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Antigen specific lymphocyte activity *in vitro* by peripheral blood leucocytes from Mantoux positive and negative human beings

I. COMPARISON OF QUANTITATIVE AND QUALITATIVE DIFFERENCES IN THE PPD-SPECIFIC LYMPHOPROLIFERATIVE RESPONSE OF LYMPHOCYTES FROM THE TWO KINDS OF DONORS

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SUMMARY

Lymphocytes from some PPD (purified protein derivative from tubercle bacillus) skin test negative (Mantoux negative = Mx−) human beings reacted against PPD in the lymphoproliferative assay with a time course and dose response very similar to those of lymphocytes from Mantoux positive (Mx+) individuals. Other Mx− persons were PPD non-responsive in the lymphoproliferative assay.

The PPD response of (immunoglobulin = Ig) Ig anti-Ig column passed lymphocytes (T-cells) from Mx−/LP+ (LP+ = lymphoproliferative) persons was significantly reduced whereas the *in vitro* PPD response of T-lymphocytes from Mx+/LP+ was the same or increased. Purified B-lymphocytes from all kinds of tested individuals did not respond *in vitro* against PPD.

Serological investigations indicated that one of the reasons for the negative skin reaction of individuals whose lymphocytes gave a positive lymphoproliferative response against PPD *in vitro*, is that such individuals had recirculating PPD of high molecular weight (>900,000) and/or PPD anti-PPD antibody complexes in the serum. These substances could block the PPD-specific T-lymphocytes.

INTRODUCTION

The *in vivo* skin test has been used frequently both experimentally and clinically to ascertain the cell-mediated immune responsiveness of normal human beings and patients with a variety of diseases (Rocklin, Rosen & David, 1970). However, this test suffers from the disadvantage of sensitizing the subject (Brody, Overfield & Hammes, 1964; Thestrup-Petersen, 1974). Lymphocyte proliferation and production of migration inhibitory factor have been shown to provide *in vitro* models of cellular immunity and these *in vitro* assays have the advantage that the subject need not be sensitized before the assay (Dutton & Eady, 1934; Mills, 1966; Borel, David & Schlossmann, 1970; Rocklin *et al.*, 1970; Hanna, Ferraresi & Leskowitz, 1973; Rubin & Wigzell, 1974; Chaparas, Good & Janicki, 1975). Most of the Scandinavian population has been vaccinated against the BCG (Bacillus Calmette-Guérin) and generally

Abbreviations used in this paper: B-cells, non-thymus processed lymphocytes; BCG, Bacillus Calmette-Guérin; BSS, balanced salt solution; CRBC, chicken red blood cells; E-cells, cells forming spontaneous rosettes with SRBC; EA, sheep erythrocytes coated with rabbit 7S anti-SRBC antibodies; EAC, SRBC coated with rabbit, anti-SRBC antibodies and mouse complement; FCS, foetal calf serum; LP, lymphoproliferative; Mx, Mantoux; ORBC; Ox erythrocytes; PBL, peripheral blood leucocytes; PHA, phytohaemagglutinin; PPD, purified protein derivative from tubercle bacillus; PWM, pokeweed mitogen; RFC, rosette-forming cells; SRBC, sheep red blood cells; T-cells, thymus processed lymphocytes; YS3, yersinia enterocolitica serotype 3.

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peripheral blood leucocytes (PBL) from normal Scandinavians will show a PPD (purified protein derivative from tubercle bacillus) specific lymphoproliferative response *in vitro*. Furthermore, human PBL will respond *in vitro* by proliferation to various mitogens such as phytohaemagglutinin (PHA) and pokeweed mitogen (PWM). These mitogen responses have been used to establish functional characteristics of human T- and B-lymphocytes under normal and pathological circumstances (Meuwissen *et al.*, 1968; Douglas, Kamin & Fudenberg, 1969; Rocklin *et al.*, 1970; Wu, Lawton & Cooper, 1973; Chess, MacDermott & Schlossmann, 1974; Greaves, Janossy & Doenhoff, 1974).

We initiated a study on the cellular requirements for the *in vitro* proliferative response of human PBL against PPD. During the course of these studies we found PPD skin-test negative (Mantoux negative) human beings whose lymphocytes reacted 'normally' against PPD in the lymphoproliferative assay, i.e. the time course and dose responses of these lymphocytes were very similar to those of lymphocytes from Mantoux positive human beings (Blomgren, 1975; Nilsson, 1972). So far we have found four persons that were Mantoux negative and whose lymphocytes were PPD non-responsive *in vitro* (Mx−/LP−).

The Mantoux negative, lymphoproliferative positive (Mx−/LP+) persons were from the Tuberculin Department at Statens Seruminstitut. The three individuals might be hyperimmune to PPD, due to daily sensitization with PPD during manufacture. This observation questions whether the Mantoux test and the *in vitro* lymphoproliferative response against PPD always reveals similar immunological reactivities, i.e. whether both *in vivo* and *in vitro* assays of cellular immunity are autonomous T-cell responses, and thus can be used interchangeably as measurements of T-cell function, or whether they are merely T-cell dependent responses (Rubin & Wigzell, 1974; Blomgren, 1975; Bloom & Shevach, 1975).

Studies on guinea-pig lymphocytes have shown that the secondary antigen-specific lymphoproliferative response is performed by T-lymphocytes in an autonomous manner (Rubin & Wigzell, 1974; Hertel-Wulff & Rubin, 1976). However, whether B-lymphocytes can respond also to secondary antigen *in vitro* by proliferation, either alone or helped by other lymphocytes (e.g. T-lymphocytes or macrophages), is still an open question. Some reports have indicated that mouse, guinea-pig, chicken and human B-lymphocytes can proliferate *in vitro* (Elfenbein, Shevach & Green, 1972; Kichner *et al.*, 1972; Osborne & Katz, 1973; Blomgren, 1974), or participate in the proliferative response of mixed lymphoid cell populations (Mugraby, Gery & Sulitzeanu, 1974; Oppenheim, 1972), or even proliferate themselves provided that soluble T-cell factors are added (Blomgren, 1975).

The present report deals with our attempts to clarify the reason(s) for the negative skin-test of individuals whose lymphocytes gave a positive lymphoproliferative response against PPD *in vitro*.

MATERIALS AND METHODS

1. *Lymphocyte donors.* Healthy members of the laboratory staff of different departments at Statens Seruminstitut, of both sexes and aged 25–40 years, served as blood donors. All had been vaccinated with BCG and they were skin-tested for PPD reactivity before being used as donors (for the first time only).

2. *Lymphocyte preparations.* Freshly drawn venous blood was defibrinated by shaking for 10 min with glass beads. Lymphoid cells were separated by centrifugation on a Ficoll–Isopaque (FIP) gradient as described previously (Böyum, 1968; Jondal, Holm & Wigzell, 1972). The nucleated cells were suspended in either Eagles minimum essential medium F13 (Grand Island, N.Y.) or RPMI 1640 medium (Grand Island, N.Y.). T- and B-lymphocytes were separated by means of either rosette sedimentation (Jondal, 1974) or passage through Ig anti-Ig coated columns (Rubin & Wigzell, 1974). In short, lymphocytes were incubated with washed sheep erythrocytes (SRBC) in heat-inactivated (56°C for 1 hr) foetal calf serum (FCS). The ratio between lymphoid cells and SRBC was 1:10. The mixture was centrifuged at 200 *g* for 6 min, incubated first for 15 min at 37°C and then further at 4°C for 45 min. Then, the cell pellet was carefully suspended and centrifuged on a FIP gradient. The cells forming spontaneous rosettes with SRBC (E-cells) sedimented, whereas other cells stayed in the fluid interface. The former cells, after lysis of SRBC with 0·84% ammonium chloride, will be called E-cells and the latter cells will be called B-cells. Lymphoid cells to be passed through an Ig anti-Ig column were adjusted to a cell concentration of 5×10^6 to 2×10^7/ml and they were passed through the column at room temperature at a flow rate of 1–2 ml/min. The passed cells will be called T-cells.

3. *Determination of the frequency of T- and B-cells.* The frequency of T-cells was determined by their capacity to form spontaneous rosettes with SRBC as described above, except that the cell pellet was incubated at 4°C overnight before rosette

counting. B-cells were identified by means of two different rosette techniques: (1) The EA-RFC (rosette forming cell) assay, i.e. lymphocytes forming rosettes against SRBC or ox erythrocytes (ORBC) coated with rabbit 7S antibodies. Lymphoid cells and EA complexes were mixed at a ratio 1:10 and immediately centrifuged at room temperature for 4 min at 200 *g*. Cells which had bound three or more EA complexes to their surface were scored as RFC. (2) The EAC-RFC assay, i.e. lymphocytes forming rosettes against SRBC or ORBC, coated with rabbit 19S antibodies and C5 deficient mouse serum. Lymphoid cells and EAC-complexes were mixed at a ratio of 1:10, incubated for 30 min at 37°C, centrifuged for 4 min at room temperature at 200 *g* and resuspended vigorously with a Pasteur pipette before RFC counting.

4. *Antigens and mitogens.* Purified protein derivative (PPD) was kindly provided by the Tuberculin Department (lot. no RT 32, 1 mg/ml). The fractionation diagram of this PPD on a Sephadex G 200 column can be seen in Fig. 1. Yersini

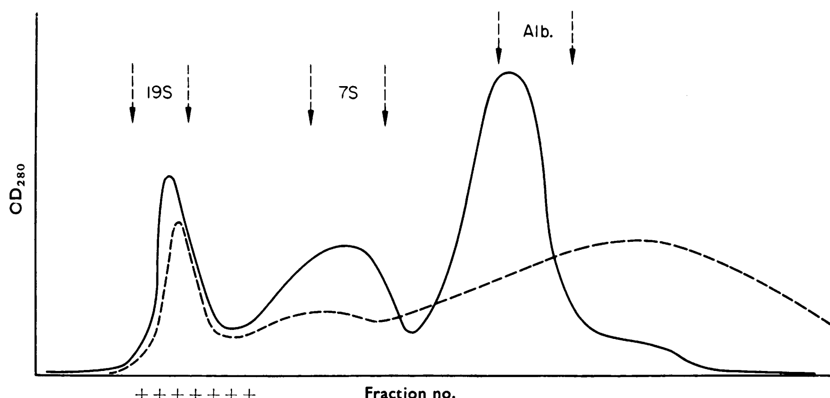

FIG. 1. Sephadex G200 fractionation diagram of normal human serum (full line) and of our preparative PPD (dotted line). Elution buffer = BSS. Crosses under the abscissa indicate PPD activity in serum from Mx− /LP+ individuals. 19S = fractions concentrated to give a 19S serum pool; 7S = fractions concentrated to give a 7S serum pool, Alb = fractions concentrated to give an albumin serum pool.

enterocolitica antigen, serotype 3 (YS3) was kindly provided by the Diagnosis Department, Statens Seruminstitut and 1:20 dilutions were made from stock solution (standard 8). Phytohaemagglutinin (PHA) was obtained from Difco Laboratories (lot. no. 3110-57). Freezed-dried PHA was reconstituted with 5 ml of distilled water and 1:10 dilutions were made from the 5 ml stock solution. Pokeweed mitogen was obtained from Gibco Laboratories (lot. no. 536). Freezed-dried PWM was reconstituted with 5 ml of distilled water and 1:10 dilutions were made from the stock solution.

5. *Lymphoproliferative assay.* Lymphocytes were cultured in plastic tubes (Falcon Cat. no. 2001) in a volume of 1 ml of 10% human AB serum in F13 Eagles or RPMI 1640 medium, supplemented with 2 mM glutamine, 100 i.u./ml of penicillin and 10 μg/ml of Gentamycin. Antigens, mitogens or cells were added to the tubes in 0·1 ml volumes and the cultures were incubated for 2–8 days at 37°C/5% CO_2 atmosphere, the last 18 hr in the presence of 1 μCi of ^{3}H-labelled thymidine (The Radiochemical Centre, Amersham, England, Cat. no. TRA 120). Uptake of radioactivity was measured by liquid scintillation counting (Hertel-Wulff & Rubin, 1976), after cell harvest on a semiautomatic harvester (Scantron, Norway). Mean radioactivity, expressed as counts per minute (ct/min) was determined from triplicate cultures by calculating the geometric means. Isotope uptake obtained in cultures without antigen or mitogen were subtracted from values obtained in the corresponding cultures with antigen or mtiogen. (Δcpm) represented radioactivity attributable to the lymphoproliferative response.

6. *PPD-specific haemagglutination assay.* The haemagglutination assay and the haemagglutination inhibition assay were carried out in microtitre plates as described previously (Rubin & Wigzell, 1974). Titration medium = 1% normal rabbit serum in balanced salt solution (BSS). PPD was coated to sheep or chicken erythrocytes by means of carbodiimide as described previously (Golstein *et al.*, 1973).

7. *Antisera and their fractionation.* Sera from persons whose lymphocytes were tested, were heat-inactivated (56°C for 30 min) and sterile-filtered through Millipore filters. They were separated into 19S, 7S and albumin fractions by passage through Sephadex G200 and then concentrated to original volumes by negative pressure dialysis (Rubin, 1971). PPD was coated to Sepharose 4B beads by means of cyanogen-bromide as described previously (Rubin & Wigzell, 1974). Antisera or their fractions to be adsorbed for anti-PPD activity were allowed to penetrate the gel, incubated for 30 min at room temperature and then eluted with BSS (Rubin, 1971).

RESULTS

1. *Methological considerations*

Firstly, we investigated optimal conditions for our *in vitro* proliferative assay. We found that a cell

concentration of 10^6/ml was optimal, $2–5 \times 10^5$ cells/ml was suboptimal and 10^5 cells/ml was the lower limit of the assay. Optimal antigen or mitogen concentrations were also investigated and the following values were found optimal PPD=10 µg/ml, YS3=1:200 dilution, PHA=1:100 dilution, PWM=1:50 dilution. Time course experiments showed that most lymphocyte suspensions tested had optimal antigen-specific responses on days 5–7, optimal PHA response on day 4. The PWM response showed a biphasic curve as found by others (Zeylemaker *et al.*, 1974). The early peak (on day 3) seems to be a specific T-cell response, whereas the late peak (on day 6) seems to be a B-cell dependent response Greaves *et al.*, 1974; Zeylemaker *et al.*, 1974; see Table 1). Thus, since the PHA response has been

TABLE 1. Effect of E-RFC-gradient centrifugation on the PHA and PWM responses of human peripheral blood lymphocytes (Δct/min)*

		E-RFC separated	
Mitogen	Non-separated	Interface	Pellet
PHA	26,100	400	27,600
PWM (day 3)	36,100	700	24,500
PWM (day 6)	37,300	18,500	19,100

* Results as expressed as Δct/min. See Materials and Methods.

shown to be an autonomous T-cell response (Chess *et al.*, 1974; Greaves *et al.*, 1974; Høier-Madsen & Rubin, 1974), and the day 6 response against PWM would seem to be a B-cell dependent response, we have used these two mitogenic responses as functional markers for T- and B-lymphocytes, respectively, in the cell separation studies to be described.

2. *Initial experiments with lymphocytes from Mantoux positive and negative persons*

During the selection of lymphocyte donors we discovered that most of the people working in the Tuberculin Department were Mantoux negative. However, when testing the PPD-specific lymphoproliferative response of these individuals, it was observed that their lymphocytes responded against PPD *in vitro* as well as, or sometimes faster than, lymphocytes from normal Mantoux positive donors. There were no significant differences in the *in vitro* response against YS3, PHA or PWM of lymphocytes from the two types of donors. The frequency of T-cells and B-cells was also very similar among the two types of donors. Thus it would seem that the *in vitro* reactivity and the *in vivo* unresponsiveness of persons from the Tuberculin Department was a specific phenomenon.

3. *Are serum factors responsible for the PPD negative skin test?*

Sera from twenty-six normal Mx+/LP+ persons, from four Mx−/LP− persons and from the three Mx−/LP+ persons were assayed for content of anti-PPD antibodies (haemagglutination) and for content of free PPD (haemagglutination inhibition) using a hyperimmune rabbit anti-PPD antiserum as positive control serum. Only Mx+/LP+ persons had anti-PPD antibodies in their serum (mean $\log_2$ titre$=3{\cdot}1\pm0{\cdot}9$ (s.d.=one standard deviation)) and these antibodies were of the 19S antibody class. However, the three Mx−/LP+ individuals had PPD in their serum in concentrations of 3·5 µg/ml ±1°7 (SD). Since normal Mx+/LP+ individuals do contain anti-PPD antibodies, the Mx−LP+ persons may have their serum-PPD in the form of PPD anti-PPD complexes, a proposal supported by the fact that all serum-PPD is found in the 19S fraction of a Sephadex G200 diagram (Fig. 1). Sera from Mx+/LP+ and Mx−/LP+ persons were tested also for their effect on the lymphoproliferative response of autologous or of 'allogeneic' lymphocytes. In order to ascertain that anti-PPD antibodies would have a specific effect on the PPD-specific lymphoproliferative response (Oppenheim, 1972), we tested our control rabbit anti-PPD antiserum ($\log_2$ titre against PPD-CRBC$=9{\cdot}0$) added to cultures of

TABLE 2. Effect of rabbit anti-PPD antibodies on the *in vitro* lymphoproliferative response against PPD*

Serum†	Stimulants					
				μg PPD per culture		
	C	PHA	YS3	0·1	1·0	10
Normal	544	5550	3167	7	1716	2882
Anti-PPD	691	4851	2600	2732	3416	4834
1:100	—	(88)	(82)	—	(199)	(168)
Anti-PPD	702	4175	2329	1520	3419	4406
1:2000	—	(75)	(75)	—	(199)	(153)

* Results are expressed as Δcpm. See Materials and Methods. Figures in parentheses indicate percentage Δcpm of experimental values in relation Δct/min from cultures with control serum.
† Rabbit anti-PPD antiserum had a $\log_2$ titre against PPD-CRBC=9·0 (1:512).

TABLE 3. Lymphoproliferative response of lymphocytes from MX−/LP+ persons in different concentrations of autologous serum*

Serum dilution	T.C.			J.M.		
	C	PHA	PPD	C	PHA	PPD
10% AB-serum which does not contain PPD-antibodies	4421	71,310	64,982	2715	92,057	35,017
1:10	8003	123,326	112,615	5305	108,264	36,947
	(190)	(172)	(172)	(195)	(117)	(106)
1:1000	3993	93,600	87,483	3991	101,349	51,076
	(90)	(131)	(134)	(147)	(110)	(90)

* See Table 2.

TABLE 4. Lymphoproliferative response of lymphocytes from MX+/LP+ in different concentrations of serum from MX−/LP+ persons*

Serum dilution†	J.H.D.			E.O.K.		
	C	PHA	PPD	C	PHA	PPD
Serum from T.C. (Mx−/LP+)	1727	19,853	54,610	617	29,067	21,711
1:10	3591	16,965	79,251	1158	18,695	22,568
	(208)	(85)	(145)	(188)	(65)	(103)
1:100	3076	14,661	26,781	756	12,980	22,909
	(178)	(74)	(49)	(123)	(45)	(106)
1:1000	1767	12,882	62,757	814	14,613	17,744
	(102)	(65)	(115)	(132)	(50)	(82)

* See Tables 2 and 3.

lymphocytes from Mx+/LP+ or Mx−/LP+ persons. Table 2 shows that rabbit anti-PPD antibodies have a specific augmenting effect on the PPD-specific lymphoproliferative response. On the other hand, serum from Mx−/LP+ individuals would seem to have no or only a slightly non-specific augmenting

effect on the lymphoproliferative response of lymphocytes from both Mx−/LP+ and Mx+/LP+ individuals (Tables 3 and 4). Sera from Mx+/LP+ persons had no detectable effect on the lymphoproliferative response. The effect of rabbit anti-PPD antiserum, but not of sera from Mx+/LP+ or Mx−/LP+ individuals, on the *in vitro* PPD-specific response, could be removed by adsorption onto a PPD-Sepharose column. Furthermore, the active molecules in the rabbit anti-PPD antiserum were situated in the Sephadex G200 7S fraction, whereas the active principle in the sera from Mx−/LP+ persons was situated in the 19S fraction. Thus, it would seem that the factor(s) which influences the *in vivo* reaction of PPD-specific T-lymphocytes is recirculating PPD of the 19S class and/or PPD anti-PPD antibody complexes.

4. *Are T-lymphocytes the responding cells in the PPD-specific lymphoproliferative response of lymphocytes from Mantoux positive and negative persons?*

Next we investigated whether T-lymphocytes were the responding cells in the PPD-specific lymphoproliferative response of lymphocytes from Mx+/LP+ and Mx−/LP+ persons. Seven experiments with five different Mx+/LP+ donors were performed where *in vitro* PPD, PHA and PWM responses were determined before and after passage through Ig anti-Ig coated columns. The control proliferative responses against T-cell mitogen PHA and 'B'-cell mitogen PWM functioned according to expectation (Table 5). The frequency of E-RFC was increased and the frequency of EAC- and EA-RFC was very

TABLE 5. Effect of Ig anti-Ig column passage on the PHA and PWM responses of human peripheral blood lymphocytes (Δct/min)

Nitrogen	Non-passed	Ig anti-Ig passed*
PHA	34,700	37,800
PWM (day 3)	36,600	33,380
PWM (day 6)	38,900	17,300

* Cell recovery after column passage=62·8%.

TABLE 6. Effect of Ig anti-Ig column passage on the proportion of T and B cells from peripheral blood lymphocytes (% RFC±SE)

RFC assay	Cell type Tested	Non-passed	Ig anti-Ig* Passed
E-RFC	T	67·8±2·50	85·8±2·05
EA-RFC	T, B, K	21·6±1·96	0·50±0·09
EAC'-RFC	B	23·5±2·48	0·84±0·20

* Mean recovery after column passage=59·3%±6·1 (one standard error of the mean).

significantly reduced. This holds true for lymphocytes from both Mx+/LP+ and Mx−/LP+ persons (Table 6). However, when it comes to the PPD responses differences emerge. In all seven experiments with lymphocytes from Mx+/LP+ persons the PPD response was the same or increased following Ig anti-Ig column passage (Fig. 2) whereas the *in vitro* PPD response of lymphocytes from Mx−/LP+ persons after Ig anti-Ig column passage was significantly reduced (Fig. 2). Since also macrophage are very efficiently depleted by the Ig anti-Ig columns (Hertel-Wulff & Rubin, 1976; Sønderstrup, Rubin & Freisleben-Sørensen, 1976) we would conclude that the *in vitro* PPD response of T-lymphocytes from Mx+/LP+ persons could be classified as an autonomous T-cell response. However, the *in vitro* PPD

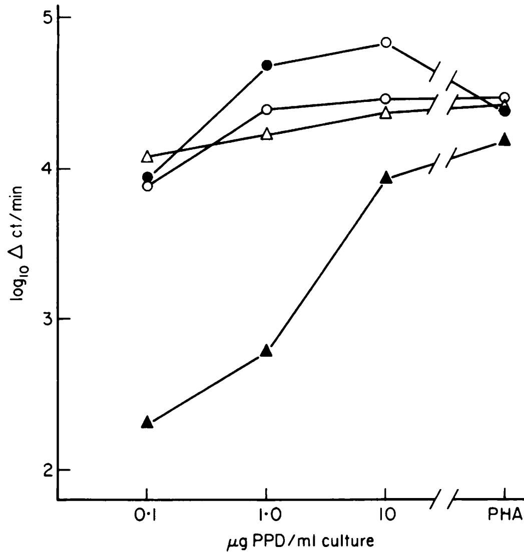

FIG. 2. PPD specific lymphocyte proliferation of non-passed lymphocytes (open symbols) and Ig–anti-Ig column passed lymphocytes (closed symbols) from one Mx+/LP+ individual (circles) or from one Mx+/LP+ individual (circles) or from one Mx−/LP+ individual (triangles). Background log cpm for Mx+/LP+ = 2·156±0·081, for Mx−/LP+ = 2·209±0·049.

response of lymphocytes from Mx−/LP+ persons requires a more complex explanation: (1) T-lymphocytes from these persons may require macrophages or other accessory cells in order to be stimulated *in vitro* (Hertel-Wulff & Rubin, 1976; Sønderstrup *et al.*, 1977 submitted for publication), (2) T-lymphocytes are unresponsive and B-lymphocytes are the responding cells, (3) PPD-specific T-lymphocytes are actually adsorbed onto the columns due to passive coating with Ig (via PPD anti-PPD complexes).

5. *Do B-lymphocytes respond or participate in the PPD-specific lymphoproliferative response?*

Experiments performed by Blomgren (1975) with lymphocytes from Mx+/LP+ persons have suggested that B-lymphocytes participate in the PPD-specific lymphoproliferative response *in vitro*. Therefore, we investigated whether B-lymphocytes from our Mx−/LP+ and Mx+/LP+ persons could proliferate *in vitro* against PPD. In addition, one 'real' Mx−/LP− person was included in this study. B-lymphocytes were separated by the E-RFC sedimentation technique. However, none of the B-lymphocyte populations tested (depleted for E-cells by more than 95%, see Table 7) responded *in vitro* against PPD (Fig. 3). Functional control studies on these cell populations showed that the B-lymphocytes did not respond against PHA but against PWM (Table 1). The E-cells responded against PPD, PHA and PWM almost as did Ig anti-Ig column passed T-cells (compare Tables 1 and 5) except

TABLE 7. Effect of rosette-gradient centrifugation on the proportion of T and B cells from peripheral blood lymphocytes (% RFC± SE)

RFC assay	Cell type tested	Non-separated	Separated by E-RFC and FIP*
E-RFC	T	60·1±4·76	1·64±0·44
EA-RFC	T, B, K	38·0±2·83	59·5±3·98
EAC′-RFC	B	25·1±1·42	66·1±3·58

* FIP = Ficoll–Isopaque gradient centrifugation.

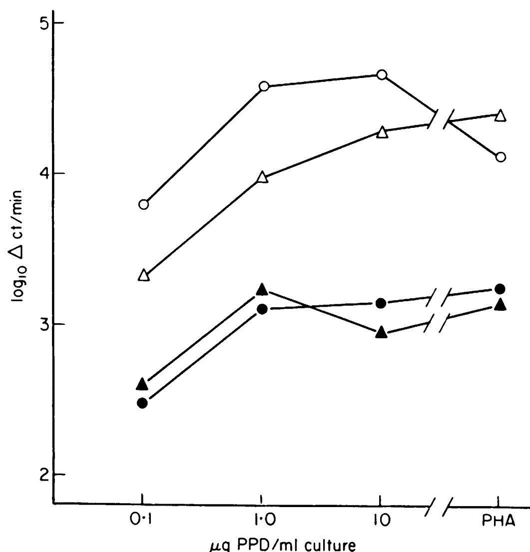

FIG. 3. PPD specific lymphocyte proliferation of non-purified lymphocytes (open symbols) and purified B-lymphocytes (E-RFC gradient separated) (closed symbols) from one Mx+/LP+ individual (circles) or from one Mx−/LP+ individual (triangles). Background log ct/min for Mx+/LP+ = 2·349 ± 0·087, for Mx−/LP+ = 2·417 ± 0·057.

that E-cells from Mx−/LP+ persons responded somewhat better than T-cells (to be published). The E-cells do contain monocytes whereas the T-cells contain very few if any monocytes (Zeylenmarker *et al.*, 1974; Hertel-Wulff & Rubin, 1976; Sønderstrup *et al.*, 1976).

DISCUSSION

The present report deals with our attempts to clarify why some people with PPD-specific T-lymphocytes are non-responsive skin tested with PPD *in vivo*.

There are many possible explanations for such a phenomenon, e.g. (1) blocking anti-PPD antibodies, (2) desensitizing, freely circulating PPD, (3) specific or non-specific suppressor cells, (4) classical tolerance of PPD-specific T-lymphocytes, and hyperimmune PPD-specific B-cells being the proliferative cells *in vitro*. The Mx−/LP+ group of individuals had no anti-PPD antibodies in the serum as detected by our passive haemagglutination assay. Thus, anti-PPD antibodies directly blocking the PPD injected intradermally seems to be an unlikely explanation. In accordance with this interpretation was the finding that human anti-PPD antibodies have little if any effect on the PPD-specific lymphoproliferative reaction *in vitro*. However, both the haemagglutination inhibition assay and the lymphoproliferative assay gave evidence that serum from Mx−/LP+ individuals contained PPD of the type found in the 19S peak of a G200 fractionation diagram. Thus, the possibility exist that the recirculating PPD-specific T-lymphocytes in Mx−/LP+ individuals are blocked by free PPD and/or by PPD-anti-PPD complexes in PPD excess (also found in the 19S fraction).

This conclusion is supported by our fractionation experiments where PBL were passed through Ig anti-Ig columns. Such columns, which were shown previously to retain most if not all antigen-specific suppressor cells (Rubin, 1976), reduced the PPD-specific lymphoproliferative response of the passed cells (from Mx−/LP+ individuals) very significantly, especially the response to small amounts of PPD (Fig. 2). If antigen-specific suppressor cells were the regulators of the negative skin reaction in these persons we would have expected a very significantly augmented PPD-specific lymphoproliferative response of Ig anti-Ig column passed lymphocytes. As a control, passed cells from Mx+/LP+ persons showed PPD-specific lymphoproliferative responses equal to or higher than non-passed cells. Thus,

PPD-specific T-lymphocytes (or a majority of these cells) from Mx−/LP+ individuals would seem to be adsorbed onto Ig anti-Ig columns. Our present working hypothesis is that the majority of PPD-specific T-lymphocytes (those with high avidity antigen-specific receptors and/or Fc receptors) from Mx−/LP+ individuals are made 'Ig positive' by the interaction with PPD anti-PPD antibody complexes. Such PPD anti-PPD antibody complexes may be shed from the lymphocyte membrane *in vitro* (either passively or actively) due to the deficit in antigen–antibody complexes in the medium compared to the person's serum. Alternatively, the binding of antigen–antibody complexes to the Fc receptors on the T-lymphocyte membrane does not necessarily interfere with the binding, between PPD and the PPD-specific T-lymphocyte receptor. If nothing else, this hypothesis has its beauty in the relative ease with which it can be tested experimentally.

The fourth possible explanation, that PPD-specific B-lymphocytes were the proliferating cells *in vitro*, especially when testing the PPD-specific lymphoproliferative response of lymphocytes from Mx−/LP+ individuals, was proven wrong. Neither B-cells from these persons nor B-cells from Mx+/LP+ and Mx−/LP− (unpublished) persons would respond with cell division *in vitro* after PPD encounter if T-cell depletion was 90% or more. Blomgren (1975) has shown that B-cells from Mx+/LP+ individuals can proliferate after PPD encounter, but only if the PPD is given as a complex or as a mixture with T-cell factors. B-lymphocytes may be stimulated by T-mitogens by a similar mechanism (Elfenbein & Gelfand, 1975).

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