

## SELECTIVE TRIGGERING OF HUMAN T AND B LYMPHOCYTES IN VITRO BY POLYCLONAL MITOGENS\*

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Lectins and other polyclonal lymphocyte stimulants have been used extensively both to analyze early events in cellular activation and clinically as aids to the assessment of immunocompetence (1-5). Earlier studies of the response of human blood lymphocytes to lectins provided suggestive evidence for heterogeneity of responding lymphocyte populations (6, 7). Chessin et al. (6) showed that the lymphoblast population evident in pokeweed mitogen (PWM)<sup>1</sup> (*Phytolacca americana*) were separable into two broad categories; type I cells which were indistinguishable from virtually all lymphoblasts activated by phytohemagglutinin (PHA) (*Phaseolus vulgaris*), and type II cells which could not be found in PHA cultures and were distinguished by their higher cytoplasm to nucleus ratio, and lack of periodic acid-Schiff-positive (presumably glycogen) granules.

These observations were made at a time when the dichotomy of lymphocytes into T and "bursal"-equivalent-derived (B) axis differentiation pathways (8) was not fully appreciated. Nevertheless, subsequent studies strongly supported the interpretation that T cells responded to PHA whereas both T and B responded to PWM. In particular, analyses of the proliferative and biosynthetic responses to PHA and PWM with lymphocytes from both normal donors (9) and patients with selective immunodeficiencies (10-12) provided compelling support for the view that lectins could selectively trigger T and/or B lymphocytes. This concept has been established beyond question in animal model systems, particularly for the chicken (13-15) and mice (3, 4, 16) where appropriate manipulations are feasible and definitive cell markers and cell separation techniques are available. In mice for example, soluble PHA stimulates T cells, lipopolysaccharide (LPS) B cells, and PWM T plus B cells. This selectivity may require some qualification, however, in terms of the culture conditions used and timing of the experiment and more especially of cellular interactions when T and B cells (plus macrophages) are mixed together. Recently, marker systems and separation methods have become available which permit the identification and purification of human T- and

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<sup>1</sup> Abbreviations used in this paper: B, "bursa-equivalent"—derived lymphocytes; CLL, chronic lymphocytic leukemia; Con A, concanavalin A; D-MEM, Dulbecco's modified minimal essential medium; EAC, erythrocyte-antibody-complement; ES, Earle's saline; FCS, fetal calf serum; HS, human serum; LPS, lipopolysaccharide; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SEB, staphylococcal enterotoxin B; SRBC, sheep red blood cells.

B-lymphocyte populations (17, 19). This new technology provides a logical direct approach to establish the precise selectivity of polyclonal stimulants for different populations of human lymphocytes.

We have studied the response capacity of T and B lymphocytes to a variety of lectins and other polyclonal stimulants when cultured as purified cell populations or alternatively when cultured as mixed T-B lymphocyte populations. The identity of the "starting" cell populations and the activated lymphoblasts has been carefully established using a panel of seven discriminating cell surface markers. Our results confirm the earlier studies of Chessin et al. (6) and Douglas et al. (10), and although some interesting species differences are evident the selectivity of polyclonal stimulants for human T and B lymphocytes is very similar to that observed earlier in rodents.

#### *Materials and Methods*

*Cell Sources.*—Cell separations were performed on human tonsil and spleen cell suspensions. Tonsils were available daily from the University College Hospital ENT Theatre and provided a convenient source of over  $2 \times 10^8$  viable lymphocytes/tonsil. Spleens were obtained from accident cases in the London area and in two instances from Hodgkins patients (shown subsequently to have no splenic involvement). Lymphocyte suspensions were prepared as previously described (20) using an Ultra-Turrax homogenizer and filtration through glass wool. The working tonsil cell suspensions in Earle's saline (ES) consisted of over 90% viable cells of which more than 95% were lymphocytes, less than 3% phagocytosed polystyrene particles in the presence of 50% fetal calf serum (FCS), and only 1–2% had monocyte/macrophage morphology. The proportions of phagocytic cells in spleen suspensions were not routinely determined.

*Reagents for Selective Identification of Human T and B Cells.*—Anti-T-cell serum (20) was a rabbit antihuman brain (cerebrum) serum absorbed with erythrocytes, liver, chronic lymphocytic leukemic (CLL) cells (5X), and acute myeloid leukemic cells (2x). Anti-B-cell serum (21) was a rabbit anti-CLL serum absorbed with erythrocytes, liver, and thymus cells (5x). Anti-T-lymphoblast serum was a pig antifetal thymus serum absorbed extensively with tonsil cells. This serum reacts effectively with every thymocyte, and stains no small T or B cells. It also fails to react with lymphoid cell lines and leukemic (lymphoid and myeloid) blast cells.<sup>2</sup> Evidence for the selectivity of these antisera was derived from double-staining and mixed fluorescence-rosette tests using antiimmunoglobulin (Ig) as a base-line marker for human B lymphocytes and is published in detail elsewhere (20, 21). The anti-Ig used was a rabbit serum raised against human IgG myeloma protein and was absorbed with human erythrocytes. Heat-aggregated human IgG was prepared by heating fluorescein-labeled serum IgG for 20 min at 60°C as described by Dickler and Kunkel (22). Aggregates were ultrasonicated immediately before use as indicators of Fcγ receptors on B cells.

*Enumeration of Human T and B Cells.*—Enumeration of lymphocytes binding anti-T and B antibodies and anti-Ig was carried out using an indirect test with fluoresceinated goat anti-rabbit Ig or goat anti-pig Ig. Observations were made using a Vickers M41 Photoplan fluorescence microscope (Vickers Instruments, Inc., Malden, Mass.) equipped with an HBO-mercury lamp, incident illumination, and dichroic mirrors. The EAC test for C3 receptors present on a subset of B lymphocytes (23, 24) was carried out by sensitizing sheep erythrocytes (SRBC)

<sup>2</sup> Greaves, M. F., G. Brown, and S. Lister. 1974. The expression of T and B-lymphocyte surface markers on human leukemic cells. Manuscript submitted for publication.

with 1:64 rat 19S anti-SRBC serum at 4°C for 60 min and subsequently coating with fresh mouse serum (absorbed with SRBC) at a 1:5 dilution for 30 min at 37°C. Controls consisted of uncoated SRBC and SRBC coated with antibody only. The EAC test was performed on a rotatory shaker at 37°C as described by Ross et al. (23). The E-rosette test for SRBC binding by T lymphocytes (25–27) was carried out by mixing 50λ lymphocytes ( $10^7$ /ml) plus 50λ heat-inactivated FCS (absorbed with SRBC) plus 100λ 2% SRBC (less than 2-wk old). This mixture was centrifuged at 175 g at 20°C and the rosetted pellet left at room temperature for 60 min before careful resuspension by tapping. E and EAC rosettes were enumerated by observations either on unstained wet preparations in a hemocytometer, or as in the case of activated lymphoblast suspensions by staining in suspension with 0.1% toluidine blue (M. Pepys, personal communication) and mounting on a slide. Cells binding three or more SRBC were considered as rosette-forming cells. In some experiments polystyrene particles were added to identify mononuclear phagocytes.

Lymphoblasts from cultures were washed three times before assay with cell surface markers. Cell aggregates were broken up by vigorous pipetting in cold ES and in occasional experiments by using an Ultra-Turrax homogenizer. Lymphoblasts were classified as such in marker experiments by virtue of their large size (>2 times the control small lymphocyte diameter), by the lack of phagocytic capacity, and by their general optical properties.

In all experiments the lymphoblasts reactive with given markers on day 3–4 of culture were calculated in absolute numbers. This type of analysis provides an approximate guide to the responding cell pool. Accurate estimates are rendered more difficult by cell death and proliferation during the culture period.

*Purification of Human T and B Cells.*—T lymphocytes were purified by a nylon wool column filtration method (28), based on that described by Julius et al. (29) for rodent cells. Recently Eisen et al. (30) have reported the use of a similar method for purifying human T cells from blood.

B lymphocytes were purified by sedimentation of E (i.e., T) rosettes on Ficoll-isopaque (28, 31). Table I summarizes the cell surface characteristics of the cell populations used in this study.

*Culture Media.*—Two different media were used. Dulbecco's modified minimal essential medium (D-MEM) was supplemented with 10% heat-inactivated human AB serum. RPMI-1640 medium (Flow Laboratories, Ltd, Irvine, England) was supplemented with 10% fetal bovine serum (batch no. 0186, Biocult Laboratories, Ltd, Paisley, England) unless otherwise stated. Both media were further supplemented with glutamine (300 mM) and antibiotics (penicillin, 200 U/ml; streptomycin, 100 μg/ml). Under the culture conditions used the pH of the two media are slightly different (D-MEM, pH 7.0; RPMI, pH 6.8).

*Culture Conditions.*—Culture vessels with flat bottoms were used. For studies in which responses were evaluated by isotope uptake a microplate method was applied (32). The plates (Microtest II, catalogue no. M-29-ART Flow Laboratories, Ltd) contained 96 wells, each with 28 mm<sup>2</sup> surface area (of bottom). The culture volume was 0.25 ml and the initial cell number was  $3 \times 10^5$  lymphocytes/0.25 ml ( $1.2 \times 10^6$ /ml), unless otherwise stated. The cell suspensions were distributed by a Repette syringe (Jencons, Hemel Hempstead, England) and stimulants were added by a Hamilton microsyringe (100 μl) adjusted into a modified repeating dispenser (Hamilton; Howe & Co. Ltd, London, England). For cytological analysis  $2.4 \times 10^6$  lymphocytes/2-ml medium were cultured in plastic vials (bottom area 180 mm<sup>2</sup>, catalogue no. 118/S, Sterilin Ltd, Richmond, England). The plates, covered with lids, and vials were placed into a desiccator and gassed with 5% CO<sub>2</sub> in air. The microcultures were usually set up in triplicate and the vials were set up in duplicate.

*Stimulants Used.*—PHA (purified PHA, Burroughs Wellcome Labs Ltd, Beckenham, England) and LPS from *Salmonella marcescens* (purified by Westphal method catalogue no. 3130–25–8 Difco Laboratories, East Molesey, England) were used in different doses and val-

TABLE I  
Cell Surface Markers and Cell Populations Used

Cells	% Reactive cells						Mononuclear phagocytes
	T lymphocyte markers		B lymphocyte markers			Aggregated IgG	
	anti-T sera	(E)SRBC	anti-B sera	anti-Ig	EAC(C3)		
<b>Tonsil</b>							
Unseparated	52.8 ± 1.9	54.8 ± 1.6	44.5 ± 2.3	43.2 ± 1.8	34.0 ± 2.3	43.1 ± 3.1 (7)	0.5-2 (5)
T cells: single cycle*	95 (4)	94 ± 0.8 (27)	1.8 (4)	2.1 ± 0.3 (27)	0.2 (2)	1.8 (3)	<0.1 (5)
B cells: single cycle†	1.3 (3)	1.9 ± 0.3 (6)	97.1 (4)	96.7 ± 1.5 (6)	79.3 (3)	86.4 (3)	3.1 (2)
double cycle‡	—	<0.05 (5)	—	97.6 (5)	—	—	3.2 (2)
<b>Spleen</b>							
Unseparated	—	34.6 (5)	—	41.2 (2)	—	—	8.0 (1)
B cells: double cycle	—	<0.05	—	—	—	—	—

\* Yield of T cells, 49 ± 2.9. Mean values ± standard error.

† Yield of B cells, 84 ± 3.1.

‡ Yield of B cells, 75.8 ± 8.

|| Phagocytosis of latex particles over a 60-min period in the presence of 50% FCS. Cells examined in suspension by phase contrast and as stained preparations after smearing (cytocentrifuge).

ues observed at optimal doses (1-2 µg/ml and 30-60 µg/ml, respectively) are given. Concanavalin A (Con A, Pharmacia Fine Chemicals, Ltd, London, England) was used in optimal stimulating doses (3 µg/ml). Pokeweed mitogen (PWM) was purified from plant stems (*Phytolacca americana*) by the method of Börjeson et al. (6). The redissolved TCA-precipitated material was used and applied in a 50 µg/ml dose, unless otherwise stated.<sup>3</sup>

*Measure of Isotope Incorporation.*—After 48 h of cultivation 1 µCi [<sup>3</sup>H]thymidine (50 mCi/mmol spec act, prepared from TRA 120, The Radiochemical Centre, Amersham, England) was added to the microcultures. In one experiment 0.1 µCi [<sup>3</sup>H]thymidine of high spec act (20 Ci/mmol, TRK 120, Radiochemical Centre) was added to some cultures. 16 h later the suspension from each well was assayed for thymidine uptake as previously described (32, 34).

*Autoradiography.*—After 60 h of incubation [<sup>3</sup>H]thymidine (100 mCi/mmol, 8 µCi/ml) was added to the cultures. After an additional 16-h incubation the number of surviving small and large cells (lymphoblasts) were determined. To analyze the proportion and structure of labeled cells within the blastoid cell population, smears were prepared with a cytocentrifuge (Shandon Scientific Co., Camberley, England) Filter papers were wetted before adding the cell suspensions. Two autoradiographs/culture were prepared by the stripping film technique (Kodak AR.10 film, 5 days of exposure). Smears were finally stained with hematoxylin and eosin.

## RESULTS

*Analysis of Purified Tonsil T and B Cells Activated by Mitogens.*—Fig. 1 illustrates the results of analysis of purified tonsil T and B cells activated by various mitogens for 3 days in D-MEM/human serum (HS) medium. As shown

<sup>3</sup> Staphylococcal enterotoxin "B" (SEB) was a gift from Dr. W. Adler and was a purified protein preparation (33).

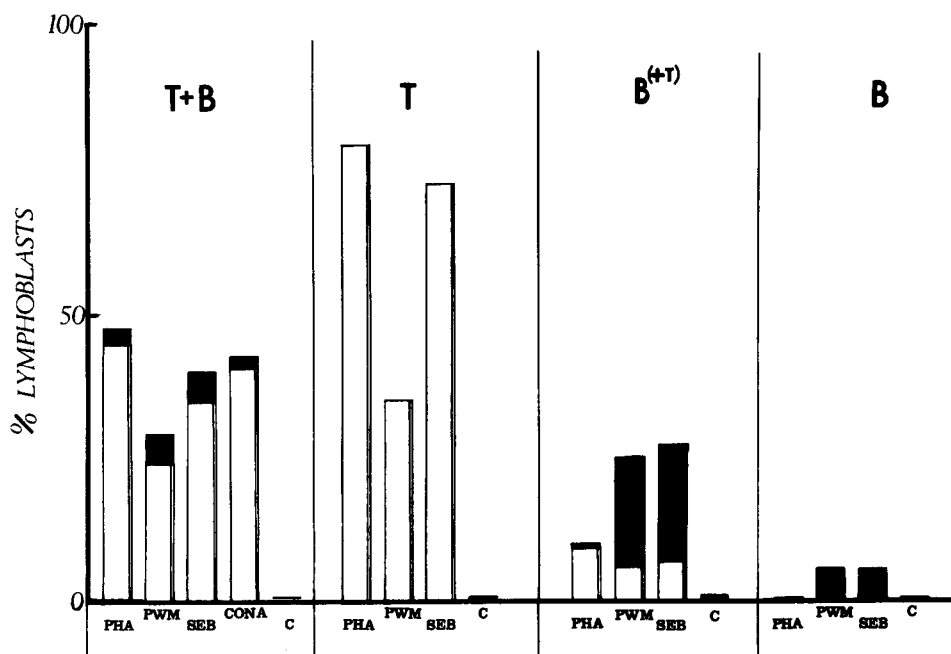


FIG. 1. Response of tonsil T and B lymphocytes to polyclonal mitogens. □, lymphoblasts verified as T type by surface marker analysis. ■, lymphoblasts verified as B type by surface marker analysis. Absolute numbers of lymphoblasts were counted and the level of response expressed in relation to the initial number of cells in the culture. C, control; T + B, unseparated tonsil cells; T, purified T cells (see Table I); B, purified B cells (double cycle) (see Table I); and B(+T), purified B cell (single cycle) (see Table I).

in Fig. 1, T cells are the predominating responsive cells in T-B mixtures. In PHA- and SEB-stimulated cultures 92-96 and 85-93%, respectively, of blastoid cells were of T-cell origin. The PWM response was slightly variable and T blasts contributed 70-83%; while the majority of the remaining large blastoid population (17-30%) was carrying surface Ig (B blasts). No LPS response was evident in any experiments with tonsil cells. Similar results were obtained in five further experiments.<sup>4</sup> Purified T lymphocytes responded well to PHA, PWM, and SEB. More than 99% of lymphoblasts in these cultures were verified as T lymphoblasts by surface marker analysis.

The purified B-cell population response depended to a considerable extent on the degree of T cell "contamination." In cultures with single cycle-purified B cells which contained 1-3% T cells a considerable response was evoked by PHA, SEB, and PWM. Analysis of cell surface phenotype of these lymphoblasts revealed, however, that a significant proportion were T cells, presumably

<sup>4</sup> Virtually identical results were obtained in three experiments with ficoll-isopaque-purified blood lymphocytes. Greaves and Janossy, unpublished observations.

derived from the residual small number of contaminating T lymphocytes. This was particularly so in PHA cultures where approximately 90% of lymphoblasts were in fact T cells. When B cells were more extensively purified by double cycling (see Materials and Methods) a different result was obtained. The PHA response was completely absent and the PWM and SEB were considerably reduced. Responses to the latter two mitogens were, however, significant and more than 95% responding lymphoblasts had the B-cell surface phenotype in three such experiments.

In further experiments we have performed cell surface marker analysis of lymphoblasts in mixed T-B lymphocyte cultures stimulated by low, intermediate, and high (with respect to [<sup>3</sup>H]thymidine uptake) doses of PWM, PHA, and SEB. The B-cell contribution to these responses was slightly greater at lower doses, however, it only ever accounted for more than 20% of the responding cell pool when PWM was the mitogen used.

*The T-Cell Dependency of Thymidine Uptake in Short-Term Cultures of Tonsil Lymphocytes with PHA.*—The results of cell surface marker analysis of activated lymphoblasts in tonsil cultures indicated that the response of T plus B lymphocytes was predominantly expressed by the former cell type and responses observed in “purified” B-cell populations attributable at least in part to contaminating T cells. The T-cell dependence of the PHA response was further established by a study of the uptake of thymidine by activated cells. When purified T and B lymphocytes were remixed together at varying proportions and stimulated by PHA it is clear that the response observed is proportional to the T-cell numbers (Fig. 2). The exact relationship between counts of radioactivity and numbers of responding T cells is however not precisely linear.

*Elaboration of Culture Conditions for B-Lymphocyte Activation.*—In the second part of this study we employed conditions which we had reason to believe would be more likely to permit good B-lymphocyte responses. Namely the use of RPMI FCS medium and spleen cells. The proportion of B cells in tonsil T- plus B-cell cultures responding to PHA and SEB is slightly elevated in our hands (up to 20%) by the use of the above medium. Experiments in mice have indicated that spleen B lymphocytes respond better to mitogenic stimuli (PWM, LPS) than B cells in lymph nodes, Peyer’s patches, and blood (M. Doenhoff, unpublished observations).

Thomas and Phillips (personal communication) have made similar observations although their B-cell response to PHA (35, 36) is considerably greater than what we observe under any conditions (see Discussion).

*Surface Marker Analysis of Activated Splenic Lymphocytes.*—The results of an analysis of activated splenic lymphocytes RPMI/FCS are given in Fig. 3. An excellent PHA response was observed in T- plus B-cell cultures; B lymphocytes contributed only 14% of the responding cells. The results with SEB as mitogen were virtually the same as with PHA. The PWM response differed significantly from the tonsil response, in involving a much higher proportion of B cells (85% of the lymphoblasts were B cells).

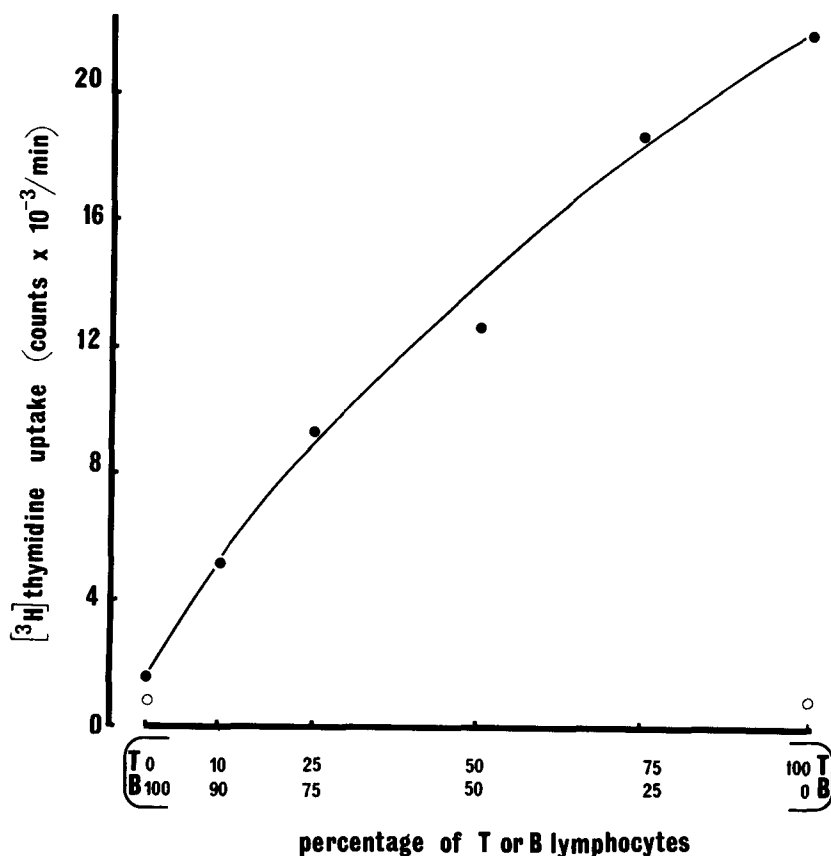


FIG. 2. Thymidine uptake in PHA-stimulated tonsil lymphocyte cultures containing variable proportions of T and B cells. Double cycle-purified B cells and single cycle-purified T cells mixed at variable proportions before culture. Total lymphocyte numbers the same in all cases.

Double cycle-purified B cells responded well to PWM, and activated lymphoblasts were virtually all verifiable B cells by surface marker analysis (see also below). Small B-cell responses to LPS and SEB were detected but no B cells responded to PHA under these conditions.

*The Proliferative Responses in Spleen Cultures.*—Two different approaches were used. First, the uptake of [<sup>3</sup>H]thymidine was determined during 48–64 h of culturing. Second, cells were labeled with [<sup>3</sup>H]thymidine during 60–76 h and then autoradiographic analysis was carried out.

As can be seen in Fig. 4 the thymidine uptake in the early cultures (48–64 h) of mixtures of T-B cells was highest in cultures with activated T lymphocytes (in PHA- and SEB-stimulated cultures, particularly in D-MEM/HS medium). There was excellent thymidine uptake in PWM-stimulated cultures, and on the basis of the high number of B lymphoblasts in these cultures (cf. Fig. 3), it can

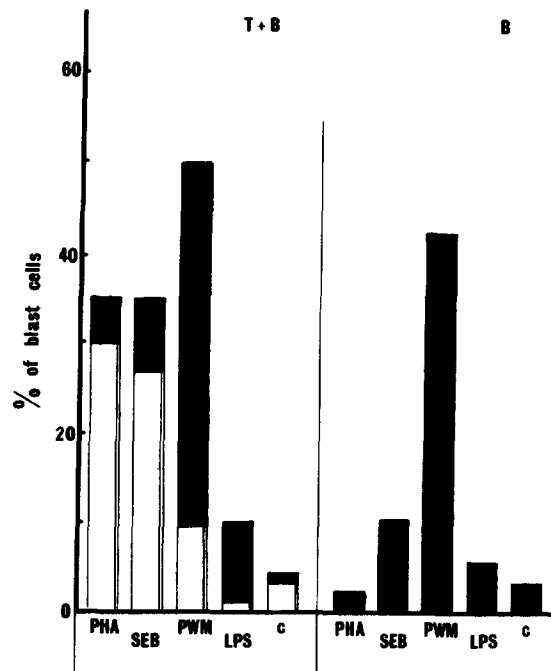


FIG. 3. Response of splenic T and B lymphocytes to polyclonal mitogens in RPMI 1640 medium. Legend as in Fig. 2. Double cycle-purified B cells (see Table I).

be assumed that activated B cells have in fact contributed considerably to the observed uptake of thymidine. LPS was a relatively poor stimulant of human spleen lymphocytes.

It is interesting, however, that there was a relatively low thymidine uptake in the pure B-lymphocyte cultures stimulated by all mitogens in such an early phase of the response, in spite of the remarkable blast transformation in the PWM-stimulated B-cell cultures. One possible interpretation of this data is that in the absence of T cells the onset of proliferative response of B cells to PWM is delayed for a few hours as compared to PWM-stimulated cultures of T and B cells. This was suggested by the autoradiographic analysis when cultures were labeled with [ $^3\text{H}$ ]thymidine during a slightly later period (60–76 h). If cultures of double cycle-purified B cells were incubated with PWM, a high proportion, but not all, of the activated large lymphoblasts were labeled (Fig. 5 c). This picture was altogether very different from that of PHA-incubated pure B-cell cultures, where only very few lymphoblasts could be seen and even fewer of them were labeled (Fig. 5 b). Occasionally, labeled blasts could be seen in unstimulated control cultures, too (Fig. 5 a).

*Persistence of Surface Markers on Activated T and B Lymphoblasts In Vitro.*— Since the panel of cell surface markers we, and others, have used have been



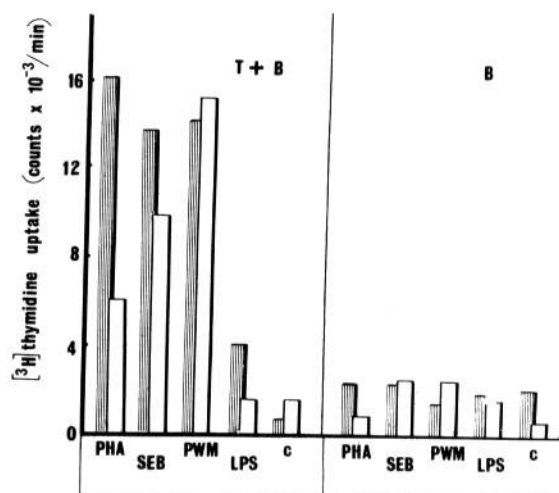


FIG. 4. Proliferation response of splenic lymphocytes to polyclonal mitogens. Legend as in Fig. 1. ▨, Dulbecco's MEM/HS; and □, RPMI 1640/FCS. B, double cycle purified (Table I).

developed and assessed for specificity primarily on the basis of their reactivity with small lymphocytes the approach used in this study provides a useful means to establish the persistent expression of the various markers when cells are cultured and pass through mitotic cycles (37). Our standard experiments have employed anti-T-cell serum and E rosettes as routine T-cell markers, and anti-Ig and anti-B-cell serum as B-lymphocyte markers. In additional experiments, however, we have added the three additional markers mentioned in the Materials and Methods section, namely antithymocyte and T-lymphoblast serum, EAC rosettes (for B-cell subset), and aggregated IgG for B cells. The pooled results from a series of experiments are given in Fig. 6. It should be noted that the slight expression of T-cell markers in B-cell responses is due to the presence of a small proportion of T lymphoblasts in LPS-stimulated un-separated (T and B) spleen cells. Less than 1% of PWM-activated B cells have any of the three T-cell markers. These results confirm the selectivity of the markers established on noncultured small lymphocytes and moreover indicate that all markers are stable, continuously synthesized, and not lost when cells become activated and enter the cell cycle. However, since only a subset of B lymphocytes express the C3 receptor before activation (Table I), we cannot rule out the possibility that some B cells gain or lose this receptor during activation. All seven markers have given similar results when applied to continuous lymphoblast cell lines.<sup>5</sup> One slight, but very interesting, discrepancy does emerge from this study however, which is the apparent expression on a small minority of activated T cells of binding sites for aggregated immunoglobulin.

<sup>5</sup> Greaves, M. F., G. Brown, and M. Epstein, manuscript submitted for publication.

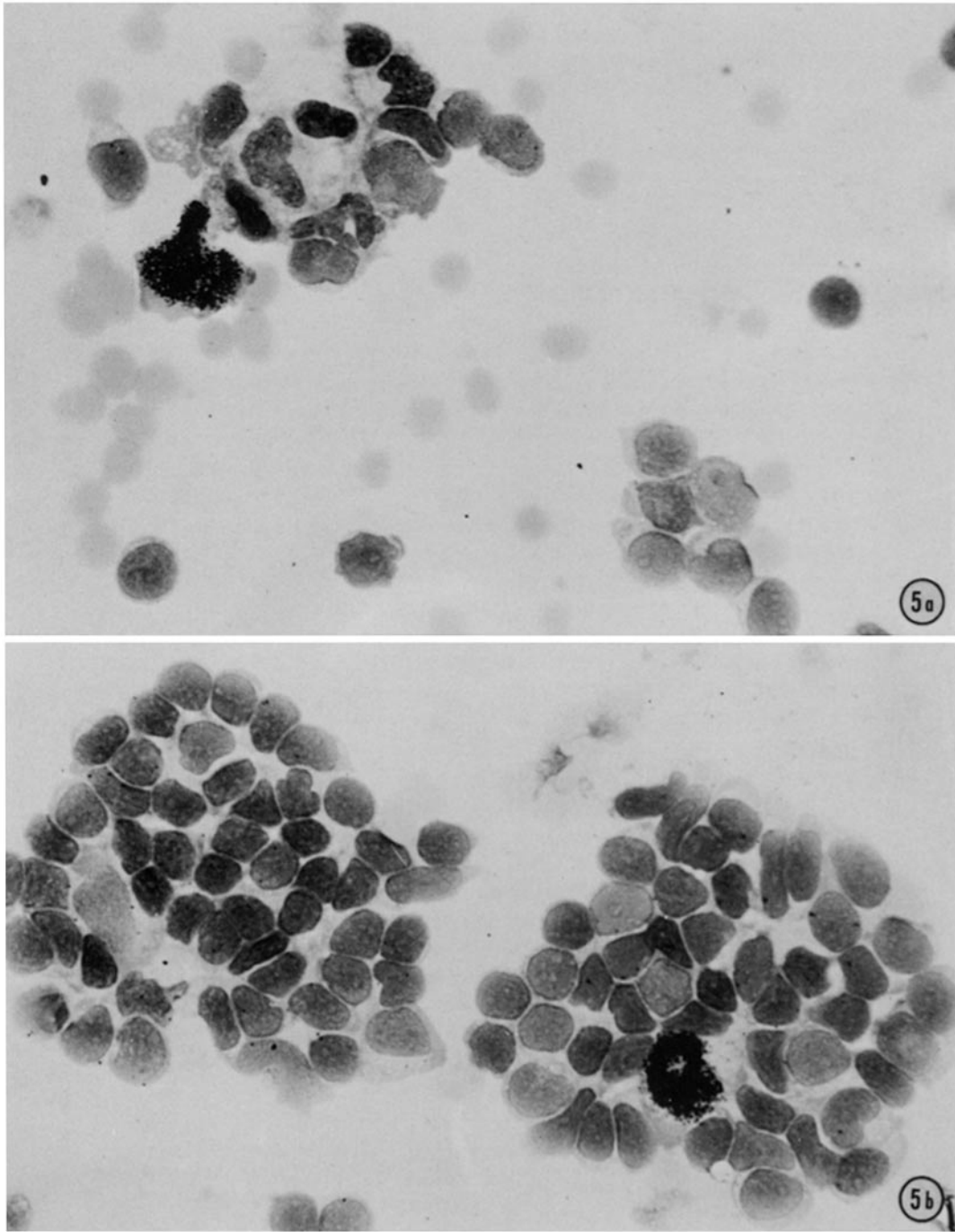
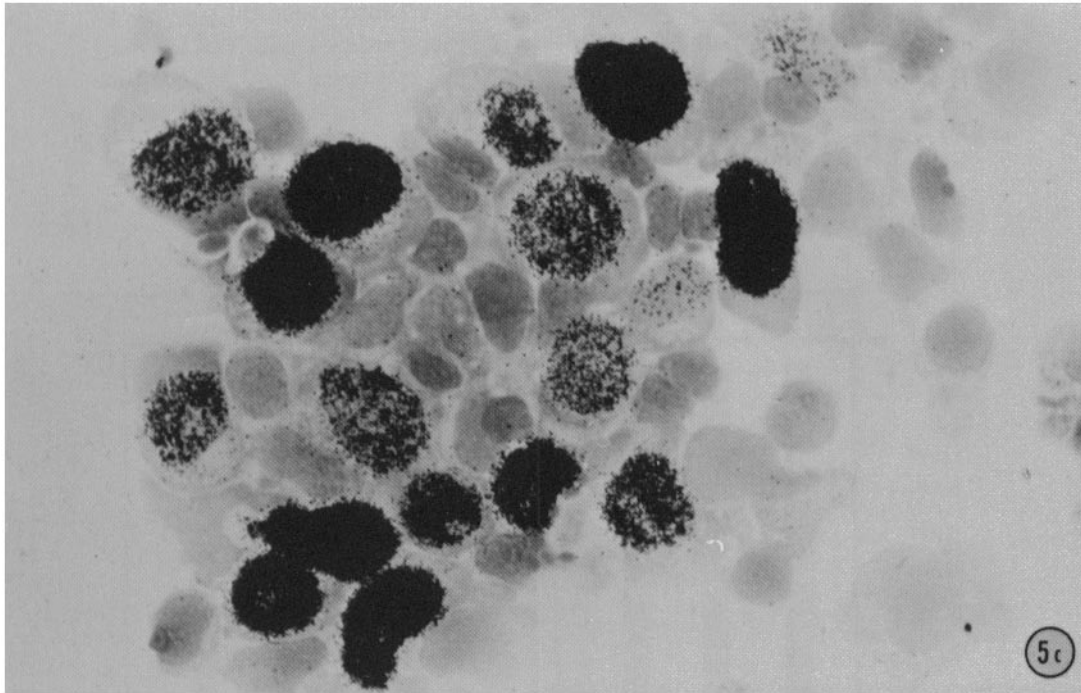


FIG. 5. Autoradiographic analysis of mitogen-activated splenic lymphocytes (a) Control (unstimulated cultures). (b) PHA-stimulated culture. (c) PWM-stimulated culture. (see Materials and Methods for conditions of experiment).  $\times 860$ .



We have previously observed a similar result with T lymphoblasts present in the tonsil suspensions immediately after initial preparation (20).

#### DISCUSSION

The purpose of this study was to map the previously described heterogeneity of PHA and PWM responses of human lymphocytes onto the well established dichotomy of lymphocytes into T- and B-cell categories. Our precedent was the already established selective response of these two major lymphocyte populations, in mice, to various polyclonal stimulants (3, 4, 16, 38). The application of a panel of seven discriminating markers for cell surface molecules on T and B lymphocytes combined with cell purification methods have provided a reliable basis for the identification of T- and B-axis cells responding to lectins and bacteria-derived mitogens. Activation of purified T and B cells over a 4-day period in vitro revealed that the cell surface markers used were consistently expressed in a selective manner and stable during the cell cycle.

We have restricted our attention to the relatively early culture period of the first 4 days, because this is when we consider it easier to establish quantitative relationships. It is readily apparent that the T- and B-lymphocyte response to polyclonal mitogens in man is influenced by the source of lymphocytes, the cellular composition of the culture (i.e. proportions of T cells, B cells and pos-

sibly other cells including macrophages), the culture media, and the particular mitogen used. The experimental model is, in other words, complex and subject to a variety of qualifications. In Table II we have attempted to summarize the relative response capacity of T and B cells to various polyclonal mitogens under our culture conditions.

When T and B lymphocytes from blood, tonsil, or spleen are cultured together the contribution of T and B cells to the PHA and PWM responses is rather what would be suspected from earlier, less direct, studies in man (6, 9)

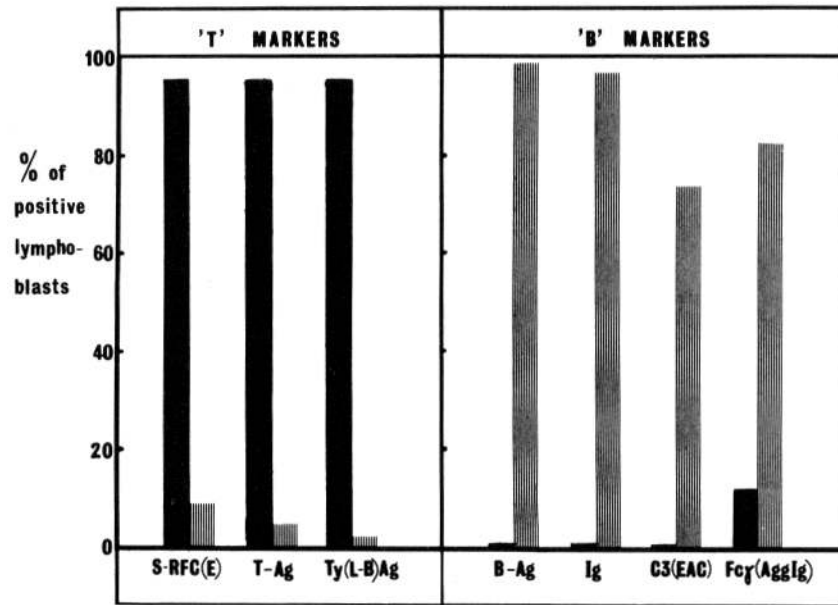


FIG. 6. Expression of T- and B-lymphocyte cell surface markers on activated lymphoblasts. ■, T lymphocytes from tonsil activated by PHA, Con A, PWM, and SEB (pooled data). ▨, Spleen cells activated by LPS and purified spleen B lymphocytes activated by PWM.

TABLE II

*Activation of Human T and B Lymphocytes by Polyclonal Mitogens, Summary Statement*

	Spleen			Tonsil				Blood; mixed	
	Mixed		Pure	Mixed		Pure		T	B
	T	B	B	T	B	T	B		
PHA	++++	+	-	++++	+	++++	-	++++	+
PWM	+++	+++	+++	+++	++	+++	+	+++	+
SEB	++++	+	+	++++	+	+++	+	++++	+
LPS	-	+	+	-	-	-	-	-	-

and parallel studies in mice (3, 16, 38, 39). The PHA response is dominated by the T-lymphocyte component, whereas the PWM response is more of a mixture. The relative contribution of B cells to the PWM response depends on cell source and culture medium used, and was most pronounced when spleen cells were cultured in RPMI plus FCS medium. Although the B-cell contribution was consistently low in PHA cultures; we do not exclude the possibility suggested by other experiments in man (35) and mice (40, 41) that different culture conditions or time of assay (i.e., late) might provide a different picture in which activated B cells are more prominent. What is important to consider however is whether conditions for exclusive T-cell activation are available and the extent to which any observed B-cell responses are T-cell dependent. We consider that the first point is in part fulfilled by the results we described, namely that when T and B cells are grown in D-MEM/HS medium for 3 days over 95% of the activated lymphoblasts are T cells. Combined marker analysis plus autoradiography will be required to establish whether the very small B-cell component of response actually contributes anything at all to the response as measured by thymidine incorporation.

T cells purified from tonsil responded well to PHA, PWM, and SEB. We conclude that this response is independent of macrophages although a facilitating role of the 5% non-T and non-B cells in these cultures has not been excluded. The exact proportion of human T cells responding to these polyclonal stimulations has not been assessed; however our results and previous studies in rodents (32) suggest that greater than 50% probably do respond. This value could however vary with the source of T cells and differential T-subset distribution (2). It is interesting to note that T cells that have responded previously to major histocompatibility antigens may be unreactive to polyclonal mitogens (42). B lymphocytes purified from spleen and tonsil responded to PWM although only the former source provided a highly responsive population.

The splenic B-cell proliferative response to PWM is delayed with respect to that of T cells, which differs from our previous observations in mice where B cells, if anything, respond rather sooner than T cells as judged by chromosome marker analysis (43). Other studies of PWM responsiveness in man also suggest that the optimal B-cell response may be delayed relative to the T-cell responses (10, 44).

Under conditions where B cells are responding well to PWM in terms of lymphoblast formation and thymidine incorporation (Fig. 6), there are weak responses to LPS and SEB; this is in contrast to studies in mice where LPS is a more potent B-cell mitogen (3-5). Spleen and tonsil B cells do not respond at all under our culture conditions to PHA, although a small response was evident (Fig. 1) in cultures with 1-3% residual T cells. In this situation, however, the majority of the activated lymphoblasts are in fact T cells. This result suggests that marginal B-cell responses to PHA as seen in cultures of T and B cells are probably T-cell dependent. This interpretation is supported by the demonstra-

tion (Fig. 3) that the PHA response in artificial graded T- and B-mixed cultures is proportional to the T-cell proportion.

These are perhaps the results one might have anticipated from parallel experiments in rodent and chicken systems and from studies of selective immunodeficiency diseases in man. However, our inability to stimulate B cells with PHA, in the absence of T cells, contrasts with the recent results of Phillips and colleagues (35, 36), who have reported that human tonsil B cells absorbed into Sephadex anti-Ig columns and eluted by digestion of the absorbent support by dextranase, do in fact respond by lymphoblast activation and DNA synthesis. These authors also report a considerably more substantial contribution of B cells to the PHA response in T-B cell mixtures. The finding that a large proportion of PHA-activated blood-derived lymphoblasts have readily demonstrable Ig light chain determinants (45, 46) may also be taken to reflect a considerable contribution of B cells to PHA-induced responses of mixed T- and B-cell cultures. The reason for these discrepancies is unclear. We think it unlikely that our culture conditions are inimical to B-cell responses since we have the positive control of the PWM response, at least with spleen B cells. In addition our media and general conditions are in fact the same as those used by Phillips and colleagues. While we are reluctant to rule out that minor variations in culture conditions can radically effect B-cell responsiveness, we consider that several other interpretations of the reported PHA response of purified B cells (35, 36) are possible: (a) That some B cells respond to PHA in the presence of T cells. Our results with T-B mixtures support the possibility, however, as shown above when B-cell cultures were "contaminated" with only 2-3% T cells the majority of the small pool of responding lymphoblasts were T cells. Some evidence for activation of mouse B cells by soluble lectin (Con A) in the presence of supernates of activated T cells has been presented (4, 47), however, in our experience these synergistic effects are quite small in relation to a "direct" B-cell mitogen such as LPS or PWM (43). (b) That B cells will respond to PHA in the presence of adequate numbers of macrophages. Evidence for macrophage facilitation of PHA responses is available (48) and recent work suggests that human B-cell activation detectable after 7 days in culture may be induced by PHA in the presence of macrophages (49). This is also unlikely to be the explanation for the discrepancy in results under discussion, since both ourselves and Phillips and colleagues used similar cell preparations from tonsils. (c) We consider it necessary to exclude that B cells purified by elution from insoluble anti-Ig absorbents are not in fact subliminally stimulated by anti-Ig antibodies. Clear precedents for such effects have been reported (50, 51). "Priming" of B cells by anti-Ig might well render them responsive to subsequent challenge with PHA. Clearly, B cells have PHA binding sites (52) and are not obligatory nonresponders as evidenced by experiments with insolubilized PHA (53).

It is relevant to this discussion that cytotoxic anti-human T-cell sera have been found to completely abolish the PHA response, providing further evidence that under most conditions the PHA response is certainly thymus dependent (references 54 and 55, and footnote 6). Finally, the response to PHA is effectively absent in cultures of lymphocytes from patients with thymic

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<sup>6</sup> M. Cooper, personal communication.

aplasia (56, 57). Although the remarkably quick return of PHA responsiveness after thymus grafting (references 56 and 57, and footnote 7) poses intriguing problems regarding the origin and nature of responsive cells, these observations are clearly indicative of the thymus dependency of PHA responses.

We conclude that our results confirm the earlier suggestion (6, 9) that PHA primarily stimulates T lymphocytes and PWM, T and B cells. This pattern of selectivity parallels that found in the mouse (39, 40).

The PHA response can therefore be used, under controlled conditions of cellular composition, culture media, and assay, as a qualitative or semiquantitative measure of the capacity of blood-borne T cells to become activated and to divide. At present there appears to be no polyclonal mitogen that will provide the same opportunity to study B lymphocytes in blood.

#### SUMMARY

Human lymphocytes from spleen and tonsils have been cultured with a variety of polyclonal mitogens. Cultures consisted of either unseparated T and B cells or alternatively purified T or B lymphocytes. The purity of the starting cell populations and the origin of activated lymphoblasts was analyzed with a panel of seven markers which discriminate between T and B cells. The selectivity of the lymphocyte responses was influenced by cell populations in a given culture, the mitogen used, and to a limited extent on culture conditions. Purified T lymphocytes from tonsil and spleen responded to phytohemagglutinin (PHA), pokeweed mitogen (PWM), and staphylococcal enterotoxin B (SEB). Purified B cells from spleen responded well to PWM, weakly to SEB and lipopolysaccharide, but not at all to PHA. Tonsil B cells responded weakly to PWM and SEB but not to PHA. Some B lymphocytes do respond to PHA in the presence of activated T cells. These results are discussed in relation to previously reported selective responses of human cells and parallel studies in animal species.

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