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Lymphocyte chemotaxis in inflammation

X. HETEROGENEITY OF CHEMOTACTIC RESPONSIVENESS IN HUMAN T SUBSETS TOWARDS LYMPHOCYTE CHEMOTACTIC FACTORS FROM DELAYED HYPERSENSITIVITY REACTION SITE

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Summary. In previous studies, four lymphocyte chemotactic factors (LCF-a, -b, -c and -d) were isolated from delayed-type hypersensitivity (DTH) skin reaction sites of guinea-pig. In tests on guinea-pig lymphocytes, LCF-a attracted B cells, whereas LCF-b, -c and -d attracted T cells.

We now report the chemotactic responses of human lymphocytes to the guinea-pig LCFs. LCF-a strongly attracted B lymphocytes and weakly attracted T lymphocytes, whereas LCF-b, -c and -d predominantly attracted T lymphocytes. In tests on T-lymphocyte subsets, LCF-b and -d selectively attracted OKT4 subsets and theophylline-resistant T cells with helper phenotype. In contrast, LCF-c attracted OKT4 and OKT8 subsets, and both theophylline-resistant and sensitive subsets (i.e. cells with both helper and suppressor phenotype).

The results indicate that the different T-cell subsets migrate selectively into sites of inflammation.

INTRODUCTION

It is well known that the majority of lymphocytes found at delayed-type hypersensitivity (DTH) reaction sites are T cells (Claudy *et al.*, 1976; Braathen, Forre & Natvig, 1979), and that a few of the cells recognize the challenging antigen which elicits the response (McCluskey & Leber, 1974). The migration of these T lymphocytes appears to be mediated at least in part, by chemotactic factors for the cells produced *in vivo*.

As reviewed recently (Hayashi *et al.*, 1984), we have shown that four types of lymphocyte chemotactic factor (LCF-a, -b, -c and -d) can be isolated from purified protein derivative (PPD)-induced DTH skin reaction sites in the guinea-pig and are well characterized. LCF-a (molecular weight (MW) less than 5000) appeared to be a peptide derived from the IgG molecule (Shimokawa *et al.*, 1982a, b). LCF-c (MW about 160,000) and LCF-d (MW about 300,000) were considered to be different lymphocyte chemotactic lymphokines; LCF-c acted *in vivo* as a free lymphokine (Shimokawa *et al.*, 1984b), whereas LCF-d acted *in vivo* as a complex of another lymphokine (MW about 27,000) with serum protein exuded at the inflamed site (Harita *et al.*, 1983, 1984). However, LCF-b (MW about 14,000) has not been satisfactorily defined (Shimokawa *et al.*, 1984a). Furthermore, it has been found that LCF-a is active on B lymphocytes of guinea-pig, while LCF-b, -c and -d are similarly active on T lymphocytes as assessed by homologous *in vitro* assay system (Shimokawa *et al.*, 1982b, 1984a).

The current experiments were undertaken to demonstrate the different responsiveness of T and non-T lymphocytes from human peripheral blood

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toward LCF-a, -b, -c and -d, and to clarify the specific responsiveness of T-cell subsets toward these LCFs.

MATERIALS AND METHODS

Preparation of human peripheral blood lymphocytes

Human peripheral mononuclear cells were separated from heparinized venous blood taken from healthy donors by Ficoll–Conray density gradient centrifugation. The mononuclear cells in the interface were collected, washed three times, resuspended in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and 5% heat-inactivated fetal calf serum (FCS, Microbiological Associates Incorporated, Bethesda, MD), and then passed through a Sephadex G-10 column (Pharmacia, Uppsala, Sweden) to deplete monocyte, as described previously (Shimokawa *et al.*, 1983). The resultant non-adherent cells were collected by centrifugation, washed twice and resuspended at a concentration of 1×10^6/ml for chemotactic assay or of 5×10^6/ml for further separation. Additionally, blood lymphocytes taken from guinea-pigs by cardiac puncture were collected in the same manner; peroxidase-positive cells were less than 1% of the suspended cells.

Separation of T and non-T lymphocytes

T and non-T lymphocytes were separated essentially by the method of Madsen & Johnsen (1979). In brief, the monocyte-depleted lymphocyte preparations (5×10^6/ml) suspended in RPMI-1640 medium containing 10% FCS were mixed with a 10% suspension of 2-aminoethylisothiounonium bromide-treated sheep red blood cells (AET-SRBC) at a ratio of 2:1 (v/v). These mixtures were centrifuged at 200 ***g*** for 5 min and then incubated for 16–18 hr on ice. The pellet of cells was gently resuspended and then layered on Ficoll–Conray and centrifuged at 400 ***g*** for 30 min. Non-rosetting cells in the interface and SRBC-rossetting cells in the cell pellet were obtained and washed with RPMI-1640 medium. SRBC were then lysed with Tris-ammonium chloride. Rosetting cells were less than 2% positive for surface Ig assessed by indirect immunofluorescence; non-rosetting cells were more than 88% positive for surface Ig.

Preparation of theophylline-sensitive T (Tsen) cells and theophylline-resistant T (Tres) cells

Tsen and Tres cells were separated from purified T cells described above according to the method of Limatibul *et al.* (1978). In brief, purified T cells were incubated at 37° for 60 min in a water-bath with 3 mM theophylline (in final concentration). The treated T cells were then mixed with AET-treated SRBC at a ratio of 1:100, incubated at 4° for 2 hr and then separated into rosetting cells (Tres) and non-rosetting cells (Tsen) by density gradient centrifugation.

Preparation of OKT4 and OKT8 T subsets

Human purified T cells were further separated into helper/inducer and suppressor/cytotoxic T subsets using the monoclonal antibodies (Ortho Pharmaceutical Corp., Raritan, NJ) specific for each subset: OKT4 antibody specific for helper/inducer T cells and OKT8 antibody for suppressor/cytotoxic T subsets. First, both T subsets were prepared by negative selection with monoclonal antibodies and complement (C) according to the method of Thomas *et al.* (1980). Briefly, purified T cells were incubated on ice for 30 min with 1 μg of OKT4 and OKT8 antibodies per 1×10^6 cells, respectively. Afterwards, the cells were washed once, resuspended in rabbit serum as source of C diluted at 1:10 in Medium 199 (Gibco) and then incubated at 37° for 45 min. After treatment with C, the cells were, respectively, washed twice and resuspended in RPMI-1640 medium for chemotactic assay. The cell viability was assessed visually by trypan-blue exclusion microscopy.

Furthermore, OKT4 and OKT8 cells were positively selected by immune rosetting with SRBC which had been coated with rabbit anti-mouse IgG antibody by the chromium chloride ($CrCl_2$) method (Griffin, Beveridge & Schossman, 1972). OKT4 and OKT8 cells were separated to 90–93% purity as determined by indirect immunofluorescent technique.

Isolation and purification of lymphocyte chemotactic factors (LCFs)

Following our methods (Shimokawa *et al.*, 1984a), LCF-a, -b, -c and -d were extracted from 24-hr-old DTH skin reaction sites induced with PPD. LCF-a, -b and -c were first separated by gel filtration on Sephadex G-100 (Shimokawa *et al.*, 1982a). LCF-a was highly purified by gel filtration on Sephadex G-15 followed by peptide mapping (Shimokawa *et al.*, 1982b). LCF-d was separated from LCF-c by chromatography with DEAE-Sephadex and then highly purified by chromatography with CM-Sephadex, immunoadsorbent chromatography with Sepharose 4B conjugated with anti-guinea-pig IgG antibody and chromatofocusing, in that order (Shi-

mokawa *et al.*, 1984a). LCF-d, which had been separated from LCF-c by chromatography with DEAE-Sephadex, was highly purified by chromatography with CM-Sephadex followed by preparative isotachophoresis (Shimokawa *et al.*, 1984a). In the experiments, partially purified LCFs were examined; LCF-a was used after filtration on Sephadex G-15, LCF-b after gel filtration with Sephadex G-100, LCF-c after chromatography with CM-Sephadex and LCF-d after chromatography with CM-Sephadex. Protein concentrations of partially purified LCFs were estimated by the method of Lowry *et al.*, (1951) and optimal concentrations of LCFs previously determined were used for the chemotactic assay: the concentration of LCF-a was 50 μg/ml and that of LCF-b, -c and -d was about 180–260 μg/ml (Shimokawa *et al.*, 1982b, 1984a).

Chemotactic assay

This was performed by a previously described method using cellulose nitrate filters (pore size, 5 μm; Sartorius, Göttingen, FRG), and blind-well chemotaxis chambers (Shimokawa *et al.*, 1983; 1984a). Test samples, in phosphate-buffered saline (PBS), pH 7·4, were placed in the lower wells; the filters were placed on them and cell suspensions in RPMI-1640 medium at the concentration of 1×10^6 viable cells/ml obtained as described above were poured into the upper wells. After incubation of chambers at 37° for 3 hr using 5% CO_2 atmosphere, the filters were removed, fixed and stained with haematoxylin in the usual way. All the cells that had migrated into the channels of filter beyond a depth of about 40 μm from the upper surface of the filter (the starting monolayer of cells) were counted in 20 randomly selected microscopic high-power fields (HPFs, 10×40). The counting depth estimated by fine microscope adjustment on the microscope was selected to achieve a background count of 1–2 cells/HPF in the absence of chemotactic substance. Chemotactic activity was expressed as mean number of migrated cells per 20 HPFs in several duplicate experiments. Freshly prepared alkaline-denatured casein (0·1–0·5%) was used as positive chemotactic control substance for lymphocytes.

RESULTS

Chemotactic responsiveness of human peripheral blood lymphocytes toward LCFs

The first tests were designed to examine the ability of guinea-pig LCFs to attract human as well as guinea-pig lymphocytes. The LCFs attracted monocyte-depleted peripheral blood lymphocytes of man and guinea-pig, indicating that there was no species specificity (Table 1).

Subsequently, purified T and non-T lymphocytes from human monocyte depleted blood lymphocytes were tested for their chemotactic responsiveness towards these LCFs. The responsiveness was assessed using the responding cell populations which had been freshly prepared and cultured overnight at 37° in 20% FCS-supplemented medium from the same donors before assay. Table 2 shows that non-T lymphocytes (mostly B lymphocytes) respond well to LCF-a, but poorly to LCF-b, -c and -d. Furthermore, a similar selective responsiveness of non-T lymphocytes toward LCF-a occurred, even when precultured cells were used, indicating that non-T lymphocytes (B lymphocytes) may not require precultivation for demonstrating chemotactic activity. In contrast, T lymphocytes did not respond well towards LCF-b, -c and -d when freshly separated cells were used. Migration of non-cultured T lymphocytes toward PBS was also poor, when compared to that of total lymphocyte populations or non-T lymphocyte populations. On the other hand, T lymphocytes responded very well toward LCF-b, -c and -d, but poorly toward LCF-a, when the cells had been precultured overnight at 37° in 20% FCS-supplemented medium (Table 2). It is concluded

Table 1. Chemotactic response of blood lymphocytes from human and guinea-pig towards LCFs

Chemotactic factors tested	Chemotactic activity* for blood lymphocytes from				
	Human			Guinea-pig	
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2
PBS†	20	19	8	13	21
LCF-a‡	80	100	92	87	90
LCF-b§	90	120	105	89	91
LCF-c¶	105	123	130	95	103
LCF-d**	93	98	107	90	87

* Chemotactic activity of each LCF for blood lymphocytes was expressed as the mean number of migrated lymphocytes in triplicated assays in each experiment.
† Phosphate-buffered saline, pH 7·4.
‡ Separated by gel filtration on Sephadex G-15.
§ Separated by gel filtration on Sephadex G-100.
¶ Separated by chromatography with CM-Sephadex.
** Separated by chromatography with CM-Sephadex.

Table 2. Chemotactic response of T and non-T lymphocytes from human peripheral blood towards LCFs

Cells tested	Chemotactic activity*				
	PBS	LCF-a	LCF-b	LCF-c	LCF-d
Exp. 1†					
Bulk cells	33	96	95	116	92
Non-T cells	50	120	24	41	36
T cells	2	7	9	13	8
Exp. 1‡					
Bulk cells	18	101	120	140	115
Non-T cells	43	150	27	30	35
T cells	10	10	107	120	111
Exp. 2‡					
Bulk cells	12	71	88	97	71
Non-T cells	21	83	20	32	27
T cells	14	19	93	130	95
Exp. 3‡					
Non-T cells	32	97	45	44	36
T cells	15	19	78	113	95

* Chemotactic activity was expressed as the mean number of migrated lymphocytes of triplicated assays in each experiment.

† Bulk cells (unseparated cells), purified non-T and T cells were obtained from the same donors and used immediately after separation.

‡ Bulk cells (unseparated cells), purified non-T and T cells were obtained from the same donors and used after overnight preculture in FCS-supplemented media at 37°.

that human T lymphocytes require the preculture in order to demonstrate the chemotactic response, and that the T cells respond selectively toward LCF-b, -c and -d.

Heterogeneity of chemotactic responsiveness in T subsets toward LCF-b, -c and -d

The following experiments were undertaken to clarify whether specific subset of T cells would respond toward LCF-b, -c, or -d.

(1) Difference in chemotactic responsiveness of Tres and Tsen subsets. Theophylline-resistant T (Tres) cells and theophylline-sensitive T (Tsen) cells, obtained from the same donors, were cultured overnight in 20% FCS-supplemented RPMI-1640 medium, washed twice, suspended in RPMI-1640 medium at the concentration of 1×10^6 viable cells/ml, and then used for their chemotactic responsiveness.

Tres subsets respond very well to all of LCF-b, -c and -d (Table 3). In contrast, Tsen subsets respond very well to LCF-c, but poorly to LCF-b and -d, when compared to bulk T-cell preparations. It is thus concluded that LCF-b and -d can selectively promote the migration of Tres subsets, whereas LCF-c promoted equal activity for both Tres and Tsen subsets.

(2) Difference in chemotactic responsiveness of OKT4 and OKT8 T subsets. After treatment of purified T cells with normal mouse serum and monoclonal OKT4 and OKT8 antibodies in the presence of C, the respective cell preparations were cultured overnight and suspended in RPMI-1640 medium and the concentration of 1×10^6 viable cells/ml for their chemotactic responsiveness.

Table 4 shows that the cell preparations treated with OKT8 antibody and C (OKT4-enriched populations) respond very well toward LCF-b, -c and -d. In contrast, the cell preparations treated with OKT4 antibody and C (OKT8-enriched populations) respond very well to LCF-c, but poorly to LCF-b and -d, when compared to the bulk preparations. It appears that OKT4 (helper/inducer) subsets can account for most of the response of bulk T cells to LCF-b and -d. In contrast, both OKT4 and OKT8 (suppressor/cytotoxic) subsets respond equally to LCF-c. In some

Table 3. Chemotactic response of Tres and Tsen subsets toward LCF-b, -c and -d

Cells tested	Chemotactic activity*				
	PBS	LCF-b	LCF-c	LCF-d	Casein (0·1%)
Exp. 1					
T cells	21	96	121	93	180
Tres cells†	41	121	150	108	173
Tsen†	18	20	104	15	164
Exp. 2					
T cells	15	109	132	123	170
Tres cells†	11	110	115	94	184
Tsen cells†	23	28	102	25	175
Exp. 3					
Tres cells†	15	93	104	86	151
Tsen cells†	24	34	79	21	139

* Chemotactic activity was expressed as the mean number of migrated lymphocytes of duplicated assays in each experiment.

† Theophylline-resistant T (Tres) cells and theophylline-sensitive T (Tsen) cells were separated as described in the Materials and Methods.

Table 4. Chemotactic response of negatively selected OKT4 and OKT8 T subsets to LCF-b, -c and -d

	Chemotactic activity*				
Cells tested	PBS	LCF-b	LCF-c	LCF-d	Casein (0·1%)
Exp. 1					
T cells	16	112	150	131	214
OKT4 cells†	4	90	120	93	236
OKT8 cells†	18	15	118	19	211
Exp. 2					
T cells	21	81	132	93	193
OKT4 cells†	11	80	113	89	180
OKT8 cells†	14	15	105	11	183
Exp. 3					
T cells	30	104	160	115	240
OKT4 cells†	13	91	107	83	225
OKT8 cells†	19	39	102	44	237

* Chemotactic activity was expressed as the mean number of migrated lymphocytes of triplicated assays in each experiment.

† OKT4 cells and OKT8 cells were separated as described in the Materials and Methods.

assays, OKT8-enriched populations were found to respond weakly to LCF-b or LCF-d, but it was confirmed to be attributed to insufficient abrogation of OKT4 subsets by the treatment with OKT4 antibody and C. Purified T cells were separated into OKT4 and OKT8 subsets by positive selection with monoclonal antibodies as described in the Materials and Methods. Figure 1 clearly shows that OKT8 subsets respond selectively to LCF-c, while OKT4 subsets do well toward LCF-b, -c and -d.

DISCUSSION

It is well known that human lymphocytes and their subsets, like neutrophils and monocytes, show chemotactic response towards a variety of chemotatic factors formed *in vitro* (Parrott & Wilkinson, 1981), although it is the contention of several investigators (Parrott *et al.*, 1978; Shields & Wilkinson, 1979; El-Nagger, van Epps & Williams, 1981) that lymphocytes, especially T lymphocytes, must be precultured in order to demonstrate *in vitro* chemotactic activity.

The results presented here show that human peripheral blood lymhocytes respond very well toward LCF-a, -b, -c and -d isolated from DTH reaction site in the guinea-pig, as well as lymphocytes from guinea-pig, indicating that these LCFs lack species specificity. Furthermore, non-T lymphocytes from human peripheral blood clearly respond to LCF-a, but poorly to LCF-b, -c and -d. The preculture of non-T lymphocytes presented no influence on the selective responsiveness towards LCF-a. In contrast, T lymphocytes respond very well to LCF-b, -c and -d, respectively, but poorly to LCF-a, when the responding cells were precultured overnight at 37° in FCS-supplemented medium. These results are virtually identical to those obtained in the previous homologous assay system (Shimokawa *et al.*, 1982b, 1984a). Although splenic T lymphocytes from guinea-pigs could respond very well toward LCF-b, -c and -d when the responding cells

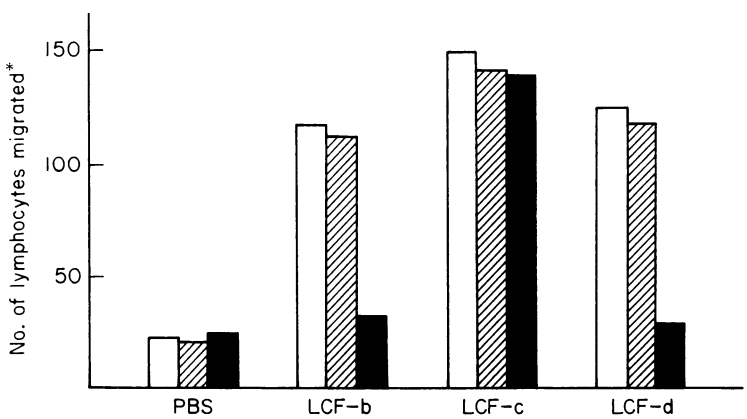

Figure 1. Chemotactic response of positively selected OKT4 and OKT8 T subsets to LCF-b, -c and -d: (□) bulk T cells; (▨) positively selected OKT4 cells; (■) positively selected OKT8 cells. * Chemotactic activity is expressed as the mean number of lymphocytes migrated in three experiments in two duplicated assays.

had not been cultured (Shimokawa *et al.*, 1984a), T lymphocytes from human peripheral blood did not show any chemotactic responsiveness to these LCFs under the same conditions (Table 2); the former cells were prepared by using anti-guinea-pig IgG ($F(ab')_2$) antibody-coated petri-dishes (Mage, McHugh & Rothstein, 1977), and the latter cells were prepared by density gradient centrifugation of rosetting cells to SRBC (Madsen & Johnsen, 1979). In an unpublished study (Y. Shimokawa, Y. Mibu and H. Hayashi), we have shown that human peripheral blood T lymphocytes, prepared using anti-human IgG ($F(ab')_2$) antibody-coated petri-dishes, can respond very well to these LCFs without preculture before assay. It is therefore indicated that the procedure for T cell preparation may influence their chemotactic responsiveness.

The present results also show that the chemotactic responsiveness to LCF-b, -c and -d is different in the subpopulations of T cells. As shown in Table 3, Tres subsets respond very well towards LCF-b, -c and -d. On the other hand, Tsen subsets show chemotactic responsiveness to LCF-c, but not to LCF-b or LCF-d. Shore, Dosch & Gelfant (1978) have shown that Tres subsets function as a helper cell for the induction of antigen-specific plaque-forming cell for target peripheral blood lymphocyte cultures, while Tsen subsets function as a suppressor cell. It is thus indicated that LCF-b and -d attract Tres cells with helper phenotype, but poorly attract Tsen subsets with suppressor phenotype, whereas LCF-c attracts both Tres and Tsen subsets equally.

Furthermore, it is apparent from Table 4 and Fig. 1 that migration of OKT4 subsets (helper/inducer phenotype) to LCF-b and -d occurs selectively, whereas LCF-c induces selective migration of OKT4 and OKT8 subsets (suppressor/cytotoxic phenotype). Since these results were obtained using positively and negatively selected T subsets, the different response of OKT4 and OKT8 subsets towards LCF-b, -c and -d could not be attributed to the binding of OKT4 and OKT8 monoclonal antibodies to the cell surface. It is thus concluded that most lymphocytes attracted by LCF-b and -d belong to OKT4 subsets, whereas the lymphocytes attracted by LCF-c belong to T subsets, including OKT4 and OKT8 subsets. However, it is unknown whether phenotypically distinct subsets of OKT4 cells respond to these LCFs, although such subsets have been identified by surface antigen (Morimoto *et al.*, 1981). In an unpublished study on chemotactic responsiveness of murine T subsets towards LCF-b, -c and -d, (Y. Shimokawa M. Hifumi, Y. Mibu and Y. Hayashi), we have found that LCF-b and -d selectively attract normal Lyt 1^+ T subsets and helper T-cell lines, whereas LCF-c attracts other T subsets, including normal Lyt 2^+ cells and cytotoxic T-cell lines.

Parrott *et al.* (1978) have shown that T cells with receptors for IgM ($T\mu$), and T cells lacking IgM or IgG receptors, migrate well in response to casein, but T cells with receptors for IgG ($T\gamma$) do not respond to casein. However, the present results clearly show that alkaline-denatured casein can promote the migration of a variety of T subsets, including OKT4 and OKT8 subsets. Van Epps, Durant & Potter (1983) have shown that both Leu-2 (suppressor/cytotoxic) and Leu-3 (helper/inducer) T subsets, prepared by negative and positive selection with monoclonal Leu-2 and Leu-3 antibodies, respond equally toward this chemoattractant. They have also shown that Leu-3 subsets respond selectively to culture supernatants from concanavalin A (Con A) stimulated human mononuclear cells and from Con A-stimulated Leu-2 subsets.

There have been some reports on the proportion of infiltrating T and B lymphocytes in human skin disorders; the majority (70–80%) of infiltrating lymphocytes are T cells in DTH reaction site to PPD or mumps antigen, and B cells represented about 10–15% of the cells (Claudy 1976; Braathen, Forre & Natvig, 1979). Afterwards, the proportion of T subsets found at inflammatory lesions has been analysed by indirect immunofluorescence technique using various monoclonal antibodies against cell surface. It has been found that mouse T cells with Lyt 1 (helper/inducer) phenotype are predominantly found at the inflammation site (Sriram *et al.*, 1982). In human tuberculin skin site, both OKT4 and OKT8 are found; the ratio of OKT4 cells and OKT8 cells was 2:1 (Poulter *et al.*, 1982; Platt *et al.*, 1983). Although further characterization of responding T subsets toward LCF-b, -c and -d remains to be analysed, LCF-b and LCF-d appear to play an important role for selective migration of T cells with helper/inducer phenotype, and LCF-c appears to be significant in the migration of T subsets with suppressor/cytotoxic phenotype.

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