Clin. exp. Immunol. (1975) **21**, 456–467.

PHA STIMULATION OF SEPARATED HUMAN LYMPHOCYTE POPULATIONS

M. R. POTTER AND M. MOORE

Immunology Division, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester

(*Received* 14 *February* 1975)

SUMMARY

Lymphocyte preparations from peripheral blood and tonsils were separated into populations enriched with T or B cells by formation of rosettes with SRBC and separation of the rosette-forming and non-rosette-forming populations. T cell-enriched populations were also prepared by nylon column filtration. Using these methods preparations were obtained which comprised 80–95% T or B lymphocytes as determined by E-rosette formation and surface immunoglobulin (Ig) staining. PHA responsiveness, measured by [^{3}H]thymidine incorporation, varied between relatively wide limits and was critically dependent on the degree of separation obtained. Relatively pure B-cell populations (<12% T cells) from blood and tonsils gave low PHA responses while preparations from blood still containing 24–38% T cells gave responses equal to or even greater than those of unseparated controls (60–78% T cells). T cell-enriched populations (80–86% T cells) responded to an equal or greater degree than controls but more efficient separation (>90% T cells) resulted in markedly reduced stimulation. There was thus no simple correlation between the degree of phytomitogen-induced transformation and the number of T cells present. It is concluded that the low response of relatively pure T-cell populations may be due to depletion of B cells or non-lymphoid cells (or both) during the separation procedures. These observations have implications for the use of PHA stimulation as a measure of T-cell activity in mixed populations such as those of human peripheral blood leucocytes.

INTRODUCTION

Distinctive cell surface properties of thymus-dependent (T) and thymus-independent (B) lymphocytes of mammalian lymphoid tissue are now well documented (Greaves, Owen & Raff, 1973). Several marker systems have been developed to selectively label human lymphocytes (Möller, 1973), of which the most commonly used are rosette formation with sheep

Correspondence: Dr M. Moore, Immunology Division, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX.

erythrocytes for identification of T cells and immunofluorescent staining with anti-immunoglobulin for characterization of B cells. More recently these techniques have provided a basis on which the respective populations might be separated: B cells have been selectively removed using anti-immunoglobulin-coated columns (Wigzell, Sundqvist & Yoshida, 1972; Chess, MacDermott & Schlossman, 1974; Schlossman & Hudson, 1973); while enriched T-cell populations have been obtained by isolation of rosette-forming cells or nylon wool column filtration (Wybran, Chantler and Fudenberg, 1973a, b; Yata *et al.*, 1973; Greaves & Brown, 1974).

Although certain *in vitro* properties in a heterogeneous mixture of cell types are frequently attributed to specific cell populations, it may not necessarily follow that the behaviour of these populations will be similar in isolation. Responsiveness of human lymphocytes to phytohaemagglutinin (PHA), as for those of mice (Takiguchi, Alter & Smith, 1971) and chickens (Greaves, Roitt & Rose, 1968) is regarded as predominantly a T-cell property (Janossy & Greaves, 1971). However, the extent to which T cell-enriched populations respond to PHA in comparison with unseparated lymphocytes and the influence of non-T cells on the transformation characteristics of these populations has not been widely examined. In this study we have attempted to correlate the degree of transformation of T and B cell-enriched populations, prepared by the more commonly used separation procedures, with the respective numbers of T and B cells estimated by surface marker analysis.

MATERIALS AND METHODS

Tonsils. Tonsils were obtained from children undergoing tonsillectomy at the Manchester Royal Infirmary. Cell suspensions were prepared by finely chopping the tonsils and teasing through a stainless steel mesh. The cell suspension was collected in Hanks's balanced salt solution (HBSS), washed by centrifugation and then resuspended in HBSS containing 10% foetal calf serum (FCS). The cell suspension was thereafter incubated in glass medical flats at 37°C for 30 min to deplete the adherent cell population (2×10^8 cells in 150 ml medical flats lying horizontally). Non-adherent cells were decanted and washed in HBSS; the resulting cell suspension consisted of 95–99% lymphocytes as determined by differential counts on stained smears.

Blood. Heparinized blood samples were obtained from the National Blood Transfusion Service, Manchester, and from laboratory personnel. Leucocyte preparations containing a high percentage of lymphocytes were obtained by Ficoll–Triosil gradient centrifugation. Blood was diluted with an equal volume of PBS and layered on top of a mixture of Triosil and 9% Ficoll. After centrifugation at 800 *g* (600 *g* in some early experiments) for 20 min, the lymphocyte-rich interfacial layer was removed and the cells washed three times in HBSS. Differential cell counts on stained smears of leucocyte preparations gave values of 86–95% lymphocytes, 3–10% polymorphs and 2–5% monocytes.

E rosettes. Sheep red blood cells (SRBC) (Wellcome Reagents Ltd, Beckenham, Kent) were washed twice in PBS and adjusted to a concentration of 2×10^8/ml in FCS (SRBC were used within 2 weeks of collection). The washed lymphocyte preparation was adjusted to a concentration of 5×10^6/ml in FCS. Equal volumes of SRBC and lymphocyte preparations were mixed (0·1 or 0·2 ml), the cells sedimented by centrifugation and incubated at 4°C for 2–18 hr. All lymphocyte preparations were tested in triplicate. The cells were then gently resuspended and examined in a haemocytometer; the percentage of rosette-forming cells was determined from the number of lymphocytes with three or more attached SRBC and the total number of lymphocytes present.

Fluorescent staining for surface immunoglobulin. Fluorescent staining for surface immunoglobulin was performed using a two-stage technique. Lymphocytes (2×10^6) were first incubated with a mixture of sheep anti-human IgG, IgM and IgA (Wellcome Reagents Ltd, Beckenham, Kent) at a dilution of 1:10 in PBS, the total volume being 0·1 ml. After 30 min incubation at 4°C the cells were washed twice with PBS and then incubated with a 1:10 dilution of rabbit anti sheep immunoglobulin labelled with fluorescein (Wellcome Reagents Ltd). Following a further 30 min incubation at 4°C the cells were washed three times in PBS and

examined under a Wild M20 fluorescence microscope. The percentage of cells showing surface fluorescence, consisting of multiple point staining was then determined.

Nylon column separation. The method used was essentially that of Greaves & Brown (1974). A 5-ml disposable syringe was packed under water with 600 mg of scrubbed nylon fibre (FT 242 Fenwal Laboratories, Illinois, U.S.A.) and a plastic tube and screw clip connected to the syringe to adjust the flow rate. The column was washed through with HBSS followed by HBSS supplemented with 10% FCS and then incubated for 30 min at 37°C before addition of the cell suspension. Lymphoid cells (10^8) were slowly run into the column (in 2 ml of HBSS/10% FCS) which was then incubated at 37°C for 30 min. The cells not adhering to the nylon fibre were thereafter eluted by gently washing the column through with HBSS containing 10% FCS. The eluted cells were finally washed in HBSS. The yield of cells obtained under these conditions represented 40–50 % of the initial T-cell population (15–25% of the total population). An aliquot of the cell suspension was not passed through the column and this was used as a control cell population.

Rosette sedimentation. SRBC and lymphocyte preparations were prepared and mixed as described for E-rosette formation except that larger volumes were used (5 ml of SRBC + 5 ml of lymphocytes). After rosette formation the mixture was diluted with an equal volume of PBS and layered on to a Ficoll–Triosil gradient as described for blood lymphocyte separation. After centrifugation at 800 *g* for 20 min at 4°C the interface (non-rosette-forming cells) and pellet (rosette-forming cells) layers were carefully removed from the gradient and collected in separate tubes. The cells were washed with HBSS and then the cell pellet resuspended in 3 ml of 0·75% NH_4Cl in Tris buffer (pH 7·2) for 5 min to lyse the erythrocytes present. The two cell preparations were then washed in HBSS. Control lymphocytes were rosetted in the same manner as the test cells and then the erythrocytes were lysed, but there was no Ficoll–Triosil separation step. The rosette-forming population contained 94–98% lymphocytes and the non-rosette-forming population 83–93% lymphocytes, as determined by differential cell counts on stained smears. The yield of cells obtained from the separated and control rosetted population represented 50–70% of the initial population.

PHA stimulation. PHA stimulation was performed in 12 × 75 mm plastic tubes (Falcon Plastics) containing 2×10^5 cells in 0·4 ml of Hepes (40 mM) buffered MEM supplemented with 10% human AB serum. All tests were performed in triplicate and PHA was used at concentrations of 0·5, 1, 2 and 4 μg/ml (Wellcome purified PHA). Blood lymphocyte preparations were incubated for 3 days and tonsillar lymphocyte preparations for 4 days. Six hours before the end of the culture period 0·5 μCi of [^{3}H]thymidine (TRK 61 Amersham) was added to each tube in 0·1 ml of the culture medium. The culture was terminated by washing with PBS then precipitating twice with 5% TCA. The precipitate was solubilized in NCS tissue solubilizer (Amersham/Searle Corporation) and transferred to scintillation fluid (toluene containing PPO and POPOP) for counting. Results were expressed as mean values of triplicate tubes in counts per minute (ct/min). The maximum [^{3}H]thymidine incorporation did not exceed 8% of the total activity added.

RESULTS

Separation of lymphocyte subpopulations

Lymphocyte preparations obtained from tonsils and peripheral blood were separated into populations enriched with T or B cells by formation of rosettes with SRBC and separating the rosette-forming and non-rosette-forming populations by Ficoll–Triosil gradient centrifugation. Populations enriched with T lymphocytes were also prepared by passage of tonsillar and peripheral blood lymphocytes through columns packed with nylon fibre. The percentage of T and B lymphocytes in the preparations were determined before and after separation using rosette formation with SRBC as a marker for T lymphocytes and fluorescent staining with anti-human immunoglobulin as a marker for B lymphocytes.

Unseparated populations of tonsillar lymphocytes contained on average $41 \pm 1\%$ (s.e.) E rosette-forming cells and $42 \pm 2\%$ immunoglobulin-bearing cells (mean values for fourteen experiments) (Fig. 1). The percentage of E rosette-forming cells, in different lymphocyte preparations, varied from 33 to 52% and the percentage of immunoglobulin-bearing cells varied from 31 to 48%.

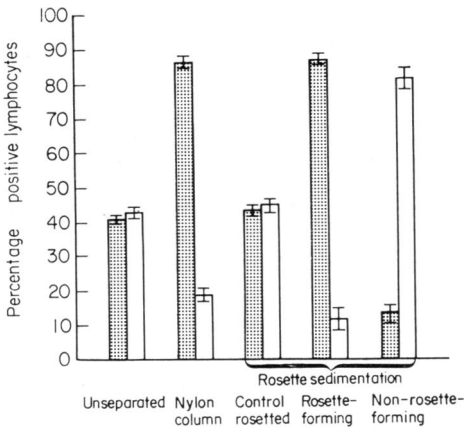

FIG. 1

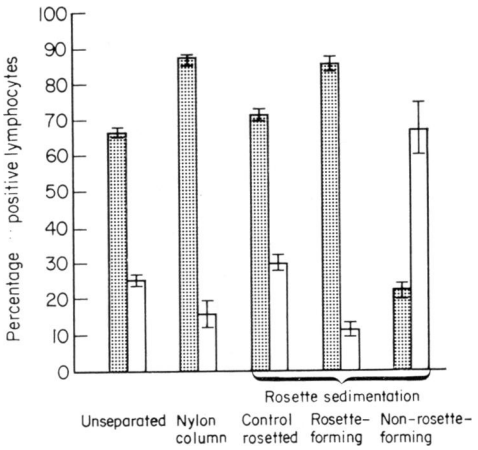

FIG. 2

FIG. 1. Separation of tonsillar lymphocyte suspensions by rosette sedimentation and nylon fibre column filtration. The percentage of T cells was determined by E-rosette formation and of B cells by fluorescent staining of surface immunoglobulin. The values for unseparated and nylon fibre column-filtered cells represent the mean (±s.e.) of fourteen experiments and the values for rosette sedimentation represent the mean of six experiments. E rosette-forming cells, stippled columns. Immunoglobulin-bearing cells, open columns.

FIG. 2. Separation of blood lymphocyte suspensions by rosette sedimentation and nylon fibre column filtration. The percentage of T cells was determined by E-rosette formation and of B cells by fluorescent staining of surface immunoglobulin. The values for unseparated populations represent the mean (±s.e.) of thirty-two blood samples and those for separated populations represent the mean of eight samples. E rosette-forming cells stippled columns. Immunoglobulin-bearing cells, open columns.

Blood lymphocyte preparations from thirty-two healthy individuals contained 58–81% E rosette-forming cells, with a mean value of 67±1%, and 18–34% immunoglobulin-bearing cells with a mean of 25±1% (Fig. 2).

When tonsillar lymphocyte preparations were separated by the method of rosette sedimentation on Ficoll–Triosil the population of cells recovered from the pellet contained on average 87±2% E rosette-forming cells and 11±4% immunoglobulin-bearing cells, while the population of cells recovered from the interface contained on average 82±3% immunoglobulin-bearing cells and 12±3% E rosette-forming cells (Fig. 1).

Separation of blood lymphocyte preparations by this method gave a pellet population containing on average 85±2% E rosette-forming cells and 10±2% immunoglobulin-bearing cells and an interface population containing 67±9% immunoglobulin-bearing cells and 22±2% E rosette-forming cells (Fig. 2).

Nylon column filtration of lymphocyte preparations from blood and tonsils produced populations containing on average 87±1% and 86±2% E rosette forming cells respectively (Figs 1 and 2).

PHA stimulation of tonsillar lymphocytes

In order to investigate the time course and the dose dependence of the PHA stimulation of tonsillar lymphocytes, cells were incubated with various concentrations of PHA and

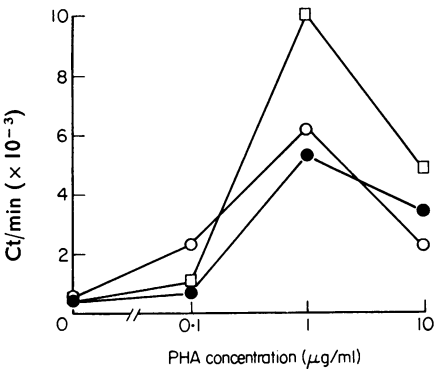

FIG. 3. Dose–response curve for PHA stimulation of tonsillar lymphocytes after 3 (○), 4 (□) and 5 (●) days of culture. [^{3}H]thymidine incorporation was measured over the final 6 hr of culture. The results are expressed as mean ct/min for triplicate tubes.

the levels of [^{3}H]thymidine incorporation were measured on days 3, 4 and 5 of culture. The results are shown in Fig. 3. Maximum stimulation was obtained after 4 days of culture with 1 μg/ml of PHA. On the basis of this observation, in subsequent experiments isotope incorporation was measured on day 4 of culture and PHA was used at concentrations of 0·5, 1, 2 and 4 μg/ml to ensure that the peak of the response was being measured.

The PHA responsiveness of tonsillar lymphocytes was measured before and after separation by rosette sedimentation and nylon fibre column filtration. The results of four separate experiments are shown in Fig. 4. Enrichment of the T-cell population by nylon fibre column filtration resulted in an increase in the amount of isotope incorporated compared with the amount incorporated by control untreated cells (Fig. 4A and C). In the two experiments performed the largest increases were obtained when PHA was used at a concentration of 1 μg/ml, at which the levels of incorporation were respectively 42 and 48% above the values for control untreated cells. At 2 and 4 μg/ml of PHA the values were between 25% and 40% above the control values, while at 0·5 μg/ml the increases were 2 and 21%. In one experiment where the cells were subjected to a second nylon column filtration the increases were between 49 and 65% at the three higher concentrations of PHA while at a concentration of 0·5 μg/ml the value was 19% (Fig. 4A).

Fractionation of the tonsil lymphocyte preparations by rosette sedimentation produced two populations with very different PHA responses. The rosette-forming population had an increased level of [^{3}H]thymidine incorporation; in two experiments the maximum incorporation was 59% and 56% above the control values and occurred at 0·5 and 1 μg/ml of PHA respectively (Fig. 4C and D) while in the third experiment a significant increase was found only at 4 μg/ml of PHA where the value was 20% above the control (Fig. 4B). The non-rosette-forming population had a much reduced level of isotope incorporation, in one experiment the values at all four PHA concentrations were reduced to between 16 and 22% of the corresponding control value (Figure 4B) while in two further experiments the values were reduced to between 18 and 39% and to between 33 and 47% of the control values (Fig. 4C and D). In two experiments the rosette-forming and non-rosette-forming populations were mixed in equal numbers as a further control for the preparation procedures. In both experiments the levels of isotope incorporation by the mixed population, at all

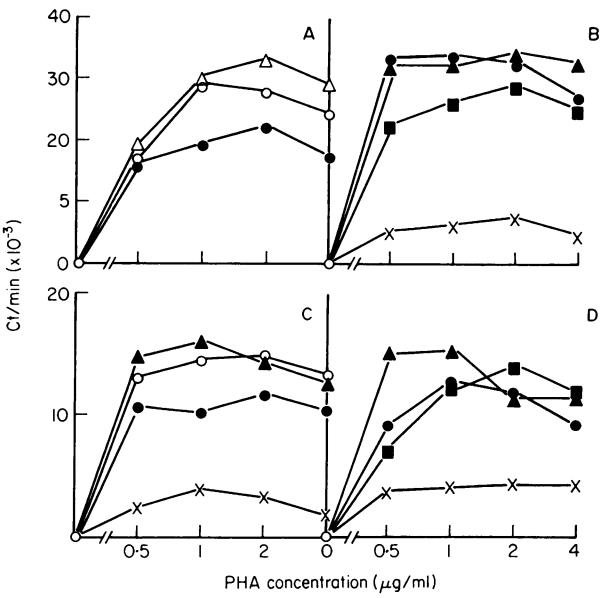

FIG. 4. PHA stimulation of separated tonsillar lymphocytes. Equal numbers of lymphocytes from each preparation (2×10^5/tube) were cultured for 4 days with various concentrations of PHA (0, 0·5, 1, 2 and 4 μg/ml) and the [^{3}H]thymidine incorporation was measured during the final 6 hr of culture. Results are expressed as the mean ct/min for triplicate tubes and each graph represents a separate experiment. The following lymphocyte populations were tested: nylon fibre column-filtered once (○); nylon fibre column-filtered twice (△); rosette-forming (▲); non-rosette-forming (×); rosette-forming plus non-rosette-forming (■); control unseparated (●).

four PHA concentrations, were within 70 and 130% of the control values. In one experiment (Fig. 4D) two values were above the control and two below, while in another experiment the incorporation was below the control at all four PHA concentrations (Fig. 4B) suggesting that there may be some reduction in the PHA responsiveness as a result of centrifugation through Ficoll–Triosil.

PHA stimulation of blood lymphocytes

The time course and the dose dependence of the PHA stimulation of blood lymphocytes was examined by culturing lymphocytes with various concentrations of PHA and measuring the amount of [^{3}H]thymidine incorporated after 1–5 days of culture (Fig. 5). By contrast with tonsillar lymphocytes the maximum response was obtained after 3 days in culture with a PHA concentration of 1–2 μg/ml. Blood lymphocyte preparations were separated into populations enriched with T and B cells by rosette sedimentation and filtration through nylon fibre columns.

The PHA responses produced by T and B cell-enriched populations prepared from blood lymphocyte suspensions showed considerable variation. In four out of eight experiments (Fig. 6A–D) the PHA response of the T cell-enriched population obtained by rosette sedimentation was considerably lower than the value for the control unseparated cells; in two experiments (Fig. 6C and D) the response was between 26 and 48% of the control values at all

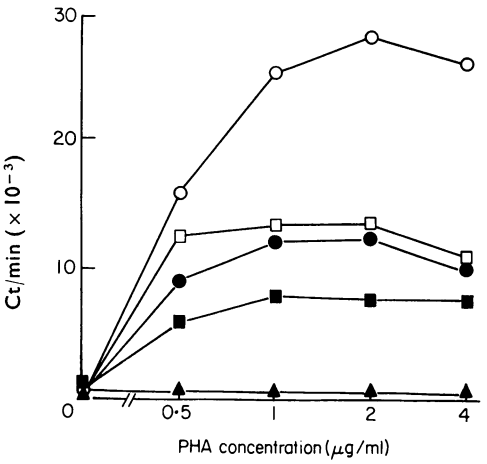

FIG. 5. Dose–response curve for PHA stimulation of blood lymphocytes on days 1–5 of culture. [^{3}H]thymidine incorporation was measured over the final 6 hr of culture. The results are expressed as mean ct/min for triplicate tubes. Day 1 (▲); day 2 (■); day 3 (○); day 4 (□); day 5 (●).

four PHA concentrations while in the other two experiments the values were between 54 and 89% of the control (Fig. 6A and B). In the remaining four out of eight experiments the rosette-forming population gave a PHA response which was equal to or greater than the control values; in one experiment (Fig. 6E) the PHA response was within 3% of the control value at all four PHA concentrations while in three other experiments the PHA response ranged from values equal to the control up to 94% above the control (with the exception of a single PHA concentration (1 μg/ml) in one experiment which was 16% below the control (Fig. 6G).

In two experiments performed with nylon column-purified blood lymphocytes the PHA response produced was below the value for control cells not passed through the column (Fig. 6B and C). In one experiment (Fig. 6C) the values were between 44 and 59% of the controls at all four PHA concentrations while in the second experiment (Fig. 6B) the values at 1, 2 and 4 μg/ml PHA were between 78 and 87% of those in controls and at 0·5 μg/ml, 5% above the control.

PHA responses of the B cell-enriched non-rosette-forming population remaining after removal of rosette-forming cells also fell into two main groups: those that where either equal to or greater than the control values on the one hand, and considerably lower than control values on the other. In four out of eight experiments the non-rosette-forming population gave a response which was only 12–56% of the control values (Fig. 6C, E, F and G) while in three other experiments the response was between 20 and 100% above the control (Figure 6A, B and H). The eighth experiment showed no clear pattern with values above and below the control (Fig. 6D).

When the PHA responses produced by the T and B cell-enriched populations are compared with the degree of purity of the preparation, as determined by the percentage of E rosette-forming cells present, it becomes clear that small variations in the composition of the preparation can produce dramatic changes in the PHA response (Table 1). The T cell-

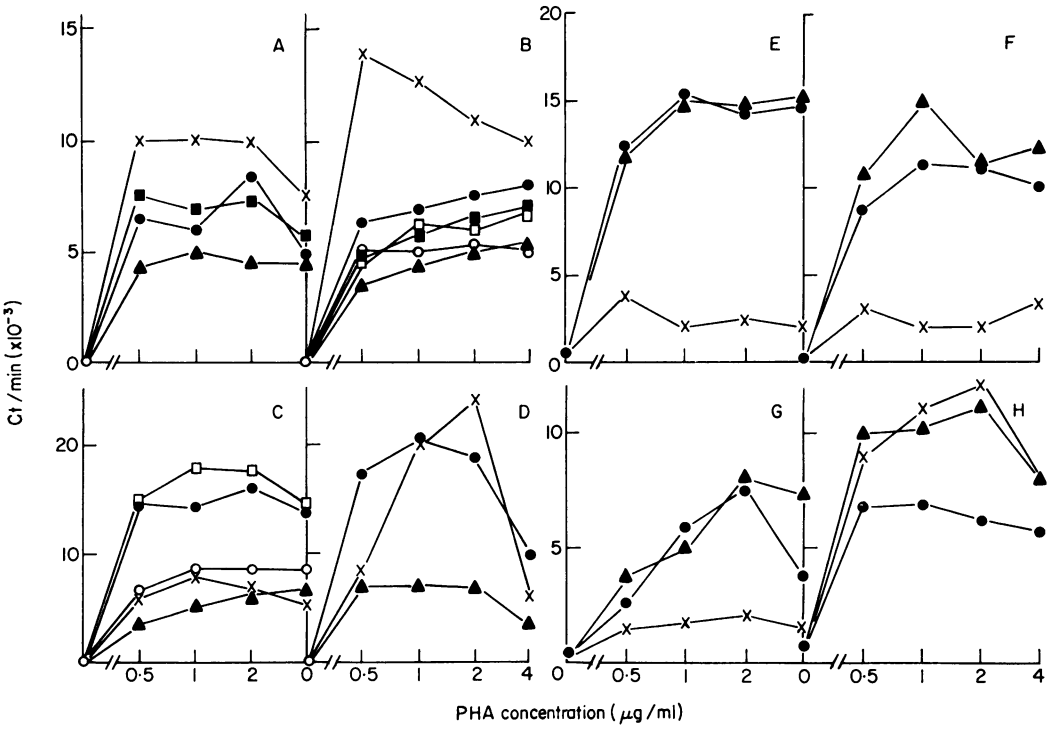

FIG. 6. A–D. PHA stimulation of separated blood lymphocyte suspensions. Equal numbers of lymphocytes from each preparation (2×10^5/tubes) were cultured for 3 days with various concentrations of PHA (0, 0·5, 1·2 and 4 μg/ml) and the [^{3}H]thymidine incorporation was measured during the final 6 hr of culture. Results are expressed as the mean ct/min for triplicate tubes and each graph represents a separate experiment. The following lymphocyte populations were tested: nylon fibre column-filtered once (○); rosette-forming (▲); non-rosette-forming (×); rosette-forming plus non-rosette-forming (■); control untreated (□); control rosetted but unseparated (●). E–H. PHA stimulation of separated blood lymphocyte suspensions. Equal numbers of lymphocytes from each preparation (2×10^5/tube) were cultured for 3 days with various concentrations of PHA (0, 0·5, 1, 2 and 4 μg/ml) and the [^{3}H]thymidine incorporation was measured during the final 6 hr of culture. Results are expressed as the mean ct/min for triplicate tubes and each graph represents a separate experiment. The following lymphocyte populations were tested: rosette-forming (▲); non-rosette-forming (×); control rosetted but unseparated (●).

enriched populations obtained by rosette sedimentation and nylon column filtration gave a low PHA response when the preparation contained less than 12% non-T cells (blood lymphocytes, experiments A–D) but preparations containing 14–20% non-T cells (blood lymphocytes, experiments E–F; tonsillar lymphocytes, experiments A–D) gave responses which were equal to or greater than the control. The B cell-enriched population obtained by rosette sedimentation also showed considerable variation in the PHA response depending on the purity of preparation. Preparations containing only 2–12% T cells (blood lymphocytes, experiments C, E, F, and G; tonsil lymphocytes experiments B–D) gave a low response to PHA whereas populations which still contained 24–39% T cells (blood lymphocytes,

TABLE 1. Percentage of E rosette-forming cells in blood and tonsil lymphocyte preparations separated by rosette sedimentation and nylon column filtration then examined for PHA responsiveness

		Percentage of E rosette-forming cells				
		Rosette sedimentation			Nylon column	
Lymphocyte source	Experiment number*	Control	Rosette-forming	Non-rosette-forming	Before column	After column
Blood	A	68·5	93·7	31·7	—	—
Blood	B	78·1	92·1	24·4	79·9	88·4
Blood	C	65·2	90·3	7·7	63·8	88·8
Blood	D	76·6	90·1	38·7	—	—
Blood	E	64·8	85·8	4·7	—	—
Blood	F	66·7	81·6	7·6	—	—
Blood	G	62·8	84·5	2·5	—	—
Blood	H	60·2	82·9	32·9	—	—
Tonsils	A	—	—	—	44·1	84·0
Tonsils	B	41·0	80·2	1·9	—	—
Tonsils	C	46·8	86·2	12·1	40·2	84·0
Tonsils	D	55·1	83·9	2·2	—	—

* These correspond to those in Figs 4(A–D) and 6 (A–H).

experiments A, B, D and H) gave PHA responses which were equal to or greater than that produced by control unseparated populations.

Two different populations of cells were used as control unseparated cells for the nylon column and rosette sedimentation procedures. The control cells for the nylon column filtration method were untreated cells as applied to the column. The control cells for the rosette-forming and non-rosette-forming populations were rosetted with SRBC in a similar manner to the test cells and the erythrocytes subsequently lysed but there was no Ficoll–Triosil separation. When the PHA responsiveness of these two control populations were compared they were found to be within the limits of experimental variation; in one experiment the control cell population which had been rosetted gave a response which was between 1 and 10% below that for the untreated control cells at all four PHA concentrations (Fig. 6C), while in the second experiment the rosetted control was 8–30% above the untreated control (Fig. 6B).

DISCUSSION

Lymphocyte populations isolated from the peripheral blood of healthy donors contained a mean of 67% T cells, as determined by E-rosette formation, and 25% B cells, as determined by staining for surface immunoglobulin. Variation in individual values was relatively wide, ranging from 58 to 81% in the case of T cells and 18 to 34% for B cells; in this respect our data are closely similar to those reported by other investigators (Jondal, Holm & Wigzell,

1972; Wybran *et al.*, 1973b; Ross *et al.*, 1973). The values for the relative proportions of T and B cells in separated lymphocyte populations do not necessarily reflect those of the corresponding cells in whole blood since Ficoll–Triosil density centrifugation gives rise to a deviation in the T cell : B cell ratio in favour of B cells (Brown & Greaves, 1974). Tonsil preparations contained a mean of 41% T cells and 42% B cells, but as with peripheral blood the range of values obtained from different tissues was wide. Greaves & Brown (1974) reported values of 55% E rosette-forming cells and 43% immunoglobulin-bearing cells in tonsil lymphocyte preparations (obtained by homogenizing tonsils and filtering the resultant cell suspension through glass wool) and concluded that 3·9% of tonsillar cells lacked both T- and B-cell markers. The apparently higher proportion of null cells in our study may be a consequence of differences in preparative procedure or alternatively, of under-estimating the number of rosette-forming cells.

Separation of lymphocyte populations by rosette sedimentation with SRBC provides a relatively simple and reproducible method of obtaining populations enriched with T and by negative selection, of B cells (Wybran *et al.*, 1973a, b; Yata *et al.*, 1973; Greaves & Brown, 1974). In the present study rosette-forming cell populations isolated from blood lymphocytes contained a mean of 85% T cells, as determined by a second E-rosette procedure, but in some experiments comprised up to 96% T cells. The T-cell content of the non-rosette-forming population was, however, more variable and contained from 2 to 39% T cells (mean 22%). Passage of peripheral blood lymphocytes through a nylon fibre column produced a population of similar T-cell composition to that obtained by rosette sedimentation, with an average of 87% T cells. This procedure has the advantage that cell suspensions are subjected to fewer washings and sedimentations and also obviates repeated exposure to Ficoll–Triosil and Tris-NH_4Cl buffer solutions. However, it suffers from the disadvantage that only a low yield of enriched T cells may be recovered on account of the fact that a significant proportion of T cells are retained by the column, with B cells. For this reason the technique is better suited for the enrichment of tonsillar T cells which are invariably more plentiful than those in the average clinical specimen (~20 ml) of peripheral blood. Using tonsil lymphocytes the rosette-forming population contained an average of 87% E rosette-forming cells while the non-rosette-forming population consisted of only 12% E rosette-forming cells. The cells obtained by nylon column filtration of tonsil lymphocyte suspensions were of similar purity (86% E rosette-forming cells) to those isolated from blood. In comparison, Greaves & Brown (1974) purified tonsillar T cells by nylon column filtration and B cells by rosette sedimentation. They obtained a somewhat more efficient separation, the T cell-enriched populations comprising 94% E rosette-forming cells and the B cell-enriched populations consisting of less than 2% E rosette-forming cells.

The PHA responsiveness of the various separated lymphocyte preparations indicated that the magnitude of the PHA response was not proportional to the number of T cells in the respective populations. For instance, B cell-enriched blood lymphocyte suspensions containing less than 8% T cells (12% in the case of tonsil lymphocytes) gave a low PHA response but populations containing 24–39% T cells gave responses which were equal to or greater than the control unseparated population comprising 60–78% T cells. T cell-enriched blood and tonsil lymphocyte populations prepared by both separation techniques gave PHA responses which were equal to, or greater than control values when they consisted of 80–86% T cells, but separated blood lymphocyte populations containing proportionally greater numbers of T cells (88–94%) responded very poorly with values considerably below those

of controls. Thus relatively pure populations of both T and B cells were characterized by diminished PHA responsiveness in contrast to mixed populations containing 24–86% T cells where phytomitogen-induced transformation was comparable to that in unseparated lymphocyte preparations.

The magnitude of the PHA response is thought to be dependent on the number of non-lymphoid cells (monocytes and polymorphs) as well as the proportion of T and B lymphocytes in the population, since the presence of adherent cells has been shown to be essential for maximal responses to mitogens (Hedfors, 1974). The reduced PHA responsiveness of the more efficiently purified T-cell populations may be due to depletion of the non-lymphoid cells along with B cells. If the contribution of the non-lymphoid element is critical, then the requisite number of cells of this type for good PHA responsiveness is evidently small since T cell-enriched populations invariably consisted of less than 6% non-lymphoid cells irrespective of whether they responded well or poorly to PHA stimulation. However, the possibility that a particular type of non-lymphoid cell with this property is depleted cannot be excluded. In purified B-cell populations, on the other hand, this non-lymphoid element is present as 7–17% of the total cells, slightly more than in unseparated preparations.

There have been conflicting reports concerning the PHA and concanavalin A (con A) responses of T and B cell-enriched populations isolated from human peripheral blood. Some investigators using the rosette sedimentation method have obtained T cell-enriched populations which responded well to PHA stimulation and B cell-enriched populations which responded poorly (Wybran *et al.*, 1973a, b; Yata *et al.*, 1973). By contrast, Zeylemaker *et al.* (1974) employing similar techniques obtained T cell-enriched populations with diminished and B cell-enriched populations with increased responsiveness to PHA compared with control unseparated preparations. In the present study the degree of phytomitogen-induced transformation depended critically on the cellular composition of the enriched populations.

Blood lymphocyte preparations have also been separated into T and B cell-enriched populations by passage through immunoabsorbent columns bound with rabbit anti-human Fab (Chess, MacDermott & Schlossman, 1974). In these experiments the T cell-enriched population showed slightly less PHA stimulation than unseparated populations while the B cell-enriched population gave a reduced but still substantial response. Similarly stimulation by con A of blood lymphocyte preparations depleted of B cells by passage through columns coated with anti-human immunoglobulin was diminished compared with that of unseparated controls (Hedfors, 1974). However in this case, increased stimulation to values greater than that of the control population was achieved by addition of adherent cells. Other investigators have separated tonsil lymphocyte preparations into T cell-enriched populations by nylon fibre column filtration and B cell-enriched populations by rosette sedimentation; mixtures of these two populations were found to give PHA responses which were proportional to the number of T cells present (Greaves, Janossy & Doenhoff, 1974). This correlation is reflected in the present study to the extent that T cell-enriched tonsil lymphocyte preparations responded well to PHA and B cell-enriched preparations poorly. The absence of a corresponding relationship in blood lymphocyte preparations may be a consequence of greater variations in the efficacy of separation or differences in participation of non-lymphoid cell components.

This study indicates that the level of PHA stimulation of mixed cell population bears no simple relationship to the number of T cells present. The major component of the

response as measured by [^{3}H]thymidine incorporation is probably T-cell transformation, although some reports suggest that B cells may also be stimulated (Chess *et al.*, 1974). The proportion of B lymphocytes and non-lymphoid cells in the mixture can clearly influence the magnitude of the response suggesting that non-T cells may act as accessory cells. It is important, therefore, to exercise caution in interpreting PHA stimulation as a measure of T-cell activity when using mixed cell populations such as blood lymphocyte preparations.

This work was supported by grants from the Medical Research Council and the Cancer Research Campaign. We are indebted to Miss Susan Chaddock and Mrs Ann Lewis for skilled technical assistance.

REFERENCES

BROWN, G. & GREAVES, M.F. (1974) Enumeration of absolute numbers of T and B lymphocytes in human blood. *Scand. J. Immunol.* **3,** 161.

CHESS, L., MACDERMOTT, P.M. & SCHLOSSMAN, S.F. (1974) Immunologic functions of isolated human lymphocyte subpopulations. I. Quantitative isolation of human T and B cells and response to mitogens. *J. Immunol.* **113,** 1113.

GREAVES, M.F. & BROWN, G. (1974) Purification of human T and B lymphocytes. *J. Immunol.* **112,** 420.

GREAVES, M., JANOSSY, G. & DOENHOFF, M. (1974) Selective triggering of human T and B lymphocytes *in vitro* by polyclonal mitogens. *J. exp. Med.* **140,** 1.

GREAVES, M.F., OWEN, J.T. & RAFF, M.C. (1973) *T and B Lymphocytes: Their Origins, Properties and Roles in Immune Responses.* Associated Scientific Publications, Amsterdam.

GREAVES, M.F., ROITT, I.M. & ROSE, M.E. (1968) Effect of bursectomy and thymectomy on the responses of chicken peripheral blood lymphocytes to phytohaemagglutinin. *Nature (Lond.),* **220,** 293.

HEDFORS, E. (1974) Activiation of peripheral T cells of sarcoidosis patients and healthy controls. *Clin. exp. Immunol.* **18,** 379.

JANOSSY, G. & GREAVES, M.F. (1971) Lymphocyte activation. I. Response of T and B lymphocytes to phytomitogens. *Clin. exp. Immunol.* **9,** 483.

JONDAL, M., HOLM, G. & WIGZELL, H. (1972) Surface markers on human T and B lymphocytes. I. A large population of lymphocytes forming nonimmune rosettes with sheep red blood cells. *J. exp. Med.* **136,** 207.

MÖLLER, G. (1973) Editor. T and B lymphocytes in humans. *Transplant. Rev.* **16.**

ROSS, G.D., RABELLINO, E.M., POLLEY, M.J. & GREY, H.M. (1973) Combined studies of complement receptor and surface immunoglobulin bearing cells and sheep erythrocyte rosette forming cells in normal and leukemic human lymphocytes. *J. clin. Invest.* **52,** 377.

SCHLOSSMAN, S.F. & HUDSON, L. (1973) Specific purification of lymphocyte populations on a digestible immunoadsorbent. *J. Immunol.* **110,** 313.

TAKIGUCHI, T., ALTER, W.H. & SMITH, R.T. (1971) Cellular recognition *in vitro* by mouse lymphocytes *J. exp. Med.* **133,** 63.

WIGZELL, H., SUNDQVIST, K. & YOSHIDA, T. (1972) Separation of cells according to surface antigens by the use of antibody coated columns: fractionation of cells carrying immunoglobulin and blood group antigens. *Scand. J. Immunol.* **1,** 75.

WYBRAN, J., CHANTLER, S. & FUDENBERG, H.H. (1973a) Human blood T cells: response to phytohaemagglutinin. *J. Immunol.* **110,** 1157.

WYBRAN, J., CHANTLER, S. & FUDENBERG, H.H. (1973b) Isolation of normal T cells in chronic lymphocytic leukaemia. *Lancet,* **i,** 126.

YATA, J., DESGRANGES, C., TACHIBANA, T. & DE THÉ G. (1973) Separation of human lymphocytes forming spontaneous rosettes with sheep erythrocytes. *Biomedicine,* **19,** 475.

ZEYLEMAKER, W.P., ROOS, M.T.L., MEYER, C.J.L.M., SCHELLEKENS, P.T.A. & EIGSVOOGEL, V.P. (1974) Separation of human lymphocyte subpopulations. I. Transformation characteristics of rosette forming cells. *Cell. Immunol.* **14,** 346.