

Effect of Encephalitogenic Protein, PPD, and Tetanus Toxoid on Leukocyte Migration in Agarose

A Study of "Cross-Reactivity"

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The reactivity to three antigens: bovine encephalitogenic protein (EP), PPD, and tetanus toxoid, was studied with blood leukocytes from healthy humans using Clausen's (5) leukocyte migration in agarose technique. There is an obvious correlation between the reactivity to EP (all concentrations studied) and to low concentrations of PPD; and between the reactivity to low concentrations of EP and low concentrations of tetanus toxoid. After vaccination with tetanus vaccine, a marked increase in reactivity to the toxoid sometimes occurred; at the same time, a marked reactivity to EP appeared. Various explanations are discussed: a true immunological cross-reactivity, that the correlations are due to a variability in individual response with respect to lymphokine production, and that BCG and tetanus vaccinations produce an adjuvant effect increasing a pre-existing low reactivity to EP.

Key words: cell migration inhibition; cross reactions; encephalitogenic basic protein; immunity cellular; tetanus toxoid; tuberculin.

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An immunological reactivity to basic encephalitogenic protein (EP) of human or bovine origin has been described in patients with multiple sclerosis (cf. literature review by Källén *et al.*, (6)). Sometimes, these reactions have been described also when cells from healthy subjects were used. We studied this problem with the aid of Clausen's (5) leukocyte migration in agarose technique and demonstrated that cells from patients with multiple sclerosis showed on average stronger reactivity than did cells from healthy subjects, although a definite reaction was sometimes found also with the latter, perhaps most pronounced at relatively low antigen concentrations at the test (4, 6). Various explanations of this finding can be suggested. One is that reactivity is the result

of previous minor nervous system lesions: it has been amply demonstrated that after such major lesions a strong reactivity to EP can develop (7). Another possibility is that the observed reactivity to EP represents a cross-reactivity to some other common antigen, e.g., PPD. We investigated this possibility further in the present study.

MATERIAL AND METHODS

Heparinized venous blood was drawn from 37 healthy subjects. Cells from 20 of them were used to study, with the agarose technique, reactivity to EP and PPD, both antigens tested in a range of concentrations. Cells from 17 subjects were used for an analogous study of EP and tetanus toxoid.

Seven of the latter blood donors were vaccinated with tetanus toxoid vaccine after the performance of the test, and a second test was made 2–3 weeks later; in one instance also after 5 weeks.

EP was prepared as described by Bergstrand (1) and was a gift from Dr Bergstrand. PPD was obtained from Statens Seruminstitut (Copenhagen), and the tetanus toxoid (with merthiolate as a preservative) and the tetanus vaccine (without diphtheroid toxoid and adsorbed to aluminium phosphate) were obtained from SBL (Stockholm).

The agarose migration test was performed as described by Clausen (5) and Bergstrand et al. (3). Leukocytes were separated with dextran separation, and the cells were incubated with the various antigens for 30 min. They were then distributed in wells punched in six agar plates. Each cell sample was studied in up to 14 parallel incubations: two of them were made without antigen added (controls, only saline added), in the remaining wells were put cells treated with EP at one of three concentrations (0.1, 50, or 200 $\mu\text{g/ml}$) or with PPD at one of nine concentrations (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, or 100 $\mu\text{g/ml}$); or alternatively, tetanus toxoid at one of four concentrations (0.01, 0.1, 1, or 10 $\mu\text{g/ml}$). All antigen solutions were prepared in saline, but the tetanus toxoid solution contained merthiolate as preservative. The concentration of merthiolate had no effect on cellular migration, which was demonstrated with two wells containing cells treated with a merthiolate solution of the same concentration as that used when diluting the tetanus toxoid. In one experiment, EP 50 $\mu\text{g/ml}$ had to be excluded; in five, PPD 0.03, 0.3, 3, and 30 $\mu\text{g/ml}$ were excluded; in a further two, only PPD 3 and 30 $\mu\text{g/ml}$ were excluded. These exclusions were necessary because of scarcity of cells. For one subject, the value of tetanus toxoid 0.1 $\mu\text{g/ml}$ had to be excluded owing to a technical mishap.

The area of leukocyte migration was measured after approx. 20 h and was

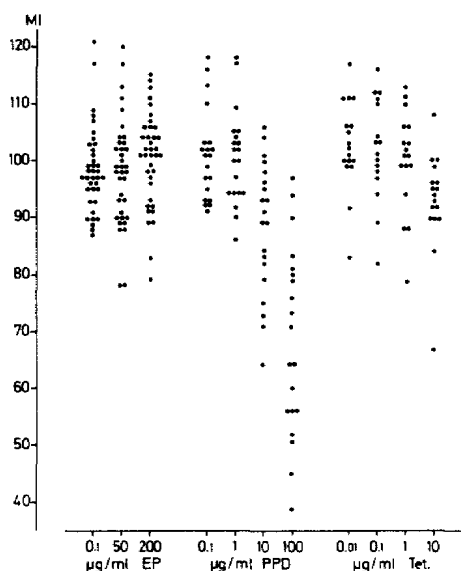


Fig. 1. Migration indices (MI) with three antigens: bovine encephalitogenic protein (EP), PPD, and tetanus toxoid (Tet.) at different antigen concentrations.

expressed in arbitrary units (one unit = 0.08 mm^2). For a discussion on the statistical treatment of this type of data see (3, 4).

RESULTS

Fig. 1 shows the recorded migration indices ($\text{MI} = \text{area of migrated cells after antigen treatment} / \text{area of migrated cells after saline treatment} \times 100$) for the various antigen concentrations. EP had no marked effect on cellular migration but in some experiments, values exceeding the 90–110 interval are seen. This interval can be regarded as the rough confidence interval for non-reactivity (to some extent varying with the absolute size of the control migrations). Some MI values even lie outside the 80–120 interval. Migration stimulations and inhibitions occurred.

Some subjects' cells react with stimulations also to low concentrations of PPD, but almost all react with a marked inhibition at high PPD concentrations.

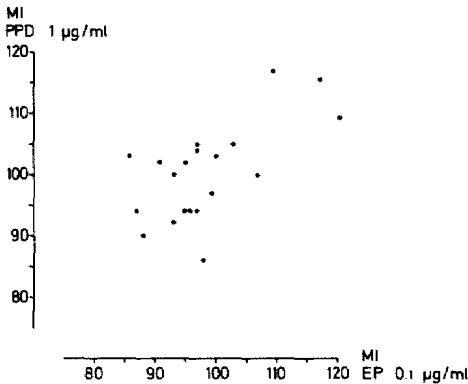


Fig. 2. Correlation between MI registered with PPD 1 µg/ml and EP 0.1 µg/ml.

The diagram also shows a definite reactivity to tetanus toxoid in some of the 17 subjects before re-vaccination. The low antigen range gives a stimulation in some and an inhibition in others, the highest antigen concentration in most instances gives a tendency to migration inhibition.

The MI values recorded for the 20 subjects studied with EP 0.1 µg/ml and PPD 1 µg/ml

are plotted against each other in Fig. 2. There is a definite indication of a positive correlation between the two sets of data. Correlations so studied can be considerably distorted by the same control values being used for the calculation of both sets of MI data. Instead, a statistical analysis was made using the absolute migrated areas, expressed in units, and using (mean of) controls (X_1), EP-treated cells (X_2), and PPD or tetanus toxoid treated cells (X_3). All possible concentrations of EP and the other two antigens were then studied with a partial correlation analysis. In this way, the possible correlation between X_2 and X_3 can be investigated, eliminating the effect of X_1 . The correlation coefficient obtained (r) can then be tested with t -tests for the hypothesis $r=0$ at $(n-3)$ d.f., where n is the number of experiments.

Table 1 presents the r values obtained and indicates their statistical significance. Fig. 3 plots the relation between the r values and the concentration of EP, PPD, and tetanus toxoid. There is an obvious positive correlation between all three EP concentrations and the low PPD concentrations, most marked

Table 1

Partial correlation coefficients (r) between migrated areas in the presence of encephalitogenic protein (EP) and PPD or tetanus toxoid (Tet.) at various concentrations.

Antigen concentration, $\mu\text{g/ml}$		0.1 $\mu\text{g/ml}$ EP			50 $\mu\text{g/ml}$ EP			200 $\mu\text{g/ml}$ EP		
		r	t	d.f.	r	t	d.f.	r	t	d.f.
PPD	0.01	0.63	3.3**	17	0.29	1.2	16	0.51	2.4*	17
	0.03	0.83	5.1***	12	0.37	1.4	12	0.42	1.6	12
	0.1	0.69	3.9**	17	0.22	0.9	16	0.43	2.0	17
	0.3	0.74	3.8**	12	0.49	2.0	12	0.74	3.8**	12
	1	0.81	5.7***	17	0.34	1.4	16	0.53	2.6*	17
	3	0.55	2.1	10	0.68	2.9	10	0.71	3.2**	10
	10	-0.13	0.5	17	-0.13	0.5	16	-0.17	0.7	17
	30	-0.28	0.9	10	-0.34	1.2	10	-0.48	1.7	10
100	-0.25	1.1	17	-0.30	1.3	16	-0.34	1.5	17	
Tet.	0.01	0.47	2.0	14	0.35	1.4	14	0.02	0.1	14
	0.1	0.73	3.8**	13	0.34	1.3	13	-0.21	0.8	13
	1	-0.03	0.1	14	0.19	0.7	14	-0.22	0.9	14
	10	0.24	0.9	14	0.32	1.3	14	-0.24	0.8	14

t -tests for $r=0$ with statistical significance: * $0.05 > P > 0.01$, ** $0.01 > P > 0.001$, *** $P < 0.001$.

d.f. = degrees of freedom.

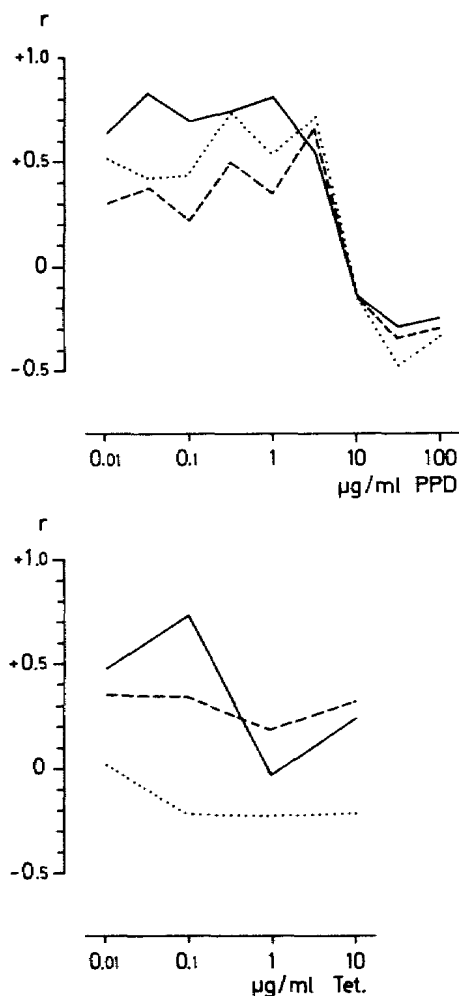


Fig. 3. Partial correlation coefficient (r) between migration areas in the presence of PPD and EP (upper graph) or tetanus toxoid (Tet.) and EP (lower graph) at various antigen concentrations. Whole-line gives values for EP 0.1 $\mu\text{g/ml}$; dashed line, values for EP 50 $\mu\text{g/ml}$; dotted line, values for EP 200 $\mu\text{g/ml}$.

for EP 0.1 $\mu\text{g/ml}$. At high PPD concentrations, an indication of a negative correlation is seen but does not reach statistical significance. A significant correlation between tetanus toxoid and EP is found only at tetanus toxoid concentration 0.1 $\mu\text{g/ml}$

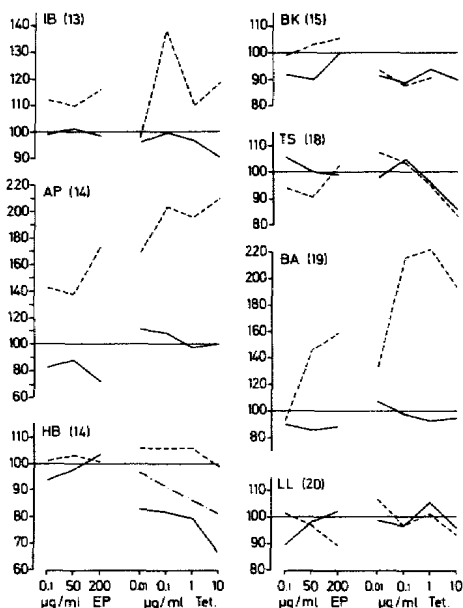


Fig. 4. Graphs showing M1 values for EP and tetanus toxoid (Tet.) at various concentrations in seven subjects. Whole-line graphs give pre-vaccination values and dashed graphs post-vaccination values (figure after initials gives number of days elapsed from vaccination with tetanus toxoid). For H.B., a dashed-and-dotted graph gives values a further 3 weeks later. Note increase in reactivity for both EP and tetanus toxoid in at least three graphs: I.B., A.P., and B.A.

and EP 0.1 $\mu\text{g/ml}$, but is possible also at that EP concentration and tetanus toxoid 0.01 $\mu\text{g/ml}$. The graph for EP 50 $\mu\text{g/ml}$ never reaches statistical significance and the graph for EP 200 $\mu\text{g/ml}$ indicates no correlation whatever.

Seven subjects were vaccinated with tetanus toxoid after the first *in vitro* test had been performed and were then re-tested 13–20 days later with tetanus toxoid and EP. Fig. 4 shows the results, expressed as M1. In three of the subjects (I.B., A.P., B.A.) who did not positively react to the toxoid before vaccination, strong reactivity, as stimulations of the migration, developed after vaccination. In the pre-vaccination tests, all three showed no or only slight inhibition with

EP, but in the post-vaccination test, marked reactivity to EP had developed.

A further three subjects (B.K., T.S., L.L.) showed no marked changes after the vaccination in the reactivity to the toxoid or to EP. The seventh subject (H.B.), when vaccinated, definitely reacted to the toxoid. This had nearly disappeared when he was re-tested 14 days later. As the pre-vaccination test gave a reaction of migration inhibition, this result indicates that the vaccination induced a stimulative effect. A third test 5 weeks after vaccination showed a return to an inhibition.

DISCUSSION

Leukocytes from apparently healthy subjects sometimes react to EP in various *in vitro* tests. Yung et al. (12) directly demonstrated lymphocytes with specificity to EP in the circulation of normal guinea pigs and healthy humans. EP reactivity can also be demonstrated with the leukocyte in agarose technique used in the present study.

There is a marked positive correlation between reactivity to EP and to low PPD concentrations, in both cases apparent as migration stimulations. There is a positive correlation in a similar way between a low EP and a low tetanus toxoid concentration. Furthermore, when subjects are vaccinated with tetanus toxoid, a marked increase in reactivity *in vitro* to the toxoid sometimes occurs, and simultaneously, an increased reaction to EP can be seen — both apparent as stimulations. Various explanations of these observations can be discussed.

One possibility is a true cross-reactivity between the antigens involved. This is perhaps especially relevant for EP and PPD. Many observations in the literature suggest that this might exist. McDermott et al. (9) claimed that they could filter out lymphocytes reactive to EP with a Bio-Gel column loaded with PPD: they tested the cells in the macrophage electrophoretic mobility test. Bergstrand & Källén (2) showed that peritoneal macrophages from

guinea pigs injected with Freund's complete adjuvant (which gives a strong immune reaction to PPD) can be enhanced in their migration by a part of EP (peptide 1-42), and certain antigenic sites responsible for the cross-reactivity could be identified. Vandembark et al. (11) reported dermal reactivity to EP in guinea pigs injected with Freund's complete adjuvant only, and these animals can even develop slight brain lesions of the type characteristic for experimental autoimmune encephalomyelitis, the usual sequela to immunization with EP (8). Less evidence exists for a possible cross-reactivity between tetanus toxoid and EP. It is true that rare neurological complications to tetanus toxoid vaccination have been described (10) but their pathogenesis is obscure.

A second possible explanation of the correlations is that there are individual differences in the capacity to produce lymphokines after antigenic stimulation. The result of a leukocyte migration test is determined by two opposing lymphokines: an enhancing factor (LEF), and an inhibiting factor (LIF). Some subjects might be unusually good LEF producers. When their cells are stimulated with low concentrations of PPD, with tetanus toxoid, or with EP, migration enhancement will occur and a correlation will be obtained in the absence of true cross-reactivity but based on individual variations in *type* of test response. Against this explanation argue the much stronger correlations seen between low PPD and high EP concentrations than between low tetanus toxoid and high EP concentrations, whereas correlations with low EP concentrations are similar for PPD and tetanus toxoid. An unspecific mechanism of the type described is hard to reconcile with this observation.

A third possibility is that BCG and tetanus toxoid vaccinations have resulted in an adjuvant effect, increasing a pre-existing weak cellular immunity to EP. According to this hypothesis, a healthy individual would carry a clone of cells reactive to EP. Under certain conditions, e.g., after vaccination with tetanus toxoid or with BCG, this clone

will expand and cause a reactivity in the test. The strongest support of this possibility is obtained from the post-vaccination data with tetanus toxoid, but they can, of course, also be explained by a true cross-reactivity.

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