

Leucocyte migration inhibition in diphtheria toxoid hypersensitivity

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A donor who was highly reactive to diphtheria toxoid (DT) in delayed hypersensitivity and in lymphocyte transformation showed scant evidence of antigen-induced inhibition in the direct leucocyte migration agarose test. Other donors, weak or negative to DT in skin test and transformation, did show evidence of inhibition. Although the migration test is useful in assessing cellular reactivity to tubercular antigen, these results question its suitability for DT.

Key words: diphtheria toxoid; leucocyte migration inhibition; lymphocyte transformation; tuberculin.

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In the direct leucocyte migration agarose test, buffy-coat cells that have been preincubated with or without antigen are placed in wells cut in agarose plates from which they migrate under the agarose layer. Inhibition of migration induced by antigen in this system is regarded as an *in vitro* expression of cellular immunity (2). Inhibition is brought about by the lymphokine leucocyte inhibition factor (LIF) acting on polymorphonuclear leucocytes (7).

In this report we present results which throw doubt on the validity of this correlate in respect of hypersensitivity to diphtheria toxoid (DT) in man.

MATERIAL AND METHODS

Subjects

Blood for this study was obtained from healthy adult male volunteers. One of the five donors used in tests with tubercular antigen had received BCG at school (D.A.). B.R. had tuberculosis as a child.

All donors report that they received immuni-

sation against diphtheria in childhood except B.R., who had the disease, and J.O. A family contact of J.O. had diphtheria in 1947. The age of donors is given in Table 1.

Skin tests

Delayed hypersensitivity to tuberculin was assessed by intradermal injection of 0.2 µg of PPD (Evans Medical, Liverpool). Sensitivity to DT was assessed similarly by means of Schick control injections at the dose used for that test. J.O. received the control reagent distributed by Massachusetts Department of Health while other donors, in a later series, received Wellcome reagent. The latter also received the Schick test toxin to which they were uniformly negative, indicating the presence of circulating anti-toxin.

The injections were kindly carried out by Professor H. Valdimarsson or Dr. J. A. Lunn, the reactions being read both by them and by one of us (M.R.S.). The responses had the characteristics of delayed hypersensitivity. There were no reactions in the first 30 min. Reactions built up after 8 h, reaching a peak at

36–72 h. They were measured at 48 h in terms of induration and erythema. The positive reactions reported here were strong and declined slowly over several weeks.

The tests with PPD and the Schick control response in J.O. were carried out at least 1 year before *in vitro* experiments. The other DT skin tests were performed at the end of the study.

Lymphocyte transformation test

This was carried out on microplate cultures of blood leucocytes as described previously (8). Antigens used were PPD and DT. PPD, a freeze-dried preparation of human tuberculin (Ministry of Agriculture, Weybridge), was dissolved in culture medium and diluted as required. DT (diphtheria toxoid from Wellcome-BJGL 736) was dialysed exhaustively against phosphate-buffered saline before dilution in culture medium. Transformation index is the ratio of incorporation of tritiated thymidine after 6 days of culture in the presence of antigen to that occurring in the absence of antigen.

Leucocyte migration agarose test

The procedures used were based on those for the “direct” agarose test as set out by Bendixen et al. (2).

Heparinised blood (10 i.u./ml) was subjected to dextran sedimentation (8) and the leucocytes

obtained were washed three times in Medium 199 (Gibco Europe-115EE) containing 20 mm HEPES by centrifugation at 200 *g* for 10 min at room temperature. The cells were resuspended at 7 × 10⁶/ml of medium and 1 ml aliquots were dispensed into conical plastic tubes (100 × 16 mm, Sterilin). Following centrifugation, the cells were taken up in 30 µl of medium supplemented further with heat-inactivated horse serum (10 %) and penicillin and streptomycin (30 i.u. of each/ml) and with antigens (see above) as required. The cells were incubated at 37°C for 30 min in an atmosphere of 5 % CO₂ before transfer to wells in the agarose plates, which were then placed in the incubator for 16 h.

Plates were of 50 mm diameter (Sterilin) and contained 5 ml agarose (BDH-33006). Agarose was dissolved at 1 % in Medium 199 (Gibco Europe-118H) supplemented with sodium bicarbonate (2 g/l) and with HEPES, antibiotics and horse serum as before. The well diameter was 2 mm.

After the migration period, plates were flooded with 5 % glutaraldehyde for 2 h. The agarose was then lifted off the plate leaving the fixed cells adhering to the plastic. The cells were stained in Giemsa and the plates were left inverted to dry. The area of migration was measured by planimetry following projection onto paper and includes that of the well. Migration in the absence of antigen stimulation was in the range of 6–10 times the area of the well. Reading of the plates was carried out “blind”.

Table 1
Delayed hypersensitivity and lymphocyte transformation responses of cell donors

Cell donor (age)	Skin reaction (mm)		Transformation index	
	Mantoux	Schick control	PPD	DT
A.L. (33)	–	0/0	–	1.1
B.R. (47)	30/90	–	44.5, 64.4	1.0, 1.4
D.A. (22)	–	–	9.8, 11.6	–
J.O. (46)	–	65/>100	16.9, 8.1	30.7, 75.8
M.A. (21)	–	0/0	2.9, 0.8	2.5, 5.1
M.Y. (43)	–	0/0	–	1.1
V.O. (49)	10/50	0/0	338, 219	2.9, 0.8

Refer to Methods section for further information on the skin tests carried out. Skin test measurements are given at 48 h as induration/erythema. The erythematous reaction of J.O. to DT affected most of the volar surface of the forearm. Transformation results at 1 µg/ml (PPD) and 2 Lf/ml (DT) are shown from either one or two experiments.

In all experiments five plates were used each containing up to eight wells. The various groups were represented by one well on each plate. Mean areas were calculated as the root mean square and the migration index is the ratio of the mean area obtained in the presence of antigen to that obtained in its absence. The significance of differences between control and test groups was determined by Student's t-test on the square root transformed values.

RESULTS

The sensitivity of donors to PPD and DT expressed as skin test delayed hypersensitivity and/or lymphocyte transformation is shown in Table 1. In the tuberculin system both tests

show strong reactivity in B.R. and V.O. The transformation values for J.O. and D.A. are consistent with moderate skin sensitivity (8). J.O., who was extremely sensitive to DT in the skin, was also highly sensitive in transformation. The transformation results quoted were obtained at 2 Lf/ml, a concentration of DT giving optimal or near-optimal response, but only a 50% reduction in index was observed with J.O. on reducing the concentration to 0.02 Lf/ml. The other donors all showed zero or weak transformation response to DT and this is in keeping with their negative skin test data.

Leucocyte migration inhibition induced by PPD was broadly as expected from the lymphocyte transformation and skin test data (Table 2). Optimum PPD concentration for inhibition was 20 µg/ml. Donors B.R. and V.O. showed clear inhibition at this and other concentrations. The expected moderate responsiveness of D.A. and J.O. was revealed in the case of the former donor, but J.O. joined M.A. in showing negative response.

No similar relationship between the transformation or skin test data and leucocyte migration inhibition was observed for DT (Table 3). Our strong DT reactor (J.O.) showed significant inhibition in only one out of five experiments (mean index at 60 Lf/ml = 0.92), whereas M.A. (weak in transformation) and A.L. (negative) showed inhibition in two of four (mean index, 0.83) and two of two (mean index 0.71) experiments respectively. Other donors -

Table 2
Leucocyte migration inhibition in the presence of PPD

Cell donor	Migration index at different levels of PPD		
	10 µg/ml	20 µg/ml	40 µg/ml
B.R.	0.76*	0.76*	0.81
D.A.	0.98	0.79*	0.92
J.O.	0.93(A)	0.93(A), 1.01(B)	-
M.A.	0.99(A), 1.13(B)	0.95(A), 1.25(B)	1.19(B)
V.O.	0.78*	0.44**	0.51**

Where results for a particular donor were obtained from more than one experiment the individual experiments are indicated by letter.
Significant effects of antigen are indicated as follows: *, $P < 0.05$; **, $P < 0.01$.

Table 3
Leucocyte migration inhibition in the presence of DT

Cell donor	Migration index at different levels of DT			
	10 Lf/ml	30 Lf/ml	60 Lf/ml	90 Lf/ml
A.L.	0.83(A), 0.95(B)	0.76*(A)	0.72*(A), 0.71*(B)	-
B.R.	-	0.98	0.97	0.88
J.O.	1.04(A), 0.97(B) 1.00(C)	0.99(A), 0.89(D) 0.97(E)	0.88(A), 1.03(B) 0.77*(C), 0.97(D) 0.94(E)	0.95(A), 0.95(D)
M.A.	0.79**(A)	1.05(B), 0.89(C) 0.92(D)	0.71**(A), 0.92(B) 0.77**(C), 0.93(D)	0.96(D)
M.Y.	-	0.87	0.88	-
V.O.	-	1.21**	1.05	0.99

For notes see Table 2.

negative or weak in transformation – were tested once; no inhibitions were observed but there was one significant enhancement. Whereas the optimum DT concentration for inhibition appeared to be 60 Lf/ml, it seemed possible that the exceptionally high sensitivity of J.O. might only be revealed at much lower antigen levels. However, in further experiments testing 10-fold dilutions of DT down to 0.00006 Lf/ml, no evidence of inhibition was obtained.

DISCUSSION

It is difficult to explain the failure to show clear evidence of DT-induced migration inhibition in respect of donor J.O. who was highly sensitive to DT by skin test and in the transformation assay. One possibility is that at this level of sensitivity factors other than LIF are being produced by the cells in quantities sufficient to interfere in the assay. On the other hand, as LIF may not be a necessary participant in the development of a delayed hypersensitivity response, it could be that this donor has a defect in its production or action. This view is consistent with the observed lack of inhibition of donor J.O. in the tuberculin system. It should be recorded that J.O. is a healthy carrier for the autosomal recessive condition known as Friedreich's ataxia, but immunological abnormalities have not been reported in such patients (1).

A second problem arising from these data is the DT-induced inhibition that was seen in the case of two donors showing zero/weak skin or transformation reactivity (A.L., M.A.), and the solution here may lie in the ability of B lymphocytes or T lymphocytes not directly involved in delayed responses to produce LIF (5, 6). Immunisation with DT, in contrast to BCG, is associated primarily with antibody production. Moreover, significant levels of anti-DT antibody persist throughout adult life in at least 50% of the immunised population (3) due presumably to repeated re-stimulation by cross-reacting environmental antigens. This is a situation in which cells capable of responding in specific antibody production might be expected to be present from time to time in the blood (9, 10).

It is known that LIF production can proceed in the absence of cell proliferation (4).

These results, though based on a small group of donors, question the suitability of the direct leucocyte migration test for assessing cellular reactivity to DT. Further studies on LIF production to this antigen with a larger panel of donors would be of interest.

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