Migration Inhibition and Stimulation Factors Produced from Peripheral Blood Lymphocyte Cultures of Sensitised Guinea Pigs*

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SUMMARY The supernatants of peripheral blood lymphocytes of tuberculin-sensitive guinea pigs incubated with PPD were investigated using macrophage and leukocyte migration inhibition tests. Inhibition as well as stimulation of cell migration were observed. The effect upon migration cultures seemed to be dependent upon the immunological state of the host; animals of the V-group (vaccinated once without challenge) showed inhibitory activity to both macrophages and leukocytes, while those in the VC-group (vaccinated and challenged) had stimulatory activity only to leukocytes. The addition of antithymocyte serum stopped all activity (macrophage and leukocyte inhibition and leukocyte stimulation), suggesting that thymus-dependent lymphocytes are necessary for such activity.

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It is well known that sensitised lymphocytes exposed to a specific antigen produce many lymphokines, including a macrophage migration inhibitory factor (MIF).[1-5] However, stimulation of migration, rather than inhibition as was anticipated, had been observed in macrophage migration inhibition (MMI)[6,7] and leukocyte migration inhibition (LMI) tests.[8,9]

During investigation of MIF, and a leukocyte migration inhibitory factor (LIF) in the supernatants of spleen cells and peripheral blood lymphocyte cultures of tuberculin-sensitive guinea pigs, we also noted stimulation of cell migration.[10]

The presence of two factors with opposing activities may obscure the determination of either activity. Therefore, attempting to distinguish these two factors is an important step in understanding each biological function.

In the present study, we isolated MIF, LIF and a stimulation factor, and investigated the effects of an anti-thymocyte serum (ATS) on the generation of these factors.

MATERIALS AND METHODS

Animals

Female Hartley guinea pigs weighing 300-400 g were used. The animals were divided into three groups: 1) normal group (N-group); 2) V-group (vaccinated once with 1 mg of heat-killed BCG suspended in 0.1 ml of incomplete Freund's adjuvant, injected into a hind footpad). The animals were sacrificed by cardiac puncture four weeks later; and 3) VC-group (vaccinated and challenged); four weeks after vaccination, 1 mg of heat-killed BCG suspended in 0.5 ml of saline was injected into the right ventricle. The animals were sacrificed one week after the challenge. The tuberculin test was done using 10 μg PPD (Park Davis Company, U.S.A.); animals in both the V-group and VC-group showed apparant induration of more than 10 mm in diameter.

Preparation of anti-thymocyte serum

The thymus was removed from young guinea pigs (body weight 200 g). A suspension of thymocytes was prepared by using Eagle minimum essential medium (MEM) containing EDTA (packed cell volume : Eagle MEM : 0.01M of EDTA solution = 1:1:2, pH 7.2) and incubated for three minutes at 37°C. Next, the suspension was centrifuged at 3,000 rpm for 10 minutes, and the supernatant (referred to as EDTA-extract) mixed with Freund's complete adjuvant (FCA), (Difco Lab., Detroit, U.S.A.) was injected subcutaneously into two rabbits. Two weeks later, 0.5 ml (packed cells) of living thymocytes obtained from young guinea

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pigs were injected intravenously into each rabbit. The EDTA-extract mixed with FCA was injected subcutaneously into the back of each rabbit one week later and this injection was repeated once a week. Blood was collected from ear veins of the two rabbits once a week after the last injection and the serum was inactivated by heating at 56°C for 30 minutes. This serum was immunologically absorbed with an equal volume of guinea pig erythrocytes (37°C for 30 minutes), then absorbed with one fourth the original volume of guinea pig bone marrow cells (37°C for 30 minutes). Anti-thymocyte activity was determined using a series of diluted sera (e.g., 1:1, 1:10, 1:20...). The serum killed more than 95 per cent of the normal thymocytes at a concentration of 1:50, but less than 5 per cent of normal bone marrow cells at the same concentration. These findings confirmed the specificity of the anti-thymocyte serum (ATS). The ATS-treated cell suspension was prepared as follows: four volumes of lymphocyte suspension were mixed with five volumes of ATS (1:50 dilution) and one volume of fresh serum from normal guinea pigs, and pre-incubated at 37°C for 45 minutes.

Preparation of peritoneal exudate cells (PEC) and peripheral blood leukocytes

PEC and peripheral blood leukocytes obtained as shown in Figure 1 were used as indicator cells in the MMI and LMI tests. The percentages of macrophages among PEC and polymorphonuclear cells in buffy coat cells were 83-90 per cent and 75-85 per cent respectively.

Preparation of peripheral blood lymphocytes

Peripheral blood lymphocytes were obtained by Ficoll-Isopaque technique (Pharmacia Fine Chemicals, Uppsala, Sweden) as shown in Figure 1. The viability of the cells tested by the trypan blue dye exclusion method was more than 90 per cent. Lymphocyte suspensions (5-9 x 10^6/ml) obtained from the three experimental groups were incubated with or without PPD. The lymphocytes were pretreated with ATS and complement in some experiments.

Indirect MMI test and indirect LMI test

MIF and LIF activities in the culture supernatants were assayed by the capillary tube method[1,10] as shown in Figure 1. After 24 hours, the migration patterns were drawn on tracing paper with a magnification projection of 40 x. The weight of the paper was measured and per cent migration inhibition (%MI) was calculated using the following formula:

$$\%\,\text{M.I.} = \left(1 - \frac{\text{mean weight of migration area with antigen-stimulated supernatant}}{\text{mean weight of migration area with unstimulated supernatant}}\right) \times 100$$

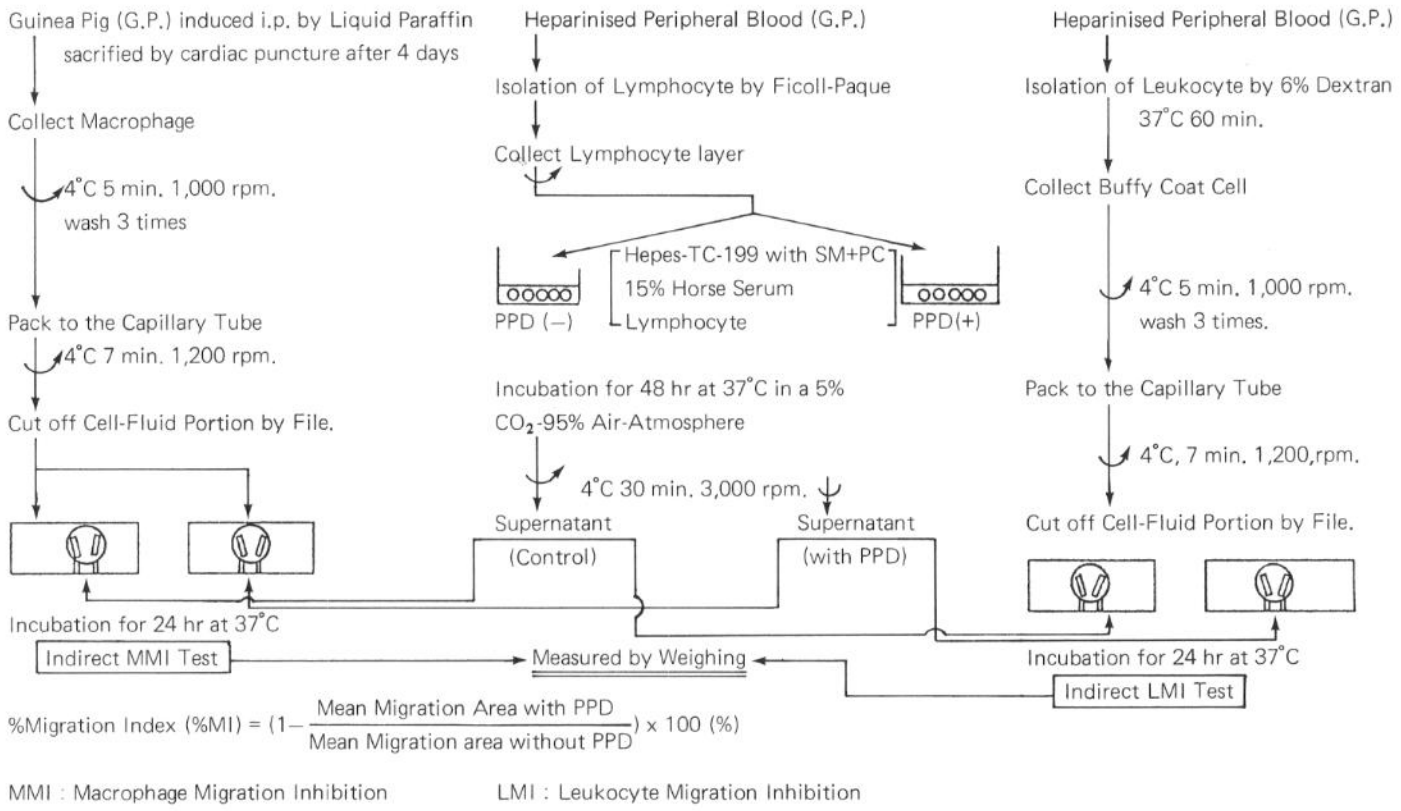

Fig. 1 Method of migration inhibition test.

The average was determined by calculating the mean value obtained in six experiments.

Sephadex gel filtration

Supernatants were obtained from cultured peripheral blood lymphocytes of the three experimental groups, concentrated to one tenth the original volume and then dialysed by PBS (pH 7.4).

The samples (3 ml) were fractionated by column chromatography (size 2.5 x 50 cm) made of 20 g of Sephadex G100 (Pharmacia, Uppsala, Sweden); PBS (pH 7.4) was used as the solvent. Bovine serum albumin (BSA, mw 68,000), ovalbumin (OA, mw 48,000), chymotrypsinogen (CT, mw 23,000) and cytochrome C (CC, mw 12,400) were used as protein markers. Each 4 ml of the eluate was collected in a tube by using a fraction collector (Coleman Type III, Sweden). Absorbance was measured at 280 nm and the effluent was separated in seven fractions (Fr I-VII) each of which was concentrated to one tenth the original volume and then the concentrates were dialysed for 48 hours using Hepes-TC-199 medium containing 15 per cent horse serum. The inner fluid was centrifuged at 3,000 rpm for 30 minutes and the supernatants were filtered through a 0.45 μm millipore filter (Millipore Corp., U.S.A.).

RESULTS

Whole supernatants

Figure 2 shows the relationship between the average of migration index for the whole supernatants and the results of the tuberculin skin reaction test. The supernatants of the V-group (V-sup) showed remarkable inhibitory activity against the migration of macrophages and buffy coat cells in parallel with the positive tuberculin skin reaction test. In the VC group (VC-sup), the macrophage inhibitory activity became relatively weak, while the buffy coat cells re-

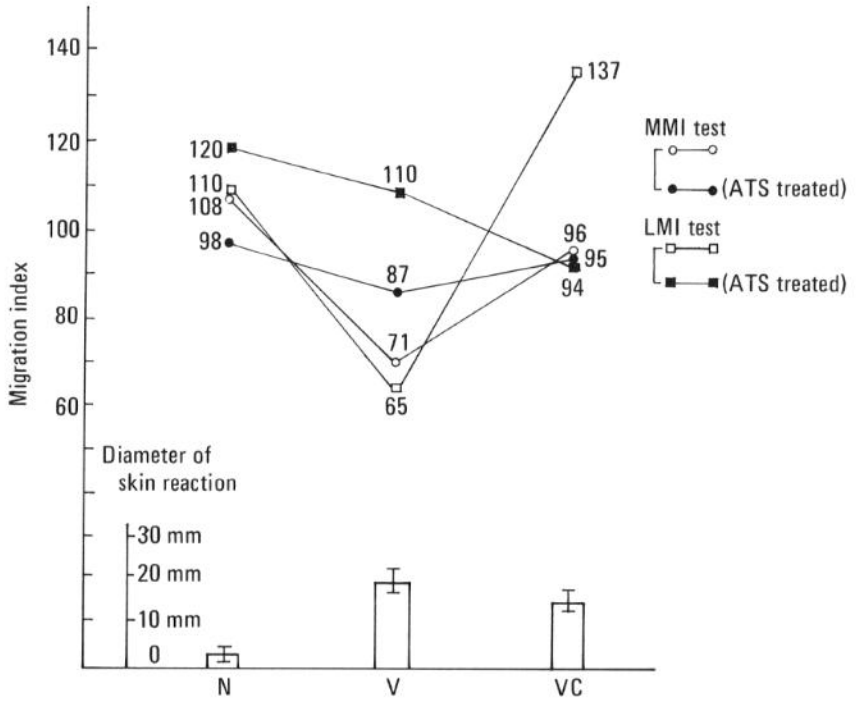

Fig. 2 Macrophage and leukocyte migration inhibitory capacity in the culture supernatants of peripheral blood lymphocytes which were obtained from N –, V – and VC-guinea pig and result of tuberculin skin reaction. (Indirect MMI test and LMI test, PPD concentration 40 μg/ml).

$$\text{Migration index} = \frac{\text{Mean migration area (sup. with PPD)}}{\text{Mean migration area (sup. without PPD)}} \times 100\ (\%)$$

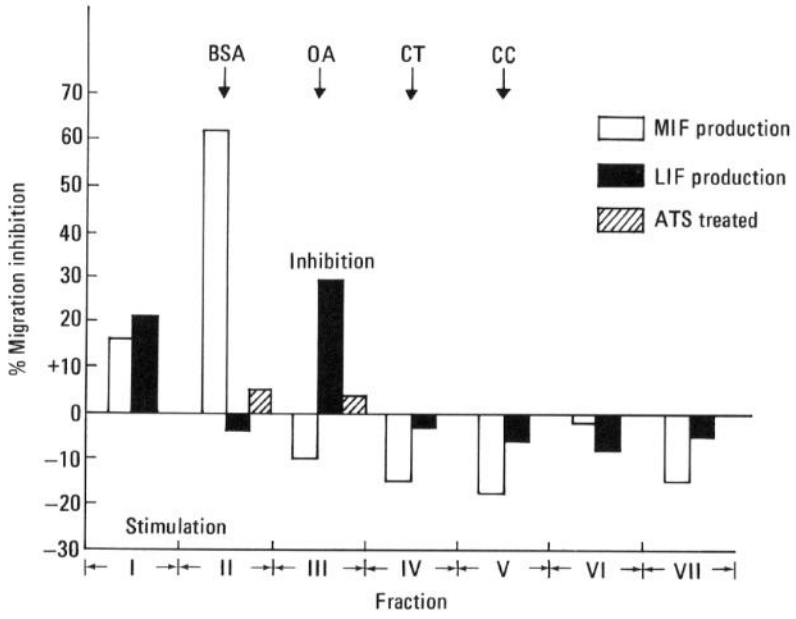

Fig. 3 Isolation of MIF and LIF from culture supernatant of V-lymphocyte by using Sephadex G-100 column chromatography.
Supernatant preparations were obtained from peripheral blood lymphocytes of V-guinea pigs cultured with PPD and filtered on Sephadex G-100 columns. Each fraction after 10-fold concentration was tested for MIF and LIF activities. Arrows indicate the positions where marker proteins were eluate; bovine serum albumin (mw 67,000) ovalbumin (mw 45,000), chymotrypsinogen (mw 23,000) and cytochrome C (mw 12,400).

markably stimulated migration; however, those animals were positive for the tuberculin skin reaction test. When peripheral blood lymphocytes were pretreated with ATS and complement both MIF (71%) and LIF (65%) production in V-sup decreased remarkably as MI was 87 per cent and 110 per cent respectively. The stimulatory effect of VC-sup was also decreased by ATS-treatment of the lymphocytes from 137 per cent to 94 per cent.

Sephadex gel filtration

As shown in Figures 3 and 4, MIF activity was detected in Fr II of V-sup (mw 67,000) and in Fr II and Fr V of VC-sup (mw 67,000 and 12,400 respectively). LIF activity was found only in Fr III of V-sup (mw 45,000). In VC-sup, however, stimulatory activity on leukocyte (buffy coat cells) migration was detected in Fr III (mw 45,000) and Fr IV (mw 23,000), but no stimulation of macrophage migration was noted.

Neither inhibition nor stimulation was seen in any fractions obtained from supernatants cultured without PPD.

DISCUSSION

Our observations indicate that there are two distinct factors which both inhibit and stimulate cell migration in the supernatants of peripheral blood lymphocytes of tuberculin-sensitive guinea pigs. Moreover, it is interesting that differences in cell migration were noted in the V-group and VC-group. The molecular weights of the fractions with MIF activity were 67,000 in the V-group and 67,000 and 12,400 in the VC-group. Fraction II in the V-group and Fractions II and V in the VC-group seem to be quite similar to the MIF described by Bloom and Bennet,[5] and Yoshida[12] respectively. In addition, no MIF was present in the V-group and VC-group after treatment with anti-thymocyte serum. Therefore, this heterogeneity in molecular weight may be due to MIF being produced by sensitised lymphocytes stimulated with PPD. On the other hand, LIF with a molecular weight of 45,000 was found only in the V-group.

These results indicate that the character and the molecular weight of LIF differ from those of MIF in guinea pigs as Rocklin[11] reported to be the case in humans.

The VC-group exhibited factors that stimulate the migration of buffy coat cells; their molecular weights were 45,000 and 23,000. There are several reports that suggest the existence of a migration stimulation factor.[6-9,13] Svejcor,[6] and Bendixen and Søborg[8,9] reported that in weakly sensitised individuals leukocyte migration may be augmented with small doses of antigen. Yoshida,[12] in examining guinea pig MIF, found that neat samples of MIF activity displayed strong inhibitory effects, yet when diluted had different levels of activity, or even no activity. Moreover, there may be inherent variability in the MIF and LIF assay system. Fox[13] pointed out that foetal calf serum (FCS) itself contained FCS-MIF and FCS-MSF. However, we have also demonstrated that the size of the spleen in the VC-group increased with remarkable cellularity and the spleen cells showed marked activity in the passive transfer of tuberculin hypersensitivity compared with those in the V-group,[14] even when the cell cultures contained horse serum, which has neither inhibitory nor stimulatory ability.

Thus, our data may mainly reflect differences in the immunological state of the host. In addition,

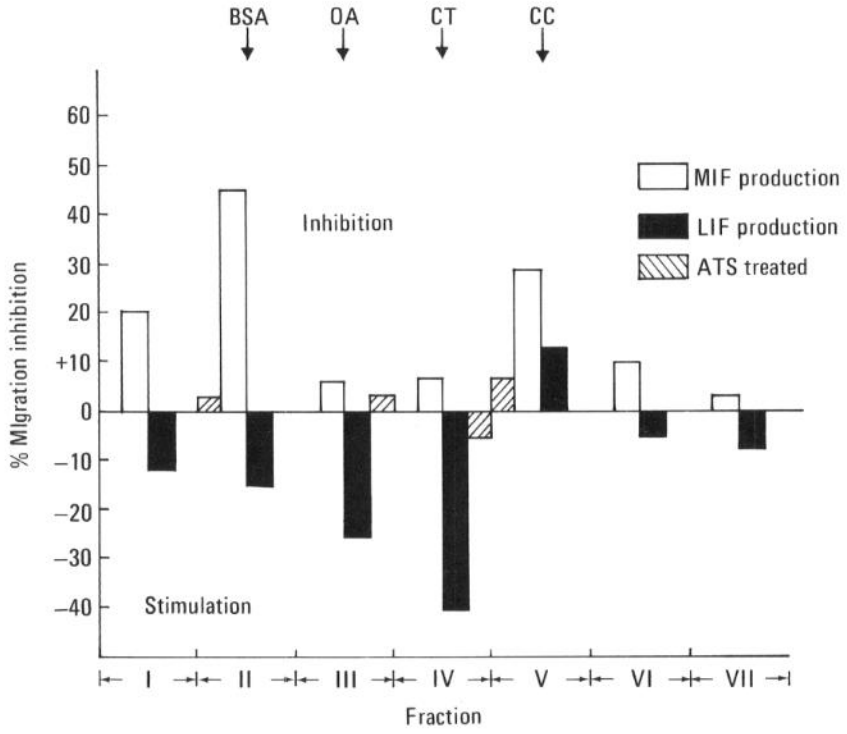

Fig. 4 Isolation of MIF, LIF and stimulating factor from culture supernatant of VC-lymphocyte by using Sephadex G-100 column chromatography

Supernatant preparations were obtained from peripheral blood lymphocytes of VC-guinea pigs cultured with PPD and filtered on Sephadex G-100 columns. Each fraction after 10-fold concentration was tested for MIF and LIF activities. Arrows indicate the positions where marker proteins were eluate; bovine serum albumin (mw 67,000), ovalbumin (mw 45,000), chymotrypsinogen (mw 23,000) and cytochrome C (mw 12,400).

stimulatory activity as well as inhibitory activity stopped after treatment with anti-thymocyte serum.

These results suggest that there are two types of antigen-induced effects on migration and each is the result of thymus-dependent lymphocyte stimulation by a specific antigen. At the same time, both types of reactions may be an expression of delayed hypersensitivity response because of the positive tuberculin skin reactions in the V-group and VC-group. However, the relationship between inhibition and stimulation remains unclear.

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