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A COMPARISON OF THE INHIBITION OF LEUCOCYTE MIGRATION AND MONOCYTE SPREADING AS *IN VITRO* ASSAYS FOR TUBERCULIN HYPERSENSITIVITY IN MAN

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SUMMARY

The ability of leucocyte migration inhibition and monocyte spreading inhibition test to detect tuberculin hypersensitivity was compared in the same twelve Mantoux-negative and fifteen Mantoux-positive persons. Tuberculin hypersensitivity expressed *in vitro* as migration or spreading inhibition, induced by 100 μg of PPD/ml, was assessed after 2 and 24, or 4 and 20 hr of incubation. A significant difference was found between negative and positive persons by migration inhibition at the early interval and by spreading inhibition at both intervals. When the two tests were compared on the basis of individual results, monocyte spreading inhibition appeared more discriminating (fewer results in the group of positive persons overlapped with those found among negative persons). Results of the monocyte spreading inhibition test correlated well with cutaneous reactions at both incubation intervals, while with migration inhibition the correlation was not so well expressed at either interval. Furthermore, a given change in skin reactivity of tuberculin-positive persons was reflected better in spreading inhibition than in migration inhibition indices. We conclude that the method of monocyte spreading inhibition compares favourably with the method of leucocyte migration inhibition, and it seems to be a suitable *in vitro* test for detection of tuberculin hypersensitivity in man.

INTRODUCTION

Owing to the steadily increasing interest in studies of cell-mediated immunity in humans, various methods are even more frequently applied in the clinical examination of patients. A good *in vitro* method for detection of cell-mediated immunity in clinics should comprise a number of features: it should be specific for cell-mediated immune reactions; it should be

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reproducible and quantitative; it should correlate with *in vivo* manifestations of the reactions. It would also be an advantage if the test could be performed quickly, using a minimum of venous blood as the source of both immunologically reactive and indicator cells. Unfortunately, none of the existing methods completely fulfils these requirements and hence present *in vitro* techniques are constantly being modified and new ones being developed.

The inhibition of leucocyte migration from capillary tubes is one of the most popular *in vitro* methods for detection of cell-mediated immunological reactions in clinics. It is, especially the micromethod described by Federlin *et al.* (1971), a convenient technique for *in vitro* detection of tuberculin hypersensitivity in man. Recently we described a new *in vitro* technique of monocyte spreading inhibition (Dekaris *et al.*, 1974) which also discriminates between Mantoux-positive and Mantoux-negative persons.

To evaluate the usefulness of this new method, we compared the results obtained in the same persons by the inhibition of leucocyte migration and monocyte spreading. Thus, the aim of this paper is to show the results of such a comparison between the two *in vitro* tests, using as a model tuberculin hypersensitivity in man.

MATERIALS AND METHODS

Donors of blood

Tuberculin allergy in healthy laboratory personal and children from the Pediatric department of 'Dr M. Stojanović' Hospital was determined by Mantoux testing. They were considered as tuberculin-negative if 72 hr after intracutaneous injection of 0·1 ml of tuberculin solution containing 0·2 μg of PPD (Statens Seruminstitut, Copenhagen), there was no redness and induration at the site of injection. Mantoux-positive persons exhibited redness and induration of 5 mm (diameter) or more, following injection of 0·02 μg of PPD. There were twelve Mantoux-negative and fifteen Mantoux-positive persons selected according to the criteria described previously (Dekaris *et al.*, 1974).

In vitro testing of tuberculin hypersensitivity

About 20 ml of venous blood were taken shortly after Mantoux testing and the plasma containing leucocytes was separated (Dekaris *et al.*, 1974). One half of the plasma was used for leucocyte migration and the other for monocyte spreading test.

The micromethod for peripheral leucocyte migration inhibition (MI) was performed as described by Federlin *et al.* (1971). Chambers consisting of a glass ring 16 mm in diameter and 5 mm high were mounted with vaseline on a glass slide and filled with either medium 199 containing 10% horse serum (control), or medium 199 containing horse serum and 100 μg/ml of tuberculin (PPD RT 32, Statens Seruminstitut, Copenhagen). Six control and six experimental capillaries were usually mounted. After 2 and 24 hr of incubation at 37°C, the leucocyte migration area was projected onto paper through a photographic enlarger, drawn, cut and weighed. The results are expressed as the migration index (MI) = [(mean migration area with antigen/mean migration area without antigen)] × 100.

The direct test of monocyte spreading inhibition (MSI), schematically presented in Fig. 1, was performed as previously described (Dekaris *et al.*, 1974). Contrary to the previous report, however, some chambers were fixed and thus became suitable both for storage and later examination under a phase-contrast microscope, and for staining and reading under a bright-field microscope. The fixation was performed as follows: chambers were formed with

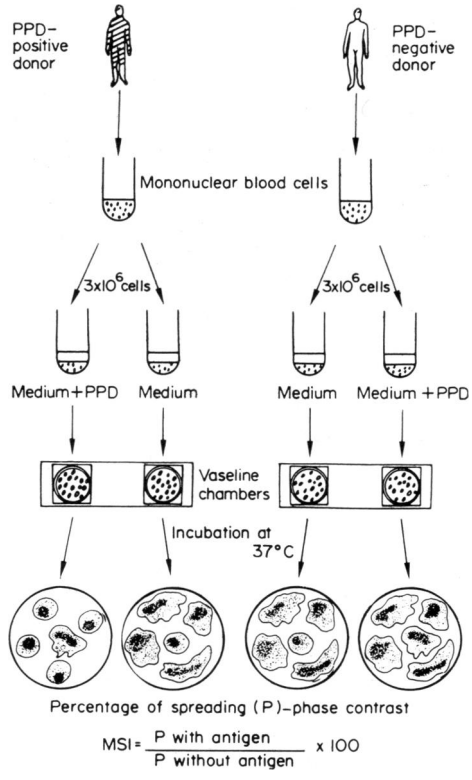

FIG. 1. Schematic presentation of the direct test of monocyte spreading inhibition. Heparinized venous blood was taken from Mantoux-positive and Mantoux-negative persons. Mononuclear cells were isolated from the leucocyte-rich plasma and adjusted to a final concentration of 3×10^6 cells/ml. One aliquot of the cells was mixed with 100 μg/ml of tuberculin and preincubated (30 min at 37°C) in medium 199 containing 2% bovine serum albumin. The other was preincubated without the antigen. After preincubation the cells were introduced into the chambers made by a vaseline ring on a glass slide. The chambers were sealed with a coverslip pressed onto the ring and incubated for 4 or 20 hr at 37°C. They were then examined with the aid of an ocular grid under a phase contrast microscope (magnification × 600). The percentage of spread cells among 100 to 200 viable monocytes was determined and the results were expressed as monocyte spreading inhibition (MSI) indices.

glass rings (16 mm in diameter and 5 mm high), mounted with vaseline on a glass slide, and the free lip of the ring was greased with vaseline. The final concentration of cells was adjusted to 1×10^6 per millilitre. After preincubation with or without the antigen (100 μg/ml of PPD), they were transferred to the chambers which were sealed with a glass coverslip. After incubation at 37°C the coverslip was removed and the content spilled out. Chambers were then filled with 2% glutaraldehyde in 0·066 M phosphate buffer (pH = 7·2) and kept at 4°C for 20 min. Finally, they were rinsed with distilled water and the glass ring was carefully removed. The remaining vaseline ring was filled with a few drops of glutaraldehyde, covered with a coverslip and scored under a phase-contrast microscope.

The difference between the migration (MI) and spreading (MSI) indices in the group of

tuberculin-positive and tuberculin-negative persons were evaluated by Student's *t*-test. The correlation between these indices and skin reactions was estimated by the correlation coefficient *r*. The ability of the *in vitro* tests to discriminate variations in skin reactivity of tuberculin positive persons was evaluated by the regression coefficient *b*. Probability for each index in the group of tuberculin-positive persons to exceed a given deviation of indices within the group of tuberculin-negative persons was determined by *z* values (Peatman, 1963).

RESULTS

In contrast to the previous report (Dekaris *et al.*, 1974), the majority of preparations of mononuclear cells used for monocyte spreading inhibition test were fixed with glutaraldehyde. However, we first established that 1×10^6 cells per millilitre, used for fixed preparations gave results comparable to those obtained with 3×10^6 cells in native (non-fixed) preparations.

The results obtained by parallel *in vitro* testing of blood leucocytes from tuberculin-postitive and tuberculin-negative persons are summarized in Fig. 2. We demonstrated different migratory abilities of leucocytes from fifteen Mantoux-positive and twelve

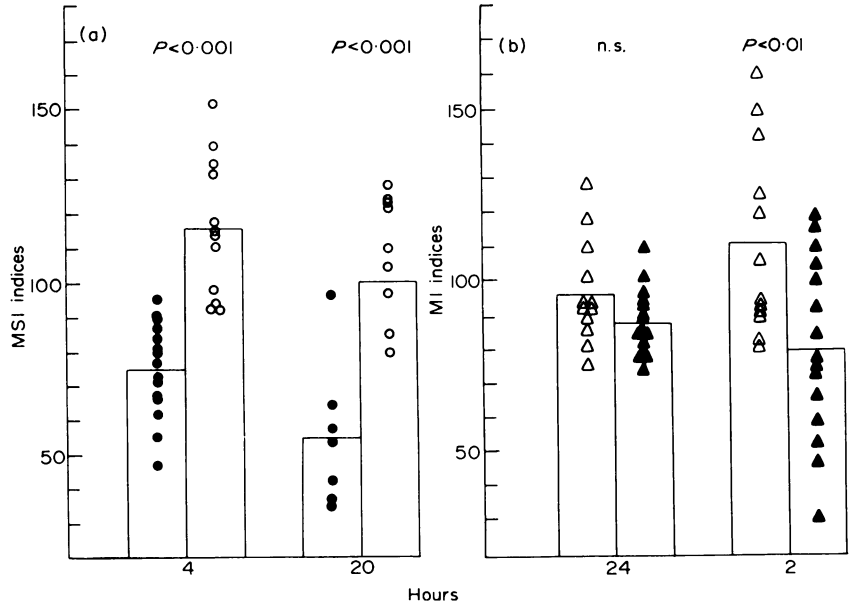

FIG. 2. The indices of (a) monocyte spreading inhibition (MSI) and (b) leucocyte migration inhibition, obtained after various intervals of *in vitro* cultivation with 100 μg PPD/ml, in tuberculin-positive (▲, ●) and tuberculin-negative (△, ○) persons. Each individual index is represented by a triangle or a circle, while the top of the column corresponds to the mean value for the group.

Mantoux-negative persons after 2 hr of *in vitro* incubation, but the difference between these two groups was not significant after 24 hr of incubation. On the other hand, monocytes from the same blood enabled us to differentiate Mantoux-positive from Mantoux-negative

persons by the spreading inhibition test, both after 4 and 20 hr of incubation. Furthermore, the distribution of individual MSI indices in Fig. 2 is better (fewer results in experimental groups overlap with control indices) than for MI indices.

In order to evaluate this difference, we have calculated the position of each individual index obtained in the experimental group within the distribution of indices found in the control group (z value). This relationship is illustrated in Fig. 3. At both early and late intervals of incubation a large majority of MSI indices fell out of the range within which 95% of control indices were distributed. Contrary to that, a preponderance of MI indices in experimental groups overlapped with control indices at 2 hr, and all of them were within the control range at 24 hr.

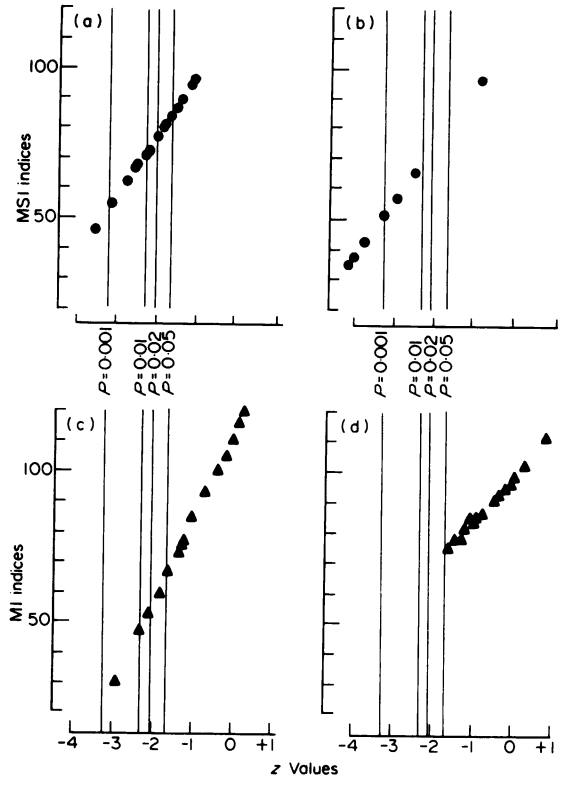

FIG. 3. Distribution of individual leucocyte migration inhibition (MI) (▲) and monocyte spreading inhibition (MSI) (●) indices of Mantoux-positive persons in relation to the normal distribution of indices found in Mantoux-negative persons (z values). MSI indices were determined after (a) 4 and (b) 20 hr, while MI indices were obtained after (c) 2 and (d) 24 hr of incubation. Vertical lines depict the limits of different levels of significance (P values). All the indices to the right of the line for $P = 0{\cdot}05$ are considered not to be significantly different from normal distribution of indices in controls. Those to the left differ from controls at the level of significance shown by the P value.

The correlation between the intensity of Mantoux reactions and leucocyte migration or MSI indices is listed in Fig. 4. At the early incubation interval the correlation coefficient r was higher for the test of spreading inhibition than for migration inhibition. At the later

interval monocyte spreading inhibition was again better correlated to skin reactions than migration inhibition. The regression coefficient *b* shows how well a given change of skin reactivity is reflected *in vitro* as a change of inhibition indices. A higher *b* value means that even a smaller change in the intensity of skin reaction is paralleled by a discernible change of inhibition indices. The *b* value was higher in the case of MSI than in the case of MI indices.

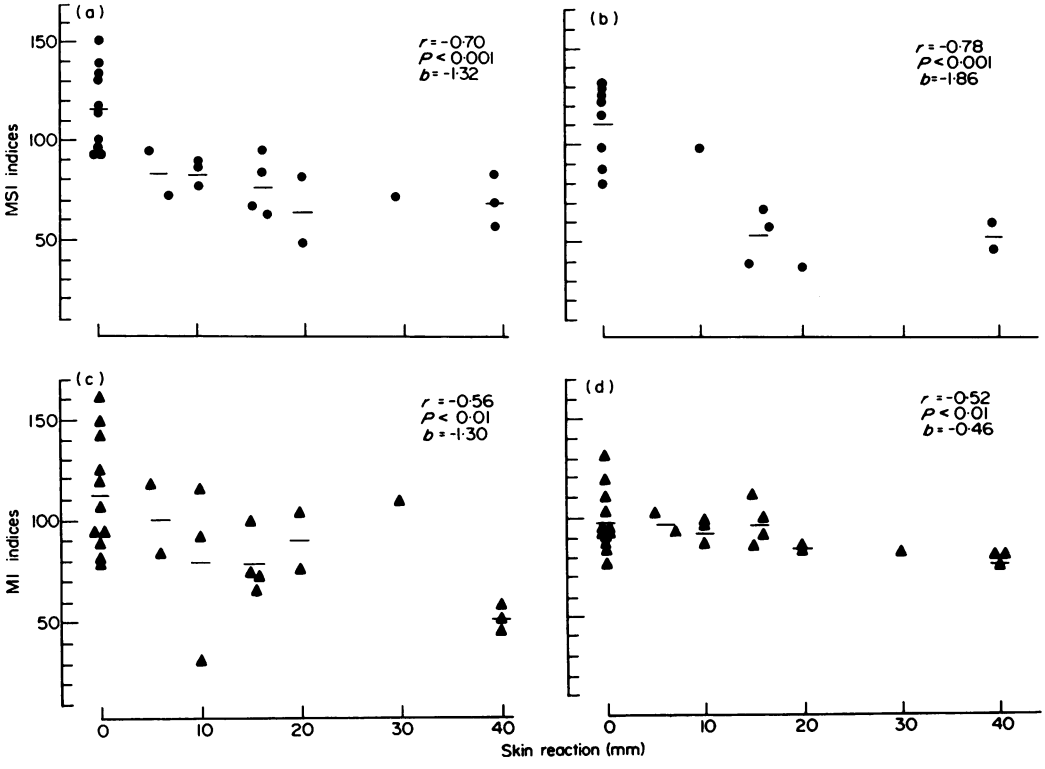

FIG. 4. Correlation between the cutaneous reaction to tuberculin and the monocyte spreading inhibition (MSI) indices (●) at (a) 4 and (b) 20 hr, or leucocyte migration inhibition (MI) indices (▲) at (c) 2 and (d) 24 hr of incubation. The parallelism between the cutaneous reaction and the migration or spreading indices after cultivation with 100 μg PPD/ml is evaluated by the correlation coefficient *r* and the regression coefficient *b*.

DISCUSSION

The technique of leucocyte migration inhibition, introduced by Søborg & Bendixen in 1967, has been extensively used for investigations of cell-mediated immune reactions in humans. However, studies of the tuberculin hypersensitivity by this technique gave differing results; some authors detected the hypersensitivity (Clausen & Søborg, 1969; Mookerjee, Ackman & Dossetor, 1969; Rosenberg & David, 1970; Clausen, 1973; Jones, 1973) and found a good correlation with cutaneous reactions (Clausen & Søborg, 1969), but others failed (Kaltreider *et al.*, 1969; Lockshin, 1969; Brogan, 1971; Claudy *et al.*, 1971; Marsman *et al.*, 1972). Since an early escape from inhibition of migration was observed by some

authors (Clausen, 1973; Brostoff, 1974), negative results might have been due to late reading (12–24 hr) of the migration areas. The micromodification of the technique represented an improvement (Federlin *et al.*, 1971; Mitchell *et al.*, 1972; Maini *et al.*, 1973; Lockshin, Waxman & Jenkins, 1973) and some investigators, previously unsuccessful with Søborg & Bendixen's technique, obtained positive results (Lockshin, Waxman & Jenkins, 1973). In extensive studies (sixty-five Mantoux-positive and forty-eight Mantoux-negative persons), for which the micromethod was used, we were able to demonstrate an inhibition of leucocyte migration in Mantoux-positive persons both after 2 and 24 hr of incubation (unpublished results). Thus, our present failure to detect tuberculin hypersensitivity after incubation for 24 hr is at variance with these results. It should be noted, however, that the group of persons tested in the present study was relatively small and a larger number might have given results comparable to the unpublished study.

Our new technique for detection of cell-mediated immunity in man (Dekaris *et al.*, 1974) was based on the macrophage spreading inhibition test (Fauve & Dekaris, 1968). To define its practical value, it seemed appropriate to compare in the same person the results obtained by this technique and the method for leucocyte migration inhibition, as one of the techniques most frequently applied in clinics for studies on cell-mediated immunity. The comparison confirmed that tuberculin hypersensitivity of Mantoux-positive persons could be detected by both techniques. However, monocyte spreading inhibition gave a more significant difference between positive and negative persons at the early incubation interval. Furthermore, it did discern the same difference after prolonged incubation, while the leucocyte migration failed to do so (at least with the number of persons in the present study).

It is very difficult to assess cell-mediated immunity in an individual person by any of the *in vitro* techniques. Namely, differences obtained when groups of positive and negative persons are compared often include a good deal of overlapping of individual results. Hence it is difficult to say whether a given inhibition index belongs to a positive or a negative person. Since this is practically very important information, we compared individual indices obtained in the migration inhibition or the spreading inhibition test with indices in the control groups. The extent and significance of overlapping of indices in the group of Mantoux-positive with those of Mantoux-negative persons was estimated by the z value. As any normal distribution is characterized by the total frequency of cases (N), centre of distribution (μ) and standard deviation (σ), it is possible to calculate the incidence of cases beyond any distance from the centre of the distribution. Thus, for any value of $(x-\mu)/\sigma$, we can find the probability that it exceeds a given deviation of results. The values for N, μ and σ of the group of Mantoux-negative persons was known and it was easy to determine the position (z values) for each index (x) in the group of Mantoux-positive persons among the indices of negative persons. This way the probability that a given index, found in positive persons, belongs within the normally distributed variate of indices in negative persons was evaluated. Such an analysis for our data revealed that a large proportion of MSI indices found in Mantoux-positive persons significantly deviates from the distribution of indices in the group of Mantoux-negative persons. This was not the case with MI indices, where the extent of overlapping was much larger. Thus, inhibition of monocyte spreading discriminated better Mantoux-positive from Mantoux-negative persons than the inhibition of leucocyte migration.

Furthermore, a firm ground for evaluation of an *in vitro* correlate of cell-mediated immunity is provided by comparison of its results with a corresponding *in vivo* manifestation.

We have already reported that there is a good correlation between indices of monocyte spreading inhibition and the intensity of skin reactions (Dekaris *et al.*, 1974). In the present study this correlation was confirmed and found to be better for MSI than for MI indices. Besides the correlation coefficient *r*, it was important to calculate the regression coefficient *b* showing the slope of the regression line drawn through the results. Obviously, it would be ideal if small variations in skin reactivity were reflected as pronounced changes in the inhibition indices (steep regression line—high *b* value). Then the *in vitro* reaction could discriminate not only positive from negative persons but also the degree of positivity. The macrophage spreading and leucocyte migration methods gave similar *b* values for the early incubation intervals, but at later intervals macrophage spreading inhibition provided better results.

Summarizing, we can say that the monocyte spreading method compares favourably with the leucocyte migration technique for detection of tuberculin hypersensitivity in man. A major technical disadvantage of the monocyte spreading method is the necessity of reading the results immediately after incubation, because of swift changes of spreading indices at room temperature (Dekaris *et al.*, 1974). This has been overcome by the use of glutaraldehyde for fixation of preparations; the slides with fixed cells could be stored for later examination, staining and photography. We also feel that the monocyte spreading method could be further improved if the optimal conditions (preincubation time, concentration of cells for preincubation, concentration of cells and incubation time within the chambers, dose of the antigen, etc.) were better defined. Investigations of this type are now in progress, as well as those defining the mechanism of the spreading inhibition. So far we know that cytophilic antibodies, or antigen–antibody complexes, do not seem to play a role in macrophage spreading inhibition in animals (Dekaris, Veselić & Tomažič, 1971), but this will have to be shown for monocyte spreading inhibition in man.

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