, the live gate was set to eliminate neutrophil and macrophage populations upon FACStar analysis, so that only the lymphocyte population was studied. Representative results are shown in Fig. 1. At 2 days, 90% of lymphocytes were T cells and approximately 46% of lymphocytes were CD 8 (+) T cells. The percentage of CD 8 (+) T cells in the lymphocyte population increased gradually and at 6 days more than 63% of lymphocytes were CD 8 (+) T lymphocytes. The increase of CD 8 (+) T cells is in contrast to the decrease of CD 4 (+) T cells. The percentage of B cells in the lymphocyte population remained unchanged. In normal rats, 55% of PEC was T cells, but the T cell number was approximately 3% of that obtained from T-9-sensitized rats, and macrophages and neutrophils were major components of the population.

The chemotactic ability of PEC Since previous studies demonstrated that regional lymph node cells exhibited differential sensitivity to LMF,[4] we examined whether peritoneal exudate lymphocytes exhibit similar differential sensitivity to LMF. Peritoneal exudate lymphocytes of normal rats obtained at 2, 4 and 6 days after tumor injection failed to exhibit a chemotactic response to either LMF-a or LMF-b. In T-9-sensitized rats, however, peritoneal exudate lymphocytes (PEL) at 2 days exhibited sensitivity only to LMF-a, whereas cells obtained at 4 and 6 days were strongly sensitive to LMF-b (Table II).

The cytotoxicity of PEL PEL obtained from normal and T-9-sensitized rats were tested for their ability to lyze T-9 cells. In normal rats, PEL obtained at 2, 4, and 6 days did not exhibit any cytotoxicity against T-9 cells. In contrast, PEL of T-9-sensitized rats were strongly cytotoxic against T-9 cells at 6 days (Fig. 2). The same cells failed to exhibit cytotoxicity against K562 cells. Tumor-infiltrating cells were also obtained from footpads as described above and tested for cytotoxicity towards K562 and T-9 cells. As shown in Fig. 2, PEL showed similar cytotoxicity against T-9 cells to tumor-infiltrating cells.

The production of LMF by PEC PEL obtained from normal rats did not produce a detectable amount of LMF, whereas PEL obtained from T-9-sensitized rats produced LMF. It should be noted that PEL produced

Table I. The Phenotype of Peritoneal Exudate Cells[a]

	Normal rats			T-9-sensitized rats		
	2[b]	4	6	2	4	6
Unfractionated	100±21[c]	100±20	60±15	300±50	700±100	1500±350
T cells	33±10	40±15	33±12	92±15	430±80	1100±250
B cells	2.3±0.5	2.9±1.0	8.2±2.5	9.6±3.1	56±10	26±8
Neutrophils and macrophages	54±9	43±10	22±5	170±40	140±35	220±60

a) Ten million T-9 cells were injected intraperitoneally in normal as well as T-9-sensitized rats.
b) Peritoneal exudate cells were collected at 2, 4, and 6 days after tumor injection. Cells were stained with R1-3B3 (anti-T) MoAb, R2-1A6 (anti-macrophage and neutrophil) MoAb or RLN-9D3 (anti-B) MoAb and were analyzed by FACStar.
c) Data are based on 3 rats. Values represent the mean cell number of PEC±SEM ($\times 10^5$).

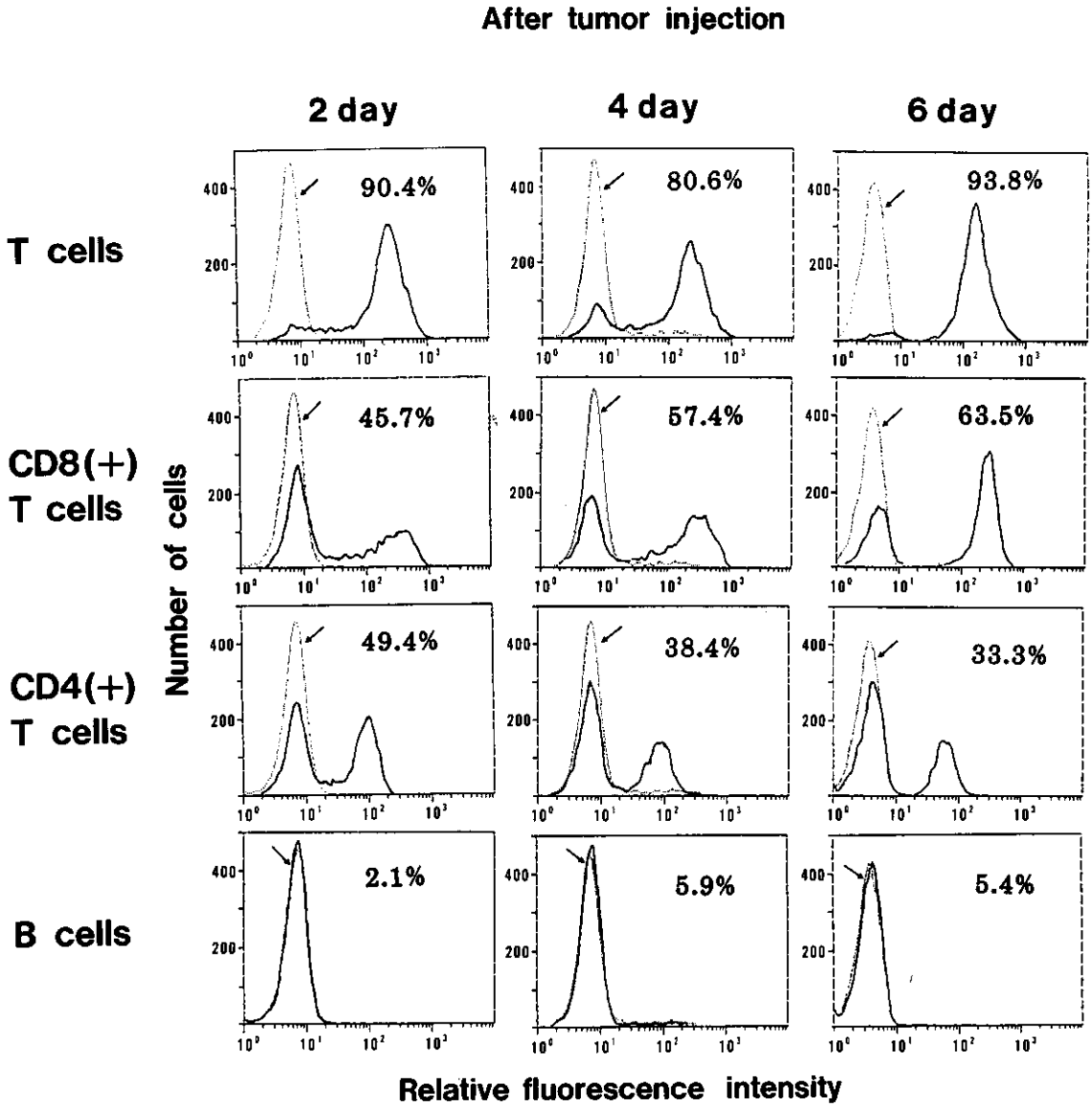

Fig. 1. Cell surface phenotype of tumor-infiltrating lymphocytes. PEC were obtained from T-9-sensitized rats at the indicated times. Upon FACStar analysis, the live gate was set so that only the lymphocyte population was studied. Arrows indicate the control sample stained with FITC-goat anti-mouse Ig.

LMF at 4 and 6 days, whereas PEL at 2 and 8 days failed to produce LMF (Table III).

The *in vivo* activity of LMF In order to test whether LMF semipurified by the methods described previously[6] is active *in vivo*, LMF was injected intraperitoneally. Although the data are not shown, PEL did not exhibit any cytotoxic activity against T-9 cells upon intraperitoneal injection of LMF into normal and T-9-sensitized rats. This result simply reflects the lack of cytotoxicity generating factor in the above system. Therefore, T-9 cells were injected into the right footpad of T-9-sensitized rats and at the time of T-9 injection, LMF was administered intraperitoneally. In this system, stimulation of CGF production was initiated by viable T-9 injection. PEL were recovered and their cytotoxic activity against T-9 cells was determined. As shown in Table IV, peak C of LMF-b attracted cytotoxic T cells, whereas the control sample, peak B of LMF-b, failed to attract cytotoxic T cells.

Table II. Migration Activity of Peritoneal Exudate Lymphocytes against LMF-a and LMF-b

	Normal rats				T-9-sensitized rats			
	2[a]	4	6	8	2	4	6	8
Peak D of LMF-a	5±1[b]	4±1	4±1	nd[c]	32±4	4±1	5±1	4±1
Peak C of LMF-b	3±1	4±1	5±1	nd	5±1	42±16	72±10	18±3

a) Peritoneal exudate lymphocytes used in this assay were collected at 2, 4, 6, and 8 days after intraperitoneal injection of T-9 cells in normal and T-9-sensitized rats.
b) Cells were assessed for their ability to migrate in response to LMF-a and LMF-b by a Boyden chamber technique. Cells were obtained from 3 rats, separately and the data are based on 3 separate experiments. Values represent the mean cell number±SEM.
c) nd; not determined.

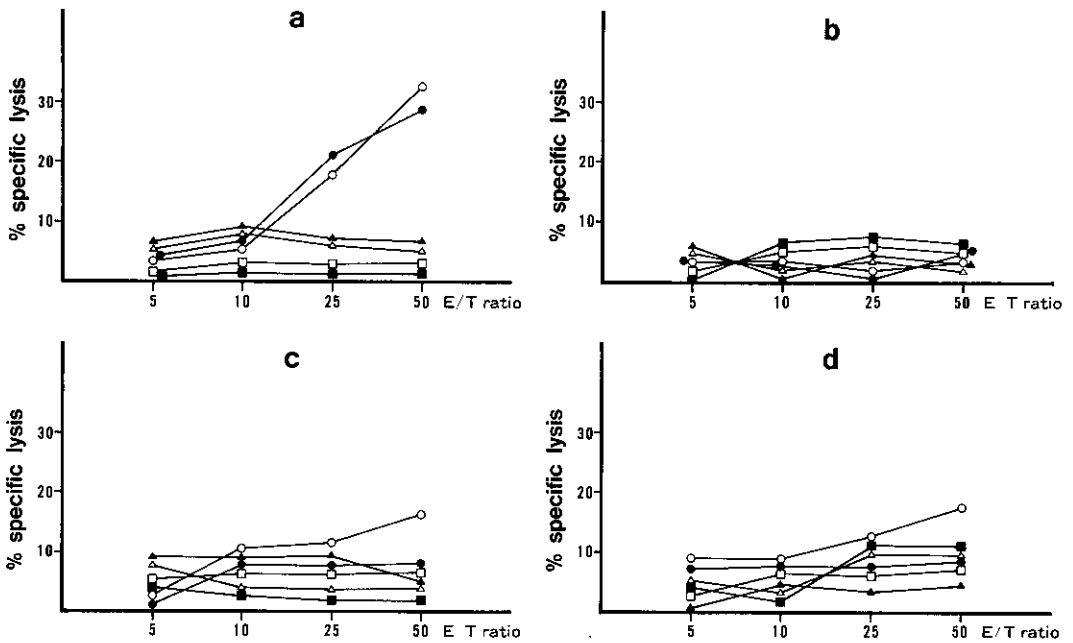

Fig. 2. Cytotoxicity of peritoneal exudate or tumor-infiltrating lymphocytes. Peritoneal exudate (open symbols) or tumor-infiltrating (closed symbols) lymphocytes were obtained from normal (b and d) and T-9-sensitized rats (a and c) at 2 (□, ■), 4 (△, ▲), and 6 (○, ●) days. Cytotoxicity was assessed by *in vitro* ^{51}Cr release assay against K562 (c and d) and T-9 cells (a and b) at various effector and target ratios.

DISCUSSION

We have demonstrated that tumor-infiltrating cells play a crucial role in the growth regulation of inoculated tumor cells. The tumor-infiltrating cells used in the *in vitro* functional study were separated from the subcutaneous solid tumor tissues by enzyme treatment. Furthermore, the number of tumor-infiltrating cells obtained from tumor masses was limited. Therefore, a system which would allow us to obtain a large number of tumor-infiltrating cells was needed. We reported here the establishment of a system which can provide a variety of functionally active tumor-infiltrating cells in large numbers. It should be noted that 25 times as many cells were recovered from T-9-sensitized rats as compared to non-sensitized rats. This phenomenon was also detected in a subcutaneous tumor system described previously.[1] The change in lymphocyte predominance from helper/inducer T cells to cytotoxic/suppressor T cells is detected

Table III. Production of LMF by Peritoneal Exudate Lymphocytes and Tumor-Infiltrating Lymphocytes[a)]

Cell	PEL				TIL			
	2 days	4 days	6 days	8 days	2 days	4 days	6 days	8 days
Normal rats	4±1[b)]	5±1	5±2	5±1	4±1	4±2	5±2	5±2
T-9-sensitized rats	5±2	52±7	32±4	4±2	6±2	40±8	42±8	6±5

a) Peritoneal exudate lymphocytes (PEL) or tumor-infiltrating lymphocytes (TIL) were obtained at 2, 4, 6, and 8 days after intraperitoneal or subcutaneous injection of T-9 cells, respectively. Cells were then cultured for 24 h at a cell density of 5×10^6/ml. Culture supernatant was recovered and assessed for LMF ability by a Boyden chamber technique.
b) Data are based on 3 separate experiments. Values represent the mean cell number±SEM.

Table IV. Cytotoxicity of Peritoneal Exudate Lymphocytes Attracted to LMF-b

Sample[a)]	Attracted PEL[b)]	Cytotoxicity against T-9 cells				Cytotoxicity against K562 cells			
		E/T ratio				E/T ratio			
		100:1	50:1	25:1	12.5:1	100:1	50:1	25:1	12.5:1
Peak B of LMF-b	T cells[c)]	8.1±2.3	5.9±4.1	5.7±2.1	5.4±3.3	12.5±2.9	8.7±4.2	5.9±2.3	4.9±3.4
Peak C of LMF-b	T cells	28.2±5.9	22.0±6.7	19.6±4.3	11.6±1.2	11.5±2.0	6.8±2.7	2.2±1.1	4.0±2.7
	R1-10B5(+) T cells	36.5±1.1	27.4±5.9	21.7±3.3	18.1±3.4	17.0±2.6	15.2±2.8	13.2±2.8	12.5±2.1

a) Semipurified peak B and peak C of LMF-b were injected intraperitoneally, into T-9-sensitized rats which had received T-9 cells into the footpad at 2 days previously.
b) T cells or R1-10B5 (+) T cells were purified from peritoneal exudate lymphocytes (PEL) as described in "Materials and Methods."
c) Cytotoxicity was not determined using R1-10B5 (+) T cell population.

in both the subcutaneous tumor system and the new system described here. The change in lymphocyte subset was previously explained in terms of the production of two distinct chemotactic factors.[4,6)] The present data clearly indicated that peritoneal exudate cells produced chemotactic factors at 4 and 6 days. Furthermore, peritoneal exudate cells exhibited different sensitivity to LMF-a and LMF-b depending upon the time when they were obtained after tumor injection. More importantly, PEC exhibited strong cytotoxic activity against T-9 cells, but not K562 cells. These data collectively indicated that the properties of PEC described here resemble those previously observed in tumor-infiltrating cells of T-9-sensitized rats.[1,4-6)] Thus, we have shown that the new system described here provides, in a large number, tumor-infiltrating cells functionally and phenotypically indistingushable from those obtained from solid tumor masses. We have also tested the *in vivo* activity of purified LMF-b. Intraperitoneal injection of purified LMF-b resulted in the specific accumulation of CD8 (+) T cells, which is in contrast to the observation that no specific accumulation of CD8 (+) T cells occurred following injection of a control sample.[6)] However, we should point out that not all lymphoid cells exhibit sensitivity to LMF-b, as shown here and previously.[4)] Spleen cells obtained from normal as well as T-9-sensitized rats exhibited good sensitivity to both LMF-a and LMF-b. In contrast, regional lymph node cells obtained from T-9-sensitized rats responded to LMF at a certain time. PEC obtained from normal rats did not show sensitivity to LMF-b. It is conceivable that the receptor for LMF is not constitutively expressed by lymphocytes, but rather the receptors are up- and down-regulated by microenvironmental stimuli. When we examined cytotoxicity of peritoneal exudate cells of T-9-sensitized rats against T-9 cells, no cytotoxic activity was detected (data not shown). This result is reasonable since mature CTL is not detected in T-9 sensitized rats as described previously.[1)] Only CTL precursor cells are detected in spleen.[2,3)] Therefore the signal for the initiation of CTL differentiation should be supplied. We provided the CTL differentiation signal by injecting viable T-9 cells into the footpads of T-9-sensitized rats together with intraperitoneal injection of LMF-b. This may stimulate the production of a soluble factor by spleen cells that is essential for the differentiation of CTL (cytotoxic cell-generating factor;

CGF), as described previously.[2,3] We obtained PEC (mainly CD8 (+) T cells) from the intraperitoneal cavity of those rats and tested them for cytotoxicity against T-9 cells. Indeed, those cells lyzed T-9 cells *in vitro*, whereas the same cells failed to exhibit cytotoxicity against K562 cells. Although we injected purified LMF-b into the peritoneal cavity, CD8 (+) T cells obtained at 4 days exhibited CTL activity, and it is conceivable that LMF-b acts on lymphocytes as well as endothelial cells, possibly by inducing an adhesion molecule. This explanation is likely, because a chemotactic factor specific for neutrophils as well as T cells (neutrophil activating protein-1; NAP-1 or tentatively called interleukin(IL)-8) augments the association of lymphocytes to endothelial venules.[12] The expression of an adhesion molecule by endothelial cells and lymphocytes exposed to LMF-b may require some time. It should be pointed out, however, that NAP-1 or IL-8 is produced by monocytes,[12] whereas LMF-a and LMF-b are produced by neutrophils and CD4 (+) T cells, respectively. Furthermore, LMF-a and LMF-b do not attract neutrophils. In addition, our recent data suggested the possibility that NAP-1 may stimulate the production of LMF-b by acting on normal neutrophils (unpublished observation). Another possibility is that differentiation of CTL precursor cells to mature CTL may require some time. In any event, we have established a new system with which we can easily obtain a large number of tumor-infiltrating cells and which is suitable for testing the *in vivo* function of LMF.

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