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The recruitment of lymphocytes into the skin by T cell lymphokines: the role of γ-interferon

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SUMMARY

Numerous lymphocytes are recruited from the blood into cutaneous DTH reactions. Alpha/beta-interferon (IFN) and its inducers can recruit lymphocytes into the skin after i.d. injection, but activated T lymphocytes, which are responsible for DTH, produce very little IFN-α/β. Our goal was to determine the major T cell lymphokine (LK) which could stimulate the migration of lymphocytes into the skin. Rats were injected i.d. with LK containing supernatants from activated T cells, and lymphocyte recruitment was measured by the accumulation of ^{111}In-labelled lymphocytes in the skin. Large numbers of labelled cells migrated into sites injected with the LKs. The major portion of the recruiting activity of the LKs coeluted with IFN-γ after hydroxylapatite and Affigel Blue chromatography, although a second recruiting factor was also found. Both the recruiting and IFN anti-viral activities were partially destroyed by pH 3. A monoclonal anti-IFN-γ antibody inhibited up to 53% of the recruitment observed after 4 h and up to 43% after 20 h. Kinetic studies showed that maximal recruitment occurred 6 h after i.d. injection of the LKs. Recombinant rat IFN-γ also stimulated lymphocyte migration into the skin. Histologically, sites injected with IFN-γ showed a mononuclear cell infiltrate. It is suggested that IFN-γ is the major mediator of lymphocyte recruitment produced by activated T cells.

Keywords Lymphokine Lymphocyte migration DTH interferon-γ

INTRODUCTION

During the course of a delayed-type hypersensitivity (DTH) reaction in the skin, lymphocytes are recruited from the blood into the inflammatory site (Hay, 1979). The lymphocytes migrate into the developing DTH reaction independent of their antigen specificity (McCluskey, Benacerraf & McCluskey, 1963), and both large lymphoblasts and small lymphocytes migrate into the inflammatory site (Hay, 1979; Platt *et al.*, 1983). Our previous studies demonstrated that a subset of small T cells preferentially migrates into cutaneous DTH reactions (Issekutz, Webster & Stoltz, 1986a). This same population of lymphocytes also migrates into skin sites injected with interferon-α/β (IFN-α/β) or inducers of IFN-α/β, such as poly I/C and virus (Issekutz, Stoltz & Webster, 1986b).

Although IFN-α/β recruits lymphocytes into the skin, it is probably not the only mediator of lymphocyte migration into an inflammatory site. During DTH reactions, T lymphocytes are activated and produce a number of lymphokines (LKs). It is not known which LKs are present at the DTH site, but T cells stimulated *in vitro* produce a diverse array of LKs including interleukin-2 (IL-2), IFN-γ, and lymphotoxin (Gillis & Smith, 1977; Youngner & Salvin, 1973; Harris *et al.*, 1981). These activated T cells do not produce much IFN-α/β (Youngner & Salvin, 1973). Therefore, it is unlikely that IFN-α/β is responsible for lymphocyte recruitment in DTH reactions. Our objective was to determine which T cell LK was the major factor recruiting lymphocytes into the skin. Based on reports that IFN-γ enhanced the binding of T cells to vascular endothelial cells (Yu *et al.*, 1985), and our findings with IFN-α/β, we hypothesized that IFN-γ may be an important LK in recruiting lymphocytes from the blood.

Rat lymphocytes were stimulated *in vitro* to undergo blast transformation and release a number of LKs, including large amounts of IL-2 and IFN-γ. The ability of the LKs to recruit lymphocytes into the skin of rats after i.d. injection was examined. Our findings suggest that IFN-γ is an important mediator of lymphocyte recruitment and although it is not the only recruiting factor in the LK supernatant, it is one of the major mediators with this effect.

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MATERIALS AND METHODS

Animals

Inbred male strain AO rats, weighing 250 g, were used in all experiments.

Reagents

The WR strain of vaccinia virus was grown on rat fibroblasts in RPMI-1640 medium containing 5% rat serum. Neuraminidase was obtained from Gibco Laboratories, Burlington, Ont., Canada, and galactose oxidase was purchased from Sigma Chemical Co., St Louis, MI. Recombinant (rec.) rat IFN-γ, rabbit anti-IFN-γ and the mouse monoclonal antibody (MoAb), DB-2, which neutralizes rat IFN-γ, were obtained as previously described by van der Meide *et al.* (1986). One neutralizing unit of anti-IFN-γ DB-2 neutralizes one unit of IFN-γ in the viral-plaque-inhibition assay.

Cell isolation

The isolation and radiolabelling of small peritoneal exudate lymphocytes has been described previously (Issekutz *et al.*, 1986a). Peritoneal exudates were induced by injecting naive rats with 5×10^7 pfu vaccinia virus i.p. Five days later, peritoneal exudate cells were obtained by lavaging the peritoneal cavity, and the small lymphocytes from these exudates were isolated on a continuous linear density gradient of Percoll (Pharmacia Fine Chemicals, Dorval, Quebec). The small, high-density lymphocytes, as determined by cell sizing on a Coulter Counter (Coulter Electronics, Hialeah, FL), were pooled, washed and radiolabelled as described below. The high-density, peritoneal exudate cells consisted of greater than 90% small lymphocytes with 5% to 8% basophils and 1% to 3% neutrophils.

Cell labelling

Lymphocytes were labelled with ^{111}In-oxine (Amersham Corp., Oakville, Ont.) as previously described (Issekutz *et al.*, 1986a). Briefly, 5×10^7 cells suspended in 0·5 ml RPMI-1640 were labelled with 3·5 μCi ^{111}In-oxine for 10 min, washed twice and resuspended in RPMI-1640 plus 10% heat-inactivated rat serum for i.v. injection. Each rat was injected with $1–2 \times 10^7$ lymphocytes labelled with $0·5–1 \times 10^6$ ct/min of ^{111}In. The viability of all the cells used was greater than 95% by trypan-blue exclusion.

Lymphokine preparation

Spleens and lymph nodes were removed from AO rats, and lymphocyte suspensions were prepared as described by Issekutz *et al.* (1986a). Lymphocyte blast transformation was induced by a modification of the procedure described by Novogrodsky & Katchalski (1973) for human lymphocytes. Rat lymphocytes were sequentially treated with neuraminidase 50 U/ml for 1 h followed by 7·5 U/ml of galactose oxidase for 45 min. The cells were then washed, and resuspended in HL-1 serum-free medium (Ventrex Corp., Portland, ME) at 2×10^6 cells per ml. After a 48-h incubation at 37° in 5% CO_2 and air, during which the cells underwent blast transformation, the supernatant from the cells was harvested by centrifugation. The LK supernatant was dialysed against 5 mM phosphate buffer, lyophilized, and redissolved in 5% of its original volume. This yielded a nearly-isotonic LK preparation (LK Prep.) that contained 0·9–1 mg/ml of protein with approximately 10,000 U/ml of IFN-γ.

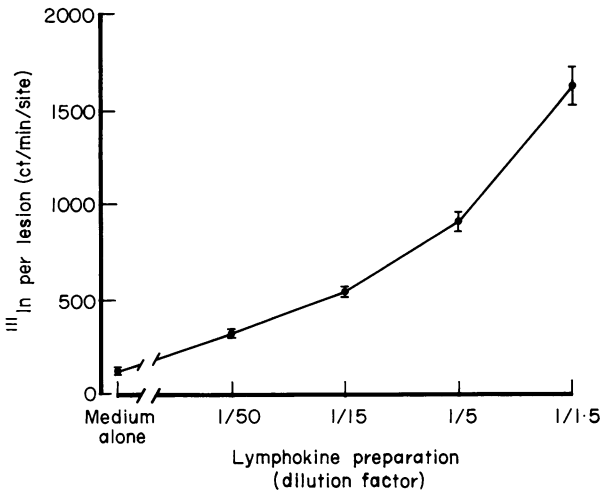

Fig. 1. Accumulation of ^{111}In-labelled lymphocytes in skin sites injected with varying dilutions of the LK Prep. Rats were injected i.d. in triplicate sites with 0·1 ml LK Prep. diluted as indicated, and labelled cells were injected i.v. Twenty hours later, the animals were killed and the ^{111}In in each lesion determined. Each point represents the mean ± s.e.m. of measurements in five animals.

Interferon assay

Interferon was assayed on rat fibroblasts using a standard viral-plaque-inhibition-microtitre assay (Campbell *et al.*, 1975). Interferon activity was expressed in units as defined by the rat IFN-α/β-reference standard provided by Lee Biomolecular, San Diego, CA. One unit of IFN is defined as the concentration that results in a 50% inhibition of plaque formation by vesicular stomatitis virus.

Protein assay

Protein concentrations were measured by the fluorometric assay employing fluorescamine (Roche Diagnostics, Vandeuril, Que.) with bovine serum albumin as a standard (Böhlen *et al.*, 1973).

Hydroxylapatite chromatography

Spheroidal hydroxylapatite (Bio-Rad Laboratories, Richmond, CA) was packed into a column (1·2 × 3 cm), washed with 1 M NaOH followed by 5 mM sodium phosphate pH 7. The LK Prep. was dialysed against the same buffer, loaded on to the column, and eluted with either a linear phosphate gradient (5–300 mM) or, in some experiments, a step gradient of 0·03, 0·1, 0·3 and 2 M phosphate buffer. Fractions, 2 ml, were collected and dialysed against phosphate buffered saline.

Affigel blue chromatography

The LK Prep. was chromatographed on Affigel Blue (Bio-Rad Laboratories) as described by Wietzerbin *et al.* (1979). Pyrogen-free, human-serum albumin was added to the LK Prep. to yield a final protein concentration of 4 mg/ml. After dialysis against 0·02 M phosphate buffer, the LK Prep. was loaded on to an Affigel Blue column (1·2 cm × 3 cm). After thorough washing with the same buffer, the column was eluted with 0·145 M NaCl, 1·5 M NaCl and finally 50% glycerol in 1·5 M NaCl.

Experimental design

Rats anaesthetized with ether were injected i.v. with ^{111}In-labelled lymphocytes, and immediately afterward, the skin on

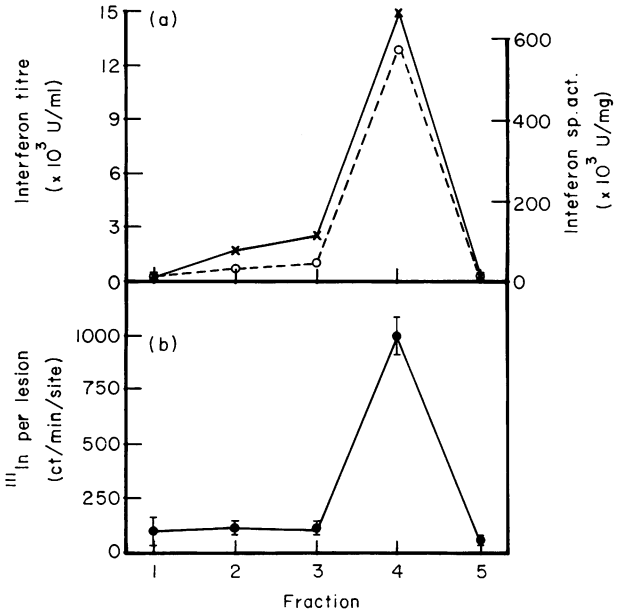

Fig. 2. Fractionation of the LK Prep. by hydroxylapatite chromatography. A 3·5 ml column was loaded with an LK Prep. containing 75,000 u of IFN-γ and washed with 5 mM phosphate buffer (Fr. 1). It was then eluted in steps with 30 mM (Fr. 2), 100 mM (Fr. 3), 300 mM (Fr. 4), and 2 M (Fr. 5) phosphate buffer. (a) IFN activity by viral plaque inhibition assay (×) and its specific activity per mg of protein (○) in each fraction. (b) Accumulation of ^{111}In-labelled lymphocytes in skin sites injected with 0·1 ml of each fraction. Each point represents the mean ± s.e.m. over control sites of triplicate lesions in one animal. Similar results were obtained with two other hydroxylapatite chromatographies, one of which employed a continuous linear gradient of phosphate buffer.

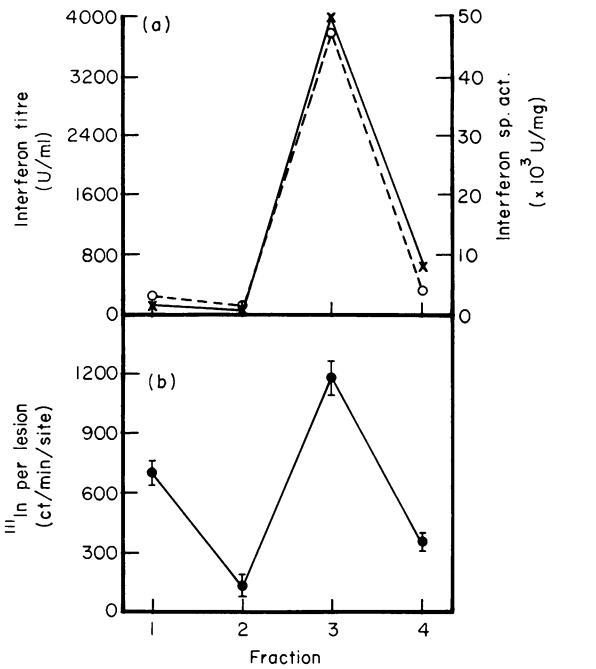

Fig. 3. Affinity chromatography of LK Prep. on Affigel Blue. A 3·5 ml column was loaded with an LK Prep. containing 70,000 u of IFN-γ in 4 ml and washed with 0·02 M sodium phosphate buffer (Fr. 1). The column was then eluted in steps with 15 ml of 0·145 M NaCl (Fr. 2), 1·5 M NaCl (Fr. 3), and finally with 50% glycerol in 1·5 M NaCl (Fr. 4). (a) IFN activity by viral plaque inhibition (×) and its specific activity (○) in each fraction. (b) Accumulation of ^{111}In-labelled lymphocytes in skin sites injected with 0·1 ml of each fraction. Each point indicates the mean ± s.e.m. over control sites of triplicate lesions in one animal. Similar results were obtained in two other rats and on one other Affigel Blue column.

the backs of the animals was shaved and 0·1 ml of the test samples were injected i.d. into three or four sites. Four additional sites were always injected with control medium. In most experiments, animals were killed 20 h later. The skin on the back of the animals was cut off, and excess blood was squeezed out. The injected areas were punched out with a leather punch and the radioactivity determined in an LKB gamma counter. In some experiments, animals were injected i.d. up to 23 h before i.v. injection of radiolabelled cells. These animals were killed 2 h after the labelled cells were given to evaluate the kinetics of lymphocyte recruitment into the skin.

RESULTS

Lymphocyte recruitment by the LK preparation

Rat lymphocytes obtained from lymph nodes and spleen were stimulated to undergo blast transformation by treatment with neuraminidase and galactose oxidase. This method was chosen since in the mouse (Johnson, Dianzani & Georgiades, 1981) and in the human (Novogrodsky & Katchalski, 1973), this enzyme treatment is strongly mitogenic, results in the production of no detectable IFN-α/β unlike some antigens (Rasmussen & Merigan, 1978), and produces large amounts of at least one important LK, namely IFN-γ. In addition, the enzymes which stimulate blast transformation are not present to contaminate the LK supernatant, as most other mitogens would be.

After enzyme treatment and 2 days in culture, the rat lymphocytes, which were initially 95% small cells, differentiated

Table 1. Effect of the monoclonal anti-IFN-γ antibody, DB-2, on lymphocyte migration induced by the LK preparation

Migration time (h)	Stimulus	^{111}In per lesion (ct/min/site)	% Inhibition
4	LK Prep.	475 ± 51*	—
4	LK Prep. + DB-2 450 nu	243 ± 16†	49
4	LK Prep. + DB-2 900 nu	224 ± 8‡	53
20	LK Prep.	768 ± 42	—
20	LK Prep. + DB-2 450 nu	568 ± 78§	26
20	LK Prep. + DB-2 900 nu	435 ± 48†	43

* Rats were injected i.d. in triplicate sites with a LK Prep. containing 300 u IFN-γ or LK Prep. plus DB-2, and labelled lymphocyte were injected i.v. Either 4 h or 20 h later, the animals were killed and the ^{111}In in each lesion determined. The values are the mean ± s.e.m. of the measurements on five animals.

† $P < 0.01$, ‡ $P < 0.001$, § $P < 0.05$ versus control.

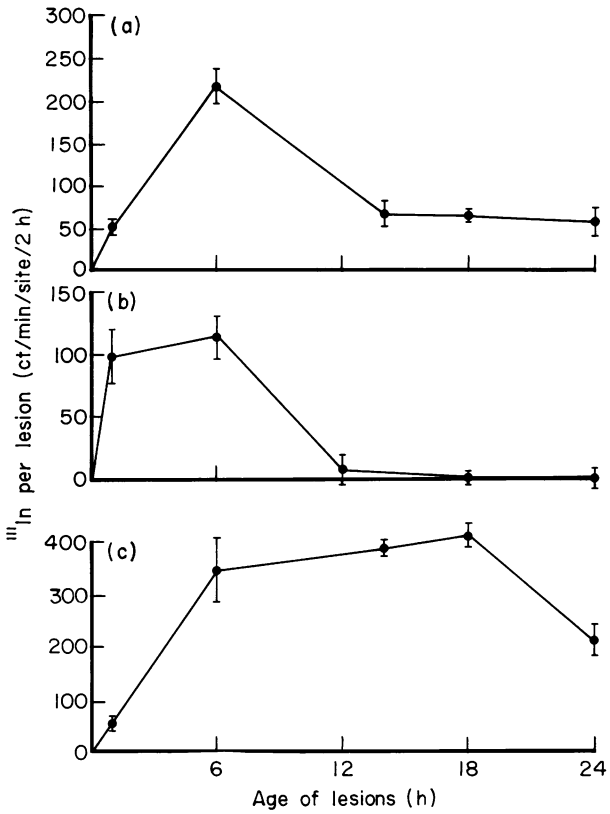

Fig. 4. Kinetics of lymphocyte migration into skin sites injected with (a) IFN-γ containing-LK Prep.; (b) IFN-α/β and (c) poly I:C. Rats were injected i.d. with each of the agents in triplicate sites. After varying periods of time, ^{111}In-labelled lymphocytes were injected i.v. and 2 h later, all animals were killed. Each point represents the mean increase in ^{111}In over control sites ± s.e.m. in three animals. Each point is plotted on the abscissa at the average age of the lesion during the 2 h the labelled cells were circulating in the animal.

Table 2. Effect of recombinant rat IFN-γ on lymphocyte migration

Injected dose (units/site)	^{111}In per lesion (ct/min/site)
0	178 ± 34 (3)*
30	393 ± 11 (3)
100	624 ± 38 (3)
300	975 ± 56 (3)
1000	1838 ± 82 (3)

* Rats were injected in triplicate sites with rec. IFN-γ at the indicated dose and labelled cells were injected i.v. The animals were killed 20 h later and ^{111}In in each lesion determined. Each value is the mean ± s.e.m.(n).

into 70% large lymphoblasts. Based on immunofluorescent staining with MoAb to rat lymphocytes, 85% were MRC OX-19$^+$ (T cells), 69% were W3/25$^+$ (T helper cells), 29% were MRC OX-8$^+$ (T suppressor cytotoxic cells) and 30% were MRC OX-6$^+$ (Ia$^+$ cells). Thus the enzyme treatment induced large numbers of both T helper and T suppressor cytotoxic cell blasts in the cultures, in keeping with a marked polyclonal activation of T cells. These cultures contained 750–1,000 U/ml of IFN. Rabbit anti-rat IFN-γ neutralized 98% and DB-2, a MoAb to rat IFN-γ, >99% of the IFN in the LK supernatant, while anti-rat IFN-α/β did not neutralize any of the IFN activity. The supernatant was dialysed and concentrated 20-fold, and the recruiting activity of this LK Prep. was determined (Fig. 1).

The LK Prep. recruited ^{111}In-labelled lymphocytes into the skin after i.d. injection with an obvious dose-response. At the highest dose tested, it produced a 13-fold increase over control sites. In order to determine which factor in the LK Prep. produced the recruitment, a number of purification and neutralizing experiments were performed.

Hydroxylapatite chromatography of recruiting activity

The LK Prep. was chromatographed on spheroidal hydroxylapatite, and the IFN concentration, and the ability of each fraction to recruit lymphocytes after i.d. injection was determined (Fig. 2). Virtually all of the IFN-γ bound to the column and over 80% of the recovered IFN-γ eluted in a single peak with 300 mM phosphate buffer together with only 2·8% of the total protein. Seventy-five percent of the recruiting activity recovered from the column was found in fraction (Fr.) 4 which contained most of the IFN-γ. However, small amounts of recruiting activity were found in Frs 1, 2 and 3 which contained little, if any, IFN-γ.

Effect of pH and heat on IFN-γ and lymphocyte recruitment

Rat IFN-γ is acid labile (Dijkema *et al.*, 1985). Since a number of other LKs, notably IL-1 and IL-2, are not destroyed by low pH (Maizel & Lawrence, 1984), the effect of treating Fr. 4 of the hydroxylapatite column at pH 3 for 24 h was evaluated. In addition, because endotoxin is a potent lymphocyte-recruiting agent in our assay (unpublished observation), the effect of incubating Fr. 4 at 100°C was determined. PH 3 decreased the viral-plaque-inhibition activity of the IFN-γ in Fr. 4 by 71% (15,000 to 4,400 U/ml) and the recruiting activity from 1,444 ± 165 ct/min/site to 757 ± 57 ct/min/site, i.e. by 48%. Heat totally destroyed the viral-plaque-inhibition activity and 88% of the recruiting activity. Assay for endotoxin by the limulus amoebocyte lysate assay demonstrated the presence of less than 0·01 ng/ml of endotoxin. Thus, acid treatment was in keeping with IFN-γ being one of the major recruiting factors. However, low pH decreased the viral-plaque-inhibition activity to a greater extent than the recruiting activity, suggesting that the recruitment in Fr 4 was not exclusively due to IFN-γ.

Affigel blue chromatography of the LK preparation

The LK Prep. was chromatographed on Affigel Blue. Figure 3 shows that rat IFN-γ eluted with 1·5 M NaCl, similar to the elution reported with mouse IFN-γ (Wietzerbin *et al.*, 1979). Fraction 3, which contained 90% of the recovered IFN-γ from the column, exhibited substantial lymphocyte-recruiting activity. However, Fr. 1, which contained the material that did not

bind to Affigel Blue also caused significant recruitment. Thus, there were at least two lymphocyte-recruiting activities. One was associated with IFN-γ and the other was independent of IFN.

Effect of anti-IFN-γ on lymphocyte recruitment by the LK preparation

The effect of the anti-IFN-γ MoAb, DB-2, on the lymphocyte-recruiting activity of the LK Prep. was determined (Table 1). Lymphocyte recruitment measured after 4 h was inhibited by 53% with DB-2, while after 20 h, there was up to 43% inhibition. This suggested that at least 50% of the early lymphocyte recruitment was caused by IFN-γ.

Kinetics of lymphocyte recruitment induced by LK prep

Our previous results demonstrated that, after the i.d. injection of IFN-α/β, lymphocytes are very rapidly and transiently recruited into the skin, while IFN inducers such as poly I/C and virus, have a delay in their effect on lymphocyte recruitment presumably because of the necessity to stimulate IFN-α/β production in the skin (Issekutz *et al.*, 1986b). Therefore the kinetics of lymphocyte recruitment by the IFN-γ containing-LK Prep. was compared with that of IFN-α/β and poly I/C (Fig. 4). Within 2 h of i.d. injection of the LK Prep., lymphocytes were recruited into the skin, and maximum recruitment occurred in lesions that were 5 to 7 h old. There was less but significant migration into lesions that were 14 h of age or older up to 24 h. The kinetics of migration to the LK Prep. were clearly different from that observed with either IFN-α/β or poly I/C. Recruitment by the LK Prep. was slower than that of IFN-α/β but was much more sustained, since IFN-α/β had no effect from 12–24 h. Similarly, the kinetics by the LK Prep. were very different from that of the IFN inducer poly I/C, which peaked at 10–18 h after i.d. injection.

Effect of recombinant IFN-γ on lymphocyte recruitment

The studies with the LK Prep. suggested that IFN-γ was a major lymphocyte recruiting factor; therefore, the effect of highly purified rec. rat IFN-γ was tested. Table 2 shows that rec. IFN-γ recruited lymphocytes into the skin. Lymphocyte migration to rec. IFN-γ was comparable to that observed with the LK Prep.

Histological changes produced by the LK prep

Skin sites were injected with the LK Prep. and after 20 h they were resected and examined histologically. Nearly all of the cells which migrated into the skin sites were mononuclear leucocytes and many were small lymphocytes (not shown).

DISCUSSION

This study demonstrates that the i.d. injection of a lymphokine-rich supernatant of activated T cells recruited lymphocytes into the skin. Hydroxylapatite chromatography and affinity chromatography on Affigel Blue suggested that the majority of the recruiting activity co-fractionated with IFN-γ although some recruiting activity was also found in fractions without any IFN-γ. Endotoxin was not responsible for the lymphocyte migration.

Treatment of the LKs at low pH, destroyed 71% of the IFN activity measured by virus plaque inhibition, and nearly 50% of the lymphocyte-recruiting activity. This decrease in recruitment was in keeping with a pH labile factor, such as IFN-γ. If one assumes that the 29% of the IFN not destroyed at pH 3 retained its recruiting activity, then up to 70% of the recruitment by the LKs could be ascribed to IFN-γ.

The MoAb to rat IFN-γ, DB-2, inhibited 53% of the recruiting activity of the LKs after 4 h and up to 43% after 20 h. This suggested that at least half of the early lymphocyte migration was the result of IFN-γ. The second factor which was found in the LK Prep. after affigel blue chromatography may be responsible for the remaining recruiting activity. Preliminary experiments have shown that this second recruiting factor has a slower kinetic pattern than IFN-γ. This may account for the decreased inhibition observed with DB-2 at 20 h.

Recombinant rat IFN-γ stimulated lymphocyte migration into the skin and it was as active as the LK Prep. Since a portion of the migration induced by the LK Prep. was not due to IFN-γ, the strong response to rec. IFN-γ suggests that it may be more active than native IFN-γ. This is similar to the increased activity of mouse rec. IFN-γ as compared to native IFN-γ in activating macrophages to enhanced H_2O_2 production (Nathan *et al.*, 1983).

In summary, these findings suggested that of the major LKs produced by activated T cells, IFN-γ was one of the major lymphocyte-recruiting factors.

Numerous studies have demonstrated that lymphocyte binding to the high endothelium in lymph nodes is a first step in migration into these tissues (Chin, Carey & Woodruff, 1982; Butcher, Scollay & Weissman, 1980). It has also been shown that in inflammatory sites, the morphology of the endothelium can change to that of high endothelium (Freemont *et al.*, 1983), and express new antigens (Cotran *et al.*, 1986). One agent which can alter antigens on endothelial cells is IFN-γ. It can induce HLA-DR expression (Pober *et al.*, 1983), and can cause the expression of an antigen (MECA-325) found on high endothelium in lymph nodes (Duijvestijn, Schreiber & Butcher, 1986). Yu *et al.* (1985) have shown that IFN-γ treatment enhances the binding of blood lymphocytes to endothelial cells *in vitro*. Our studies *in vivo* extend these *in vitro* findings by demonstrating that IFN-γ can also stimulate lymphocyte migration out of the blood into the skin.

Yu *et al.* (1985) have shown that 6 h of pretreatment with IFN-γ is required for maximal lymphocyte-endothelial binding. Our kinetic studies showed that the maximum rate of migration stimulated by the LK Prep. also occurred at 6 h. These kinetics suggest that IFN-γ acted directly on the endothelial cells as compared with poly I/C, which acted indirectly through IFN-α/β.

Finally, our results on IFN-γ extend our previous observations with IFN-α/β (Issekutz *et al.*, 1986b). Both types of IFN are able to recruit lymphocytes from the blood into an inflammatory site. During the initial phase of virus infections, IFN-α/β, which is produced by a variety of cells, may be responsible for lymphocyte migration into the infected tissue. Our studies suggest that in a DTH reaction, IFN-γ produced by lymphocytes activated by the antigen, may play a similar role.

ACKNOWLEDGMENTS

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