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LYMPHOCYTE CHEMOTAXIS IN INFLAMMATION. IV. ISOLATION OF LYMPHOCYTE CHEMOTACTIC FACTORS FROM PPD-INDUCED DELAYED HYPERSENSITIVITY SKIN REACTION SITE IN THE GUINEA-PIG, WITH SPECIAL REFERENCE TO A FACTOR CHEMOTACTIC FOR B CELLS

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Summary.—Lymphocyte chemotactic factors were extracted from PPD-induced delayed hypersensitivity skin reaction sites in guinea-pigs. The presence of 3 chemotactic factors (LCF-*a*, LCF-*b* and LCF-*c*) was suggested by gel filtration on Sephadex G100. LCF-*a* was purified by gel filtration on Sephadex G-15 followed by peptide mapping. This substance was considered to be a thermostable and dialysable peptide; it appeared to be effective for B cells but not for T cells.

LYMPHOCYTE CHEMOTACTIC FACTORS (LCFs) produced *in vitro* including lymphokines (Ward, Offen and Montgomery, 1971; Ward *et al.*, 1977), products from immunoglobulins by enzymatic treatment (Higuchi, Honda and Hayashi, 1975; Higuchi, Ishida and Hayashi, 1979) and product from complement (C5a) (El-Naggar, van Epps and Williams, 1980) have been described. However, it seems important to investigate the isolation and characterization of chemotactic factors produced *in vivo* to clarify the mediation of leucotaxis in inflammation, although there remains the difficult problem of obtaining sufficient amounts of chemotactic factors from tissues. In an accompanying communication, we report that guinea-pig lymphocytes were attracted chemotactically to skin extract from purified protein derivative (PPD)-induced delayed type hypersensitivity (DTH) reaction sites and that very marked chemotactic activity was shown in extracts from 24-h-old lesions, corresponding to the intensity of lymphocyte reaction in skin sites (Shimokawa *et al.*, 1982).

The purpose of the present communication is to describe how the 3 types of lymphocyte chemotactic factor (LCF-*a*, LCF-*b* and LCF-*c*) can be isolated from extract of DTH 24-h-old lesions and LCF-*a*, among these LCFs, may be a peptide chemotactic for B cells.

MATERIALS AND METHODS

Induction of DTH reaction.—Male Hartley guinea-pigs weighing 300–350 g were used. The animals were immunized by intradermal injection of BCG (a total of 1·6 mg in physiological saline; Japan BCG Corporation, Tokyo) in the footpad, thigh and nape of the neck. Six weeks later, DTH reaction was induced with 0·1 ml of PPD (100 μg/ml in physiological saline, Ministry of Agriculture's Veterinary Laboratory, Weybridge, England) on the flank, erythema and induration being maximal at 24–30 h. For extraction, the animals were injected intradermally with 0·1 ml PPD/site at 10 sites on the flank.

Preparation of skin extract.—This was performed following the previously described method of Hayashi *et al.* (1962). DTH reaction skin sites at 24 h were excised immediately after the animals had been killed by severing of the carotid arteries. The skin (8–10 g/animal) was cut into several pieces and frozen at −80°. The The pieces of frozen skin were cut into slices about 50 μm thick with a freezing microtome, the slices being dehydrated with 3 changes of

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10-fold volume of cold acetone and powdered. Powdered skin (2–3 g/animal) was extracted with a 10-fold volume of 0·067M phosphate buffer, pH 7·4, at 4° for 4 h. After centrifugation, the supernatant was passed through a Millipore filter (pore size 0·45 μm) and used for chemotactic assay for lymphocytes.

Cell preparation.—Lymphocytes were obtained from the spleens of normal Hartley guinea-pigs weighing about 400 g for chemotactic assay. Spleens obtained aseptically were teased in cold Hanks' balanced salt solution (HBSS) and squeezed gently with sterile slides. The cell suspension was freed of tissue particles by being passed through a nylon mesh, centrifuged at 700 rev/min for 10 min and suspended in RPMI 1640 medium (Grand Island Biological Company, Grand Island, New York) containing 10% heat-inactivated foetal calf serum (Microbiological Associates Incorporated, Bethesda, Maryland) and supplemented with L-glutamine (300 μg/ml). The resulting cell suspension was filtered through a glass wool column to remove non-lymphoid cells (Ford, 1978), centrifuged and resuspended at a concentration of 4×10^6/ml in RPMI 1640 medium containing 1% human serum albumin (Sigma, St Louis, Missouri) and supplemented as described above.

In some experiments, T- and B-cell preparations were performed by the method of Mage, McHugh and Rothstein (1977). In brief, polystyrene Petri plates, tissue culture grade, 8 cm nominal diameter, were coated with anti-guinea-pig IgG antibody by incubation at 4° for 16–20 h with antibody solution containing 1 mg/ml protein. After these dishes had been washed 5× with HBSS, 5 ml of lymphocyte suspensions (5×10^6/ml) suspended in HBSS containing 10% foetal calf serum were added to the antibody-coated Petri plates and were allowed to settle at 37° for 1 h. Afterwards, the plates were swirled, non-adherent cells were removed and cells adherent to antibody-coated surface were removed by pipetting additional medium on to the cell monolayer. The non-adherent cells were less than 6% positive for surface Ig by indirect immunofluorescence and believed to be mostly T cells. The adherent cells were more than 93% positive for surface Ig and were believed to be mostly B cells.

Chemotactic assay.—This test was done by a previously described method using Millipore filters (SSWPO 1300, pore size 3 μm, Millipore Filter Company, Bedford, Massachusetts) and a chamber containing 2 1ml compartments (Higuchi *et al.*, 1975). Test samples, in 0·067M phosphate buffer, pH 7·4, were placed in the lower compartment; the filter was placed over it and the lymphocyte suspension obtained as described above was poured into the upper compartment. The chambers were incubated at 37° for 3 h using a 5% CO_2 atmosphere. After the filters had been removed, fixed and stained with haematoxylin in the usual way, all cells that had migrated into the filter channels deeper than about 40 μm from the upper surface of the filter (the starting monolayer of cells) were counted in 20 randomly selected microscopic fields (10× 40). The counting depth was selected so as to achieve a background of 1–2 cells/high-power field to the absence of chemotactic substance. Chemotactic activity was shown as mean number of migrated cells per 20 microscopic fields in several duplicate experiments.

Column chromatography.—Chromatography was done on columns of Sephadex G-100 and Sephadex G-15 obtained from Pharmacia, Uppsala, Sweden, according to the details given below. Estimation of the approximate mol. wt was performed by gel filtration on Sephadex G-100. Bovine serum albumin (monomer, mol. wt 67,000; dimer, mol. wt 134,000; Armour, Kankakee, Illinois), cytochrome c (mol. wt, 12,500; Sigma, St Louis, Missouri) and bacitracin (mol. wt, 1461; Sigma) were used as standard substances.

Peptide mapping.—This was performed essentially according to the method of Katz, Dreyer and Anfinsen (1959). Descending paper chromatography of LCF-a, as described below, was done with a chromatocab (Toyo Corporation, Tokyo) utilizing the organic layer of a mixture of *n*-butanol:acetic acid:water (4:1:5) for 16 or 24 h at room temperature. After that, high-voltage paper electrophoresis was performed on a Toyo electrophoresis apparatus, model HPE-406, equipped with a cooling system (Toyo Corporation, Tokyo) and operated at 40–50 V/cm at 10° using pyridine-acetate buffer, pH 3·7. Fluorescamine solution (1 mg/100 ml in acetone) was sprayed lightly on to the paper and fluorescent spots were detected by viewing under a long-wave (336 μm) ultraviolet lamp by the method of Mendez and Lai (1975). Each strip (3 × 5 cm) of paper was eluted with 30% acetic acid. The samples were re-lyophilized 2× from 5 ml of distilled water and dissolved in 0·067M phosphate buffer, pH 7·4, for chemotactic assay.

RESULTS

Separation of LCF-a, b and c from skin extract on Sephadex G-100

As previously described, skin extract from 24-h-old DTH sites exhibited extremely potent lymphocyte chemotactic activity, suggesting that the extract may present the most favourable sample for the isolation of LCFs. The extract, (3 ml) concentrated by a Diaflo membrane UM-2 to give an absorbance between 25 and 30 at 280 nm, was applied to a Sephadex

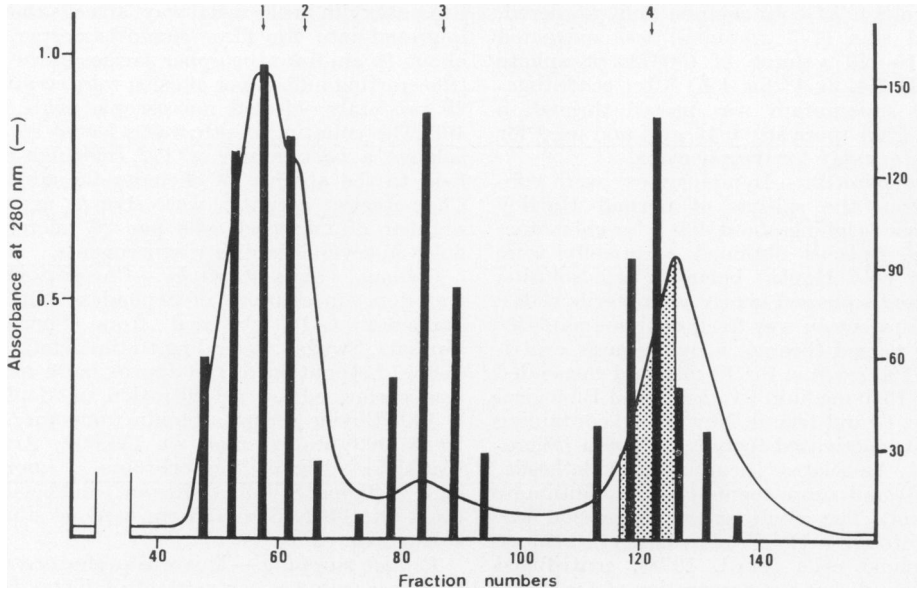

FIG. 1.—Demonstration of lymphocyte chemotactic activity in chromatographic components, extracted from 24-h-old lesions (in sensitized animals) and eluted on Sephadex G-100. Chemotactic assay was done on non-diluted fractions. Three chemotactic peaks were revealed; the third component contained LCF-*a*, the second component LCF-*b*, and the first component LCF-*c*, The third component (stippled area) was used for further purification of LCF-*a*. 1, Bovine serum albumin dimer; 2, bovine serum albumin monomer; 3, cytochrome c; 4, bacitracin.

G-100 column (3 × 50 cm) equilibrated with 0·067M phosphate buffer, pH 7·4. The flow rate was 20 ml/h and 3g effluent fractions were collected for chemotactic assay.

As can be seen in Figure 1, there were 3 chromatographic components. The total yield was about 98% of the applied samples; the first component comprised 47%, the second 7%, and the third 39%. Chemotactic activity for lymphocytes was detected in each chromatographic component; the chemotactic substance found in the third component was named LCF-*a*, that in the second component, LCF-*b*, and that in the first component LCF-*c*.

LCF-*a* was dialysable, because most of the chemotactic activity disappeared by dialysis against 0·067M phosphate buffer, pH 7·4 for about 16 h. The elution volume of LCF-*b* corresponded to that of cytochrome c; its mol. wt was assumed to be about 14,000. The mol. wt of LCF-*c* was assumed to be more than 70,000 by comparison with the elution profile of bovine serum albumin.

Gel filtration of LCF-a on Sephadex G-15

Since we (H.H.) have confirmed that the third component noted above exhibits no chemotactic activity for macrophages and neutrophils (Hayashi *et al.*, 1978; Honda and Hayasi, 1982; Honda *et al.*, 1982), this component was considered to be most convenient for the purification of LCF-*a*. The third component (stippled area in Fig. 1) was concentrated by rotary evaporator to give an absorbance between 4 and 6 at 280 nm. A 3ml portion of the sample was eluted on a Sephadex G-15 column (1·5 × 60 cm) equilibrated with 0·067M ammonium bicarbonate, pH 8·0. The flow rate was 6 ml/h and 3g effluent fractions were collected. These fractions were re-lyophilized and then dissolved in 0·067M phosphate buffer, pH 7·4, for chemotactic assay.

As shown in Figure 2, there were 6 chromatographic components. The total yield, measured as the absorbance at 280 nm, was about 96% of the applied samples. Chemotactic activity for lympho-

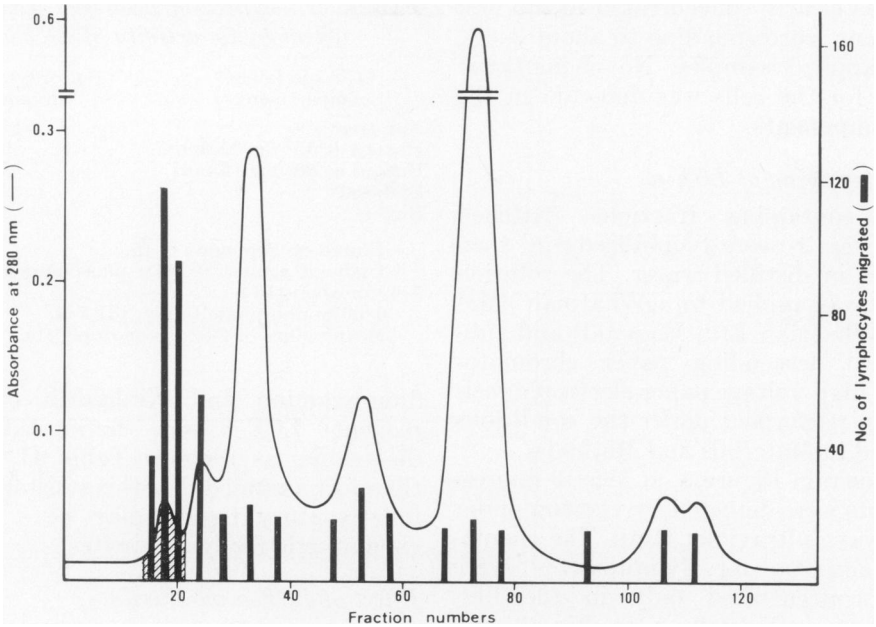

FIG. 2.—Gel filtration of LCF-*a* (stippled area in Fig. 1) on Sephadex G-15. Chemotactic assay was done on non-diluted fractions. The first component (hatched area) was used for peptide mapping of LCF-*a*.

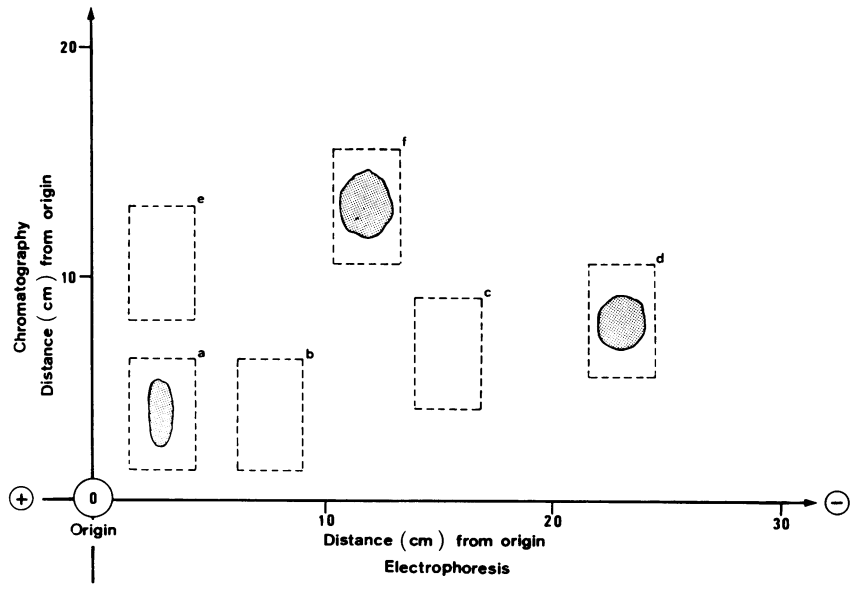

FIG. 3.—Peptide mapping of LCF-*a*. The active fraction (hatched area in Fig. 2) was lyophilized and dissolved in distilled water. The solution was applied on a paper and subjected to descending chromatography followed by electrophoresis. Each spot was detected with fluorescamine solution and each strip of paper (a, b, c, d, e, and f; 3 × 5 cm) was eluted with 30% acetic acid.

cytes was clearly concentrated in the first component, corresponding to about 2–4% of the applied samples. No chemotactic activity for the cells was detected in the other components.

Peptide mapping of LCF-a

LCF-*a*-containing fractions (hatched area in Fig. 2) were lyophilized and then dissolved in distilled water. The solution (0·1 ml) was applied to a Whatman 3MM paper (Whatman Ltd, England) and subjected to descending paper chromatography; high-voltage paper electrophoresis was then performed under the conditions described in Materials and Methods.

As shown in Figure 3, at least 3 fluorescent spots were detected by viewing under a long-wave ultraviolet lamp. The chemotactic activity for lymphocytes were clearly concentrated only in the first fluorescent spot (strip a in Fig. 3 and Table I), moving 2·4–3·5 cm towards the

TABLE I.—*Chemotactic activity of LCF-a after peptide mapping*

Samples in lower compartment	No. of lymphocytes migrated[c]
Strip a[a]	103
Strip b[a]	22
Strip c[a]	19
Strip d[a]	17
Strip e[a]	23
Strip f[a]	15
Buffer [b]	19

[a] Each strip, as shown in Figure 3, was eluted with 30% acetic acid, re-lyophilized and then dissolved in 0·067M phosphate buffer, pH 7·4, for chemotactic assay for lymphocytes.
[b] 0·067M phosphate buffer, pH 7·4.
[c] Mean values of 3 assays in duplicate.

cathode and descending 2·4–4·8 cm from the origin; its quantity seemed to correspond to about 22% of the applied samples. No chemotactic activity was detected in the other strips, including the second (strip d in Fig. 3 and Table I) and the third (strip f in Fig. 3 and Table I) fluorescent spots.

Physicochemical properties of LCF-a

Purified LCF-*a* (Fig. 3) was positive to fluorescamine and Ninhydrin or Folin reagent. LCF-*a* was thermostable and dialysable, as seen in Table II. It was therefore assumed that this substance from inflammatory tissues may be a peptide chemotactic for lymphocytes.

TABLE II.—*Effect of heat and dialysis on chemotactic activity of LCF-a*

LCF-*a* in lower compartment	No. of lymphocyste migrated[d]
Non-treated[a]	115
Heated at 56° for 30 min	97
Heated at 80° for 15 min	86
Dialysed[b]	21
Buffer[c]	22

[a] Eluted on Sephadex G-15.
[b] Dialysed against 0·067M phosphate buffer, pH 7·4, for about 16 h.
[c] 0·067M phosphate buffer, pH 7·4.
[d] Mean values of 2 assays in duplicate.

Effect of LCF-a on B cells

Chemotactic activity of LCF-*a* (Figs 2 and 3) was assayed for cells adherent to anti-IgG-coated Petri dishes, and non-adherent cells, prepared by the method of Mage *et al.* (1977). Schreiner and Unanue (1975) reported that ligand (anti-Ig) surface Ig interaction resulted in a stimulation of motility which was brief and disappeared about 30 min later. Therefore, fractionated cells (adherent and non-adherent cells) were used for chemotactic assay after being washed 3× and settled for about 60 min.

As summarized in Table III, LCF-*a* was clearly active for adherent cells (mostly B

TABLE III.—*Effect of LCF-a on adherent and non-adherent cells*

Samples in lower compartment	No. of lymphocytes migrated[d]		
	Unfractionated	Adherent[c]	Non-adherent[c]
LCF*a*[a]	72	89	36
0·1% casein	105	143	138
Buffer[b]	19	24	26

[a] Eluted on Sephadex G-15.
[b] 0·067M phosphate buffer, pH 7·4.
[c] Prepared by the method of Mage *et al.* (1977); adherent cells were more than 93% positive for surface Ig and non-adherent cells were less than 6% positive for surface Ig.
[d] Mean values of 3 assays in duplicate.

cells) but not for non-adherent cells (mostly T cells). On the other hand, 0·1% alkaline-denatured casein as a control material was similarly active for both adherent and non-adherent cells, as described by O'Neill and Parrott (1977).

DISCUSSION

The present observations showed that at least 3 types of LCFs (LCF-*a*, LCF-*b* and LCF-*c*) can be isolated from skin extracts of 24-h-old DTH reaction sites induced with PPD by gel filtration on Sephadex G-100 and that LCF-*a* can be purified by gel filtration on Sephadex G-15 followed by peptide mapping (Figs 1–3). This substance was considered to be a thermostable and dialysable peptide chemotactic for lymphocytes. Peptides chemotactic for leucocytes have been described. Schiffmann, Corcoran and Wahl (1975) reported a synthetic *N*-formyl-methionyl peptide chemotactic for neutrophils and macrophages. Goetzl and Austen (1975) described a tetrapeptide from human lung tissue chemotactic for eosinophils.

The present results demonstrated that LCF-*a* was effective for adherent cells (mostly B cells) but not for non-adherent cells (mostly T cells). Although further characterization of the lymphocytes responding chemotactically to LCF-*a* remains to be analysed, the observations suggest that LCF-*a* may be a peptide chemotactic for B cells. Our recent observations have suggested that both LCF-*b* and LCF-*c* may be similarly effective for non-adherent cells but not for adherent cells (Shimokawa, Harita and Hayashi, 1979). There are reports on the proportion of infiltrating T and B cells in human skin disorders; in DTH reaction site to PPD or mumps antigen, most (70–80%) of the infiltrating lymphocytes are T cells, while B cells account for about 10–15% of the total (Claudy *et al.*, 1976; Braathen, Førre and Natvig, 1979). These findings suggest that LCF-*a* may play an important role in B-cell migration and LCF-*b* and LCF-*c* may play an important role in T-cell migration in the DTH reaction to PPD.

The precursor of LCF-*a* remains to be ascertained. However, there is the difficult problem of obtaining sufficient amounts of LCF-*a* from inflamed tissues. Higuchi *et al.* (1975, 1979) have shown that a peptide, among the dialysable peptides released from IgG molecule by mild digestion with neutrophil neutral thiol protease, exhibits distinct chemotactic activity for lymphocytes. It is of special interest that this peptide, purified by gel filtration on Sephadex G-50 and Sephadex G-15 followed by high-voltage paper electrophoresis, was thermostable and effective for B cells but not for T cells, clearly resembling LCF-*a*. However, fine comparison of this peptide with LCF-*a* must await further studies.

LCFs with a mol. wt (approximately 14,000) similar to that of LCF-*b* have been described. Houck and Chang (1977) found a factor in thymus extract of vaccinated calves, and Higuchi *et al.* (1975) produced a factor *in vitro* from rabbit IgM by neutrophil neutral thiol protease. However, it seems difficult to compare LCF-*b* with these factors, because LCF-*b* cannot be purified satisfactorily.

LCFs with a mol. wt (more than 70,000) similar to that of LCF-*c* have been reported. Cohen *et al.* (1973) detected 2 factors in skin extract from DTH reaction induced with *o*-chlorbenzoyl chloride; the major chemotactic peak was sedimented in the region of an IgG marker, and the minor chemotactic peak was sedimented in the region of an albumin marker. However, comparison of LCF-*c* with their factors awaits further study, because the LCF-*c* was not satisfactorily purified.

Recently, we have found that two lymphocyte chemotactic lymphokines (α and β) can be released from PPD-stimulated guinea-pig lymphocytes; the mol. wt. of α-lymphokine was about 160,000 and that of β-lymphokine about 27,000 (Harita, Shimokawa, and Hayashi, 1982). We have demonstrated that LCF-*c* is a

mixture of LCF-*c* and LCF-*d* by its further purification; the mol. wt. of LCF-*c* was about 160,000 and that of LCF-*d* about 300,000 (Shimokawa, Harita, and Hayashi, 1981). LCF-*c* was found to exist in a free form and LCF-*d* in a complex with serum protein in the DTH reaction site.

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