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LEUKOCYTE MIGRATION IN AGAROSE, A STUDY ON MULTIPLE SCLEROSIS

Evidence for Qualitatively Different Responses to Intact Encephalitogenic Protein and to Peptides Derived from it

By

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Recent studies by Berg et al. (2) and Bergstrand et al. (9) demonstrated that basic encephalitogenic protein (EP)* or parts of it could influence the migration of leukocytes from patients with multiple sclerosis (MS) or some other neurological diseases that damage the nervous system. Clausen's (13) direct leukocyte migration in agarose technique was used as a test system. This is often regarded as an *in vitro* method for detecting immunologically competent cells sensitized to the studied antigen. We used concentrations of EP between 10 and 250 $\mu\text{g/ml}$ or equimolar concentrations of peptides derived from EP. This range is suitable in animal studies on cell mediated immunity *in vitro* using the macrophage migration inhibition assay (5) or the lymph node cell transformation technique (7).

Another study (10) showed that PPD could influence the

* Unusual abbreviations used in this paper: ALS = amyotrophic lateral sclerosis. EAE = experimental autoimmune (allergic) encephalomyelitis. EP = basic encephalitogenic protein of bovine origin. Gp-EP = basic encephalitogenic protein of guinea-pig origin. LEF = leukocyte migration enhancement factor. LIF = leukocyte migration inhibition factor. MIF = macrophage migration inhibition factor. PPD = purified protein derivative.

leukocyte migration at concentrations much below those normally used. PPD is generally added at a concentration of $5 \mu\text{g/ml}$ or more. We found definite effects in some cases with concentrations of about $0.01 \mu\text{g/ml}$. We therefore extended our study of the reactivity of human leukocytes to EP to a concentration range below that previously used, in order to find out whether reactions do occur; and, if so, whether patients with MS behave differently in this respect from healthy subjects or patients with other neurological diseases.

MATERIAL AND METHODS

Blood Donors

Heparinized blood was drawn from 13 healthy subjects, none of whom had been exposed to EP during laboratory work; 12 patients with MS; and 15 patients with other neurological diseases involving neural destruction—of these, four had amyotrophic lateral sclerosis (ALS), four had intracranial tumours (two gliomas, two meningiomas), and seven had had a cerebral infarction 2–3 weeks earlier. None were being treated with immunosuppressive drugs.

Antigen Preparations

EP was prepared from bovine brain substance, as described by Bergstrand (3). Descriptions of the preparation of peptides are also available (3, 4). The numbering of the amino acid residues has been changed according to the revised primary sequence (12).

Direct Agarose Migration Technique

We used the technique described by Clausen (13), as performed in our previous studies (9). Briefly, leukocytes were separated from heparinized blood by sedimentation with dextran, and small aliquots were incubated for 30 min with different antigen solutions or—as controls—with saline used as dilutant. After incubation, the cell material from each incubation tube was distributed among six wells punched out in six different agarose plates. The distribution was randomized, as previously described (9). Each test comprised two controls and eight incubations with EP at the following concentration: 0.03, 0.1, 0.3, 1, 3, 10, 50, and $250 \mu\text{g/ml}$. Four peptides derived from EP were also tested at concentrations equimolar with EP, $0.3 \mu\text{g/ml}$: peptide 1–42, peptide 43–88, peptide 89–169, and peptide HNB-89–169, where HNB marks a chemical blocking of the tryptophan residue with 2-hydroxy-5-nitrobenzyl bromide. One aliquot of cells was incubated with PPD at $50 \mu\text{g/ml}$ (PPD obtained from Statens Seruminstitut, Copenhagen), the object being to obtain a positive control. As a rule, leukocyte migration was markedly inhibited with PPD.

Indirect Agarose Migration Technique

In some experiments, an indirect method was used, a modification of Clausen's (14) two-step method. Leukocytes from patients with MS were incubated in a medium composed of 30 per cent heat-inactivated normal human serum in Parker 199 (SBL, Stockholm) for 4 hours at 37° C in the presence or absence of EP. Three concentrations of EP were used: 0.1, 1, and 10 $\mu\text{g/ml}$. Controls were prepared with only saline added. After the end of the incubation period, the cells were washed carefully three times by centrifugation, each time with 10 ml of prewarmed Parker 199. The cells were then suspended in a medium composed of 20 per cent heat-inactivated horse serum in Parker 199, incubation being continued for 4 days. The cells were then spun down, and the supernatant was re-centrifuged for 10 min at $1000 \times g$ and stored frozen. The supernatants were tested with leukocytes from healthy subjects as follows: cells from one subject were distributed in four aliquots, each suspended in one supernatant (one control, three from tubes where EP treatment had been given at three different concentrations). The cells were incubated for 30 min at a cell density of 2.2×10^5 cells/ml, and were then treated as in the direct method.

RESULTS

Direct Technique—Mean Effect of EP

Fig. 1 shows the mean migration index at different EP concentrations for the three groups studied: healthy subjects, patients with MS, and patients with other neurological diseases. At high concentrations of EP, the mean migration index for all three groups tends to approach the value 100, suggesting non-reactivity to EP. However, this is not so, as cells from patients (with MS or with other neurological diseases) often deviate from normal reactivity, either showing a stimulated or an inhibited migration (2, 9).

All three groups of subjects show a stimulation of migration with a maximum effect at 0.1 and 0.3 $\mu\text{g/ml}$ of EP. The reaction tends to be stronger in the group with MS than in the other groups. The mean degree of stimulation amounts to only 7 to 13 per cent. As stimulation can be seen in all three groups, a non-specific effect of the low amount of basic protein added can be suspected. We therefore tried to discover whether the enhancement was due to the mere presence of EP in the incubation medium at the performance of the migration test, or

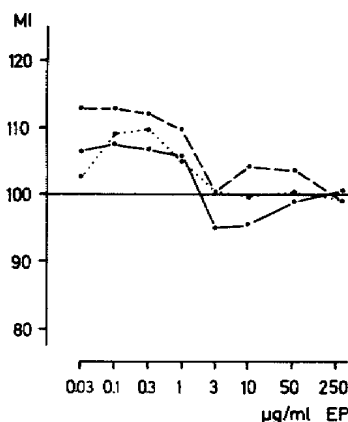


Fig. 1.

Mean migration index (MI) in direct technique and at different EP concentrations. Leukocytes from healthy subjects —, from patients with MS ----, and from patients with other neurological diseases

whether it was due to soluble products appearing after the exposure of the cells to EP. The indirect migration method was used to study the reactivity of cells from nine patients with MS.

Indirect Technique

Fig. 2 shows the results of these nine tests. At 0.1 µg/ml of EP, no stimulation was seen in three experiments, a moderate enhancement in three (110–120 per cent), and a marked enhancement in three (120–130 per cent). The percentages are based on the means of two separate experiments using cells from two different healthy donors.

The mean migration index at 0.1 µg/ml of EP was 112.5 ± 4.4 (S.E.M.), which differs significantly (Student's *t*-test) from 100 ($t = 2.85$ at 8 d.f., $P = 0.02$). EP at 1 µg/ml stimulated in a few experiments, but the mean migration index was 106.8 ± 3.9 ; thus not significantly different from 100. EP at 10 µg/ml gave an enhancement in two experiments and

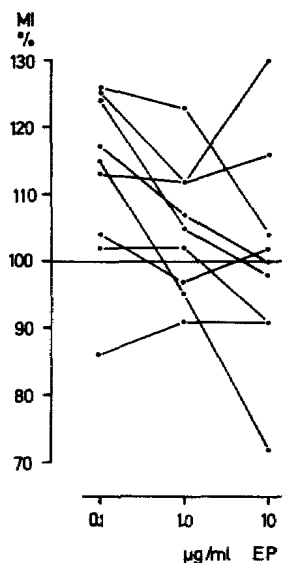


Fig. 2.

Migration index (MI) in experiments performed with the indirect technique and cells from healthy subjects as marker cells. Supernatants from cultures of leukocytes from patients with MS. Cultures stimulated at three different EP concentrations; MI-values from each of nine experiments combined with lines.

a marked inhibition in one: the mean migration index was almost exactly 100.

These results suggest that the enhancement registered with low EP concentrations in the direct technique is also due to the production of soluble factors that affect leukocyte migration, and not to a direct, non-specific effect of EP on the locomotion of the latter cells.

Direct Technique—Statistical Treatment of Data

As we discussed earlier (2, 9, 10), there are two main sources of technical variation in the direct technique: that arising from variation between different wells in the agar plates,

TABLE 1

Variances around Regression Lines $Y = b \cdot X$, where Y is the migrated area (mean of 6 determinations) in the presence of EP at a certain concentration, and X is the mean area from two such sets of migrations in the absence of antigen (controls). The regression coefficients (b) are tabulated, and F -tests are performed between the recorded variances and the expected variance calculated from double determinations of control incubation ($100 (\frac{1}{2} + 1) = 150$). Tests performed with blood from healthy subjects.

Concentration of EP, $\mu\text{g/ml}$	Recorded variance	$F_{12;40}$	P	b
0.03	1327.5	8.85	0.001	1.07
0.1	472.3	3.15	0.01-0.001	1.12
0.3	245.4	1.64	0.2-0.05	1.10
1	125.2	< 1	—	1.08
3	133.4	< 1	—	0.97
10	232.8	1.18	> 0.2	0.98
50	232.8	1.55	0.2-0.05	1.00
250	168.0	1.12	> 0.2	1.01

filled with material from one incubation tube; and that arising from differences between different incubation tubes containing the same antigen solution. The latter variation has been shown to be significantly larger than the former, representing the main technical variance. Possible differences between the results of migration after the incubation with different antigens and without antigen should, therefore, be evaluated against that "error" variance. It can be estimated from 40 pairs of identically performed control incubations, and is found to be almost exactly 100. The standard deviation thus amounts to 10 units. If cells from all healthy subjects were similarly affected at a certain concentration, the observed migration areas should disperse around the expected value in a degree compatible with the described error variance. Table 1 compares the actual findings. It can be seen that, at least at concentrations of 0.03 and 0.1 $\mu\text{g/ml}$, there is a significantly higher dispersion. This finding also argues against a direct, non-specific, stimulating effect of EP, as it means that different subjects

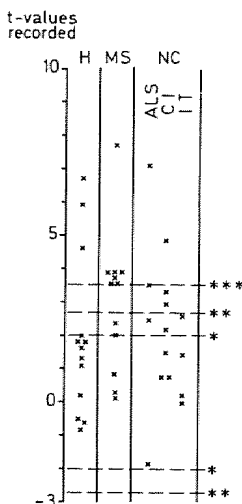


Fig. 3.

Recorded t-values at comparisons between migration with EP 0.1 and 0.3 $\mu\text{g/ml}$ and with controls (cf. text) H = healthy subjects, MS = patients with MS, NC = patients with other neurological diseases. ALS = amyotrophic lateral sclerosis. CI = cerebral infarction. IT = intracranial tumour. Limits at different

P-values: * $P = 0.05$, ** $P = 0.01$, *** $P = 0.001$.

react in different ways to the antigen at these low concentrations.

The hypothesis that certain subjects react to EP and others do not was then tested. The difference between the mean migration area recorded with 0.1 and 0.3 $\mu\text{g/ml}$ of EP was compared with the mean control area for each subject. These differences should distribute with an error of $10 \sqrt{1/2 + 1/2} = 10$ units, and t-tests were performed with 10 as standard deviation, based on 40 d.f. Fig. 3 presents the results. Of 13 healthy subjects, three showed highly significant stimulations, and one just reached the 95 per cent limit; of 12 patients with MS, seven showed a highly significant and two a possibly significant stimulation; of 15 patients with other neurological dis-

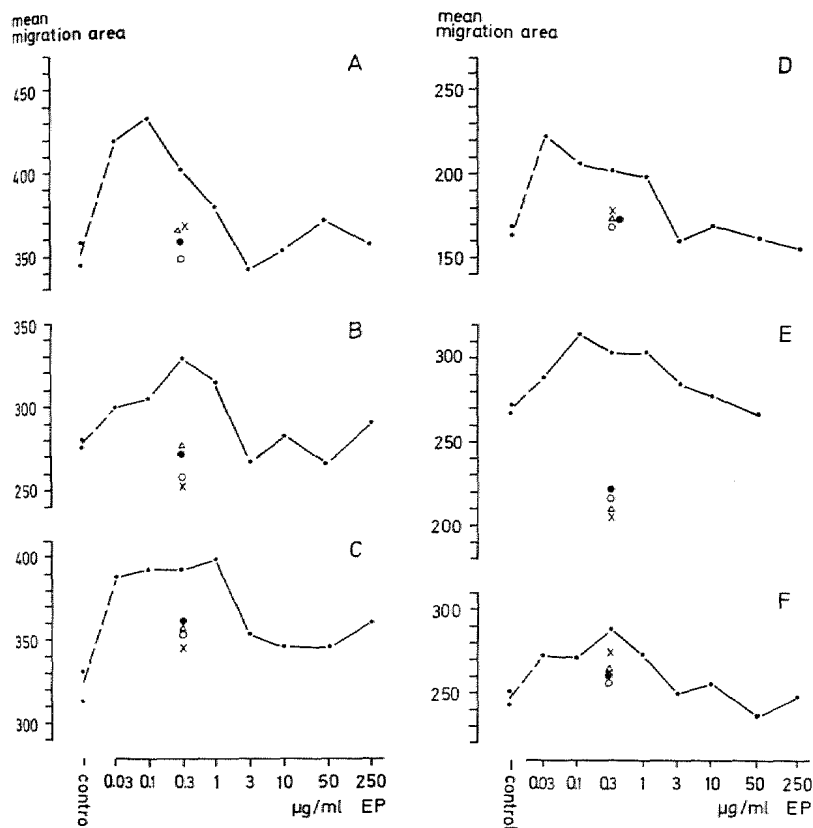


Fig. 4.

Recorded mean migration areas for leukocytes from six subjects tested with the direct technique and at different concentrations of EP (line) or peptides at concentrations equimolar with EP 0.3 µg/ml. Δ = peptide 1-42, $\times$ = peptide 43-88, $\circ$ = peptide 89-169, $\bullet$ = peptide HNB-89-169.—Healthy subjects in A and E, patients with MS in B and D, patients with ALS in C, patient with cerebral infarction in F.

eases, significant stimulations were seen in five, and possibly significant stimulations in three. If mean t -values with errors were calculated for those not reaching the 95 per cent limit, the following means were obtained: healthy subjects—mean $t = 0.68 \pm 0.36$; patients with MS—mean $t = 0.45 \pm 0.20$; patients with other neurological diseases—mean $t = 0.40 \pm 0.23$. A slight stimulation might have occurred also in these tests, but it could be random.

Thus it is obvious that EP markedly stimulates the migration of leukocytes in tests from certain subjects. This seems to be more common in patients with MS or other neurological diseases, but it occurs also in healthy subjects (3/13). Fig. 4 A–F illustrates six cases with marked reactions to EP in this concentration range.

Effect of Peptides

Fig. 4 illustrates the reactions seen with peptides derived from EP. The peptides were tested at a concentration equimolar with $0.3 \mu\text{g/ml}$ of EP. In two experiments shown in this figure, no clear-cut reaction was seen with any of the peptides (Fig. 4 A and D). In two experiments, all peptides or some of them inhibited leukocyte migration (Fig. 4 B and E). In two experiments, some of the peptides enhanced migration (Fig. 4 C and F).

The effect of each peptide was tested with t -tests with the hypothesis that the difference from the mean control area was zero. As only one set of migrations was made with each peptide, the error of the difference will be $10 \sqrt{1/2 + 1} = 12.2$ units. Fig. 5 shows the t -values recorded for the four peptides and the three categories of subjects studied. Only tests where the cells had reacted to EP at $0.3 \mu\text{g/ml}$ are included ($P < 0.05$), i.e. 20 tests. As four peptides were studied at each test 80 t -tests were performed. At least four should reach the 95 per cent limits—17 did. At the most, a few tests should reach the 99 per cent limits—11 did.

Of these tests, an enhancement occurred in three (two with

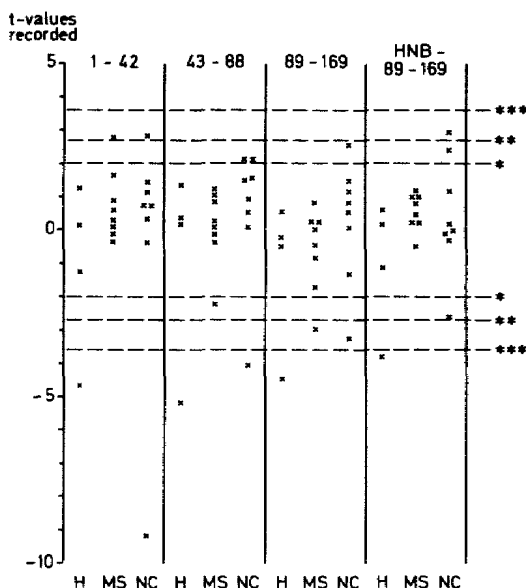


Fig. 5.

Recorded t-values at comparisons between migration with peptides 1-42, 43-88, 89-169, or HNB-89-169 and controls, direct technique. Designations as in Fig. 3. Only those tests were included in which the leukocytes were significantly stimulated by EP at 0.1-0.3 $\mu\text{g}/\text{ml}$.

peptide 1-42, one with peptide HNB-89-169), and an inhibition in eight (two with peptide 1-42, two with peptide 43-88, three with peptide 89-169, and one with peptide HNB-89-169). Six peptides reached $P = 0.001$ levels—all were inhibitions (two with peptide 1-42, two with peptide 43-88, and one each with peptides 89-169 and HNB-89-169).

In a further 14 tests with cells from other subjects, EP showed no significant effect on leukocyte migration at 0.3 $\mu\text{g}/\text{ml}$. In one, all peptides showed definite inhibition (Fig. 6 A); an inhibition was also marked with EP at higher concentrations. We have seen a similar response pattern in other cases, not included in the present study. In the second case (Fig. 6 B),

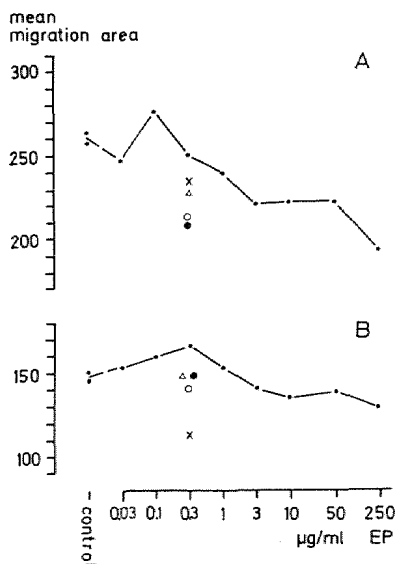


Fig. 6.

Recorded mean migration areas from leukocytes from a patient with MS (A) and a patient with a cerebral infarction (B), tested with the direct technique and at different concentrations of EP (line) or peptides. For designations, see Fig. 4.

only peptide 43–88 gave a marked inhibition of migration. We regard this as artefactual (c.f. discussion in 9). In the remaining 12 tests, no reactions to peptides were seen.

HL-A Type and Responsitivity to EP

Table 2 compares the reactivity for EP at 0.1–0.3 µg/ml with the HL-A type of the donor of cells. Only the distribution of four HL-A characters is given: 3, 7, W10, and 27. The three former HL-A types have been shown to have a positive or a negative correlation with MS (c.f. 1, 11, 18). HL-A 27 is strongly associated with certain arthropathies, notably pelvispondylitis. This material comprises only 12 patients with MS and 12 healthy subjects. Of the latter, the three strongly reac-

TABLE 2

Summary of Distribution of HL-A 3, 7, W10, and 27 in Subjects who did ($P < 0.001$) or did not ($P > 0.01$) react to 0.1–0.3 $\mu\text{g/ml}$ of EP (three Healthy Subjects and eight Patients with MS) or showed a Reaction to Peptides at this Concentrations but not to EP (one Patient with MS).

Group	P value	No. of subjects	HL-A type							
			7+	7-	3+	3-	W10+	W10-	27+	27-
Healthy	>0.01	9	4*	5	1	8	3	6	0	9
	<0.001	3	0	3	0	3	1	2	2	1
MS patient	>0.01	4	1	3§	1	3	1	3	0	4
	<0.001	8	4	4	4	4	0	8	0	8

* one had $0.05 > P > 0.01$.

§ two had $0.05 > P > 0.01$.

tive subjects were all HL-A 7 negative; none carried HL-A 3; one had W10; and the other two had HL-A 27. Of the 12 patients with MS, the eight strongly reactive patients were equally divided between HL-A 7 positive and negative. Five MS patients had HL-A 3, and four of them reacted to EP. One non-reactor was W10. No MS patient had HL-A 27.

DISCUSSION

Lymphocytes sensitized to a specific antigen, and treated *in vitro* with that antigen, release factors that affect the migration *in vitro* of macrophages and polymorphonuclear leukocytes. In most experimental systems, these factors inhibit migration, but sometimes enhancements have also been described, especially when human leukocytes were used as marker cells, either in the capillary tube technique or the agarose technique. The existence of two different factors has been demonstrated (22), one enhancing (LEF) and the other inhibiting (LIF) leukocyte migration. LIF differs from MIF, a factor that inhibits the migration of guinea-pig macrophages (20). The quoted studies thus demonstrate that a number of soluble factors affecting cell migration are released after antigen stim-

ulation of lymphocytes, and the proportions of these factors might determine the net result in the migration test (c.f. 15).

Differences might also exist in the mechanisms which determine the release of inhibiting and enhancing factors. If the effects on leukocyte migration in our experiments are due to such soluble factors, some of our data suggest that the nature of the antigen used is of importance. In most of our experiments (20/34), EP at a concentration of 0.3 $\mu\text{g}/\text{ml}$ gave a significant migration stimulation; but in the same experiments, one or more of the peptides tested at equimolar concentrations could give a significant inhibition of migration. The interpretation of this observation could be that EP induced the release of migration enhancement factors which could overwhelm the inhibiting factors also released. Peptides, on the other hand, could not stimulate the lymphocytes to produce or release enough enhancement factors; therefore, the inhibiting effect dominated.

A temporal difference in the production of LIF and LEF was previously demonstrated (22), and it has been suggested that migration enhancement is the effect of a weaker antigenic stimulation than is migration inhibition (21). Our results suggest that the nature of the antigen is also of importance. Possibly, the higher antigenic valency of the native protein, compared with that of the peptides derived from it, can be of significance. The internal cross-reactivity described in EP (8, 16) can give the protein the characteristics of an antigen composed of repeating units, which can also be of importance. However, the general validity of the present observation must be tested with other suitable antigens and antigen fragments before accepting the concept of different mechanisms for the production or release of enhancing and inhibiting factors.

Previous papers (2, 9) showed that leukocytes from patients with MS or with some other diseases reacted to EP at 10 to 250 $\mu\text{g}/\text{ml}$ in a different way than leukocytes from healthy subjects. The low-dose effect discussed in the present paper was seen also with cells from some healthy subjects, but more often with cells from patients with MS or other neuro-

logical diseases involving neural destruction. There are several possible explanations for the very strong reactions seen with cells from three apparently completely healthy subjects. Evidence for a cross-reactivity between part of EP and PPD has been presented with the macrophage migration inhibition test (6); and a cross-reactivity between part of EP and measles antigens or PPD has been demonstrated with the macrophage electrophoretic mobility test (c.f. 17). The last-mentioned antigens are omnipresent, and a cross-reactivity with EP would perhaps better explain an effect seen in most healthy subjects. But again the uncertainty caused by the possible balance between enhancing and inhibiting factors makes a great variability possible.

Another possibility is that some individuals carry certain immune response genes (Ir-genes) which facilitate an immunological reaction with EP. If such genes are somehow related to MS, as has been suggested by studies of serologically and lymphocyte-defined genes localized to the same chromosome region as the Ir-genes (c.f. reviews in 18, 18a), the higher incidence of positive reactions in patients with MS could be explained. This hypothesis finds some support in a recent study by McFarlin et al. (19). They demonstrate that Lewis rats—susceptible to experimental autoimmune encephalomyelitis (EAE)—also develop skin reactivity to guinea-pig basic encephalitogenic protein (Gp-EP) after challenge with Gp-EP, and that *in vitro* their cells react to Gp-EP. Whereas Brown Norway rats—resistant to EAE—fail to develop skin reactivity or *in vitro* responsitivity to Gp-EP when challenged with Gp-EP. Both rat strains produce antibodies against Gp-EP, but the antibody titre is much higher in the Lewis strain than in the Brown Norway strain. These observations suggest that the immune response gene necessary for the development of EAE also controls T-cell response against Gp-EP.

In an effort to obtain an indication of a similar correlation between the presence of specific HL-A genes—suspected of being closely coupled to Ir-genes—and reactivity to EP, the

distribution of HL-A 3 and 7 was compared between subjects who did react with EP and those that did not react or reacted only poorly. These two HL-A specificities were chosen, as they have been shown to be over-represented in MS (c.f. 1, 18). It has also been claimed that W10 is under-represented in MS (11). Our material is too small to permit firm conclusions, but it is suggestive that none of the three healthy subjects who showed strong reactivity to EP carried HL-A 3 or 7; and that one of the four MS patients who did not show significant reactions to EP carried both 3 and 7. There are thus no indications that these two specificities are positively correlated to reactivity to EP in the test system used. Interestingly enough, two of the three healthy subjects who showed strong reactivity with EP had HL-A 27, a characteristic present in less than 10 per cent of Caucasians and which has been associated with some other diseases, notably ankylosing spondylitis.

SUMMARY

The effect of low concentrations of bovine encephalitogenic protein on the migration of human peripheral leukocytes in agarose was studied. A concentration of 0.3 $\mu\text{g/ml}$ of the protein stimulated the migration of cells from many donors, including some healthy subjects. An indirect technique suggested that the migration enhancement is due to the production of soluble factor, possibly corresponding to the leukocyte migration enhancement factor described by others. The frequency of subjects whose cells could be stimulated and the recorded degree of stimulation tended to be higher in a group of patients with multiple sclerosis than in a group of healthy subjects. When the effect of some of the main peptide fragments of the protein was studied on cells that were stimulated by the intact protein, one or more of these peptides sometimes induced the opposite effect: a migration inhibition. There is, apparently, a complex balance between enhancing and inhibiting factors acting on leukocyte migration *in vitro*; and the character of the antigen seems to be one important factor.

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