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LYMPHOCYTE ACTIVATION.

VI. A RE-EVALUATION OF FACTORS AFFECTING THE SELECTIVITY OF POLYCLONAL MITOGENS FOR MOUSE T AND B CELLS

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SUMMARY

In the culture and assay systems described here the majority of mouse spleen cells stimulated by soluble concanavalin A and phytohaemagglutinin have been shown to be T cells, whereas bacterial endotoxin (LPS) stimulates B cells almost exclusively. These observations appear to be independent of the tissue source, the length of the culture period, and the presence of supernatants from other cultured T cells. Pokeweed mitogen (PWM) is capable of stimulating both T and B cells, the relative numbers of each probably being affected by the length of the culture period and variability between different PWM samples. Preliminary evidence is presented for mature LPS-responsive cells being able to persist for long periods in the mouse peripheral lymphoid system. The importance of using culture conditions that allow selective stimulation of T and B cells is discussed, especially in relation to their accurate quantitation.

INTRODUCTION

It has been shown that under certain conditions of *in vitro* lymphocyte culture, the 'non-specific' or 'polyclonal' mitogens concanavalin A (Con A) and phytohaemagglutinin (PHA) can preferentially, if not exclusively, stimulate mouse thymus-derived (T) cells (review by Greaves & Janossy, 1972). In contrast, bacterial endotoxin (LPS) has been shown to stimulate mouse bone marrow-derived (bursa equivalent, B) lymphocytes (Gery, Krüger & Spiesel, 1972; Andersson, Sjöberg & Möller, 1972a; Peavy *et al.*, 1972; Greaves & Janossy, 1972) and Pokeweed mitogen (PWM) appears to be able to stimulate both B and T cells (Greaves & Janossy, 1972; Piguet & Vassalli, 1973).

The exact conditions under which preferential or exclusive stimulation of T cells by Con A and PHA occurs *in vitro* are not well documented. One proviso appears to be that both

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reagents have to be presented to the cells in a soluble form and that when they are covalently bound to a solid matrix, activation of substantial numbers of B cells occurs (Greaves & Bauminger, 1972; Andersson *et al.*, 1972b). More recent reports have suggested other conditions in which large numbers of B cells are activated by Con A and PHA. Thus, mouse B cells appear to be activated (a) by PHA when the period of culture is prolonged (Piguet & Vassalli, 1972), or (b) by PHA stimulation of lymphocytes in serum-free medium (Vischer, 1972), or (c) by Con A in the presence of factors produced by cultured thymocytes or T cells (Andersson *et al.*, 1972a; Andersson, Möller & Sjöberg, 1972c). Phillips & Roitt (1973) have also reported that substantial numbers of purified human B cells are capable of responding directly to soluble PHA, though this finding has not been confirmed (Greaves, Janossy & Doenhoff, 1974).

A previous analysis using chromosome markers on PHA-stimulated mouse blood lymphocytes indicated that virtually all dividing cells were of thymic origin (Doenhoff *et al.*, 1970), but in view of the recent conflicting results it was felt that there was a need for a re-investigation of some of the factors said to effect the selectivity of the *in vitro* response of mouse spleen-borne T and B lymphocytes to polyclonal mitogens. Quantitative assessment of lymphocyte responsiveness has been performed using tritiated thymidine ([^{3}H]T) and identification of the responding cells was effected either by detection of chromosome markers in dividing cells, or by fluorescent staining of surface immunoglobulins on blast cells.

MATERIALS AND METHODS

Two strains of CBA mice were used, namely CBA/Lac (hereafter called Lac) and CBA/H.*T6T6* (*T6T6*), cells of the latter strain being distinguished in metaphase preparations by the presence of two minute chromosomes. The strains are syngeneic in that permanent skin graft acceptance between them has been found (Leuchars, unpublished observations).

Experiments involving chromosomally marked cells were performed on radiation chimaeras prepared by thymectomizing 8-week-old Lac males, subjecting them to 850 rads total body irradiation 1 week later, and reconstituting them haematopoietically with 5×10^6 Lac bone marrow cells. On the same day as the irradiation and bone marrow injection, T-cell reconstitution was achieved in one of four different ways: in Group A by implantation of a neonatal *T6T6* thymus lobe under the kidney capsule followed by its removal 30 days later; in Group B by injection of 50×10^6 freshly isolated thymocytes from 4-week-old, otherwise untreated *T6T6* mice;* in Group C by implantation of a *T6T6* thymus graft which was left intact for the duration of the experiment; and in Group D by injection of 50×10^6 *T6T6* spleen cells from normal mice. Spleen cells from these mice were taken for culture 50–70 days after irradiation and reconstitution, except for results given in Table 1.

Experiments involving non-chimaeric lymphocyte populations were performed using either spleen cells from untreated 8-week-old Lac mice, or thymus cells, either from untreated mice or from mice given 2·5 mg hydrocortisone acetate (Hydrocortisyl, Roussel) 48 hr previously.

Cell suspensions for culture were prepared from spleen, lymph node, thymus or blood. The three former tissues were removed aseptically, and constituent cells teased out into

* Previous results show that at least 90% of dividing cells in PHA-stimulated blood lymphocyte cultures were *T6T6* (i.e. of known thymic origin) in mice prepared as in Groups A and B; hence the use of this animal model here.

culture medium, washed once and resuspended at a concentration of 2×10^6 cells/ml. The technique for exsanguinating mice and separating blood lymphocytes for culture has been described previously (Doenhoff *et al.*, 1970). Cell suspensions were distributed for culture into one of three types of receptacle: (a) 0·25 ml into each (flat base) well of a microplate (Microtest II, Flow Laboratories, catalogue number M-29-ART), giving a total of 5×10^5 cells over a 28 mm^2 surface area (Janossy *et al.*, 1973); (b) 1·0 ml into curved base plastic tubes (Falcon tubes, 12 × 75 mm, Gateway International, catalogue number 2003). Both these types of vessel were used for assessing [^{3}H]T uptake; (c) for cytological analysis 2 ml were aliquoted into flat, based culture vials (Sterilin Ltd, catalogue number 118/S) giving a concentration of 4×10^6 cells over 180 mm^2 surface area.

The medium used throughout was RPMI 1640 containing 10% foetal calf serum (FCS) (Flow Laboratories, batch number 42070), 300 mM glutamine, 200 units/ml penicillin and 150 μg/ml streptomycin. The foetal calf serum batch was selected for its ability to support maximal stimulation by mitogen, while allowing only low 'background' activity in unstimulated cultures.

The mitogens used were: concanavalin A (twice crystallized, Miles-Yeda, Israel); phytohaemagglutinin (Wellcome, purified grade); lipopolysaccharide B from *E. coli* 055:B5 (Difco); and Pokeweed mitogen was prepared from plant stems of *Phytolacca americana* according to the method of Börjeson *et al.* (1966). For the first preparation (see text to Fig. 3) the trichloracetic acid (TCA) precipitate was redissolved in water, while in the second batch (Figs 5–8) the TCA precipitate was dissolved in 0·2 N NaOH. Both preparations were dialysed and filtered before use in cultures. Lentil Mitogen (LM) was isolated from seeds of *Lens culinaris* by the method of Howard & Sage (1969). Optimum concentrations of mitogen were Con A (3 μg/ml), PHA (1·5 μg/ml), LPS (50 μg ml), PWM (approximately 50 μg/ml) and LM (20 μg/ml), as assessed by maximal [^{3}H]T uptake on day 3.

Cultures were incubated in an atmosphere of 7% CO_2, 10% O_2 and 83% N_2 at 37°C.

Supernatants from thymocyte cultures were obtained by incubating: (a) thymocytes from untreated 4-week-old Lac mice for 48 hr in Petri dishes in RPMI 1640 with 10% foetal calf serum (Supernatant 1); (b) by incubating thymus cells (CRT) from cortisone-treated mice in the same medium with 5 μg/ml Con A for 24 hr, removing excess Con A from the cells with 100 mg/ml α-methyl glucoside and then reincubating them for 24 hr in fresh medium which was used later as Supernatant 2 (see Andersson *et al.*, 1972c); (c) by treating spleen cells in a similar manner to the CRT in 'b' (Supernatant 3).

Thymidine uptake by cultured cells was measured by adding 4μCi [^{3}H]T of low specific activity (50 mCi/mmol) to each millilitre of culture suspension and after 16 hr transferring the suspension quantitatively to wet filter discs (Whatman, GF/C 2·5 cm). The discs were washed under suction twice with saline, twice with 10% trichloracetic acid solution and twice with methanol, transferred dry to scintillation vials, treated with hyamine and counted as before (Janossy *et al.*, 1973; Janossy & Greaves, 1971, 1972). The resulting figures were expressed in terms of counts per minute (cpm) per ml of cell suspension.

Metaphase arrest for cytological analysis was achieved by adding colcemid (Ciba) to the cultures at a concentration of 10^{-7} M 16 hr prior to harvesting. Metaphase spreads were prepared by the method of Ford (1966), and where possible 100 cells/culture were analysed.

Indirect immunofluorescence staining of cultured cells was performed using the suspension technique of Möller (1961) and described previously in Greaves, Bauminger & Janossy (1972).

For statistical purposes in Figs 2, 3, 5, 6 and 8 the mean result for each group has been bracketed by one standard error (s.e.) each way.

RESULTS

(1) *Culture conditions*

In a preliminary experiment to define some of the culture conditions which allow optimum stimulation of lymphoid cells *in vitro*, spleen cells from normal mice were cultured with Con A in medium with or without FCS, in flat or round-base culture vessels. The concentration of Con A was reduced ten-fold in serum-free cultures, as suggested by Cutinho *et al.* (1973) to obtain optimum stimulation under these conditions. The response was assessed by measuring uptake of radioactivity from low specific activity thymidine added 16 hr previous to harvesting in each of three assays performed during a 90-hr time course, and the results are shown in Fig. 1. It can be seen that cultures with or without serum in round-base tubes are relatively poorly stimulated, as are serum-free cultures in flat-base tubes.

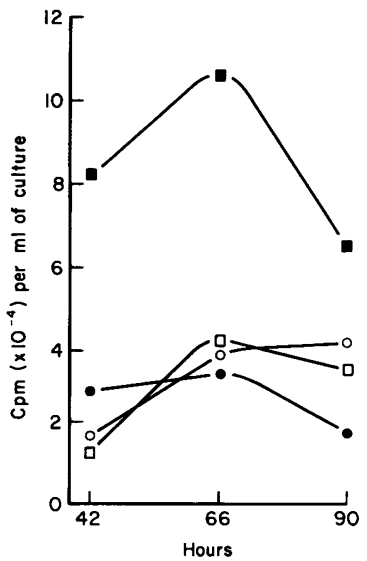

FIG. 1. Response of spleen cells from normal CBA mice to Con A when cultured in: (■) flat-base tubes with FCS; (□) flat-base tubes without FCS; (●) round-base tubes with FCS; (○) round-base tubes without FCS. Concentration of Con A with FCS = 5 μg/ml; concentration of Con A without FCS = 0·5 μg/ml.

Detection of a large difference between cultures with and without FCS is probably dependent on the use here of [^{3}H]T of low specific activity (50 mCi/mM). It has previously been shown that the use of thymidine of high specific activity (10 Ci/mM) does not permit effective discrimination between cultures containing very different numbers of responding cells (Janossy *et al.*, 1973). The failure of cells in round-base tubes to respond well even in the presence of serum may be explained by the cells falling into the centre of the concave tube base. This could give rise to a high cell density, which might exert an inhibitory effect on

lymphocyte growth (Janossy & Greaves, 1971). Optimum activity was observed only in flat-base tubes with serum in the medium, and these culture conditions have therefore been adopted throughout this series of experiments.

(2) *Identification of transformed and dividing cells*

In the first experiment involving the use of radiation chimaeras, spleen cells from mice in each of groups A, B and C were cultured with Con A, PHA, PWM or LPS, or were left unstimulated. Tritiated thymidine was added after 54 hr and the cultures harvested 16 hr later (Fig. 2). Further samples of similarly stimulated 3-day cultures were analysed for the

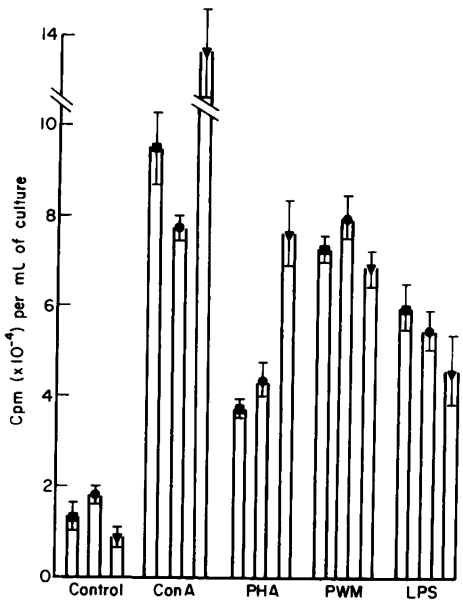

FIG. 2. Response of spleen cells from chimaeras of Groups A (■), B (●) and C (▼), in unstimulated cultures (controls) and to stimulation by Con A, PHA, PWM and LPS at optimum concentrations (see Materials and Methods section). Response was assayed by [^{3}H]T uptake from 54 to 70 hr.

karyotype of dividing cells and for cell surface membrane immunoglobulin. In addition LM was studied cytologically, and all the results for cytology and immunoglobulin staining are given in Fig. 3.

It can be seen that according to the results on thymidine uptake all mitogens caused significantly increased uptake of radioactivity compared to unstimulated controls. Cells from animals in Group C tended to have higher counts when stimulated by Con A and PHA, when compared to Groups A and B, most probably because their T-cell pool is reconstituted to a greater extent by the intact thymus graft (Doenhoff *et al.*, 1970).

In terms of cytological analysis there is little difference between Con A, PHA or LM with respect to the percentage of *T6T6* cells (i.e. cells of known thymic origin), found dividing in cultures from the three groups of mice, and in Groups A and B this value was approximately 80%. The percentage of transformed cells staining with fluorescent anti-immuno-

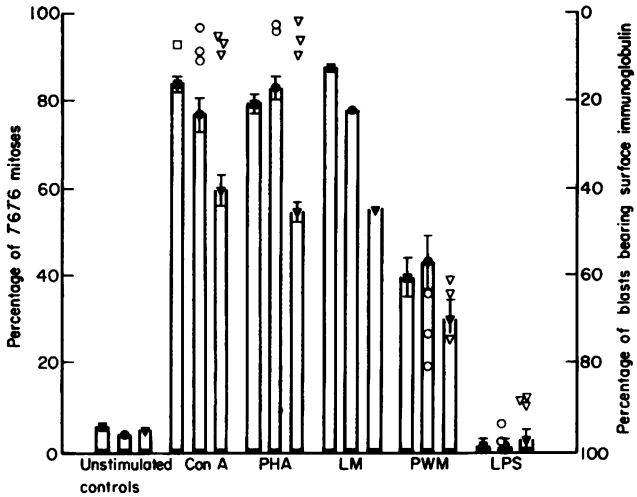

FIG. 3. Response of spleen cells from chimaeras of Groups A (□), (■), B (○), (●) and C (▽), (▼), assayed in terms of the percentage of *T6T6* cells in division at 70 h of culture, (solid symbols) and the percentage of transformed blasts bearing membrane immunoglobulin (open symbols, one per mouse).

globulin serum in representative cultures stimulated by Con A or PHA was approximately 10% throughout the three groups. It is noteworthy in Group C (thymus graft intact mice), that although the percentage of *T6T6* cells found dividing in response to PHA and Con A is lower than in A and B, there is no commensurate increase in immunoglobulin-bearing blast cells, indicating that the Lac cells dividing here are probably not B cells, but are cells of bone marrow karyotype which have been 'processed' by the thymus (Doenhoff & Davies, 1971). The transformed cells in Groups A and B, which have neither the *T6T6* chromosomes nor are stained with anti-immunoglobulin, and which constitute approximately 10% of the total, are perhaps also T cells, some of which were introduced with the bone marrow inoculum and some of which survived irradiation (Doenhoff *et al.*, 1970).

Three-day cultures of LPS-stimulated cells have less than 5% of cells of known thymic origin dividing in them and about 90% of the transformed cells carry surface immunoglobulin.

The results for PWM are intermediate, indicating that this lectin stimulated both B and T cells, as expected from previous work (Greaves & Janossy, 1972). It is noted that the summation of the percentage of lymphoblasts staining for surface immunoglobulin and the percentage of *T6T6* cells in mitosis in Group B cultures stimulated with PWM gives a figure greater than 100%; and although the same sum for Group C yields a figure of approximately 100%, no allowance is thereby made for T cells of Lac karyotype which are present in mice with their thymus graft intact. The reason for this anomaly is not known, but it is possible that some of the immunoglobulin secreted from the stimulated B cells (Greaves and Janossy, 1972), is in turn absorbed by means of Fc receptors onto the surface of some activated T cells in the culture (e.g. Yoshida & Andersson, 1972).

It is worth noting, in view of the evidence suggesting that Con A may activate a subpopulation of T cells which is unresponsive to PHA (Stobo & Paul, 1973), that in cultures of spleen cells from Group C mice there is no large difference between Con A and PHA in

terms of the percentage of *T6T6* cells in division. This corroborates the postulate of Stobo and Paul that any difference between T cells which respond to Con A alone and those which respond to both mitogens is not related to the length of time which the cells have spent in the periphery after having been released from the thymus.

Of the dividing cells analysed in unstimulated (control) cultures, over 90% were of bone marrow karyotype, and Andersson, Nordling & Häyry (1973) also intimate that background mitotic activity in spleen cell cultures is not of T-cell origin, but it is not known whether these dividing cells are in any way similar to the B cells stimulated by LPS or to the transformed B cells found in PHA and Con A cultures. In view of the observation that 'background' mitotic activity involves mainly bone marrow-derived cells it is perhaps surprising that PHA cultures of cells from mice in Groups A and B, which in terms of thymidine uptake are approximately only half as active as equivalent Con A cultures, do not differ from the latter by having a significantly lower percentage of *T6T6* cells in division. Nevertheless, the percentage of *T6T6* cells found responding to PHA in spleen cell cultures is approximatly 10% less than that found in blood cell cultures of similar animals, (Doenhoff *et al.*, 1970), and there are approximately 10% of immunoglobulin bearing blasts in the former (Fig. 3).

Although these lymphoblasts could be derived from a B-cell population which is directly stimulated by Con A and PHA, or which is activated by products of activated T cells, an alternative possibility is that *in vitro* 'background' mitotic activity may be able to continue

TABLE 1. The percentage of *T6T6* cells dividing in Con A and PHA-stimulated cultures of peripheral blood, spleen and lymph node cells obtained from each of four mice of Groups A (thymus graft removed 30 days after implantation) and C (thymus graft intact). Group A mice were cultured 150 days and Group C mice 110 days after thymus graft implanation

Mouse	Group A					
	Blood		Spleen		LN	
	Con A	PHA	Con A	PHA	Con A	PHA
1	86	83	72	83	72	78
2	80	91	70	81	73	80
3	77	91	78	79	80	86
4	87	88	76	75	77	89
Mean ± s.e.	82·5 ± 2·4	88·3 ± 1·9	74 ± 1·9	79·5 ± 1·7	75·5 ± 2	83·3 ± 2·7
Mouse	Group C					
	Con A	PHA	Con A	PHA	Con A	PHA
1	48	49	41	39	46	38
2	48	43	39	31	37	39
3	45	50	41	34	38	31
4	38	37	34	28	25	32
Mean ± s.e.	44·8 ± 2·4	44·8 ± 3	38·8 ± 1·6	33 ± 2·4	36·5 ± 4·4	35 ± 2·1

simultaneously with mitogen-induced activity of T cells. Thus a higher 'background' mitotic rate in spleen than in blood cultures could account for the apparent discrepancy between the two tissues noted above, and for a previous observation that PHA-stimulated cultures of spleen cells and to a lesser extent lymph node cells from Group C mice contain a lower percentage of dividing *T6T6* cells than blood cell cultures from the same mice (Doenhoff *et al.*, 1970).

The latter observation has, however, also led to the suggestion that cells emerging from the thymus are first found in a PHA-responsive state in the peripheral lymphoid tissues, particularly the spleen, and that only later are they found in the blood (Cantor, 1972).

In order to investigate the phenomenon further blood, spleen and lymph node cells obtained from mice in Groups A and C (four animals per group) were individually cultured with either PHA or Con A for cytological analysis. The results are given in Table 1, and it can be seen that, although the response pattern is similar for both Con A and PHA, once again spleen cell cultures in particular, and to some extent lymph node cell cultures, have a lower percentage of *T6T6* cells in division than blood cell cultures. The difference here is not as marked as previously (Doenhoff *et al.*, 1970) but because it still occurs in mice which have had no thymus tissue for at least 100 days, the result is perhaps less likely to be due to T-cell maturation processes.

An alternative explanation may be sought in terms of the relative background mitotic rates of the various tissues, and it follows that the lower the mitotic rate induced by a T-cell mitogen such as Con A or PHA, the higher the relative contribution of background mitotic activity to the total response provided that both types of activity do continue independently of each other *in vitro*.*

(3) *Persistence of responsiveness of lymphocytes in vitro*

Results in the previous section indicate that the culture conditions employed did allow growth of Con A-responsive T cells and LPS-responsive B cells, at least when each cell type was stimulated at the time of culture initiation. It could, however, be argued that in the absence of direct stimulation at the beginning of culture death of one or more cell types occurs. To investigate this possibility, spleen cells from normal CBA mice were set up in similar culture conditions, and Con A or LPS added to the medium at various times afterwards. Tritiated thymidine was added to the cultures at a standard time (50 hr) after addition of the mitogen, and the cultures harvested 16 hr later. The results are given in Fig. 4, and indicate that although responsiveness to Con A declines fairly rapidly after 12 hr of culture, the magnitude of the response to LPS is maintained at a relatively constant level throughout the time course. It would thus appear that using the culture conditions described here, the survival and growth of LPS-responsive cells compares favourably with that of Con A-responsive cells. The death or loss of function of B cells other than those responsive to LPS is, of course, not excluded.

* The ability of unstimulated mouse blood, spleen and lymph node cells to utilize radioactive thymidine was assessed semi-quantitatively by giving a 2-hr pulse of 10 μCi [^{3}H]T of high specific activity (26 Ci/mM) to 3-day cultures initiated with 2×10^6 cells/ml medium. Tissues from four mice were assayed separately and four tubes of medium without cells were included as controls. The results in cpm/ml culture ($\pm$s.e.) were: medium alone, 340 ($\pm$14); blood cells, 3276 ($\pm$570); spleen cells, 10158 ($\pm$2428); lymph node cells, 1774 ($\pm$274).

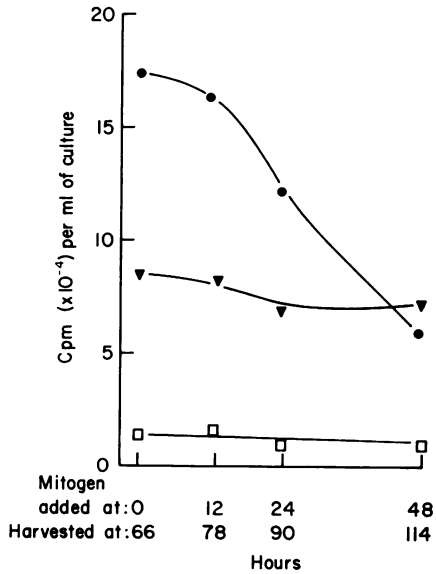

FIG. 4. Response of normal spleen cells to Con A and LPS added at various times after culture initiation and harvested at a standard time after mitogen addition, Con A (●), LPS (▼), unstimulated controls (□).

(4) *The effect of an extended culture period*

A recent report by Piguet & Vassalli (1972) suggested that if the culture period was extended beyond 3 days, bone marrow-derived cells, which they identified using chromosome markers, were found dividing in increasing numbers in PHA cultures and mixed lymphocyte reactions. Andersson *et al.* (1973) have already reported that they were unable to arrive at the same conclusion as a result of their work on the mixed lymphocyte reaction. To explore the possibility more extensively with respect to 'polyclonal' mitogens, spleen cells from three groups of radiation chimaeras were cultured during a 5-day period with Con A, PHA, PWM and LPS, and the response assessed daily in terms of [^{3}H]T uptake and the karyotype of dividing cells. The Group B (thymocyte-reconstituted) mice that are made use of here are broadly similar to the animals used by Piguet & Vassalli (1972). In addition, mice of Groups A (thymus graft removed at 30 days) and D (spleen cell-reconstituted) were studied.*

The results of cytological analysis for Group A are given in Fig. 5 and for Group B in Fig. 6, and it is apparent that both types of chimaera are similar in their response to each of the four mitogens over the 5-day time course. At least 80% of the cells dividing in response to Con A and PHA from day 3 onwards are of known thymic origin, and the response of *T6T6* cells to LPS, in contrast, is never more than 10% of all dividing cells. Tritiated thymidine uptake results are given in Tables 2 (Group A) and 3 (Group B). Throughout the

* Chimaeras injected with spleen cells were included in this experiment as a control for the thymocyte-injected group, since it was considered possible that the *in vitro* response of T cells derived from a freshly isolated thymocyte population may differ from that of a population introduced into the chimaera in a more mature state such as spleen-derived T cells, or that produced in the chimaera itself by functional thymus tissue. It was not anticipated that the result given by spleen cells from Group D animals would invalidate them as a control group (Results, section 6).

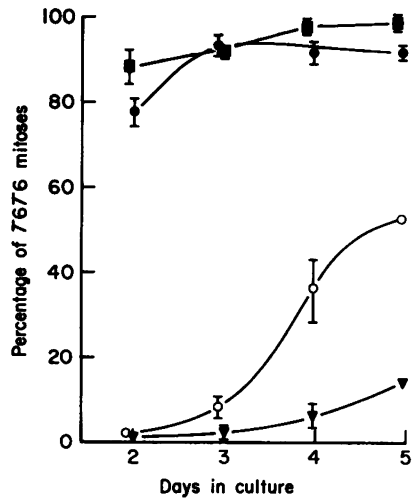

FIG. 5. Response of spleen cells from Group A chimaeras analysed daily for 5 days for the percentage of *T6T6* cells dividing in response to Con A (●), PHA (■), PWM (○) and LPS (▼).

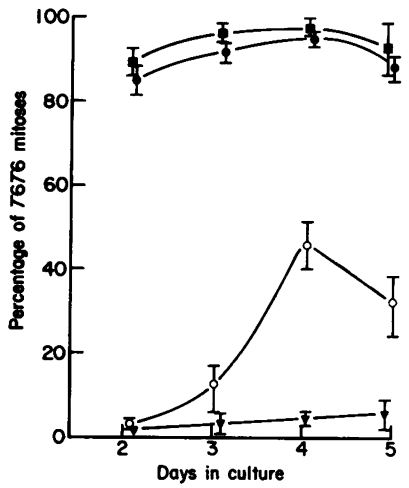

FIG. 6. Response of spleen cells from Group B chimaeras analysed daily for 5 days for the percentage of *T6T6* cells dividing in response to Con A (●), PHA (■), PWM (○) and LPS (▼).

time course, stimulated cultures of both groups show substantially greater uptake of radioactivity than control unstimulated cultures. There is an indication from these results that the percentage of *T6T6* cells dividing in response to Con A and PHA on day 2 is lower than on subsequent days. This may be due to *in vivo* spleen cell mitotic activity, presumably of bone marrow origin, continuing for a limited period in culture. It is, however, apparent that there is no significant decrease with time of the percentage of dividing thymus-derived cells. In other experiments, blood from Group C chimaeras was pooled and the lymphocytes

TABLE 2. [^{3}H]T uptake by spleen cells from Group A mice stimulated with various mitogens over a 5-day time course. Figures are given as cpm per ml of cultured cell suspension

Time of thymidine uptake (hr)	Mitogen				
	None	Con A	PHA	PWM	LPS
24–36	6800	20100	18000	14200	23600
48–60	6000	45200	27200	38200	41200
72–84	9200	92000	33200	34800	31200
96–108	6800	31000	22800	24500	24000

TABLE 3. [^{3}H]T uptake by spleen cells from Group B mice stimulated with various mitogens over a 5-day time course. Figures are given as cpm per ml of cultured cell suspension

Time of thymidine uptake (hr)	Mitogen				
	None	Con A	PHA	PWM	LPS
48–60	6800	29200	16000	36400	28400
72–84	9200	52400	27200	35760	34400
96–108	7200	48400	23200	23600	21200

cultured with PHA for 11 days, a procedure facilitated by changing the medium on alternate days (Doenhoff, unpublished observations). It was found that the percentage of *T6T6* cells dividing on day 11 was 57%, compared with 59% on day 3.

(5) *The response to PWM*

The batch of PWM used for the experiment, the result of which is given in Figs 4 and 5, in contrast to the sample used for Fig. 3, stimulated very few *T6T6* cells to divide by day 3 in culture, though an increased number of these cells were found dividing on the 4th and 5th days in response to the second PWM sample. Although one could postulate that the cause of this time-dependent variation is a T-cell stimulatory factor produced by B cells, it has alternatively been proposed that the mitogenicity of PWM preparations is due to two or more components (Loor, personal communication) and each of these may be found to activate different lymphocyte populations. Thus, the disparity between the two batches of PWM used here, as well as the variation with time shown by the second batch may be a result of the differences in potency or quantity of each of the mitogenic fractions in different PWM preparations. A preliminary analysis of the second PWM sample was performed by testing its ability to stimulate [^{3}H]T uptake by cortisone-resistant thymocytes, and spleen cells from deprived mice. The results are depicted in Fig. 7, and it can be seen that by 42 hr following PWM stimulation, the spleen cells have reached maximum activity,

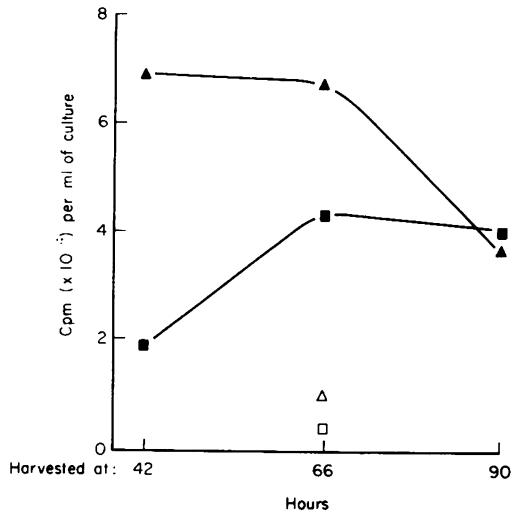

FIG. 7. Response of CBA cortisone-resistant thymocytes (■), and spleen cells from T cell-deprived CBA mice (▲) to PWM of the same batch as used in Figs 5–8 (△). (□) Unstimulated controls. The response was measured in terms of [^{3}H]T uptake.

whereas the peak response of the CRT is delayed. This result is an indication that the mitogenicity of this batch of PWM is greater towards B cells than T cells during the initial stages of the response, a conclusion which is not contradicted by analysis of the response of chromosomally marked cells (Figs 5 and 6).

(6) *The response of chimaeras reconstituted with spleen cells*

The third group of chimaeric cells studied over a time course *in vitro* were those taken from mice injected with 50 million *T6T6* spleen cells (Group D). The results for cytological analysis of dividing cells are given in Fig. 8. Unlike the results for Groups A and B (Figs 5 and 6 respectively) it is apparent that a high proportion (70–75%) of the cells responding to LPS and PWM are derived from the spleen cell inoculum.

The LPS-responsive cells in these mice could have been introduced in a mature form as a constituent of the injected spleen cells, and the result could be an indication of the ability of LPS-responsive cells to persist in mouse peripheral lymphoid tissue over long periods of time (at least 50 days in this instance), in a manner similar to that suggested for T cells (Doenhoff *et al.*, 1970). Other preliminary studies on mice subjected to repeated injections of [^{3}H]T for 30 days indicate that LPS-responsive cells have an *in vivo* mitotic rate as low as that of PHA-responsive cells (Mathur & Janossy, unpublished observations) though Sprent & Basten (1973) have suggested that thoracic duct B cells have a shorter intermitotic interval than T cells. However, it has also been observed that spleen-derived stem cells can compete effectively with marrow-derived stem cells, albeit for a limited period, in regenerating irradiation-damaged haematopoietic tissue (Wallis, unpublished observations), and therefore some or all of the LPS-responsive *T6T6* cells observed in Group D chimaeras could be the progeny of spleen-borne stem cells. The 25% of cells in these LPS cultures are presumably derived from the bone marrow inoculum injected at the time of irradiation.

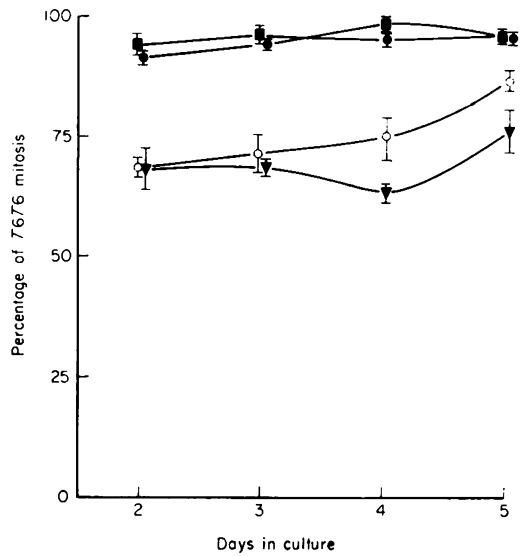

FIG. 8. Response of spleen cells from Group D chimaeras analysed daily for 5 days for the percentage of *T6T6* cells dividing in response to Con A (●), PHA (■), PWM ( ) and LPS (▼).

(7) *The effect of supernatants from T-cell cultures*

The ability of mitogens in their soluble form to activate T cells, but to activate T and B cells when they are covalently linked to solid substrates has been documented previously (Greaves & Bauminger, 1972; Andersson *et al.*, 1972b). There are also reports of factors produced by thymocytes rendering B cells capable of stimulation by soluble T-cell mitogens such as Con A (Andersson *et al.*, 1972a, c). Much of this latter work appears to have been

TABLE 4. [^{3}H]T uptake by spleen cells from T cell-deprived mice stimulated with various concentrations of Con A, with or without various dilutions of supernatant taken from cultured thymocytes (see Materials and Methods section). [^{3}H]T uptake by the same cells stimulated with LPS are also given

	No supernatant	Supernatant 1 diluted by:			Supernatant 2 diluted by:		
		8×	4×	2×	8×	4×	2×
Con A (μg/ml) added to deprived spleen cultures							
0·00	6610	—	12,020	10,800	12,400	12,360	11,300
0·75	6580	4800	9160	8320	—	—	—
2·50	7450	6160	7340	9080	12,960	10,680	—
5·00	5440	6800	6480	7380	9320	6360	10,200
7·50	7520	4640	4020	4150	6000	7000	6560
25·00	6540	6320	3720	3100	—	—	—
LPS	101,450	84,400	85,560	93,000	—	87,000	—

performed in systems in which the thymocytes and B-cell source were not syngeneic. A preliminary investigation of this system using syngeneic thymocyte and B-cell sources has been attempted here on CBA mice. Thus, supernatants from unstimulated normal thymocyte cultures incubated for 48 hr (Supernatant 1) or from Con A-stimulated CRT cultures (Supernatant 2, see Materials and Methods section), were diluted 2-, 4- and 8-fold with fresh medium and used to culture spleen cells taken from T cell-deprived CBA mice, with Con A present at various concentrations. Control cultures included those with neither supernatant, nor Con A present, and those with Con A or LPS alone. Assessment of [^{3}H]T utilization was performed on day 3 of culture. The results are given in Table 4 and it can be seen that irrespective of the combination of thymocyte supernatant and Con A concentration, radioactivity uptake is never more than double that of cultures without stimulant, compared to a 13–15-fold increase when LPS is present.

Andersson *et al.* (1972a) also showed B-cell stimulatory activity with supernatants obtained from spleen cells involved in mixed lymphocyte reactions. In the present experiments supernatants from Con A-stimulated normal CBA spleen cells (Supernatant 3) were added at a 1:2 dilution with fresh medium to spleen cells from Group A chimaeras to test for B-cell stimulatory activity. Con A at a concentration of 5 μg/ml was added to the chimaera cell cultures and cytological analysis of dividing cells performed on day 3 of culture. No consistent decrease in the percentage of dividing *T6T6* cells was observed as a result of the addition of various supernatants, four control cultures ranging from 83–91% dividing *T6T6* cells, and five cultures with supernatants ranging from 80–94% *T6T6*.

It thus appears that with the methods used here supernatants from cultured thymocytes or T cells have only a marginal ability, if any, to facilitate B-cell stimulation by Con A. This seems to be the situation when the supernatants are tested for stimulatory activity either on a T-cell deprived spleen cell population and the response compared with that given by LPS acting on the same cells; or on a lymphocyte population consisting of both T and B cells and the response of B cells would be detectable by a change in the *T6T6*: Lac ratio of dividing cells. The observations recorded here do not exclude the possibility that T cells stimulated by antigens or suboptimal doses of mitogen release factors which facilitate B-cell maturation (Schimpl & Wecker, 1972) which may be detected by methods other than those used here, such as immunoglobulin synthesis (Andersson *et al.*, 1972a).

DISCUSSION

The results presented here confirm evidence already in the literature showing that the main cell type activated by PHA and Con A is of thymic origin. When looked for, cells with B lymphoblast characteristics constituted no more than 10% of the total activated cell population in PHA- and Con A-stimulated spleen cell cultures. This proportion does not appear to be substantially affected either by the length of time the cells are cultured, nor by the presence of supernatant medium taken from cultures of other thymus-derived cells. It cannot be resolved from present evidence whether the transformed B cells in these cultures are activated directly by mitogen, or by other factors inherent in the culture system.

It has also been confirmed that bacterial lipopolysaccharide stimulated B cells almost exclusively and that PWM can activate both B and T cells. It is not definitely known whether the B and T cells which can be transformed by mitogens *in vitro* are representative of the total B and T cell pools found *in vivo*, or whether only subpopulations can be activated

in vitro. Stobo & Paul (1973) have evidence for differences between Con A and PHA-responsive T cells and Greaves & Janossy (1972) have shown that the immunoglobulin class synthesized by B cells activated by PWM is exclusively IgM. The latter observation could mean that only a limited state of maturity is achieved by B cells when they are activated *in vitro* or alternatively that this lectin stimulates only an IgM-producing subpopulation of the total B-cell pool.

Many of the conflicting results concerning the characteristics of lymphocyte growth *in vitro* may be attributable to variability in the reagents in current use, particularly those of biological origin. The different results obtained with the two preparations of PWM used in the experiments reported here are an obvious example, and it is not known whether the slightly different methods used for preparing each batch satisfactorily accounts for the discrepancies. A difference probably at the animal model level, is also evident between the results given in Fig. 3 and those of Gery *et al.* (1972) for the percentage of *T6T6* cells dividing in response to PHA in cultures from mice with their thymus graft intact (Group C). The lower value (*circa* 30% *T6T6*) obtained by Gery could be due to thymus graft having been implanted 1 week after irradiation and bone marrow reconstitution, rather than on the same day as in the animals described here. Until such discrepancies can be adequately accounted for it will probably be necessary for the cell types responding in each culture system to be regularly identified, particularly if *in vitro* systems are to be of any use in the quantitation of lymphocyte classes *in vivo*.

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