Mechanism of Buffy Coat Migration Inhibition Test

The direct one-step (buffy coat) migration inhibition test (DMI)[1,2] has been widely used as a measure of cellular immunity and has been equated[3,4] with the indirect, two-step[5,6] assay for migration inhibition factor (MIF) production (IMI). In the IMI test successful performance requires living lymphocytes[7] capable of protein synthesis[8]. The present paper demonstrates that the DMI test performed with soluble antigen also requires lymphocytes; that the test is sensitive to the action of puromycin; that autologous serum enhances the inhibition response; and that this enhancement can be removed by dilution of the serum. We confirm the contention[2,9,10] that strict control of multiple technical variables is necessary to achieve reproducible results.

Materials and methods. Subjects chosen for blood donation were healthy normal laboratory personnel with negative or positive intermediate strength tuberculin (PPD) tests and tuberculin positive patients with active tuberculosis.

The DMI tests were carried out as specified by MAINI[2,10] with gravity sedimentation of lightly heparinized blood, 4 washes of buffy coat cells, packing of cells in non-siliconized 20 µl capillaries (halved), migrating cells in 10% horse serum with 300 µg/ml Weybridge PPD in plastic chambers and measuring migration areas as the limits of the outer migration ring. Serum source and concentration, PPD source and concentration, number of washes, size of capillary, and method of measurement of migration were varied as indicated. Leukocyte types were differentially isolated by Ficoll-Hypaque gradient centrifugation[11]. Puromycin at 10^{-6} *M* was added in some experiments. In this paper migration index (MI) is calculated as average area of migration in presence of antigen (test) divided by average area of migration in absence of antigen (control).

Results. In PPD positive subjects DMI was consistently positive (average MI = 0.66, range 0.53–0.82) and consistently negative in PPD negative subjects (average MI = 0.98, range 0.83–1.24). In PPD positive subjects 10 µl capillaries gave inconsistent results. Parke-Davis PPD lot number 974775 (now exhausted; a gift of Dr. H. B. DEVLIN) most clearly separated in vitro the PPD positive and negative subjects. Parke-Davis PPD lot number 997135C and Weybridge PPD lot number 288 (a gift of Mr. D. B. LEE) produced less dramatic inhibition; greater consistency among leukocyte donors was found with Weybridge product. Inhibition in positive tests was seen after 3 and 4 leukocyte washes, but not after the first two washes, and was not seen after 4 washes of leukocytes obtained from PPD negative subjects. Migration of sensitive leukocytes in the presence of autologous serum or plasma (AS) produced much greater inhibition than did migration in the presence of horse serum (HS)[12]: in a representative experiment, MI = 0.20 in 20% AS, 0.72 in 5% AS-5% HS, 0.72 in 10% HS, and 0.60 in 20% HS. The effect of AS was easily diluted. (Inhibition of non-senistive leukocytes sporadically occurred in 20% HS; 10% HS was therefore routinely used). Heat inactivation of AS (56 °C for 30 min) did not alter the results. Dose response curves to PPD were demonstrable between 100 and 600 µg/ml. Clearest separation of donor populations occurred at 300 µg/ml. Measurement of inner migration rings[13] was found to be subjective and inconsistent and was not used.

Puromycin abolished the inhibition seen in experiments carried out in HS and partially but not completely abolished inhibition seen in experiments carried out in AS (Figure 1). (Control migrations were not significantly affected by puromycin[14]).

The migration in HS of purified (94%) polymorphonuclear leukocytes (PMN) from skin test positive donors was not inhibited by antigen; inhibition was seen when the whole buffy coat cell population was used, or when lymphocytes were added back to the purified PMNs (Figure 2).

Discussion. Sensitized human lymphocytes cultured in the presence of soluble specific antigen produce a substance, MIF, which inhibits the migration of cells from peritoneal exudates[5,6], lung washings[15], or pre-cultured

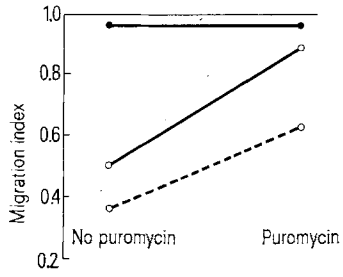

Fig. 1. Effect of puromycin. Migration indices of leukocytes from a PPD-sensitive donor tested in media-containing horse serum (○—○) and autologous serum (○---○) in the presence and absence of $10^{-6}M$ puromycin. Effect on a PPD-insensitive donor in horse serum is shown for comparison (●—●).

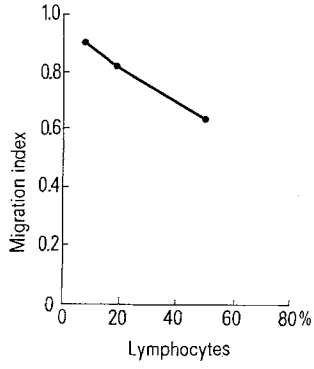

Fig. 2. Effect of different percentages of lymphocytes on migration index of leukocytes from a representative PPD-sensitive subject.

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peripheral blood cells[16–19]. When *particulate* antigens are used with buffy coat cells, lymphocytes must be present for positive responses[20,21]; in the mixed lymphocyte culture system production of an MIF capable of inhibiting buffy coat cell migration has been demonstrated[22]. Elaborate mechanisms have been proposed to demonstrate the presence of macrophages in positive soluble antigen buffy coat cell tests[13,23]. Nontheless it remains an assumption, not critically proven, that *soluble* antigen induced buffy coat cell migration inhibition is mediated by MIF. The present experiments support this assumption by demonstrating the requirement for lymphocytes and for puromycin-sensitive protein synthesis.

Autologous serum and plasma clearly increase the inhibitory response of cells from skin-test positive donors, suggesting that immunoglobulin-antigen complexes can inhibit buffy coat cell migration as they can macrophage migration or that cytophilic antibody is measured in the migration test[24–26]. The failure of puromycin to block inhibition completely when AS is present is also consistent with at least a partial effect of immune complexes independent of protein synthesis. However findings that the AS-dependent response can be removed by dilution and that successsive washes of leukocytes augment rather than diminish the positivity of the test indicate that serum antibody is not the sole determinant of the DMI response.

When certain conditions are met, the DMI test is reproducible, which is at variance with our earlier experience[27]. We attribute the difference to changes in antigen dose, capillary size, and species of origin of sera in the media.

Résumé. La présence de lymphocytes et la synthèse de la protéine sont nécessaires à la production du MIF (migration inhibition factor). Pour le test de migration des leucocytes du sang périphérique, nous avons pu montrer en utilisant un antigène soluble (PPD) et du sérum de cheval, qu'il n'y a point d'inhibition de migration en l'absence des lymphocytes ou en présence de puromycine. Quand le milieu contient du sérum de sujet testé, l'inhibition n'est que partiellement sensible à la puromycine.

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Preliminary Report on the Influence of the Plasma Upon the Effect of ADP on Arterial and Venous Platelets

In order to explain whether the phenomenon previously observed[1], i.e. that arterial platelets show less marked responsiveness to ADP than venous ones, we undertook experiments to check whether the different platelet behaviour in platelet-rich plasma (PRP) is inherent to platelets or to plasma.

Methods. Arterial blood was collected in open-chest animals from the left ventricle of artificially ventilated rats and venous blood from the right ventricle. PRP was obtained as previously described[1]. ADP-responsiveness of washed platelets resuspended in different milieux was tested (see foreward).

The platelets were washed twice with a modified Tyrode's solution: 8 g NaCl, 0.2 g KCl, 1 g $NaHCO_3$, 1 g/l glucose, pH adjusted to 7.35 with NaOH. This solution does not contain calcium, magnesium or phosphate.

Washing was carried out as follows: PRP (3 ml) was diluted in a plastic centrifuge tube with 20 ml of modified Tyrode's solution and then centrifuged at 800 *g* for 8 min at room temperature. The supernatant was discharged and the pellet again resuspended and centrifuged.

After 2 washings: a) platelets were resuspended either in Tyrode's modified solution or in platelet-poor plasma (PPP) freshly obtained from arterial or venous blood; b) the samples were centrifuged at 100 *g* for 5 min in order to eliminate contaminating red blood cells and small aggregates. Platelet count was finally adjusted to a number of 700.000/µl; c) ADP-induced aggregation was estimated as previously described[1]. The lapse of time between a) and c) was about 1 h.

Results. As can be seen (Figure 1a) twice washed 'venous' platelets resuspended in Tyrode's modified solution do not clump upon addition of ADP (6 experiments). Similar responses have been obtained from 'arterial' washed platelets resuspended in Tyrode's modified solution (8 experiments). When the platelets were resuspended in PPP freshly obtained, they recovered ADP-responsiveness, the extent of which was related to the type of plasma. There is evidence (Figure 1, b, c) that 'venous' washed platelets resuspended in PPP obtained from arterial blood (6 experiments) show a less-marked ADP-induced aggregation than 'arterial' washed platelets resuspended in PPP obtained from venous blood (9 experiments).

In 7 experiments we resuspended washed platelets in their own PPP; the ADP-responsiveness was quite similar to that of initial PRP.

Considering that the clumping power of twice washed platelets is lost in Tyrode's modified solution but restored by incubation in plasma, we studied the course of this recovering (8 experiments).

Figures 2 and 3 show the results obtained at different time intervals from the resuspension of platelets in plasma. It can be seen that ADP-responsiveness increases with the incubation time of 'arterial' or 'venous' platelets with the venous or arterial plasma, respectively.

Discussion. Experiments with washed platelets indicate that washing abolishes any clumping power, while resuspension in plasma restores it. So there should be a plas-

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