Clin. exp. Immunol. (1972) **11**, 535–541.

EVALUATION OF THE LEUCOCYTE MIGRATION TEST AS A MEASURE OF DELAYED HYPERSENSITIVITY IN MAN

SUPPRESSION OF MIGRATION INHIBITION BY PUROMYCIN

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(*Received* 25 *January* 1972)

SUMMARY

The response of peripheral blood leucocytes to PPD was studied *in vitro* by means of the leucocyte migration test, using Mantoux-positive and -negative subjects. The migration of leucocytes from 83% of the positive subjects was significantly inhibited when a concentration of 100 μg PPD/ml was used in the chambers. Leucocytes from the majority of these subjects were also inhibited at a lower concentration of 50 μg PPD/ml and one strongly positive subject showed inhibition at all concentrations tested, down to 1 μg PPD/ml. Stimulation of migration was not observed with leucocytes from any of the positive subjects even when the lowest concentration of PPD was used. Inhibition of migration with PPD was prevented by the addition of puromycin, suggesting that the response is dependent on protein synthesis and may be mediated by a soluble factor produced by sensitized lymphocytes.

INTRODUCTION

There has been considerable interest recently in the leucocyte migration test as a measure of cellular hypersensitivity (Søborg, 1967; Clausen & Søborg, 1969; Lockshin, 1969; Kaltreider *et al.*, 1969; Mookerjee, Ackman & Dosseter, 1969; Rosenberg & David, 1970; Falk, Collste & Möller, 1970; Federlin *et al.*, 1971). Most of these studies have examined the relationship between PPD-induced inhibition of leucocyte migration and the delayed skin reaction to this antigen. The results reported have varied considerably. A number of workers have demonstrated a correlation between inhibition of migration and the presence of a positive skin reaction (Clausen & Søborg, 1969; Rosenberg & David, 1970; Mookerjee *et al.*, 1969; Federlin *et al.*, 1971). Although Rosenberg & David (1970) and Federlin *et al.* (1971) could find no relationship between the magnitude of the *in vitro* and *in vivo* reactions, Clausen & Søborg (1969) were able to demonstrate a close correlation between the degree of

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inhibition and the size of the skin reaction. In some experiments (Lockshin, 1969; Kaltreider *et al.*, 1969) inhibition appeared to be completely unrelated to the *in vivo* response.

We have used the leucocyte migration test to diagnose graft rejection (Eddleston, Williams & Calne, 1969; Smith *et al.*, 1969; Eddleston *et al.*, 1971) and to study cell mediated immunity in autoimmune liver disease (Smith *et al.*, 1972). While inhibition of migration did seem to reflect sensitization to the antigens used, stimulation of migration was frequently observed and was difficult to interpret. Søborg (1967) demonstrated stimulation of migration when host sensitivity was low and when small amounts of antigen were used. Federlin *et al.* (1971) also reported stimulation of migration at low antigen concentrations. Because of our interest in the clinical application of the leucocyte migration test and in view of the discrepancies reported in the literature we decided to re-investigate the relation between the leucocyte migration test and delayed skin reactions using PPD. Antigen-induced inhibition of peritoneal exudate cell migration in animals can be prevented by the addition of puromycin to the migration chambers (David, 1965). This probably acts on sensitized lymphocytes to prevent the formation of migration inhibitory factor (MIF) and we have studied its effect on antigen-induced inhibition of leucocyte migration in man.

MATERIALS AND METHODS

Skin tests

Mantoux testing was carried out immediately after blood had been taken for the leucocyte migration studies in forty healthy volunteers using preservative-free PPD (Weybridge). The area of skin induration in response to 1 μg of PPD (1/1000 Mantoux) was measured at 48 hr and a mean diameter >5 mm was taken as a positive reaction. Those found to be negative were tested again using 10 μg PPD (1/100 Mantoux).

Migration technique

The migration technique used, which is a modification of the one described previously (Smith *et al.*, 1969) was as follows. Twenty millilitres of venous blood were taken in a plastic syringe containing 200 units of preservative-free heparin and 2 ml of 6% dextran. The red cells were allowed to sediment in the syringe for about 60 min at 37°C and the leucocyte-rich supernatant was then transferred to a siliconized centrifuge tube. The leucocytes were separated by centrifugation at 350 *g* for 5 min, washed three times in Hanks' balanced salt solution, and the cell button was resuspended in Waymouth's medium containing 10% foetal calf serum. Microcapillaries (10-μl volume) were filled with the leucocyte suspension and plugged with critoseal. After centrifugation at 350 *g* for 5 min the tubes were cut below the cell/fluid interface, and were mounted in pairs in disposable tissue culture chambers (Sterilin Ltd). Control chambers were filled with 0·5 ml Waymouth's medium containing 10% foetal calf serum, and test chambers with Waymouth's medium containing 10% foetal calf serum and concentrations of preservative-free PPD ranging from 1–300 μg/ml. In every experiment at least four capillaries were set up in control medium and at each concentration of antigen. After sealing the chambers with cover slips, they were incubated at 37°C for 20 hr and the areas of migration were then measured by means of a projecting microscope and a planimeter. All measurements were done without knowledge of the result of the Mantoux test. The migration index was calculated by dividing the mean area of migration in the presence of antigen by the mean area of migration in control chambers. In these

studies, the mean coefficient of variation for replicate migrations was 6·9% with a range from 1% to 13%.

Effect of puromycin

Leucocytes from sixteen Mantoux-positive and three Mantoux-negative subjects were used. Four groups of chambers were set up for each subject, each group consisting of two control chambers containing no PPD and two test chambers containing 100 μg PPD/ml. Puromycin was added to three of the groups in doses of 15 μg/ml, 7·5 μg/ml, and 5 μg/ml.

RESULTS

Of the forty volunteers studied, twenty-four gave a positive Mantoux reaction. In twenty-three this was obtained at a 1/1000 Mantoux test and in one at a 1/100 test. A definite relationship was seen between the positive or negative status of the subject and the migration index of their leucocytes when the concentration of PPD in the test chambers was 100 μg/ml (Fig. 1). At this concentration, values for the migration index of leucocytes from

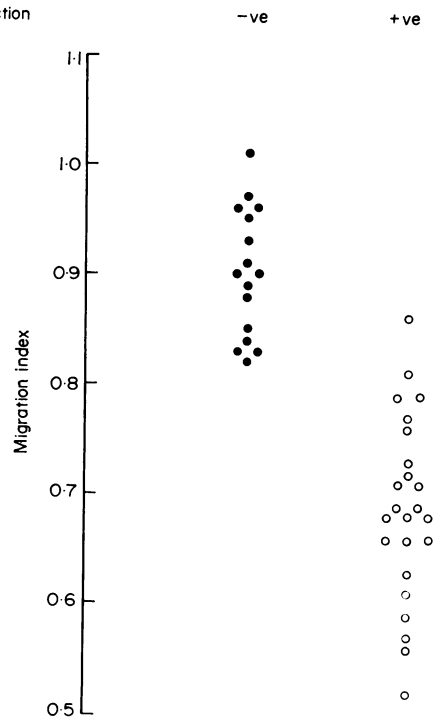

FIG. 1. Results of the leucocyte migration test in Mantoux-positive and -negative subjects using PPD (100 μg/ml) as the antigen.

negative subjects fell within the range of 0·82–1·01. The lower limit of the normal range (mean − 2 S.D.) was 0·79 and the migration index of leucocytes from twenty of the twenty-four positive subjects was below this value, the lowest being 0·52. This agreement between a positive skin reaction and inhibition of migration could not be extended further to a correlation between the degree of inhibition and the diameter of induration (Table 1).

TABLE 1. A comparison of the leucocyte migration index and the diameter of skin induration in Mantoux-positive subjects. No. 19 positive at 1/100 dilution, others at 1/1000 dilution

Subject No.	Mean diameter of skin induration (mm)	Migration index (100 μg PPD/ml)
1	6	0·66
2	9	0·68
3	9	0·73
4	10	0·68
5	11	0·79
6	11	0·68
7	13	0·71
8	14	0·66
9	14	0·81
10	14	0·59
11	15	0·86
12	15	0·56
13	20	0·71
14	20	0·69
15	23	0·76
16	25	0·61
17	30	0·76
18	73	0·52
19	15	0·66

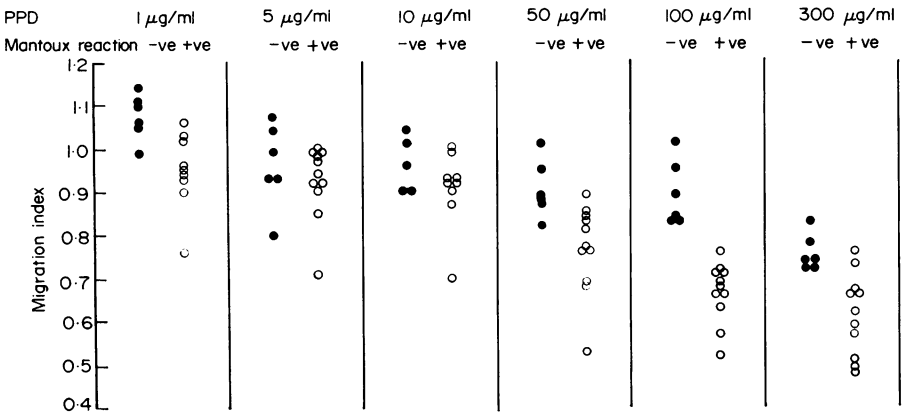

FIG. 2. Measurements of the migration index in Mantoux-positive and -negative subjects using different concentrations of PPD.

Blood from eleven Mantoux-positive and six Mantoux-negative subjects was used to study the effect of different concentrations of PPD (1, 5, 10, 50, 100 and 300 μg/ml), on the area of migration. The results showed that even at the lower concentrations no stimulation of leucocyte migration was observed in the blood taken from Mantoux-positive subjects (Fig. 2). In the case of one strongly positive subject inhibition of migration occurred at all

concentrations of PPD. With the remainder, significant inhibition was observed at concentrations greater than 10 μg/ml. The migration of leucocytes from Mantoux-negative subjects was not significantly inhibited by PPD except at the highest concentration studied.

Puromycin was found to affect the migration of leucocytes in the absence of antigen. Although the effect was variable, at a concentration of 15 μg/ml the area of migration was reduced by about 50% and at the two lower concentrations by 20–30%. All three concentrations of puromycin prevented the inhibition of migration normally observed when leucocytes from Mantoux-positive subjects were incubated in medium containing PPD (Fig. 3).

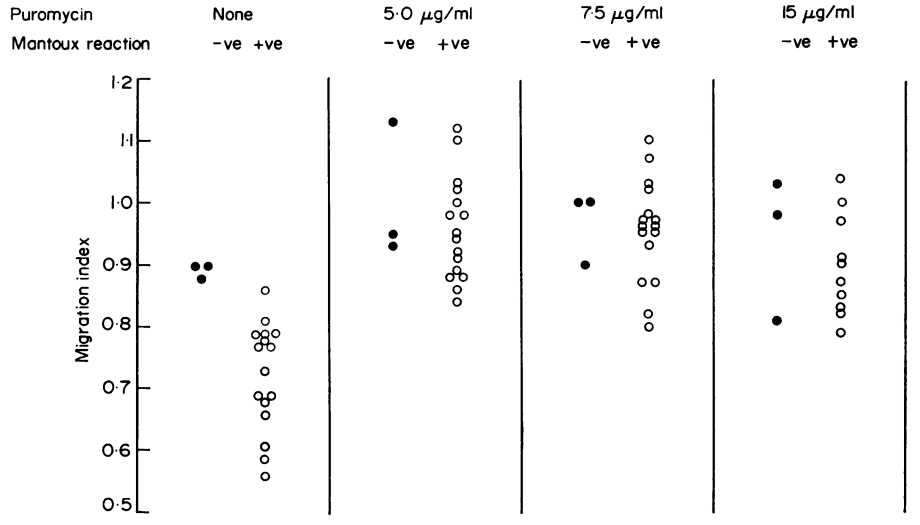

FIG. 3. The effect of three concentrations of puromycin on the migration index in Mantoux-positive and -negative subjects with PPD (100 μg/ml) as the antigen.

DISCUSSION

The difference in the results obtained by different workers using the leucocyte migration test could reasonably be explained by differences in technique. Various tissue culture media and sera additives are used and speeds of centrifugation differ greatly, in addition to variations in many seemingly more minor details of the procedure. Svejcar & Johanovsky (1965) have suggested how differences in technique might influence the results. They studied migration of cells from guinea-pig spleen fragments over a period of 18 hr, taking measurements at hourly intervals. When cells from Mantoux-positive guinea-pigs were used they found that these cells showed initial stimulation of migration when PPD was present in the medium, but later during the period of incubation the migration of these cells was inhibited. They suggested that the length of the stages may vary according to experimental conditions and some workers might therefore observe the final stage of inhibition, while others observe either the temporary balanced state of apparently equal migration or possibly the early stimulation.

Although our results show definite inhibition of migration of cells from Mantoux-

positive subjects in the three highest concentrations of PPD, the degree of inhibition did not correlate with the size of the skin reaction. This is not altogether unexpected since inhibition of migration is believed to occur as the result of the action of a soluble factor (produced by the lymphocytes after contact with specific antigen) on the granulocytes present (Søborg, 1969), whereas the *in vivo* response is a complex reaction involving all the lymphokines (Dumonde *et al.*, 1969).

Søborg (1968) has demonstrated that stimulation as well as inhibition of migration is indicative of *in vivo* sensitization. Using Brucella antigen, he has shown that the result obtained is dependent on both antigen concentration and the degree of sensitivity of the cell donor. High antigen concentrations caused inhibition of migration of cells from all Brucella-positive subjects. Low antigen concentrations tended to result in stimulation of cell migration if the cells had been obtained from weakly or moderately sensitive subjects, while cells from highly sensitive subjects were still inhibited. Federlin *et al.* (1971) reported stimulation of leucocyte migration in a small number of Mantoux-positive subjects at a low concentration of PPD (3 μg/ml). In our studies with the leucocyte migration test, using antigens prepared from liver and donor leucocytes in the case of patients receiving liver transplants (Eddleston *et al.*, 1971), and from liver, kidney, and salivary tissue in the case of patients with autoimmune diseases (Smith *et al.*, 1972), stimulation of migration was observed in some cases, and on the basis of other evidence this appeared to correlate with weak sensitization. In the PPD studies described here, using the modified technique, we did not observe stimulation of migration with leucocytes from Mantoux-positive subjects, and indeed in one subject who gave a particularly strong Mantoux reaction, inhibition occurred throughout the range of PPD concentrations, even with 1 μg PPD/ml. In other experiments we have occasionally observed stimulation using PPD, but these results were not obtained consistently.

David (1965) demonstrated that puromycin at concentrations which interfered with protein synthesis, could abolish the antigen-induced inhibition of migration of peritoneal exudate cells from Mantoux-positive guinea-pigs. Only analogues of puromycin which inhibited protein synthesis prevented antigen-induced inhibition of migration. Inhibition of peritoneal exudate cell migration in animals has been shown to be mediated by a migration inhibitory factor (MIF) elaborated by sensitive lymphocytes when cultured with antigen (Bloom & Bennett, 1966). Preliminary work by Søborg (1969) and Rosenberg & David (1970) has indicated that a soluble lymphocyte-produced factor may be operating in the leucocyte migration test. Our results with puromycin would be consistent with their observations, and also suggest that preformed cytophilic antibody and antigen–antibody complexes do not contribute to PPD-induced leucocyte migration inhibition.

The results we have obtained with PPD in the modified test system are in agreement with our findings in studies with other antigens. It would appear that although a small proportion of antigen-sensitive individuals may not show significant inhibition, the leucocyte migration test in the majority of subjects does give an indication of *in vivo* sensitivity to a specific antigen.

ACKNOWLEDGMENTS

We are grateful to Miss Angela Kemp for excellent technical assistance, and to King's College Hospital School of Nursing for their co-operation. We thank the Wellcome Trust for their continued support.

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