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Temporary Suppression of Lymphocyte Transformation after Tuberculin Skin Testing

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Summary. Sixteen normal subjects were studied to see if a tuberculin skin test (Mantoux) would induce changes in their cell-mediated immune response (CMIR) as determined by a lymphocyte transformation test (LTT). In ten Mantoux-positive persons, a significant suppression of the responses to stimulation with both purified protein derivative (PPD) and phytohaemagglutinin (PHA) occurred. The suppression was seen within a week of the skin test. In three Mantoux-negative persons, an increase was seen in the response to stimulation with PPD, together with a significant suppression of the response to stimulation with PHA. Antibodies to PPD were found in low titres, but the titres did not change during the period of suppression of CMIR. The results show that a transient suppression of CMIR can be induced in normal individuals by the application of a small dose of antigen.

INTRODUCTION

It is known that the cell-mediated immune response (CMIR) is changed in certain diseases and under certain therapeutic procedures. A typical example of this is the temporary suppression or disappearance of the tuberculin reaction (Brody, Overfield, and Hammes, 1964). These *in vivo* observations have been followed by findings of suppression of the transformation of lymphocytes to stimulation with phytohaemagglutinin (PHA) in several diseases. Some investigators tend to regard this as an intrinsic defect in the lymphocytes (Al-Sarraf, and Vaikevicius, 1971; Hagen, Frøland, and Weberg, 1972), while others have suggested that serum or plasma may contain factors that suppress the response to PHA stimulation (Edwards, Rowland, and Lee, 1973; Gatti, 1971; Steward, 1973; Whittaker, Rees, and Clark, 1971). The finding of impaired CMIR carries important implications with regard to the pathogenesis of malignant diseases and infections of viral or fungus origin.

We have been interested to study whether exposure to antigen would induce changes in an existing CMIR in normal persons. We used a well-known CMIR, i.e. that observed in skin testing with purified protein derivative (PPD), and assessed the CMIR in the persons concerned by the lymphocyte transformation test (LTT). Blood was drawn for the LTT just before, and at various intervals after, a skin test with PPD. The LTT were performed on exactly the same lines on the individual days of blood sampling.

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MATERIALS AND METHODS

A total of sixteen healthy subjects, twelve men and four women, were studied. Their ages ranged from 20 to 44 years and averaged 29·8 years. None of them were under any kind of medical treatment.

In Denmark, BCG vaccination is given to all normal persons at a young age. A group of ten persons (Group A) knew that they had a positive skin test to PPD (Mantoux). Three persons (Group B) believed that they were negative responders. All three had previously received BCG vaccination on two or three occasions without developing a positive Mantoux reaction. After the skin test in the investigation, they all showed a positive Mantoux reaction, but, in contrast to the persons in Group A, the reaction did not become positive until after 24 hours. Group C consisted of three persons, of whom only one had been BCG-vaccinated (U.H.). He was tested with 1 tuberculin unit (TU) on each forearm and gave a completely negative reaction at both sites. He therefore participated as a Mantoux-negative person. One week later he showed a positive response to the injection of 1 TU given at the start of the investigation.

Skin testing

The standard solution of purified protein derivative (PPD RT 23 with 0·005 per cent Tween 80) from Statens Seruminstitut, Copenhagen, was used. A volume of 0·1 ml containing 1 TU equivalent to 0·02 μg of PPD was injected intradermally on the extensor side of the forearm. Readings were done at 24 and 72 hours. An induration of 5 mm or more in diameter was considered a positive reaction.

Lymphocyte transformation test (LTT)

Blood was taken for the LTT just before the skin testing (day 0) and then on days 1, 3, 7, 17 and 21. Approximately 30 ml of venous blood was drawn into a test tube containing phenol-free heparin in order to produce a final concentration of 20 i.u./ml. The blood was diluted 1:1 with TC-199 containing 5 per cent normal inactivated human AB Rh-positive serum and heparin 20 i.u./ml). An amount of 7–8 ml of diluted blood was placed on 3 ml of a mixture of Isopaque–Ficoll (Böyum, 1968). The tubes were centrifuged at 400 ***g*** at room temperature for 30 minutes, and the mononuclear cells could then be withdrawn. The cells were washed twice in TC-199 with 5 per cent serum as before. The cell suspension was diluted in TC-199 with 15 per cent pooled normal, inactivated human AB Rh-positive serum without heparin to a concentration of 1×10^6 lymphocytes per millilitre. Portions of 2·0 ml of this solution were placed in culture tubes, 15 × 150 mm. PPD (RT 32) supplied by Statens Seruminstitut, Copenhagen, and purified phytohaemagglutinin (PHA-P, Wellcome MR 68) were added in concentrations of 1 μg and 10 μg/ml. In some experiments an extract from *Candida albicans A* was added to the cultures in a concentration of 1 and 10 μl/ml. The culture tubes were incubated in a water bath at 37°. The pH was adjusted with HEPES buffer, 0·05 M, in the medium. Cultures were made in triplicate or, if only a few cells were available, in duplicate. The duration of culture was 5 days for PPD-stimulated and *C. albicans*-stimulated cultures, and 3 days for PHA-P-stimulated cultures. At 24 hours before harvesting of the cultures, 0·18 μCi of [2-^{14}C]thymidine (specific activity > 50 μCi per mM) was added, and harvesting was performed using a modification of a semi-automatic multiple-sample processor (Harzmann, Bach, and Bach, 1972). The cells were collected on Whatman glass-fibre filters (GF/83) and washed with normal saline. The filters were then transferred to plastic counting vials to which

10 ml of dioxan scintillation fluid was added. The samples were counted in a Tri-Carb scintillation counter, and the results shown as disintegrations per minute (d/min) after correction for quenching and efficiency. The background activity was not subtracted. A positive reaction was defined as a result higher than twice the control value.

Haemagglutination test

Sera were examined for antibodies against PPD using tanned sheep red blood cells (SRBC) (Boyden, 1951). After absorption with SRBC, the sera were titrated in a microtitre system with doubling dilutions, starting with a 1:10 dilution.

RESULTS

In Group A, all ten subjects developed a positive Mantoux reaction within 24 hours. In Group B (three subjects), the Mantoux reaction became positive only after 24 hours (slow responders). In Group C (three subjects), two were completely negative, while one (U.H.) developed a positive Mantoux reaction, although he had been a completely negative responder 1 week earlier.

The results from stimulation with PPD and PHA-P at concentrations of 10 μg/ml are shown in Tables 1 and 2, respectively. The results from control cultures are shown in Table 3. A statistical analysis of the results using a two-way analysis of variance was done. The results are given in Table 4.

TABLE 1

RESULTS FROM LYMPHOCYTE TRANSFORMATION TESTS (LTT) ON DIFFERENT DAYS AFTER A SKIN TEST WITH 1 TU. THE CULTURES WERE STIMULATED WITH PPD (10 μg/ml)

Group	Subjects	Day 0	Day 1	Day 3	Day 7	Day 17	Day 21
A*	J.V.F.	15·0	0·7	5·5	8·1	n.d.§	19·7
	B.T.	38·3	0·6	0·2	27·6	66·2	n.d.§
	P.A.	7·0	7·1	1·1	0·1	3·7	18·9
	T.H.	24·8	0·3	9·3	0·1	10·7	13·0
	S.H.	7·4	1·1	2·3	0·2	11·8	16·2
	K.T.P.	21·9	11·5	10·2	0·2	0·3	28·3
	P.B.	11·5	7·7	8·8	8·4	2·0	3·6
	C.C.	21·9	18·7	17·8	27·4	23·5	13·9
	K.M.	18·0	13·0	13·0	12·8	21·8	4·5
	S.M.	45·3	17·8	19·0	26·2	30·0	21·0
	$\bar{x} \pm$ s.e.	21·1 ± 12·6	7·9 ± 7·2	8·7 ± 6·6	11·1 ± 11·9	18·9 ± 20·5	15·5 ± 7·9
B†	K.J.	5·8	5·1	19·2	9·3**		
	E.J.	3·3	11·0	7·5	6·5**		
	A.J.M.	12·2	8·1	11·4	9·9		
	$\bar{x} \pm$ s.e.	7·1 ± 4·6	9·6 ± 2·1	12·7 ± 6·0	8·6 ± 1·8		
C‡	S.G.	n.d.¶	2·6	10·8	6·1		
	M.M.	n.d.¶	1·5	3·4	3·1**		
	U.H.	5·4	17·6	25·3	23·4		
	$\bar{x} \pm$ s.e.	5·4	7·2 ± 9·0	13·2 ± 11·1	10·9 ± 11·0		

Results are expressed as mean disintegration per minute $\times 10^{-3}$ per 2×10^6 lymphocytes.

* Group A = Mantoux-positive persons.
† Group B = Mantoux-positive persons (slow responders).
‡ Group C = Mantoux-negative responders.
§ Not done.
¶ Not done due to infection of cultures.
** Estimated values.

TABLE 2

RESULTS FROM LTT ON DIFFERENT DAYS AFTER A SKIN TEST WITH 1 TU. THE CULTURES WERE STIMULATED WITH PHA-P (10 μg/ml)

Group	Subjects	Day 0	Day 1	Day 3	Day 7	Day 17	Day 21
A*	J.V.F.	79·0	79·4	88·1¶	107·9	n.d.§	145·5
	B.T.	92·5	24·7	70·6	73·9	126·3	n.d.§
	P.A.	118·9	126·8	89·1	104·3	107·4	101·6
	T.H.	125·3	112·1	112·6	147·6	127·2	108·6
	S.H.	111·7	87·9	100·6	139·9	90·3	86·1
	K.T.P.	75·9	56·7	67·4	87·7	82·6	104·3
	P.B.	79·7	86·1	27·1	89·9	67·0	58·8
	C.C.	72·1	81·6	73·6¶	68·9	41·1	50·2
	K.M.	116·7	98·0¶	97·7	102·9	121·7	94·9
	S.M.	95·1¶	105·3	52·7	91·5	84·3	111·2
	$\bar{X} \pm$ s.e.	96·7 ± 20·0	85·9 ± 29·0	78·0 ± 25·3	101·5 ± 25·6	94·2 ± 29·3	95·7 ± 28·5
B†	K.J.	81·4	44·1	89·4	60·6¶		
	E.J.	99·2	11·9	39·1	38·9¶		
	A.J.M.	106·0	33·2	23·5	43·2		
	$\bar{X} \pm$ s.e.	95·5 ± 12·7	29·7 ± 16·4	50·7 ± 34·4	47·6 ± 11·5		
C‡	S.G.	95·5	79·8	79·0	133·8		
	M.M.	99·2	56·7	49·7	102·6¶		
	U.H.	123·7	75·8	101·1	119·2		
	$\bar{X} \pm$ s.e.	106·1 ± 15·3	70·8 ± 12·3	76·6 ± 25·8	118·5 ± 15·6		

Results are expressed as mean disintegrations per minute × 10^{-3} per 2×10^6 lymphocytes.

* Group A = Mantoux-positive persons.
† Group B = Mantoux-positive persons (slow responders).
‡ Group C = Mantoux-negative responders.
§ Not done.
¶ Estimated values.

In Group A, there was a significant suppression of the LTT response to PPD and PHA-P in both concentrations used. This suppression was seen even on the 1st day after skin testing. At day 7, the results from the PHA-P-stimulated cultures had returned to the initial values, whereas this was seen after 17 days in the PPD-stimulated cultures. A different duration and degree of suppression was found for each person, but the pattern was consistent. Some exhibited a reduction of nearly 100 per cent in the LTT to PPD at 10 μg/ml.

TABLE 3

MEAN VALUES OF CONTROL CULTURES ON DIFFERENT DAYS AFTER A SKIN TEST WITH 1 TU

Group	Duration of culture (days)	Day 0	Day 1	Day 3	Day 7	Day 17	Day 21
A*	3	950 ± 480	600 ± 260	740 ± 400	630 ± 160	970 ± 590	825 ± 450
	5	1390 ± 870	1400 ± 730	1730 ± 1535	1180 ± 835	1500 ± 990	1235 ± 715
B†	3	470 ± 180	440 ± 310	850 ± 75	285 ± 65		
	5	350 ± 45	350 ± 135	1230 ± 815	800 ± 660		
C‡	3	850 ± 475	445 ± 275	695 ± 225	695 ± 320		
	5	340	885 ± 540	1090 ± 225	1070 ± 120		

Results are expressed as mean disintegrations per minute × 10^{-3} per 2×10^6 lymphocytes.

* Group A = Mantoux-positive persons.
† Group B = Mantoux-positive persons (slow responders).
‡ Group C = Mantoux-negative responders.

TABLE 4

A TWO-WAY ANALYSIS OF VARIANCE TEST OF VARIATION IN RESULTS FROM DAYS 0, 1, 3 AND 7, AFTER A SKIN TEST WITH 1 TU

Antigen/mitogen per ml	Group*		
	A	B	C
PPD (1 μg)	S, $P<0.01$	I, —	I, —
PPD (10 μg)	S, $P<0.01$	I, —	I, —
C. albicans (1 μl)	I, —	n.d.	n.d.
C. albicans (10 μl)	I, —	n.d.	n.d.
Control cultures	I, —	I, —	I, —
PHA-P (1 μg)	S, $P<0.05$	v.c. —	S. —
PHA-P (10 μg)	S, $P<0.05$	S, $P<0.05$	S, $P<0.01$
Control cultures	S, —	I, $P<0.05$	S, —

* S = suppression of responses in LTT; I = increase of responses in LTT; v.c. = variable changes; — = No significant change; n.d. = not done.

Four subjects showed very low responses on day 7, but there was no indication of a technical explanation of this. PHA values from these subjects were not affected. Normally, a culture period of 5 days gives maximum uptake of thymidine into lymphocytes stimulated with PPD in our system. For six subjects in Group A, PPD-stimulated cultures were also harvested after 3 or 4 days. This was done to find out if there was a change in the correlation between the duration of culture and maximum uptake of thymidine. Such a change was not found. In four subjects (Group A), the lymphocytes were stimulated with *C. albicans* antigen. The results in d/min showed a slight increase after skin testing, but the increase was not significant.

In Group B and Group C, the results showed an increase in the response to stimulation with PPD (Table 1). The response to stimulation with PHA-P showed a suppression, which was significant (Table 4). The subjects in Group B showed some variation in response. One of them (E.J.) seemed to be more in accordance with Group C, while the others (A.J.M., K.J.) showed changes like those seen in Group A. In Group C, one subject (U.H.) had a strongly positive response to PPD in a LTT. This was probably due to the skin test performed 1 week earlier, to which his Mantoux reaction had been negative.

The subjects in Group A were investigated for haemagglutinating antibodies to PPD. Only titres of 1 : 10 and 1 : 20 were found, and some showed negative results. No changes in titre occurred during the period of observation.

DISCUSSION

The results indicate that changes in CMIR of short duration can be induced by the application of a small dose of antigen (1 TU) to the skin. It appears from Table 4 that different patterns of changes exist. In Mantoux-positive persons a suppression of lymphocyte transformation occurred on stimulation with PPD. Conversely, in Mantoux-negative subjects an increase was observed. In all three groups, the response to PHA stimulation was significantly suppressed.

It is known from investigations in animals that induction of suppression of humoral and cellular immune responses can be induced by antibodies and antigens (Axelrad, 1968; Rowley, Fitch, Axelrad, and Pierce, 1969; Asherson, Zembala and Barnes, 1971; Heller

and Siskind, 1973; Diener and Feldmann, 1970). We have no reasons to believe that the content of antibodies in the serum could have induced the suppression observed. We did not find any sign of alterations in the antibody titres against PPD after the skin test in the subjects studied. Moreover, the observed suppression of lymphocyte transformation on PHA-stimulation does not agree with the specificity of antibody-mediated suppression.

It is an important question whether a skin test with PPD induces changes in the distribution of PPD-sensitive lymphocytes in the peripheral blood. Some investigations in animals using ^{125}I-labelled PPD have shown an increase in PPD-binding lymphocytes in peripheral blood after BCG vaccination and skin testing with PPD (Donald and Swanson Beck, 1971; Hjort, Beutner and Witebsky, 1968). A maximum of PPD-sensitive cells was found at the end of the 1st week after skin testing. The results obtained render it likely that blood drawn for the LTT after skin testing has a higher percentage of PPD-sensitive cells. Will these cells be able to respond to a second stimulation with PPD in a LTT, or are they blocked? Judging from our observation in one subject (U.H., Group C), it seems that they may under certain circumstances be able to respond. He was skin tested 1 week before the start of the study, exhibiting a completely negative reaction. He gave a fairly high response in d/min to PPD stimulation in the LTT as compared with the other subjects in the group (Table 1). Hughes (1968) found an increase in the number of blast cells in a LTT with PPD-stimulation in one normal, BCG-vaccinated person in whom a skin test with PPD (2 μg or 1000 TU) was done. The responses in the LTT were at a maximum 5 days after skin testing. His observations are in agreement with our results in Group B.

Other investigations are not in accordance with these findings. The application of an antigen to an animal previously sensitized to that antigen induces a temporary loss of recirculating, antigen-sensitive T cells, most pronounced during the 1st week after exposure (Jacobsson and Blomgren, 1973; O'Toole and Davies, 1971). Asherson and Barnes (1973) investigated DNA synthesis in mouse lymph node cells stimulated in cultures with an antigen which had been applied to the animals 12 days before. He found a distinct reduction in the synthesis of DNA. PHA stimulation of the cells was not done. These findings lend support to the suppression of lymphocyte transformation to PPD stimulation observed in Group A.

Recently, Dwyer and Kantor (1973) suggested that the CMIR is regulated by a feed-back mechanism involving suppressive factors excreted from stimulated lymphocytes. They found a non-specific, transient suppression of CMIR in guinea-pigs after antigen application to sensitized animals. Outteridge and Lepper (1973) demonstrated, both *in vivo* and *in vitro*, immunosuppressive factors released after tuberculin skin testing in cattle.

Our observations of changes in the responses to antigen and mitogen in the LTT after skin testing seem to be in agreement with the results of the authors just mentioned. The final outcome appears to be dependent on the existence of sensitivity of the person tested, and on the dose and nature of the antigen applied in cultures. The slight increase in the response from cultures stimulated with an extract of *C. albicans* and the increase seen in Groups B and C in PPD-stimulated cultures show that not all receptors on the lymphocytes are blocked.

The clinical implications of these findings are to be investigated. If repeated exposure to small doses of antigen is able to induce prolonged suppression of CMIR, this may be a contributory factor in the development of diseases in which the existence of CMIR is believed to play a role.

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