Leukocyte Migration Inhibition Test (LMIT) in Systemic Lupus Erythematosus

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Abstract A leukocyte migration inhibition test (LMIT) utilizing the agarose gel technique was performed with native DNA as an antigen in ten patients with systemic lupus erythematosus (SLE) and five normal subjects. Irrespective of disease activity, supernatants obtained at different time intervals during lymphocyte culture in eight patients with SLE showed significant alteration of migration, either enhancement or inhibition, of normal leukocytes. However, supernatants in the control experiments produced no significant alteration of migration. Polyacrylamide gel electrophoresis of supernatants obtained from the SLE group revealed that the inhibitory activity was present in the albumin region, whereas the enhancement activity was found in the β-globulin region. These results indicate that the hitherto employed estimation of the leukocyte migration inhibition test based on the total activity of these two factors is insufficient for accurate evaluation of chemical mediators from sensitized lymphocytes and that the separation of these two factors may be important for a greater understanding of cellular immunity.

Recently Weisbart et al (6), performing LMIT in normal subjects with PPD as an antigen, recognized a factor which opposed the action of the leukocyte migration inhibitory factor (LMIF) and they termed this factor the leukocyte migration enhancement factor (LMEF). However, the LMEF has still not been found in cases of SLE.

Performing indirect LMIT utilizing the agarose gel technique in patients with SLE in the presence of native DNA as an antigen, we found that supernatants obtained at different time intervals during lymphocyte culture showed significant alteration of migration, i.e., either enhancement or inhibition of migration. This fact suggests the existence of not only LMIF but also LMEF in SLE. We further tried to isolate LMIF and LMEF separately by polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Patients and controls. Ten patients with SLE, one male and nine females, aged 11 to 54 with a mean of 33 years, were studied. The diagnosis of SLE was established according to the ARA preliminary criteria for the classification of SLE (3). Seven of the patients were on steroid therapy. Five normal individuals served as controls.
Antigen. Calf thymus DNA (Worthington Biochemical Corp. Freehold N. J.) was solubilized in phosphate buffered saline (PBS). The concentration of native DNA as an antigen was 100 μg/ml of culture medium.

Leukocyte migration inhibition test. The indirect leukocyte migration inhibition test utilizing the agarose gel technique (2) was employed.

(i) Lymphocyte separation and culture with the antigen: Human lymphocytes were isolated by gradient centrifugation on Ficoll-Isopaque as described by Böyum (1). After the cells had been resuspended at a cell concentration of 2 × 10⁶ cells per ml in Eagle's minimal essential medium (Eagle's MEM) containing 10% heat-inactivated fetal calf serum, L-glutamine (1.6 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml), lymphocytes were cultured with and without antigen at 37 C. After 6 hr the tubes were centrifuged and the supernatants were decanted. Medium was added to the cell pellet and the culture was continued. The same procedure was performed after 12, 24, 48 and 72 hr. Cells were viable after 72 hr as determined by trypan blue exclusion.

(ii) Preparation of agarose medium: Fresh agarose medium was prepared after cooling to 55 C and by mixing with warm (55 C) horse serum, sterilized water and tissue culture medium 199 to provide a final concentration of 10% horse serum and 1% agarose. Penicillin (100 IU/ml), streptomycin (100 μg/ml) and Heps (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid) buffer were added. The medium was passed through a Millipore filter (pore size: 0.45 μm). Five ml aliquots of agarose medium were poured into petri dishes and allowed to harden. Wells of 2.5 mm in diameter were cut in the agarose plate.

(iii) Agarose gel technique: Before testing, aliquots of supernatants from lymphocyte culture were replenished with an equal volume of medium 199 with 10% horse serum and antibiotics. Seven × 10⁶ leukocytes which had been obtained from normal donors were resuspended in 0.2 ml of each replenished supernatant. Cell suspension was then incubated at 37 C for 30 min and adjusted to 3 × 10⁸ leukocytes per ml. Five microliter aliquots of suspension were dispensed in three wells. Tests were performed in triplicates on a single plate. Plates were incubated at 37 C for 24 hr in a CO₂ incubator. The migration index (MI) was calculated as follows by an ocular micrometer.

\[ MI = \frac{\text{mean area of migration with antigen}}{\text{mean area of migration without antigen}} \]

Viability of cells was determined by trypan blue exclusion. Viability before incubation was more than 98%. Statistical analysis was performed by Student’s t-test.

Polyacrylamide gel electrophoresis. Some supernatants which showed significant inhibition or enhancement of migration were concentrated 10-fold by polyethylene-glycol and subjected to polyacrylamide gel electrophoresis as described by Remold (4). Six glass tubes of identical size, 0.5 × 7.0 cm, were used as gel-containing columns. The gels consisted of three parts: the running gel of 1 ml, the spacer gel of 0.16 ml and the sample gel of 0.2 ml. After gel polymerization, 50 μl of non-stimulated and stimulated supernatants which had been concentrated 10-fold were placed on gel columns and subjected to electrophoresis simultaneously. The 6th gel con-
taining medium and 10% horse serum was used as a control and stained with amido black. A current of 2.0 mA per column was applied for 90 min. Gels were sectioned into three portions containing albumin, β-globulin and γ-globulin, respectively. Identical portions were pooled and eluted by electrophoresis. The eluates were dialyzed in a dialysis sac (24-A pore size) for 24 hr in Hanks' BSS. The volume of eluates was adjusted to 2 ml with Eagle's MEM and sterilized by Millipore filtration. With 0.2 ml of each solution activity was assayed in leukocyte suspensions from normal donors in the agarose system.

Criteria of activity. Patients were considered to be in a clinically active stage if 2 or more of the following items were present: a fever of more than 38 C, oral-cutaneous lesions (oral ulcers and/or skin rash), arthritis, hematologic abnormality (anemia, leukopenia and/or thrombocytopenia) or active renal disease.

RESULTS

Time-Course Studies of Migration Inhibition

Supernatants studied at intervals between 6 hr and 3 days in five controls produced no significant alteration of migration. Time-course studies on two of these subjects are shown in Fig. 1. Remaining three individuals showed similar results. However, supernatants from patients with SLE showed five different patterns of response. Type 1: Increasing inhibition of migration was initially observed, followed by enhancement of migration. There were three patients who exhibited...
this type of migration. One of them had active SLE and the other two had inactive SLE. The time course of MI produced by supernatants of lymphocyte culture of a patient (T.A.) with inactive SLE, who was taking 15 mg of prednisolone daily, is depicted in Fig. 2. Neither inhibition nor enhancement of migration was noted at 6 and 12 hr. However, at 24 hr inhibition of migration was observed with MI of 0.73 ± 0.03. At 48 hr, enhancement of migration was produced with MI of 1.33 ± 0. At 72 hr, no significant inhibition or enhancement was present. Other two patients showed similar results. Type 2: Enhancement of migration was noted before 24 hr, followed by inhibition of migration. Figure 3 shows the time course of MI produced by supernatants of a patient (F.S.) with active SLE, who was not on steroid therapy. Neither inhibition nor enhancement was observed at 6 hr. However, enhancement of migration was noted at 12 hr with MI of 1.32 ± 0.04 followed by inhibition of migration at 24 hr with MI of 0.79 ± 0. No significant inhibition or enhancement was present at 48 and 72 hr. No other patient showed this type of migration. Type 3: Neither inhibition nor enhancement of migration was observed before 48 hr. However, there ensued a period of enhancement between 48 and 72 hr. Two patients showed this type of migration, both of whom had inactive SLE. Figure 4 shows the time course of MI of a patient (K.Y.) with a daily maintenance dose of prednisolone. Enhancement of migration with MI of 1.27 ± 0.02 was noted at 48 hr and 1.27 ± 0 at 72 hr. The other patient showed a similar result. Type 4: Inhibition of migration was seen between 6 and 24 hr. This was followed by decreasing inhibition, but inhibition of migration increased again after 24 hr. There were two patients who exhibited this type of migration.
both of whom had inactive SLE. Time course of MI of a patient (A.I.) is depicted in Fig. 5. At 12 hr inhibition of migration with MI of 0.76±0.0 was observed, followed by decreasing inhibition. However, there ensued a period of increasing inhibition with MI of 0.75±0.03 at 72 hr. The other patient showed a similar type of migration. Type 5: Neither inhibition nor enhancement of migration was observed with any of the supernatants. There were two patients with inactive SLE, who showed this type of migration.

No correlation seemed to exist between clinical activity and the five migration patterns.

Separation of Migration Inhibitory and Enhancement Activity from Supernatants Utilizing Polyacrylamide Gel Electrophoresis

Supernatants in patients T.A. and F.S. were studied. The supernatants of
T.A. obtained at 24 and 48 hr and those of F.S. obtained at 12 and 24 hr were concentrated 10-fold by polyethyleneglycol and were subjected to polyacrylamide gel electrophoresis.

MI's using the eluates from each fraction of the two supernatants of patient T.A. are shown in Table 1. With the eluates from the supernatant at 24 hr, inhibitory activity with an MI of 0.88±0.02 was noted in the albumin region, while in other two fractions no activity was present. With the eluates from the supernatant at 48 hr, enhancement activity with an MI of 1.16±0.04 was noted in the β-globulin region, whereas eluates from other two fractions showed neither inhibition nor enhancement of migration. Table 2 shows MI's of the eluates from each fraction of the two supernatants of patient F.S. With the eluates from the supernatant at 12 hr, enhancement activity with an MI of 1.14±0.04 was noted in the α-globulin region, whereas no activity was noted in other two fractions. At 24 hr, inhibitory activity with an MI of 0.87±0.05 was present in the albumin fraction, whereas other two fractions showed no activity.

These results indicate that inhibition of migration shown with the original supernatant is due to an inhibitory factor which exists in the albumin region and enhancement of migration is due to an enhancement factor in the β-globulin region.

Leukocyte Migration Inhibition Test with Mixture of the Two Factors

In patient T.A., the next procedure was performed. The sterilized eluate with the inhibitory activity taken from the 24 hr supernatant and the eluate with the enhancement activity taken from the 48 hr supernatant were mixed together, and LMIT was performed with 0.2 ml of this solution. The MI of this test was 0.86±0.03. In patient F.S. the same procedure was performed using the sterilized eluates with the enhancement and inhibitory activity taken from 12 and 24 hr supernatants. The MI of this test was 0.90±0.01. These results show that competing actions of these two factors do not allow leukocytes to show either inhibition or enhancement of migration. These results also indicate that leukocyte migration is influenced by the amounts of coexistent LMIF and LMEF in the supernatants. Therefore, LMIT should be estimated taking this point into consideration and the hitherto employed method of estimation of LMIT should be re-evaluated.

DISCUSSION

In the present study we have found that both LMIF and LMEF are released in lymphocyte cultures of patients with SLE in the presence of native DNA. We would like to reconsider the hitherto employed method of estimating LMIT. We cultured lymphocytes of patients with SLE with native DNA as an antigen, obtaining supernatants at different time intervals for 3 days and assayed the activities of the supernatants on normal leukocytes. As a result, the supernatants obtained showed different types of migration, either inhibition or enhancement.

There were five different patterns of migration and it was evident that lymphocytes from patients with SLE in the presence of native DNA elaborate not only LMIF
but also LMEF. There was no correlation between clinical activity and the migration pattern.

A question arises as to why leukocytes migrate differently according to the supernatants. In other words, why are different amounts of these factors produced as time progresses? There are several points imaginable. Firstly, production of these factors may be influenced by cell interaction between lymphocytes in addition to the reaction between sensitized lymphocytes and the antigen. During lymphocyte culture, the viability of cells at 72 hr was, on an average, approximately 50% of the initial count. Alteration of viability of cells has an effect on cell interaction, which will lead to the altering production of these factors. Secondly, these factors, on one hand, may be inactivated as time progresses, while on the other hand, they may be released almost continuously throughout the culture. For that reason the concentrations of these factors alter during the culture, which will lead to different values of MI's according to supernatants. Thirdly, according to the degree of sensitization of the lymphocytes to the antigen, there may be a rhythm inherent in the lymphocytes themselves to produce these factors, which causes production of different amounts of these factors at various time intervals. To make sure that LMIF and LMEF are distinct factors we tried to isolate these factors separately, utilizing polyacrylamide gel electrophoresis. As a result it was noted that LMIF exists in the albumin region, whereas LMEF is found in the β-globulin region. This fact was compatible with the report of Weisbart et al (6), who found that LMEF was released in addition to LMIF while incubating lymphocytes of normal individuals with PPD. Our present study also supports their work. Therefore, migration of leukocytes seems to be determined by the amounts of LMIF and LMEF. For that reason production of these factors cannot be denied even in cases where no inhibition or enhancement is present in LMIT, because competing actions of these two factors may not allow leukocytes to show either inhibition or enhancement. Actually we mixed the inhibitory and enhancement activity together and employed it in LMIT and found that, as a whole, the mixed material showed neither inhibition nor enhancement of migration. Therefore, the hitherto employed method of LMIT does not always reflect the correct aspect of delayed hypersensitivity, and we have to reconsider the evaluation of the test. To correctly evaluate the cellular immunity separation of the two factors will be indispensable.

The influence of steroids on migration of leukocytes (5) has been reported. However, the relationship between the release of LMEF or LMIF and steroid therapy remains to be determined. Although steroids may play some role in releasing these factors, no significant difference was observed between patients with steroid therapy and those without in our study. This problem still remains unclarified.

REFERENCES


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