

Leukocyte Migration Inhibition with Epstein-Barr Virus Negative and Positive Cell Extracts

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Leukocyte migration inhibition of seronegative and seropositive healthy children and that of patients with infectious mononucleosis was studied in the presence of EBV-negative and -positive cell extracts. Seronegative children showed no alteration of the leukocyte migration in the presence of the extracts. EBV-positive cell extracts caused significant migration inhibition in the group of seropositive healthy children and of IM patients. The effect was specific, since the EBV-negative cell extract had no inhibitory effect. In the acute phase of infectious mononucleosis a depressed response was observed, while after recovery the same migration inhibition could be detected. The possible cause of the depressed cell-mediated immune response in the acute phase of the disease is discussed.

Keywords: EB-virus; infectious mononucleosis; leukocyte migration.

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Infectious mononucleosis (IM) is considered to be a self-limiting, benign lymphoproliferative disease, caused by the Epstein-Barr virus (EBV) (4, 6). During the disease and after recovery there are specific serological alterations (10, 14) and also changes in cell-mediated immunity (CMI) (19). Although the majority of the increased number of the mononuclear cells in the peripheral blood belongs to the T cell population (12), T cell responses have been reported to be depressed (3, 12, 18) compared with EBV-seropositive healthy persons (15). On the other hand, the T cells in IM, which are depressed in *general* function, are sensitized against EBV-positive B cells and have specific killer activity against cell lines carrying the EBV-genome (21). The sensitization of T cells in IM was also detected by leukocyte migration inhibition (LMI) assays (2, 8, 9), but the data are difficult to

interpret due to lack of specificity control, i.e. tests with EBV-negative cell line extracts. Therefore, we studied the LMI of IM patients in the presence of extracts obtained both from EBV-negative and EBV-positive cell lines.

MATERIAL AND METHODS

Patients

Twenty-one IM patients were studied. The diagnosis of IM was confirmed by the clinical symptoms and the EBV serology (7). IgM, IgG, IgA antibodies to virus capsid antigen (VCA) and α -EBNA positivity ($> 1:20$) of the sera were investigated (detailed in: Discussion). Some patients were examined twice or three times with LMI, so the number of LMI assays totalled 34 in this group. The control group comprised 20 EBV-seropositive

healthy children, having no clinical symptoms in the anamnesis and/or at the time of the study and no serological signs of acute EBV-infection, but α -VCA-IgG positivity. Sex distribution was the same in both groups. Mean age was 6.6 (1–15) years in the IM group and 6.79 (1.5–15) years in the seropositive healthy children. Eight EBV-seronegative subjects were also included as a separate group. The mean age of this group was less than the other groups (2.2 years, from 4 months to 6 years).

Antigen preparation

One EBV-negative (BJAB) and two EBV-positive (BJA-B 95/8 and P3HR) lymphoblastoid cell lines were kindly provided by Georg Klein, Karolinska Institute, Stockholm. All lines were kept in RPMI-1640 medium, supplemented with 8% heat-inactivated foetal calf serum (Gibco). Cultures were grown and the cells were harvested weekly until a sufficient amount of cells were collected for preparing the antigen(s)-containing substances. The cells were washed three times in TC-199 medium (Difco) and sonicated under microscopic control using an LKB sonicator. The resulting suspension was spun at 100,000 *g* for 30 min at +4°C. The aspirated supernatant was passed through a Millipore filter (pore size: 0.22 μ m) and stored at -20°C until use. Protein concentration was measured by Lowry's method (11). Fifty and 100 μ g/ml of antigens were the usual amounts used. In some cases, 20, 25, and 200 μ g/ml concentrations were used. LMI was studied in three cases also in the presence of 2 μ g/ml PPD powder (HUMAN, Budapest) as antigen.

Leukocyte migration inhibition

Direct microcapillary tube technique was carried out according to Søborg & Bendixen (22), micromodified by Federlin et al. (1). Briefly, leukocytes were obtained from heparinized blood after sedimentation with Dextran-250. Buffy-coat cells were washed 3–4 times with TC-199 and the cell concentration was adjusted to 3×10^6 cells for one microcapil-

lary tube (20 μ l, Hemocap, Drummond). The cell suspension was drawn into the glass capillaries. After sealing one end in flame and centrifuging at 2000 r.p.m. for 30 min, the capillaries were cut just below the cell-fluid interphase and placed on a drop of silicone grease (Dow Corning Co.) in a 0.5 ml disposable polystyrene culture chamber (Sterilin®) containing TC-199 and 10% heat-inactivated, pooled human AB-serum. In some cases the pooled AB-serum was substituted with heat-inactivated serum, obtained from a patient with IM in the acute phase (6th day) or another serum of a patient after recovery (3rd month). The appropriate amount of cell extract was added to the medium in advance. The chamber was closed with a coverslip. Three or four cultures were set up for each concentration of cell extract employed. After 24 h at 37°C, the migration areas of leukocytes were projected onto white paper, using a photographic blow-up machine. The areas were drawn, cut out and weighed. The average area of the migration with antigen (M_a) and control cultures (M_c) containing only medium + serum, from the same blood sample, determined the migration index: $MI = M_a/M_c$. MI of less than 1.0 indicates inhibition, while MI of more than 1.0, a stimulation of leukocyte migration.

Statistical analysis

Upper and lower cut-off values of the normal range were calculated on the ground of the mean $\pm$ 2 s.d. of MIs of EBV-seronegative children in the presence of 100 μ g/ml of EBV-positive extracts (Fig. 1). For group statistics Student's *t*-test was used.

RESULTS

In pilot experiments it was pointed out that BJA/B 95/8 and P3HR cell extracts have the same migration inhibitory effect in the same individuals. Therefore only one of these was used, as EBV-positive antigen, at any one time. Twenty and 25 μ g/ml of EBV-positive

extracts had no migration inhibitory effect, while 200 $\mu\text{g/ml}$ was found to be toxic, as measured with trypan-blue exclusion. For this reason the LMI of healthy seropositive children and of IM patients was studied with only 50 and 100 $\mu\text{g/ml}$ concentrations of these extracts, while in seronegative patients only 100 $\mu\text{g/ml}$ of EBV-positive extract was used.

Seronegative healthy children showed no reaction either with EBV-negative or -positive cell extracts. The average MIs were

1.06 ± 0.11 and 1.01 ± 0.11 respectively. Double s.d. from the mean in the presence of 100 $\mu\text{g/ml}$ EBV-positive extract represents the upper and lower cut-off value of the normal range using this concentration (1.22 versus 0.79) (Fig. 1).

Both concentrations of the EBV-positive cell extracts caused significant LMI (MI: 0.70 ± 0.10 with 50 $\mu\text{g/ml}$ and 0.60 ± 0.18 with 100 $\mu\text{g/ml}$) in the group of seropositive healthy children. The EBV-negative extract had no

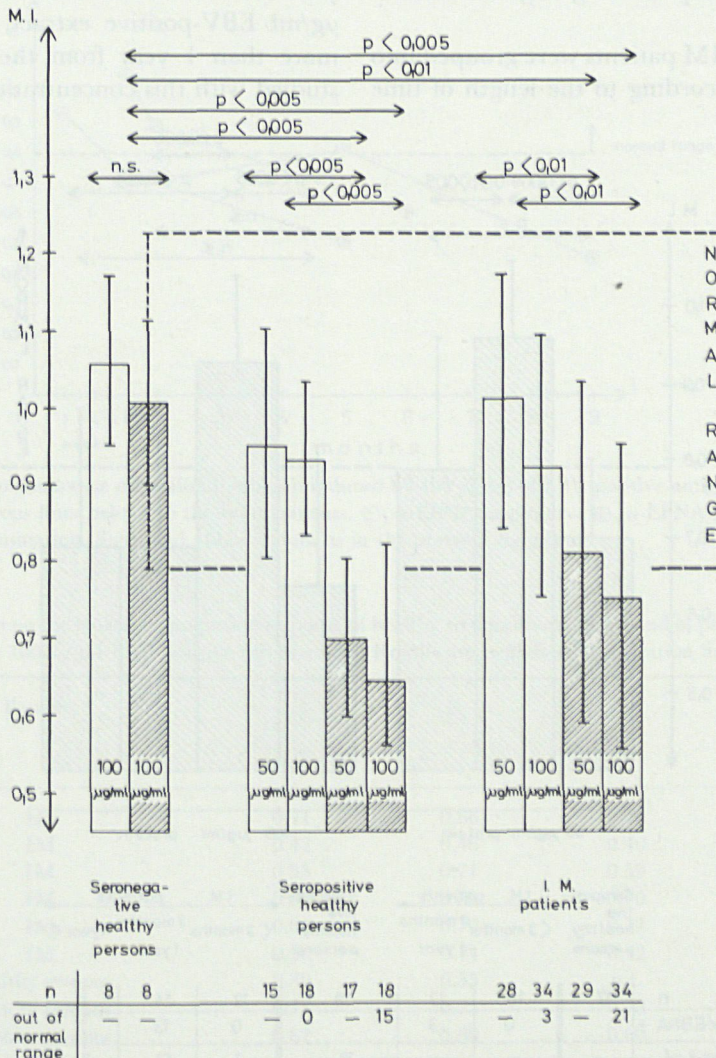
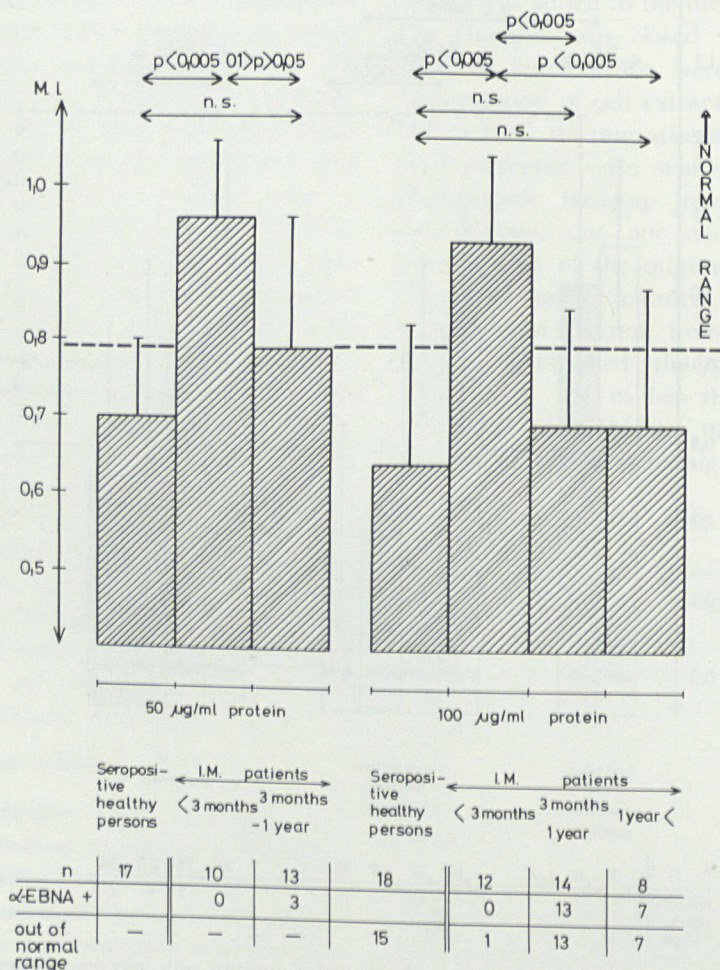


Fig. 1. Leukocyte migration inhibition of normal subjects and of patients with infectious mononucleosis (IM). MI, migration index; $\square$, EBV-negative antigen; $\blacksquare$, EBV-positive antigen; n, number of cases. Top of the columns represents the mean. The continuous vertical lines are s.d. from the mean. Horizontal dotted lines correspond to upper and lower cut-off values of the normal range. Normal range was calculated by only using 100 $\mu\text{g/ml}$ of antigen. n.s., not significant.

effect: all of the MIs were within the normal range. On the other hand, 15 out of 18 subjects showed migration inhibition in the presence of 100 $\mu\text{g/ml}$ EBV-positive extracts. No stimulation of the migration was found. Similarly, specific, but weaker LMI was observed in the group of IM patients (MI: 0.81 ± 0.22 with 50 $\mu\text{g/ml}$, 0.75 ± 0.20 with 100 $\mu\text{g/ml}$ EBV-positive extract). Twenty-one out of 34 MIs in this group were out of the normal range. Three of these 21 MIs were greater than the upper limit of normal, representing migration stimulation (Fig. 1).

If the data of IM patients were grouped into three periods according to the length of time

from the onset of the first symptoms of the disease (< 3 months, 3 months to 1 year and > 1 year), it has been found that in the patients with a history of less than 3 months all but one of the MIs were within the normal range, (mean 0.93 ± 0.11) when 100 $\mu\text{g/ml}$ EBV-positive extract was used. With longer observation periods, however, significant migration inhibition was observed in 13 out of 14 patients (3 months to 1 year) and in seven out of eight patients with a history of more than 1 year. The same reaction was found using 50 $\mu\text{g/ml}$ EBV-positive extract, though patients more than 1 year from the onset were not studied with this concentration (Fig. 2).



Serial studies were carried out in some IM patients at different times after the onset of the disease. In three cases we made three LMI assays on each patient. No correlation was found with serological data except for the α -EBNA positivity ($> 1:20$) of the patients' sera. Significant LMI appeared when the serum already showed significant α -EBNA positivity. Repeated examinations revealed the same results (Fig. 3).

In six IM patients and three healthy seropositive subjects LMI was also studied in the presence of serum taken in the acute phase of IM (S_1), and with convalescent serum (S_2) as well. S_1 abrogated LMI in six out of nine cases, while S_2 proved to be ineffective: MIs were almost the same compared with those in the presence of pooled AB-serum (S_{AB}) (Table 1). The effect of S_1 serum on the PPD-induced migration inhibition was studied in three

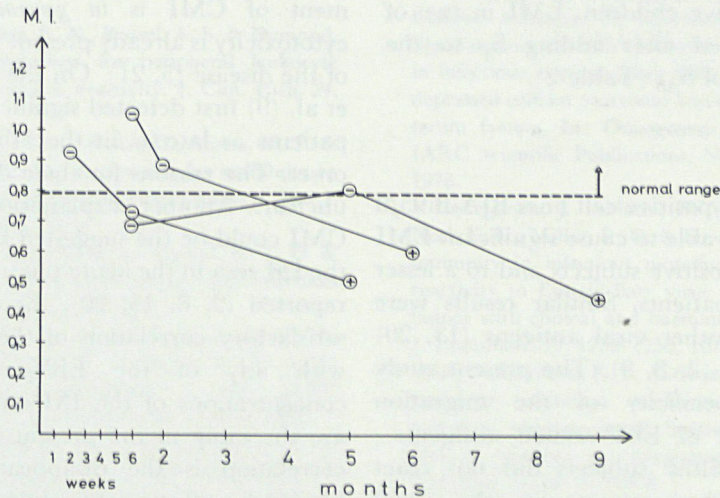


Fig. 3. Serial studies of leukocyte migration inhibition induced by 100 μ g/ml of EBV-positive antigen. Three cases were studied. The continuous lines belong to the same patients. $\ominus$, α -EBNA negativity; $\oplus$, α -EBNA positivity of patient's serum at time of examination. Signs and abbreviations as in the previous figures.

Table 1
Effect of different sera on the leukocyte migration response of healthy seropositive subjects and of patients with infectious mononucleosis, using 100 μ g/ml EBV-positive cell extracts. (Results are expressed in migration indices)

No.	Patients	S_{AB}	S_1	S_2	Blocking effect of S_1
1.	IM	0.71	0.86	0.76	+
2.	IM	0.42	0.46	0.40	-
3.	IM	0.55	0.71	0.59	+
4.	IM	0.67	0.89	0.70	+
5.	IM	0.69	0.70	0.72	-
6.	IM	0.50	0.74	0.52	+
7.	Healthy seropos.	0.39	0.55	n.t.	(+)
8.	Healthy seropos.	0.71	0.75	0.72	-
9.	Healthy seropos.	0.62	0.80	0.60	+

S_{AB} , heat-inactivated human, pooled AB-serum.
 S_1 , heat-inactivated IM serum, obtained in the acute phase of the disease.
 S_2 , heat-inactivated IM serum, obtained at the recovery.
 n.t., not tested; +, change of MI in the presence of S_1 of more than 10%; -, no effect.

Table 2

Effect of IM serum, obtained in the acute phase of the disease, on the leukocyte migration inhibition induced by 2 μ g/ml PPD. (Results are expressed in migration indices)

No.	Patients	S _{AB}	S _I	Blocking effect of S _I
1.	Healthy seropositive	0.54	0.70	+
2.	Healthy seropositive	0.62	0.60	-
3.	Healthy seropositive	0.71	0.84	+

Abbreviations and signs as in Table 1.

healthy seropositive children. LMI in two of them was blocked after adding S_I to the medium instead of S_{AB} (Table 2).

DISCUSSION

Extracts of EBV-positive cell lines BJA/B 95/8 and P3HR were able to cause significant LMI in healthy seropositive subjects and to a lesser extent in IM patients. Similar results were described with other viral antigens (13, 20) and also in IM (2, 8, 9). The present study proved the specificity of the migration inhibitory effect of EBV-related antigen(s); seronegative healthy subjects did not react with EBV-positive extracts in the usual concentrations. On the other hand, EBV-negative cell extract did not cause LMI in either group, proving that the migration inhibitory effect is closely connected with the EBV. Other antigens could not play a role since BJA/B 95/8 is an *in vitro* EBV-superinfected variant of the viral genome negative cell line, BJAB. There is no HLA-difference between the two cell lines, the only alteration is the engagement of the EBV-receptor by the virus. It is not clear yet, which one of the EBV-related antigen(s) is responsible for this specific effect. It would be worthwhile to study LMI with more or less purified antigens, e.g. purified EBNA.

The finding that LMI in IM patients developed after the acute phase of the disease is well correlated with data reported earlier (9) and those obtained with other techniques, such as *in vitro* stimulation of lymphocytes (15, 16). The cause of depressed CMI in the acute phase of IM is still obscure; although the develop-

ment of CMI is *in general* slow, specific cytotoxicity is already present in the first weeks of the disease (5, 21). On the other hand, Lai et al. (9) first detected significant LMI in two patients as late as in the 5th-6th week after onset. The reasons for these discrepancies are unclear. Another explanation of depressed CMI could be the suggested blocking effect of the IM sera in the acute phase. In the findings reported (2, 8, 15, 20), there was found no satisfactory correlation of the blocking effect with any of the EBV-related antibody concentrations of the IM sera. Our findings are the same in the present study. The only correlation is the disappearance of serum blocking effect concomitant with the appearance of α -EBNA positivity of the sera, but it could be a coincidence, since only three patients were studied serially. This question calls for further investigation. Nevertheless, the blocking effect seems to be non-specific, since in two of three cases we found an abrogation of PPD-induced migration inhibition as well.

Finally, Palit et al. (17) reported spontaneous LIF-release in the acute phase of IM. It would mean smaller migration areas in IM without using any antigen, giving a false MI, (i.e. greater than it would be in reality) and imitating a depressed CMI. In our study we have found no difference between healthy persons and patients with IM as regards the migration size without antigen (M_c). Data are not detailed. Thus it seems that the spontaneous LIF-release, measured by the sensitive two-step agarose-plate method, is not so great that it would influence the leukocyte migration inhibition detected by the less sensitive direct capillary tube technique.

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