

## Comparison of a leucocyte adherence test with the leucocyte migration inhibition test and skin reactivity to PPD

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### SUMMARY

Using PPD as antigen, a leucocyte adherence test was compared with the leucocyte migration inhibition test and with the results of skin testing in healthy subjects. In the presence of antigen, leucocyte adherence was significantly greater in skin test-positive subjects than in skin test-negative subjects who had not previously had BCG vaccination. When the test was performed in heterologous serum, skin test-negative subjects who had had previous BCG vaccination showed significantly greater leucocyte adherence in the presence of antigen than skin test-negative subjects who had not had BCG vaccination; this difference was not present in autologous serum and its abolition was attributed to a serum blocking effect. The leucocyte adherence test correlated with the results of the leucocyte migration inhibition test in heterologous serum, and also with the results of skin testing interpreted in the light of previous vaccination history. The finding of increased rather than decreased adherence in the presence of antigen, although previously reported, is unusual.

### INTRODUCTION

The leucocyte adherence inhibition test has been used as a simple test for the detection of cell-mediated tumour immunity and serum blocking factors (Halliday & Miller, 1972; Halliday, Maluish & Isbister, 1974b; Maluish & Halliday, 1974; Halliday, Maluish & Miller, 1974c; Halliday *et al.*, 1974a). It is thought that the basis of the technique is the liberation of a lymphokine from sensitized lymphocytes on exposure to the appropriate antigen. The phenomenon of leucocyte adherence inhibition may be related to other phenomena dependent on lymphokine release, such as leucocyte migration inhibition (Clausen & Soborg, 1969) and monocyte spreading inhibition (Silobrcic *et al.*, 1975). The leucocyte migration inhibition and monocyte spreading inhibition methods of assessing cell-mediated immunity have been correlated with skin reactions to specific antigens, particularly PPD, but the leucocyte adherence inhibition test has not been fully evaluated in this way.

We have compared skin reactivity to PPD with leucocyte migration and leucocyte adherence tests.

### SUBJECTS AND METHODS

Eighteen skin test-positive healthy subjects and twelve skin test-negative healthy subjects were studied. In addition, four skin test-negative subjects who had previously had BCG vaccination were considered separately.

Skin testing was performed by intradermal injection of 0.1 ml of 1 in 1000 tuberculin on the inner aspect of the forearm. The result was read at 48 and 72 hr, and a positive result was taken to be 5 mm or more induration.

Peripheral blood was obtained by venepuncture for the *in vitro* tests before skin testing was carried out.

To perform the leucocyte adherence test, 15 ml of blood was mixed with 300 u of preservative-free heparin in a glass bottle and allowed to sediment for 1 hr at 37°C. The leucocyte-rich plasma was placed in a 10 ml plastic tube and centrifuged at 1000 rev/min for 10 min. The supernatant was removed and the plug of cells shaken. 1 ml of 0.15 M ammonium chloride

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was added to lyse the red cells, and after 1 min 10 ml of Eagle's medium with 10% foetal calf serum, kept at 37°C, was added. The suspension was centrifuged at 1000 rev/min for 10 min and the cells washed three times in Eagle's medium with 10% foetal calf serum. After the final wash, the cell suspension was made up to  $2 \times 10^7$  cells per ml in Eagle's medium with 10% foetal calf serum. On the basis of preliminary experiments (see the Results section), it was decided to perform the tests at a concentration of 100  $\mu\text{g/ml}$  PPD.

Freeze-dried PPD (Weybridge) was made up into a 400  $\mu\text{g/ml}$  solution in Eagle's medium with 10% foetal calf serum.

100  $\mu\text{l}$  of the white cell suspension was added to 100  $\mu\text{l}$  of the antigen solution, 100  $\mu\text{l}$  of Eagle's medium with 10% foetal calf serum, and 100  $\mu\text{l}$  foetal calf serum in a small plastic tube. In control tubes, 100  $\mu\text{l}$  of cell suspension was added to 200  $\mu\text{l}$  of Eagle's medium with 10% foetal calf serum, and 100  $\mu\text{l}$  foetal calf serum. In order to assess the effects of autologous serum on adherence of cells, 100  $\mu\text{l}$  of cell suspension was added to 100  $\mu\text{l}$  of antigen solution, 100  $\mu\text{l}$  Eagle's medium with 10% foetal calf serum, and 100  $\mu\text{l}$  of autologous serum. All tubes were set up in triplicate and placed in an incubator at 37°C for 30 min, shaking every 5 min.

The suspension from each tube was placed on a clean haemocytometer covered with a thin glass coverslip. Each haemocytometer was incubated in a moist atmosphere at 37°C for 60 min. The nucleated cells in a predetermined pattern of squares were counted. The coverslip was then floated off in saline, and the haemocytometer washed twice by slow immersion in a beaker of saline. A second coverslip was gently placed in position, and the number of cells remaining on the same squares was again counted. All counts were done without knowledge of whether the chambers contained cells incubated in medium only, medium plus antigen or medium plus antigen and autologous serum.

For each square, the number of cells remaining after washing was expressed as a percentage of the original number of cells. For each determination of percentage adherence, at least thirty-two squares were counted and the mean was calculated. Unexpectedly, there was increased adherence of cells in the presence of antigen, and therefore the results were expressed as: percentage increase in adherence in heterologous serum =

$$\frac{\text{percentage adherence in the presence of antigen} - \text{percentage adherence in absence of antigen}}{\text{percentage adherence in the absence of antigen}} \times 100.$$

The percentage increase in adherence in autologous serum was expressed as:

$$\frac{\text{percentage adherence in the presence of autologous serum plus antigen} - \text{percentage adherence in the absence of antigen}}{\text{percentage adherence in the absence of antigen}} \times 100$$

The addition of autologous serum caused a decrease in the adherence of cells in the presence of antigen in some subjects. The percentage decrease in adherence was expressed as:

$$\frac{\text{percentage adherence in the presence of antigen} - \text{percentage adherence in the presence of autologous serum plus antigen}}{\text{percentage adherence in the absence of antigen}} \times 100$$

To perform the leucocyte migration inhibition test, 20 ml of peripheral blood was defibrinated by shaking with glass beads in sterile conditions and 10 ml of dextran 150 in normal saline was added. After sedimentation, the leucocyte-rich supernatant was centrifuged at 1000 rev/min for 10 min. The cell pellet was re-suspended in medium 199 and recentrifuged for a further 10 min at 1000 rev/min. This washing procedure was repeated three times.

The cells were resuspended finally in medium 199 to give a packed cell volume of approximately 50%. The suspension was drawn into 20  $\mu\text{l}$  capillaries (Drummond microcaps) which were heat-sealed at one end, taking care not to char the cells. The capillaries were centrifuged at 500 rev/min for 5 min to pack the cells, then were cut squarely across just below the cell-fluid interface and secured with silicone grease in Sterilin migration chambers rimmed with silicone grease. Each chamber was filled with the appropriate medium, sealed with a coverslip and incubated for 20 hr at 37°C.

Control chambers were filled with medium 199 + 10% foetal calf serum, and test chambers were filled with the same mixture plus 100  $\mu\text{g/ml}$  of PPD, this being the concentration of antigen used in the leucocyte adherence inhibition test.

Control and test chambers were set up in quadruplicate. After incubation, the image of the migration chambers was projected at constant magnification onto a screen, and the area of migration was traced around and measured by planimetry.

The percentage inhibition of migration in heterologous serum in the presence of antigen was expressed as:

$$\frac{\text{mean area of migration in the absence of antigen} - \text{mean area of migration in the presence of antigen}}{\text{mean area of migration in the absence of antigen}} \times 100.$$

The Mann-Whitney U test and the Spearman rank correlation were used in the statistical analyses.

## RESULTS

In preliminary experiments, the leucocyte adherence test was performed using venous blood from four skin test-positive subjects and two skin test-negative subjects, at concentrations of 20  $\mu\text{g}$ , 100  $\mu\text{g}$ , 200  $\mu\text{g}$ ,

300  $\mu\text{g}$ , 400  $\mu\text{g}$ , and 600  $\mu\text{g}$  of PPD per ml. In skin test-positive subjects, increased adherence was found at 20  $\mu\text{g}$ , 100  $\mu\text{g}$  and 200  $\mu\text{g}$  per ml, but above this concentration adherence decreased, and at 600  $\mu\text{g}/\text{ml}$  there were very few adherent cells. In the two skin test-negative subjects, the presence of PPD did not affect the adherence at concentrations up to 200  $\mu\text{g}/\text{ml}$ , but above this concentration adherence decreased, suggesting that concentrations above 200  $\mu\text{g}/\text{ml}$  were toxic to the cells. All subsequent tests were therefore performed using a concentration of 100  $\mu\text{g}$  of PPD per ml.

Fig. 1 shows the percentage increase in adherence in the leucocyte adherence test in skin test-positive subjects, skin test-negative subjects who had not had BCG vaccination (BCG-negative), and skin test-negative subjects who had had previous BCG vaccination (BCG-positive).

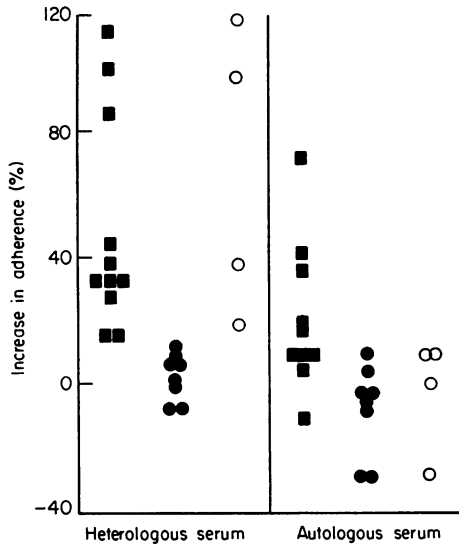


FIG. 1.

FIG. 1. Percentage increase in adherence, in the presence of PPD, of leucocytes from skin test-positive subjects (■), skin test-negative (BCG-negative) subjects (●) and skin test-negative (BCG-positive) subjects (○).

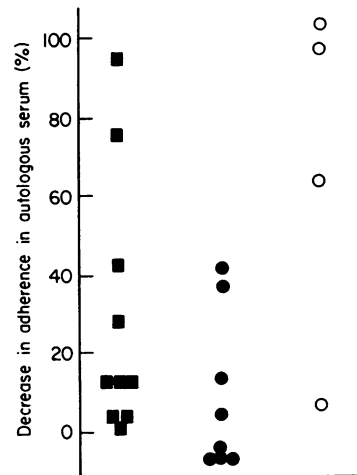


FIG. 2.

FIG. 2. Percentage decrease in adherence in autologous serum of leucocytes from skin test-positive subjects (■), skin test-negative (BCG-negative) subjects (●) and skin test-negative (BCG-positive) subjects (○).

In heterologous serum, the percentage increase in adherence in the presence of antigen was significantly greater ( $P < 0.002$ ) in skin test-positive subjects than in skin test-negative (BCG-negative) subjects and there was no overlap, but there was no significant difference ( $P > 0.05$ ) between skin test-positive subjects and skin test-negative (BCG-positive) subjects. The percentage increase in adherence in the presence of antigen was significantly higher ( $P < 0.005$ ) in skin test-negative (BCG-positive) subjects than in skin test-negative (BCG-negative) subjects.

In autologous serum, the percentage increase in adherence in skin test-positive subjects was significantly greater ( $P < 0.02$ ) than in skin test-negative (BCG-negative) subjects, but there was no significant difference between skin test-negative (BCG-negative) subjects and skin test-negative (BCG-positive) subjects.

The percentage decrease in adherence in the presence of autologous rather than heterologous serum is shown in Fig. 2. Although the addition of autologous serum caused some decrease in adherence in most patients, the decrease in adherence was most marked in the skin test-negative (BCG-positive) group, and significantly greater ( $P < 0.02$ ) than in the skin test-negative (BCG-negative) group.

The finding of increased rather than decreased adherence in the presence of antigen was unexpected. However, the same antigen, when used in the same subjects in the leucocyte migration inhibition test with heterologous serum, caused migration inhibition in the skin test-positive subjects, as expected. Fig. 3

shows the percentage migration inhibition in the three groups of subjects. The percentage migration inhibition was significantly greater ( $P < 0.002$ ) in skin test-positive subjects than in skin test-negative (BCG-negative) subjects, but, as in the leucocyte adherence test, there was no significant difference between skin test-positive and skin test-negative (BCG-positive) subjects.

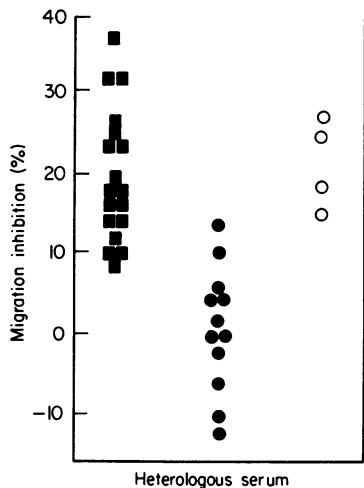


FIG. 3.

FIG. 3. Percentage migration inhibition, in the presence of PPD, of leucocytes from skin test-positive subjects (■), skin test-negative (BCG-negative) subjects (●) and skin test-negative (BCG-positive) subjects (○).

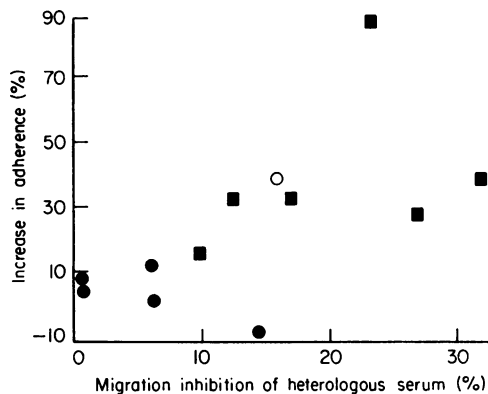


FIG. 4.

FIG. 4. Percentage increase in adherence in the presence of PPD plotted against percentage migration inhibition in the presence of PPD of leucocytes from skin test-positive subjects (■), skin test-negative (BCG-negative) subjects (●) and one skin test-negative (BCG-positive) subject (○).

Fig. 4 shows the percentage increase in adherence in the leucocyte adherence test in heterologous serum plotted against percentage migration inhibition in the leucocyte migration inhibition test in heterologous serum for twelve subjects (six skin test-positive, five skin-test-negative (BCG-negative) and one skin test-negative (BCG-positive)) who had simultaneously performed tests. The percentage increase in adherence correlates with the percentage migration inhibition ( $r = 0.69$ ,  $P < 0.05$ , Spearman rank correlation).

## DISCUSSION

Several workers have suggested that skin testing with PPD does not always give a good indication of immunity to tuberculosis, especially after BCG vaccination. Following BCG vaccination there may be enhanced lymphocyte transformation with PPD in skin test-negative subjects (Matsaniotis *et al.*, 1968; Thomas, Clements & Grzybowski, 1971) and skin test-negative subjects who have been vaccinated with BCG previously are more resistant to tuberculosis than skin test-negative subjects who have not been vaccinated (Hart *et al.*, 1967).

In our study, four skin test-negative subjects had had previous BCG vaccination and these were compared with the other skin test-negative subjects. There was a clear distinction between the leucocyte responses to PPD in the two groups, but the difference was found only in heterologous serum. It seems likely that a serum factor causes the lack of effect of PPD in autologous serum and negative skin tests in the patients previously vaccinated with BCG.

The comparison of leucocyte adherence and leucocyte migration tests in heterologous serum using  $100 \mu\text{g/ml}$  PPD as antigen suggests that there is a correlation between the results of the two tests.

However, the results of the leucocyte adherence test in heterologous serum are at variance with most other published results, in that the presence of antigen in sensitized subjects was associated with increased adherence rather than adherence inhibition. We also noted that in skin test-positive subjects,

mononuclear cells on the haemocytometer surface spread more in the presence of PPD than in its absence, whereas monocyte spreading has previously been reported to be inhibited by PPD (Silobrcic *et al.*, 1975). These unexpected results are unlikely to be due to serum factors, since the cells were washed three times and suspended in medium containing heterologous serum. Although the results of *in vitro* tests of cell-mediated immunity using PPD vary with the source of the antigen (Passaleva, Forti & Ricci 1974), PPD (Weybridge) has been used extensively and gave the expected results in the leucocyte migration inhibition test.

Increased adherence is unlikely to be due to the particular concentration of antigen used, since in our preliminary experiments increased adherence was shown at all concentrations of antigen up to 200 µg/ml, whereas others have shown decreased adherence with similar concentrations of PPD (Lampert & Dietmair, 1973; Walters, Chick & Halliday, 1974).

However, increased adherence has been described in other situations. It has been noticed in occasional patients suffering from cancer, using tumour extracts as antigen (Armistead & Gowland, 1975), and in mice inoculated with BCG, using PPD as antigen (Holt *et al.*, 1975). The phenomenon of increased adherence may be related to that of leucocyte migration enhancement (Weisbart *et al.*, 1974), in that enhancement and inhibition of adherence or migration may be related to the presence of different lymphokines produced by sensitized cells after varying periods of contact with antigen.

In our previously vaccinated skin test-negative subjects, increased adherence in the presence of PPD was blocked by autologous serum. Similar blocking of the effect of the antigen has been described in other situations. Serum factors causing a specific inhibitory effect in tests of cell-mediated immunity have been described in tuberculosis (Heilman & McFarland, 1966), chronic histoplasmosis (Newberry *et al.*, 1968), cancer (Halliday *et al.*, 1974b) and chronic dermatophytic infections (Walters *et al.*, 1974). A non-specific inhibitory effect of serum in such tests has also been described (Cooperband *et al.*, 1968; Levene *et al.*, 1969; Goldblum & Goldblum, 1972). It may be that the blocking of increased adherence by autologous serum in our experiments represents a specific blocking of cell-mediated immunity. Jensen, Kurpisz & Rubin (1977) have suggested that such blocking activity might be due to presence of PPD or PPD-anti-PPD complexes in the serum. However, since the effect of autologous serum using another antigen has not been assessed, a non-specific blocking effect cannot be excluded.

We suggest that a test of leucocyte adherence in the presence and absence of PPD correlates with the results of another *in vitro* test of immunity; when interpreted in the light of previous vaccination history and the differing effects of autologous and heterologous serum, the test also correlates with the results of skin testing. The technique we have used, however, results in leucocyte adherence enhancement rather than inhibition; this difference is unexplained.

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