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SUPERIORITY OF CORPUSCULAR BCG TO SOLUBLE PPD ANTIGEN IN THE LEUCOCYTE MIGRATION ASSAY

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SUMMARY

The recognized trend towards the standardization of the leucocyte migration assay prompted the comparison of efficacy of BCG with soluble purified protein derivative (PPD) antigens in this test. Even low BCG concentrations permit high correlations between *in vivo* and *in vitro* responses, whilst PPD doses needed to elicit comparable reactions fall within toxic concentrations. BCG-induced inhibition of leucocyte migration is abolished by the protein synthesis inhibitor, cycloheximide.

INTRODUCTION

The migration inhibition assay is an accepted *in vitro* correlate of cell-mediated immunity. Despite the fact that a number of modifications of the two-step test involving the use of human lymphocyte culture supernatants have been developed (Górski, 1974; Curtis & Hersh, 1973), it appears that peripheral leucocyte migration is particularly suited to routine clinical investigation by virtue of its simplicity and rapidity (Lawrence *et al.*, 1973). Unfortunately, this assay raises some problems both regarding its reproducibility and also the correlation with *in vivo* studies. It has been suggested that only particulate antigens are effective with this technique, whilst soluble antigens do not cause significant inhibition with non-toxic concentrations (Maini *et al.*, 1973; Zabriskie & Falk, 1970), i.e. BCG or tubercle baccilli seem to be superior to purified protein derivative (PPD) in tuberculin allergy. However, no detailed comparative studies on this intriguing problem are available.

In our previous paper we presented evidence in favour of the preincubation technique as a method giving satisfactorily reliable results (Górski *et al.*, 1973). This present work was designed to compare the results using BCG and PPD in the leucocyte migration test, since the evaluation of tuberculin hypersensitivity, the accepted model of cell-mediated immune reactions, is of utmost importance in the assessment of immunological reactivity in a variety of clinical disorders.

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MATERIALS AND METHODS

Human subjects were Mantoux-tested with increasing doses of PPD (batch RT-23, containing Tween 80, Statens Serum Institute, Copenhagen, Denmark). Most individuals exhibited a positive response to 2TU (induration at least 10 mm in diameter within 24–72 hr after intradermal injection—the Polish population is BCG-vaccinated at birth). Prior to the testing, 25 ml of venous blood was withdrawn into a heparinized syringe and allowed to sediment for 1 hr at 37°C. Leucocytes from the supernatant layer were washed three times with phosphate-buffered saline (PBS) and suspended finally in medium 199 enriched with 20% heat-inactivated calf serum (Institute for Sera and Vaccines, Lublin, Poland). The leucocyte suspension was introduced into siliconized capillary tubes (75 mm length, 1·0 mm diameter), whose ends were sealed with sterile plasticine. Capillaries after centrifugation were cut 1 mm below the cell–fluid interface and mounted in plastic chambers (Tekniska Verkstad AB, Sweden). The chambers were filled with media, closed with coverslips and left for 18 hr in humidified atmosphere containing CO_2. The migratory patterns were then outlined by projection microscopy and the migration index (MI) calculated from the ratio: MI = (mean migration area in medium with antigen)/(mean migration area in medium without antigen), where the mean area was obtained from at least four capillaries.

Antigens

BCG, supplied in ampoules containing 1 mg of lyophilized baccilli (Institute for Sera and Vaccines, Lublin) was dissolved in sterile 0·9% saline before use. This antigen was incorporated into chambers in concentrations 5, 10, 15, 25, 50 and 100 μg/ml of medium 199 + calf serum.

Preservative-free PPD (kindly provided by Statens Serum Institute) was dissolved and stored no longer than 2 weeks. Some experiments were paralleled using PPD provided by the Central Veterinary Laboratory, Weybridge. PPD was used at a concentration of 100 μg/ml which is accepted as effective, but a number of cases were studied with lower (25 and 50 μg) and higher (200, 500 and 1000 μg) concentrations.

Cycloheximide (Aktidion, Serva Feinbiochemica, Heidelberg, Germany) and puromycin (Sigma Chemicals, St Louis, Missouri) were dissolved in sterile water and stored frozen at −20°C. Every 2 weeks a new solution was prepared. Initial experiments surveyed a variety of cycloheximide concentrations for its ability to alter leucocyte migration in the presence or absence of antigens. Although 50 μg/ml of antibiotic sometimes reduced *per se* the migration of leucocytes, this dose reduced inhibition evoked by antigens, whereas 25 μg was frequently ineffective. The effect of puromycin (25 μg/ml) was also assessed, although exhaustion of supply made its use on every occasion impossible.

RESULTS

Effect of various concentrations of BCG on leucocyte migration

When lyophilized BCG antigen was incorporated into chambers, the concentration of 25 μg/ml clearly distinguished skin positive and negative populations, mean MI = 0·53 ± 0·05 and 0·94 ± 0·07, respectively ($P < 0·001$). It is noteworthy that as low as 5 μg of BCG enabled the differentiation between these two populations with the probability of $P < 0·05$ (Fig. 1).

Comparison of inhibitory activity of PPD and BCG

Fig. 2 shows indices obtained from the same individuals tested with both antigens simultaneously. Mean MI for PPD testing was $0{\cdot}75 \pm 0{\cdot}09$ and for BCG $0{\cdot}53 \pm 0{\cdot}05$, this difference was statistically significant ($P < 0{\cdot}001$). Although increasing PPD concentrations resulted in some decrease of MI at the same time these doses, starting from 300 μg/ml, were occasionally toxic to the cells from skin-negative persons. A marked variability of results was observed between individual migratory patterns when PPD was used. On the other hand, the use of BCG provided a coefficient of variation not greater than 8–10%.

Effect of protein synthesis inhibitors

Cycloheximide abolished to various extents inhibition of migration evoked by BCG and

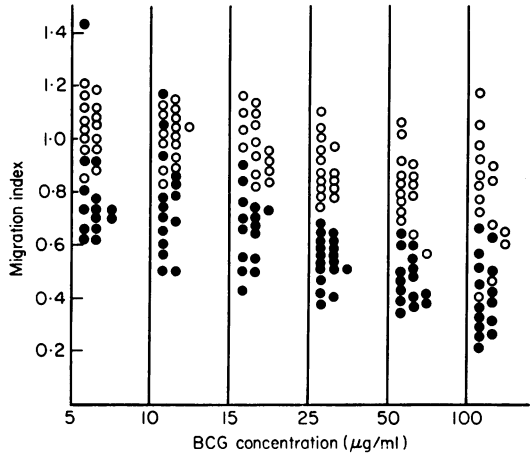

FIG. 1. The effect of increasing BCG concentrations upon leucocyte migration from skin positive (●) and negative (○) individuals.

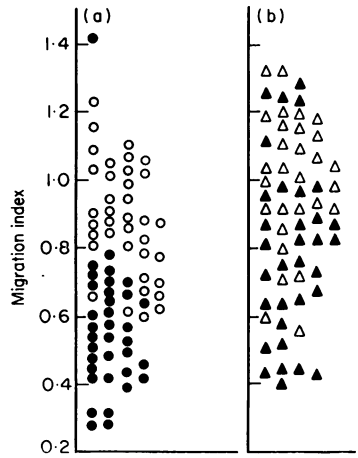

FIG. 2. The effect of cycloheximide (50 μg/ml) on (a) BCG- and (b) PPD-induced migration inhibition of leucocytes from skin positive individuals. (●) BCG (25 μg). (○) BCG+cycloheximide. (▲) PPD (100 μg). (△) PPD+cycloheximide.

PPD; in most cases, the inhibitory effect of antigen was not blocked entirely, although the differences were significant. In one individual we were unable to reduce migration inhibition even when a variety of parameters were chosen; this may indicate that in this case inhibition was due to factors other than MIF. It is worthy of note that cycloheximide and puromycin prevented stimulation of migration which we observed in a few cases; in one of them the inspection of the culture at 6 hr gave a MI of 1·42, with cycloheximide 0·98, and with puromycin 0·89. At 18 hr the indices were 0·48; 1·01 with cycloheximide, or 1·00 with puromycin, respectively. Microscopic examination of Pasteur pipette-aspirated cell fan harvested from inhibited, control and protein synthesis inhibitor-treated cultures revealed that the addition of BCG into chambers containing leucocytes from tuberculin-hypersensitive donors resulted in cell clumping, most of the cells being mononuclear. This effect was not observed either in control or in inhibitor-containing cultures.

DISCUSSION

The data presented herewith demonstrate that BCG is a more potent antigen than soluble PPD, thus enabling the clear-cut differentiation between sensitized and non-sensitized population at a lower antigen concentration. Apparently, BCG was superior to PPD in both the post-immunization state and also in cases of overt active tuberculosis included in this study. Moreover, our preliminary observations indicate that particulate BCG is more stimulatory than soluble PPD tuberculin prepared from it. It is noteworthy that BCG skin testing is a more sensitive method in weak reactors than PPD, or this form of antigen may even elicit a positive skin response in subjects exhibiting no reaction to PPD at all (Zapaśnik-Kobierska, 1968). In addition, Nilsson & Möller (1972) have pointed out that particulate PPD is more active in inducing lymphocyte activation than the soluble form of antigen. Salvin *et al.* (1973) demonstrated that BCG was more efficient than PPD in inducing MIF in BCG-infected mice.

An appropriate correlation between skin tests and an *in vitro* response, as well as susceptibility to protein synthesis inhibitors (previously demonstrated for soluble PPD by Mitchell *et al.* (1972)) also argues that a factor promoting inhibition of migration in this system is of lymphokine nature (MIF).

The low concentrations of antigen which permit a satisfactory lymphocyte response are an additional advantage of my approach, compared to the hundreds of micrograms of PPD needed to elicit a similar effect (Lockshin, Waxman & Jenkins, 1973). As have others, I too have observed a few cases of stimulation of migration. The significance of this phenomenon is still under investigation (Rauch & King, 1973); it is claimed that it may reflect a low level of sensitization. Reduction of stimulation by cycloheximide and puromycin as well as the observation that it may represent an early event lend further support to the assumption that this phenomenon also reflects cellular immunity in the leucocyte migration assay.

The observation of mononuclear cell clumping, presumably MIF-dependent, is in agreement with other authors (Read & Zabriskie, 1972).

The apparent superiority of corpuscular BCG antigen in this assay may implicate rapid processing of this form of antigen during an early stage after a final assembly of the test by mononuclear phagocytes or granulocytes (Bendixen, 1972), thus triggering a potent T-lymphocyte response.

Thus, our results support recent work of Maini *et al.* (1973) in the use of corpuscular antigen as one of the prerequisites for a standardized leucocyte migration test.

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