CELLULAR IMMUNOLOGY 12, 407–421 (1974)

Fluorescence-Activated Cell Sorting of Human T and B Lymphocytes

II. Identification of the Cell Type Responsible for Interferon Production and Cell Proliferation in Response to Mitogens[1]

LOIS B. EPSTEIN, H. WOLFGANG KRETH,[2] AND LEONARD A. HERZENBERG

Cancer Research Institute, University of California, San Francisco, California 94143, and the Department of Genetics, Stanford University School of Medicine, Stanford, California 94305

Received October 29, 1973

We have employed the fluorescence-activated cell sorter to separate pure viable preparations of human T and enriched B lymphocytes. Using such preparations, we have demonstrated that both human T and B cells can respond to PHA and PWM *in vitro* in the presence of macrophages with proliferation and the production of interferon, a mediator of cellular immunity. However, selective T cell interferon production and proliferative response can be assessed at 3 days in culture; B cell interferon production and proliferative response is delayed to 5 and 7 days. T cells or T cell products are ineffective in inducing or accelerating B cell interferon or proliferative response at 3 days. The use of 3-day T cell interferon production as a new technique for the assessment of T cell effector function and competence is suggested.

INTRODUCTION

Interferon is a mediator of cellular immunity. It has been produced *in vitro* upon exposure of human or mouse leukocytes to phytohemagglutinin (PHA) (1–3), pokeweed mitogen (PWM) (2, 3), Concanavalin A(3), and anti-lymphocyte sera (4). It is produced on an immune-specific basis *in vitro* in response to bacterial antigens by human (5) and mouse leukocytes (6), and in mouse mixed lymphocyte cultures (7). Our own studies have demonstrated that the lymphocyte is the human cell type responsible for *in vitro* interferon production in response to PHA (8, 9), bacterial (10, 11) and viral antigens (12, 13) and that the presence of macrophages augments the amount of interferon produced by the lymphocytes in these situations.

The recent development of the fluorescence-activated cell sorter (FACS) has provided a unique opportunity to sort and physically separate populations of cells on the basis of their fluorescence (14). By employing fluorescein-labeled anti-immunoglobulin reagents and adapting the FACS for sterile use, it is now possible

[1] Supported by USPHS Grants CA 11067 and GM 17367, the American Cancer Society, California Division, Special Grant #605, and by the Damon Runyon Memorial Fund, Grant DRG-1205.

[2] Recipient of a postdoctoral fellowship from the Deutsche Forschungs gemeinschaft, Germany.

to use the instrument for the separation and subsequent culture of human lymphocytes according to their surface immunoglobulin properties (15, 16).

There is already considerable evidence that the non-immunoglobulin-bearing, low fluorescence fraction of human lymphocytes separated by the FACS is indeed analogous to animal T (thymus-derived) cells (17). In the accompanying paper (16), Kreth and Herzenberg demonstrated by functional studies that such non-immunoglobulin-bearing human lymphocytes proliferated in response to PHA and PWM. In addition, Bobrove et al., have shown that >95% of such FACS-separated non-immunoglobulin-bearing lymphocytes are killed in a complement-dependent cytotoxic assay which employed a goat anti-human thymocyte antisera adsorbed with human erythrocytes and chronic lymphocytic leukemic lymphocytes (ATCS). Also, treatment of peripheral blood lymphocytes with ATCS inhibited their proliferative response to PHA in short term culture (18). Furthermore, Kreth and Herzenberg (16) demonstrated that those lymphocytes separated by the FACS, which bore surface immunoglobulin and were of high fluorescence, could develop into mature phasmacytes. Thus, they are analogous to immunoglobulin-bearing B (bone marrow-derived) lymphocytes observed in the mouse (17). By way of confirmation, less than 10% of the FACS-separated immunoglobulin-bearing lymphocytes were killed in the cytotoxic assay of Bobrove *et al.* (18).

The purpose of the present studies was (A) to employ the FACS to separate pure populations of human T and B lymphocytes; (B) to assess the ability of human T and B lymphocytes to produce interferon in response to the mitogens PHA and PWM; (C) to further define the proliferative potential of human T and B lymphocytes in response to these mitogens; and (D) to assess the role of the macrophage in the interferon and proliferative response of T and B lymphocytes to these mitogens.

MATERIALS AND METHODS

Donors. All blood donors were healthy adults with no recent history of viral illness.

Preparation of macrophage cultures. Macrophage cultures were obtained by the growth and differentiation for 7 days of glass-adherent monocytes in small Leighton tubes in McCoy's medium supplemented with 30% AB serum (19). Before use, each culture was examined microscopically and evaluated for cell number and extent of differentiation. Representative cultures were counted (9); those employed in this study contained from 2 to 5×10^4 macrophages/ml.

Preparation of lymphocytes. Blood for the isolation of lymphocytes was always obtained from the same donor who 6 days previously had donated blood for the preparation of macrophage cultures. Lymphocytes were isolated either from leukocyte-rich plasma by their passage through a sterile nylon-fiber column (9) or from blood using a Ficoll–Hypaque gradient technique (10) from which the upper layer, comprised of lymphocytes and monocytes, was collected. Erythrocytes which accompanied the column effluents were lysed by an exposure to NH_4Cl (12); morphologically identifiable monocytes which accompanied the lymphocytes in the Ficoll gradient were removed by adsorption to glass coverslips in Leighton tubes for 2 hr at 37°C. The lymphocytes were adjusted to a concentration of 1×10^6/ml in AB serum containing McCoy's medium and kept at room tempera-

ture until and during their transport to Stanford for fluorescence labeling and passage through the FACS. A portion of each sample was retained for viability studies and for comparison with the FACS-separated cells.

Fluorescent staining of lymphocytes. Upon arrival at Stanford, the lymphocytes were centrifuged at 400*g* for 7 min at 20°C and washed three times with a large volume of Dulbecco's phosphate-buffered saline (DPBS) which contained 5% fetal calf serum (FCS). The method of preparation, purification, and conjugation of the anti-immunoglobulin reagents with fluorescein has been described previously (16). For the present study, fluorescein-labeled rabbit anti-Cohn fraction II with F/P ratios between 2.3 and 3 was used for direct staining of lymphocytes with membrane-associated immunoglobulin. The specificity of staining of this reagent has been described in detail elsewhere. It is thought to react with all classes of immunoglobulin and identifies normal B lymphocytes (18). Lymphocytes were incubated with antiserum (50×10^6/mg/ml) at 4°C for 15 min. They were then washed twice through discontinuous FCS gradients (2 ml 100% FCS, 2 ml 50% FCS at 290*g* for 5 min at 4°C). Prior to separation on the FACS, cell smears were prepared and the percent of fluorescein-positive lymphocytes determined by fluorescence microscopy. If cell clumping was observed, the cell suspension was filtered through a small plug of sterile glass wool.

Processing of lymphocytes by the FACS. The mechanical and electronic design of the FACS have been described previously (14–16). For the present studies, the FACS was adapted for use under sterile conditions and all parts of the instrument that came in contact with the cells were autoclaved prior to use. Two fractions of cells were collected at close to 0°C: B cells containing $>10^5$, and T cells containing $< 5 \times 10^3$ immunoglobulin molecules per cell. Those in the intermediate range were discarded. An aliquot of the T and B cell preparations was then assessed for purity by fluorescence microscopy and the remainder of the cells was then washed three times, resuspended in AB-serum-enriched McCoy's medium at 1×10^6/ml, and returned at room temperature to San Francisco by early evening. On several occasions, cells that were stained but not yet separated by the FACS were also returned to San Francisco.

Preparation of cultures. Prior to use, an aliquot of each sample was studied for viability by trypan blue exclusion and compared with cells that had never been transported and cells that had been transported and stained, but not separated by the FACS. T or B cells were placed in Leighton tubes either in the presence or absence of macrophage monolayers and either PHA (Difco, Detroit, MI), PWM (Gibco, Berkeley, CA), or no stimulant was added. The final concentration of PHA was 33 μg/ml and that of PWM was either 24, 45, or 84 μg/ml. Cultures were prepared with McCoy's medium enriched with 30% AB serum; the concentration of T or B cells varied between 0.25 and 10×10^5/ml. The cultures were maintained in a CO_2 incubator at 37°C for 3, 5, or 7 days.

In some experiments, supernatant media from 1-day-old PHA- or PWM-stimulated combined T lymphocyte–macrophage cultures were collected, passed through a Millipore filter (0.22 μm) to remove any residual T cells, and added to cultures containing B cells and macrophages. In other experiments, varying amounts of T cells were added to combined B-cell–macrophage cultures, and then stimulated with PHA or PWM. In addition, other experiments were performed in which B lymphocytes were kept in culture with macrophages at 37°C

for 1 day, and then washed, resuspended with fresh macrophages, and stimulated with PHA or PWM.

Interferon assay and cell-harvesting procedures. Cell-free supernatant culture fluids were collected after 3, 5, and 7 days under sterile conditions on a multifiltration apparatus (Hoefer Scientific Instruments, San Francisco, CA) (12) and assayed for interferon by a viral plaque reduction method (9, 20). Dilutions of supernatant culture fluids to be tested for antiviral activity were exposed to confluent monolayers of neonatal foreskin fibroblasts for 18–24 hr at 37°C in a 5% CO_2 atmosphere. The monolayers were then washed and bovine vesicular stomatitis virus was adsorbed for 45 min at 37°C. The monolayers were overlayed with 0.9% agar in Eagle's medium with 5% bovine serum for 48 hr to allow for plaque development and stained with neutral red. Interferon titer is defined as that dilution of sample which, in a 4-ml volume, resulted in 50% reduction of viral plaques. Duplicate or triplicate samples were run for each type of culture. Control supernatants with medium plus PHA or PWM were also tested. At least two, and most often three, dilutions of each sample were run along with the controls for the interferon assay; i.e., fibroblast monolayers with virus and no sample to be tested, and fibroblast monolayers with virus and a known interferon standard. For statistical purposes interferon titers of <10 were assigned a value of 5.

In some instances, interferon titers were extrapolated to a cell concentration of 1×10^6 lymphocytes/ml and corrected for contamination by the other lymphocyte type. For example, in Expt. 8, T cell cultures were prepared with 1×10^6 cells/ml and B cell cultures were prepared with 0.3×10^6/ml; these were known to have a maximum T cell contamination of 21%. Thus, to express the interferon titer per 1×10^6 B cells after stimulation with PHA, the following calculations were made:

$$\frac{\dfrac{\text{Observed interferon titer in (M + B + PHA)}}{0.3} - 0.21 \times \text{Observed interferon titer in (M + T + PHA)}}{0.79}.$$

The proliferative response of T and B lymphocytes to mitogens was assessed by the incorporation of ^{3}H-thymidine into DNA. ^{3}H-thymidine (New England Nuclear Corp., Boston, MA; 2.1 Ci/mmole) was added 1 hr before termination of each culture. At the time of harvest, cells were collected on glass filter paper, washed, and treated with cold trichloracetic acid and methanol; the precipitate was dissolved with Nuclear Chicago Solubilizer, and counted as described previously (12).

In some instances, the counts per minute observed in cultures containing macrophages and B cells were extrapolated to a B cell concentration of 1×10^6/ml and corrected for the contamination by T cells as described above.

RESULTS

Initial observations. Our initial efforts were directed toward establishing those conditions under which the best viability of the lymphocyte preparations could be

achieved. This was of concern since as long as 40 hr were required for lymphocyte isolation, transportation, staining, separation by the FACS, and preparation for tissue culture. Using the trypan-blue-dye exclusion test, we established that lymphocytes maintained at room temperature at a concentration of 1×10^6/ml or less in McCoy's medium supplemented with 30% AB serum and transported in two-thirds-full tissue culture glass bottles had >99% viability for up to 72 hr. Factors which tended to decrease viability were the use of fetal calf serum, maintenance of the samples in ice for prolonged periods, and more concentrated cell suspensions.

Next, we had to establish that the presence of fluoresceinated anti-Cohn fraction II by itself would not interfere with the production of mitogen-stimulated interferon. In a series of four experiments, we compared the amount of mitogen-stimulated interferon produced by column-separated lymphocytes in the presence of macrophages with that obtained from similar cells which had been exposed to fluoresceinated anti-Cohn fraction II but not separated by the FACS. No difference was observed.

Percent of lymphocytes bearing surface immunoglobulin after their isolation by nylon-fiber columns or by Ficoll gradient. After exposure of the lymphocytes to fluoresceinated anti-Cohn fraction II and just prior to their separation on the FACS, they were evaluated for the percent of immunoglobulin-bearing cells. The data in Table 1 indicate that the proportion of B cells in preparations of lymphocytes isolated by their passage through nylon-fiber columns was 4–9.5% (mean, 6.5%) and in those isolated from Ficoll gradients from which monocytes had been absorbed, 15–21% (mean, 18.0%).

Purity of T and B cell preparations obtained from the FACS. After separation, the T and B cell populations were examined by fluorescence microscopy and their purity determined (Table 2). The purity of T cell preparations was always >99.9% and that of B cells, at least 70–95%. For each determination, 300–500 cells were scored, and only those cells which bore many fluorescent spots were considered to be B cells. The B cells were probably purer than the data indicate for the following reasons: (A) Cells with only one discrete spot of fluorescence were counted as negative. Some of these could represent B cells in which capping of the immunofluorescent complexes had occurred. (B) Some of the cells scored as negative might actually represent B cells in which capping and subsequent shedding of the immunofluorescent complexes had already occurred. (C) Cell clumping in some instances obscured the proper quantitation of cells carrying fluorescent label, and so only single cells were scored.

TABLE 1

PERCENTAGE OF B CELLS IN LYMPHOCYTES ISOLATED FROM NYLON-FIBER COLUMNS OR FICOLL GRADIENTS

	Method of isolation	
	Nylon-fiber column	Ficoll gradient
% of B cells	5.0; 7.0; 9.5; 8.5; 5.0; 4.0	15.0; 21.0; 18.0
Mean	6.5%	18.0%

TABLE 2

PURITY OF T- AND B-LYMPHOCYTE FRACTIONS AFTER SEPARATION BY THE FACS

	T Cells	B Cells
Values observed (%)	>99.9 (in nine experiments)	72; 85; 95; 80; 85; 83; 90; 79; 70
Mean	>99.9%	83%

The purity of the B cell fractions is related to the speed with which they are processed (i.e., to a limit, the slower, the purer) and the brilliance of their fluorescence. The speed is inversely proportional to the concentration. Thus, it is necessary to balance the desire for large numbers of separated cells with requirements for purity and the time required for processing the cells. For example, in most instances, cells were applied to the instrument at $0.8–1.0 \times 10^7$/ml, but because of the large number of cells to be processed for the final experiment, they were applied at 1.6×10^7/ml, with resulting slight loss in purity of the B cell preparation. The purity of T cell fraction is not dependent on the rate of separation, within the limits used.

Recovery of T and B cells after passage through FACS. In four separation experiments, the percent of T and B cells recovered after their passage through the FACS was determined. The values for T cells were 30–52% with a mean value of 43%, and for B cells 34–64% with a mean value of 47%. The remainder of the T and B cells were accounted for either by coincidence loss (loss of cells which could not be separated by the FACS because they were too close together to discriminate) or by trapping in a short glass-fiber filter in the line of passage of the cells.

Interferon production in cultures of PHA- or PWM-stimulated T or B lymphocytes plus macrophages. The data in Table 3 indicate that in seven experiments, human T cells cultured in the presence of macrophages produced interferon when stimulated with PHA or PWM for 3 days. During this same period, no interferon was ever detected in mitogen-stimulated cultures of B lymphocytes with macrophages. As is depicted in Fig. 1, increasing concentrations of T cells in cultures containing a fixed number of macrophages results in increasing amounts of interferon produced at 3 days. By contrast, increasing the concentration of B cells did not result in any detectable interferon. No interferon was ever detected in T lymphocyte–macrophage or B lymphocyte–macrophage cultures prepared without PHA or PWM, or in cultures of macrophages alone or in the presence of PHA and PWM.

Experiments were then performed to determine if T cells or T cell products could stimulate interferon production by B cells in the presence of mitogens. In three experiments, varying amounts of T cells were added to cultures of B cells in the presence of macrophages and either PHA or PWM and harvested 3 days later. The interferon titers observed in such cultures were never higher than would be expected from the number of T cells added to the cultures. Similarly, in three experiments in which 24-hr supernatants from cultures containing T

TABLE 3

INTERFERON PRODUCTION IN 3-DAY COMBINED T-LYMPHOCYTE–MACROPHAGE OR B-LYMPHOCYTE–MACROPHAGE CULTURES STIMULATED WITH PHA OR PWM

Experiment No.	Interferon titer[a]			
	T Lymphocytes		B Lymphocytes	
	PHA	PWM	PHA	PWM
1	144	184	N.D.[b]	N.D.
2	217	211	<10[c]	<10
3	669	398	<10	<10
4	120	135	<10	<10
5	100	112	<10	<10
6	176	80	<10	<10
7	264	128	<10	<10
Mean value ± SE of mean	241 ± 74	178 ± 40	5 ± 0	5 ± 0

[a] Interferon titer is that dilution of supernatant which, in a 4-ml volume results in 50% reduction of viral plaques. All cultures prepared without PHA and PWM had a titer of <10.

[b] N.D.: not done.

[c] For statistical purposes titers of <10 are assigned a numerical value of 5.

lymphocytes, macrophages, and PHA, PWM, or no stimulant were added to cultures of B cells with macrophages, no interferon was detected 3 days after the addition of the supernatants.

In some experiments the fluoresceinated anti-Cohn fraction II was removed from the B cells by maintaining them in culture at 37°C for 24 hr to induce capping and then by thorough washing of the cells. Subsequent stimulation of such cells with PHA or PWM in the presence of macrophages did not result in the production of interferon after 3 days.

In two experiments, cultures of T or B lymphocytes with or without macrophages and PHA or PWM were harvested not only at 3 days but also at 5 and 7 days. The data is shown in Table 4. Again, T cells produced interferon in response to PHA and PWM at 3 days, and at 5 and 7 days as well when macro-

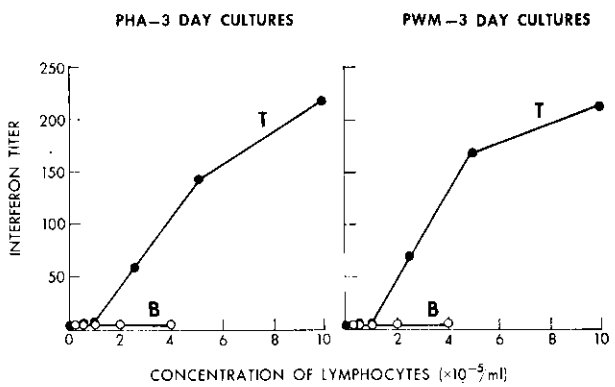

FIG. 1. Effect of increasing the concentration of T or B lymphocytes on interferon production in 3-day cultures which also contain macrophages and PHA or PWM.

TABLE 4

INTERFERON PRODUCTION BY HUMAN LYMPHOCYTES IN RESPONSE TO PHA AND PWM AT 3, 5, AND 7 DAYS IN CULTURE: EFFECT OF MACROPHAGES

	Interferon titer					
	3 day		5 day		7 day	
	Expt. 8	Expt. 9	Expt. 8	Expt. 9	Expt. 8	Expt. 9
M + T + O[a]	<10	<10	<10	<10	<10	<10
M + T + PHA	147	485	591	625	508	503
M + T + PWM	440	181	610	460	518	435
T + O	<10	<10	<10	<10	<10	<10
T + PHA	<10	<10	<10	<10	<10	<10
T + PWM	<10	<10	<10	<10	<10	11
M + B + O[b]	<10	<10	<10	<10	<10	<10
M + B + PHA	<10	87	15	350	758	713
M + B + PWM	<10	10	273	117	721	280
B + O	N.D.[c]	<10	N.D.	<10	<10	<10
B + PHA	N.D.	<10	N.D.	<10	<10	N.D.
B + PWM	N.D.	<10	N.D.	<10	<10	<10

[a] M Cultures containing macrophages. T Cultures containing T lymphocytes. The final concentration of T cells was 1×10^6/ml.

[b] B Cultures containing B lymphocytes. The final concentration of B cells was 0.3×10^6/ml. All B-cell values represent interferon titers extrapolated to a B-cell concentration of 1×10^6/ml and corrected for the presence of contaminating T cells.

[c] N.D.: not done.

phages were present. The lack of a detectable interferon response to PHA and PWM by macrophage-enriched B cells at 3 days was noted again in one of these experiments. In the other, a small amount of interferon was produced by B cells in response only to PHA at 3 days: this was the only instance out of nine experiments in which this was noted. However, by 5 and 7 days considerable B cell interferon was detected in response to both PHA and PWM, when macrophages were present. Figure 2 illustrates the fact that increasing concentrations of PWM result in increasing amounts of B cell interferon at 7 days in cultures which also contained macrophages. This study substantiates the fact that, at the final concentration of PWM used for all the other studies, i.e., 48 μg/ml, good titers of interferon are observed.

As has been observed previously with macrophages and mixed populations of T and B lymphocytes (9), the presence of macrophages augments the amount of T cell interferon at 3, 5, and 7 days, and that observed from B cells at 5 and 7 days. No interferon was ever noted in cultures prepared with T or B cells without macrophages with either PHA or PWM studied at 3, 5, and 7 days.

The interferon produced in PHA- or PWM-stimulated cultures was characterized according to methods described previously (9). It was species specific, susceptible to the action of trypsin, and resistant to RNAase or DNAase. Its

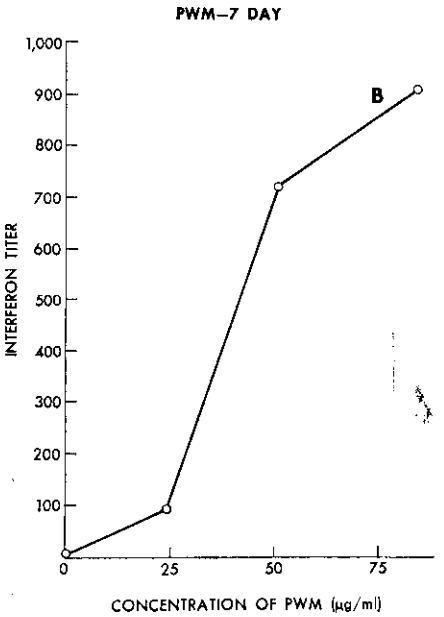

FIG. 2. Effect of increasing the concentration of PWM on interferon production by 7-day cultures containing macrophages and B lymphocytes.

activity was unaffected by ultracentrifugation at 100,000 *g*, but was depressed by heating at 56°C for 1 hr, and by a 6-hr exposure at pH 2. It did not exert its protective effect if exposed to the confluent monolayers of neonatal foreskin fibroblasts for only 2 hr, but rather required 18–24 hr.

PHA- and PWM-stimulated lymphocyte transformation in combined T or B lymphocyte–macrophage cultures. All of the cultures from which supernatant fluids were removed for the assay of interferon were also studied for the incorporation of ^{3}H-thymidine into DNA as a means of assessing the proliferative response of human T and B cells to PHA and PWM. In general, the results paralleled the observations on interferon, and also those observations noted by Kreth and Herzenberg on mitogen-stimulated cultures containing lymphocytes and monocytes (16). First of all, at 3 days in culture in all seven experiments, T cells proliferated in response to both PHA and PWM (Table 5). In these experiments, B cells did not respond to either agent at 3 days. Second, increasing the concentration of T cells in a range from 0.25 to 10×10^5 in the presence of macrophages and PHA or PWM resulted in a linear increase in ^{3}H-thymidine incorporation at three days. Doing the same with B cells did not result in any stimulation in 3-day cultures. Third, when T cells were added to cultures of B cells, no increment in ^{3}H-thymidine occurred other than that accounted for by the number of T cells present. Similarly, supernatants of nonstimulated or PHA- or PWM-stimulated T cells had a negligible effect on B-cell proliferation after 3 days. In two of three experiments, supernatants containing PWM had no effect on B cell proliferation and in the third only a 2× increase in cpm was observed. In the case of PHA, one of three experiments was completely negative and the other two caused only a 2× increase in cpm. Removal of the immunofluorescent complex from the B cells also did not affect their poor proliferative response to

TABLE 5

^{3}H-THYMIDINE INCORPORATION IN 3-DAY COMBINED T LYMPHOCYTE–MACROPHAGE OR B LYMPHOCYTE–MACROPHAGE CULTURES STIMULATED WITH PHA OR PWM

Experiment No.	CPM[a]					
	T Lymphocytes			B Lymphocytes		
	O[b]	PHA	PWM	O	PHA	PWM
1	1560	28,265	12,883	N.D.[c]	N.D.	N.D.
2	5086	23,798	12,366	0	582[d]	683
3	740	12,346	15,999	1975	3793	1605
4	258	31,620	18,419	N.D.	N.D.	N.D.
5	143	12,374	12,369	N.D.	5873	6038
6	814	25,925	17,583	981	N.D.	692
7	200	8,915	2,683	2200	2230	1642
Mean value ±SE of mean	1257 ± 665	20,463 ± 3419	13,186 ± 1990	1289 ± 505	2130 ± 1128	2132 ± 999

[a] cpm Counts per minute.
[b] Cultures prepared without PHA or PWM.
[c] N.D.: not done.
[d] The values for B lymphocytes have been extrapolated to a B-cell concentration of 1×10^6 ml and corrected for the presence of contaminating T cells.

TABLE 6

PROLIFERATIVE RESPONSE OF HUMAN LYMPHOCYTES TO PHA AND PWM AT 3, 5, AND 7 DAYS IN CULTURE: EFFECT OF MACROPHAGES

	CPM[a]					
	3 day		5 day		7 day	
	Expt. 8	Expt. 9	Expt. 8	Expt. 8	Expt. 8	Expt. 9
M + T + O[b]	759	644	921	586	491	3,488
M + T + PHA	24,345	26,766	10,827	18,517	6,892	5,359
M + T + PWM	11,207	18,362	11,890	17,312	6,045	6,335
T + O	868	2,309	752	594	417	1,345
T + PHA	8,282	13,597	4,940	7,956	1,325	6,139
T + PWM	2,818	4,024	2,844	1,549	1,132	2,124
M + B + O[c]	3,077	877	3,565	3,716	2,043	0
M + B + PHA	28,832	6,057	42,125	51,450	13,792	33,890
M + B + PWM	8,979	416	27,919	21,180	15,200	12,229
B + O	N.D.[d]	379	N.D.	4,573	2,424	2,741
B + PHA	N.D.	0	N.D.	9,076	16,031	N.D.
B + PWM	N.D.	7,079	N.D.	4,064	8,774	3,118

[a] cpm Counts per minute.

[b] M Cultures containing macrophages. T Cultures containing T lymphocytes. The final concentration of T cells was 1×10^6/ml.

[c] B Cultures containing B lymphocytes. The final concentration of B cells was 0.3×10^6/ml. All B cell values have been extrapolated to a B cell concentration of 1×10^6/ml and corrected for the presence of contaminating T cells.

[d] N.D.: not done.

PHA or PWM at 3 days. However, at 5 and 7 days, definite evidence of human B cell proliferative response to both PHA and PWM was noted, and is shown in Table 6. In these experiments, B cells had a slight proliferative response to PHA at Day 3 also, but not to PWM. The proliferative response of T cells to PHA was most marked at 3 days; to PWM between 3 and 5 days. The proliferative response of B cells to both PHA and PWM was most marked at 5 days. For both T and B lymphocytes, the peak of lymphocyte transformation preceded the appearance of highest interferon titers.

Finally, as was noted with interferon production, the presence of macrophages augmented the T cell proliferative response to both PHA and PWM at all time intervals studied, and augmented that of B cells at 5 and 7 days. In contrast with the findings on interferon, cultures from which macrophages had been omitted, i.e., T cells + PHA or PWM at 3, 5, and 7 days or B cells + PHA and PWM at 5 and 7 days, did show some degree of significant proliferative response.

Microscopic observations on the cultures. Cultures were observed by phase microscopy or Giemsa staining and the following observations made: (A) No rosette formation or attachment of lymphocytes to macrophages occurred in cultures of T cells with macrophages or B cells with macrophages when no mitogen was added. Furthermore, no evidence of lymphocyte transformation was

observed in these unstimulated cultures. (B) In 3-day cultures of T cells and macrophages prepared with PHA, large clumps of cells were noted, some with as many as 40 rosettes containing a central macrophage and many adherent T cells, in all stages of lymphocyte transformation. Much smaller clumps were observed with B cells and macrophages in the presence of PHA, but rosettes did occur. However, transformed cells were most pronounced at 5 and 7 days in PHA-stimulated B cell cultures. Furthermore, the percent of transformed cells observed was far in excess of that which could be accounted for by the small contaminant of T cells. In 3-day cultures of T cells and macrophages prepared with PWM, small clumps of cells were observed with as many as 10 rosettes per clump. Even smaller clumps were noted when B cells and macrophages were cultured with PWM. At 7 days, about three rosettes per clump were noted, and transformed cells were noted within them.

DISCUSSION

We have employed the FACS as an effective tool to obtain separate, pure populations of human T and enriched populations of B lymphocytes. These studies are helpful in our understanding of the similarities and differences between human T and B cell functions. They demonstrate that, in the human, both T and B lymphocytes in the presence of macrophages have the capacity to respond to both PHA and PWM with mediator production and proliferation, but that the response by B cells is delayed. When studied after 3 days in culture, both the production of interferon and the proliferative response to both PHA and PWM were found in the T cell population. Interferon production by B lymphocytes in the presence of macrophages was never detected at 3 days in response to PWM, or in eight of nine experiments with PHA, but did occur in response to both mitogens at 5 and 7 days. Similarly, at 3 days little or no proliferative response was noted in macrophage containing B lymphocyte cultures with PHA or PWM, but at 5 and 7 days the response was quite marked.

Although the exact mechanism for the delayed mediator production and proliferative response by B cells described herein is not understood, it is certain that the presence of fluoresceinated anti-Cohn fraction II was not a contributory factor, as removal of such had no effect on accelerating B cell responses in 3-day cultures.

Our observation that human B lymphocytes can respond to PHA even in a delayed fashion with proliferation and mediator production was of considerable interest, and was unexpected. In the present study, the transformation of human B cells in response to PHA was substantiated by both isotopic and microscopic evaluations. Furthermore, the extent of interferon production and proliferative response observed in the mitogen-stimulated cultures at 5 and 7 days was far in excess of that which could be accounted for by the presence of a small contaminant of T cells.

Extensive studies on the response of mouse lymphocytes to mitogens have indicated that although both mouse T and B cells have binding sites for PHA and PWM, only T cells proliferate in response to soluble PHA, but both T and B cells respond to soluble PWM (21–23). Furthermore, the B cell response to PWM described in the mouse is not delayed as we have shown in the human. The reasons for the differences observed in man and the mouse are not apparent.

It is of interest that Phillips and Roitt have also demonstrated the *in vitro* proliferative response of human B cells to PHA (24). They employed a technique developed by Schlossman and Hudson (25) whereby dextranase was used to release B lymphocytes from a column comprised of antiimmunoglobulin bound to Sephadex G200. They confirmed the B cell response to PHA by the fluorescence microscopic demonstration of light chain on the surface of the transformed lymphocytes found in PHA-stimulated B cell-enriched cultures. Light chains were not present on the surface of transformed lymphocytes found in PHA-stimulated T cultures.

To our knowledge, the present studies represent the first in which the human lymphocyte cell type responsible for PHA- and PWM-stimulated interferon have been defined. The conventional notion of the T cells as the cell type responsible for mediator production (26) has been confirmed, but, in addition, for interferon at least, the B lymphocyte has also been implicated. It will be of considerable interest to see if the same holds true for other mediators of cellular immunity.

One other study on the lymphocyte origin of mitogen-stimulated interferon production has been performed, and that employed a mouse system. Wallen *et al.*, using mouse spleen lymphocytes, demonstrated that the interferon production which occurred after stimulation with PHA, Con A, and PPD could be eliminated by treatment of the cells with complement and antitheta serum, but that which occurred in response to PWM could not (3). Those studies suggested that, in the mouse, T cells produce interferon in response to PHA and Con A, but B cells produce interferon only in response to PWM. Thus, they too have implicated both T and B cells in the production of interferon. Furthermore, by albumin gradient separation, they demonstrated that the cells which proliferate in response to mitogen are not necessarily the same ones which produce interferon.

Three additional observations are worthy of comment. First, the present study demonstrates interferon production only in those cultures that contain both lymphocytes and macrophages; cultures of pure T cells, B cells, or macrophages alone had no detectable interferon when stimulated with PHA or PWM or when no stimulant was added. We established in previous studies that, in such mitogen-stimulated cultures containing both lymphocytes and macrophages, that the lymphocyte was the cell responsible for interferon production, and that the presence of macrophages augments its production, presumably by presenting the mitogen to the lymphocyte in a more efficient manner than occurs in the absence of macrophages (8, 9). Evidence for the lymphocyte origin of interferon in such situations was gained from experiments in which human lymphocytes were combined with mouse macrophages in the presence of mitogen: the interferon produced was of human, not mouse origin. Furthermore, freeze-thaw killing of lymphocytes completely ablated the interferon response to mitogens, whereas freeze-thaw killing of macrophages only diminished the response.

Numerous factors elaborated by T cells have been suggested to influence B cell function (27). Such factors have been implicated in the B cell response to antigens (27) and Con A (28) and in the induction of B cell tolerance (29). In contrast, the present studies demonstrate that T cells or T cell products do not influence the ability of human B cells to produce interferon or to proliferate in response to PHA and PWM. It might be argued that the contaminating T cells

in the B cell preparations used for cultures at 5 and 7 days (in which interferon and proliferation were noted) induced the B cell response. However, only 6.3–9.0 $\times 10^4$ T cells were present in such cultures; in other experiments, supernatants from far more T cells, i.e., 10^6 T cells, were found to be ineffective in triggering B cells after 3 days.

Finally, these studies are of considerable importance for future investigations of patients with various immunologic defects. Although T cells can be enumerated by several techniques, i.e., rosette-forming ability (30), lack of surface-bound immunoglobulin, the means by which their functional competence has been assessed *in vitro* has been limited to their proliferative response to various mitogens. The present studies demonstrate an additional, new way by which T cell competence and effector function can be evaluated: by the study of the 3-day interferon response to PHA and PWM.

Use of the FACS would not be required for such studies, as the nylon-fiber-column effluents described herein contained only 4–9.5% of contaminating B cells, and could thus effectively be employed as a technique for the collection of relatively pure human T cells; this, in fact, has recently been suggested by Eisen *et al.* (31) in the human and by Julius *et al.*, in the mouse (32). Such studies could be of use in the detection, diagnosis, classification, and further understanding of various immunodeficiency states, and in other diseases in which abnormalities of T cell function are suspected. We have, for example, recently shown that certain patients with selective IgA deficiency (33) and others with chronic lymphocytic leukemia (19) have a depressed T cell interferon response to mitogens.

ACKNOWLEDGMENTS

The authors wish to thank Miriam Debby, Virginia Hill, and Ruth Waters for superb technical assistance, and Tim Merrill and Richard Stovel for assistance with the FACS.

REFERENCES

1. Wheelock, E. F., *Science* **149**, 310, 1965.
2. Friedman, R. M., and Cooper, H. L., *Proc. Soc. Exp. Biol. Med.* **125**, 901, 1967.
3. Wallen, W. C., Dean, J. H., and Lucas, D. O., *Cell. Immunol.* **6**, 110, 1973.
4. Falcoff, R., Oriol, R., and Iscaki, S., *Eur. J. Immunol.* **2**, 476, 1972.
5. Green, J. A., Cooperband, S. R., and Kibrick, S., *Science* **164**, 1415, 1969.
6. Milstone, L. M., and Waksman, B. H., *J. Immunol.* **105**, 1068, 1970.
7. Gifford, G. E., Tibor, A., and Peavy, D. L., *Infect. Immunol* **3**, 164, 1971.
8. Epstein, L. B., Cline, M. J., and Merigan, T. C., *In* "Proceedings of the 5th Leukocyte Culture Conference" (J. E. Harris, Ed.), p. 506. Academic Press, New York, 1970.
9. Epstein, L. B., Cline, M. J., and Merigan, T. C., *J. Clin. Invest.* **50**, 744, 1971.
10. Epstein, L. B., Cline, M. J., and Merigan, T. C., *Cell. Immunol.* **2**, 602, 1971.
11. Epstein, L. B., Cline, M. J., and Merigan, T. C., *In* "Proceedings of the 6th Leukocyte Culture Conference" (R. Schwarz, Ed.), p. 265. Academic Press, New York, 1971.
12. Epstein, L. B., Stevens, D. A., and Merigan, T. C., *Proc. Nat. Acad. Sci. USA* **69**, 2632, 1972.
13. Merigan, T. C., Stevens, D. A., Rasmussen, L. E., and Epstein, L. B., *In* "A Reexamination of Non-Specific Resistance to Infection" (W. Braun and J. Ungar, Eds.), p. 430. Karger, Basel, 1973.
14. Bonner, W. A., Hulett, H. R., Sweet, R. G., and Herzenberg, L. A., *Rev. Sci. Instrum.* **43**, 404, 1972.
15. Hulett, H. R., Bonner, W. A., Sweet, R. G., and Herzenberg, L. A., *Clin. Chem.* **19**, 813, 1973.

16. Kreth, H. W., and Herzenberg, L. A., *Cell. Immunol.* **12**, 396, 1974.
17. Greaves, M., and Janossy, G., *Transplant. Rev.* **11**, 87, 1972.
18. Bobrove, A., Strober, S., Herzenberg, L. A., and De Pamphilis, J. D., *J. Immunol.*, **112**, 520, 1974.
19. Epstein, L. B., and Cline, M. J., *Clin. Exp. Immunol.* in press, 1974.
20. Merigan, T. C., Gregory, D. F., and Petralli, J. K., *Virology* **29**, 515, 1966.
21. Janossy, G., and Greaves, M. F., *Clin. Exp. Immunol.* **10**, 525, 1972.
22. Greaves, M. F., Bauminger, S., and Janossy, G., *Clin. Exp. Immunol.* **10**, 537, 1972.
23. Greaves, M., and Janossy, G., *Transplant. Rev.* **11**, 87, 1972.
24. Phillips, B., and Roitt, I. M., *Nature (New Biol.)* **241**, 254, 1973.
25. Schlossman, S. F., and Hudson, L., *J. Immunol.* **110**, 313, 1973.
26. Valentine, F., and David, J. R., *In* "Progress in Immunology" (B. Amos, Ed.), p. 1527. Academic Press, New York, 1971.
27. Katz, D. H., and Benacerraf, B., *Advan. Immunol.* **15**, 2, 1972.
28. Andersson, J., Moller, G., and Sjoberg, O., *Eur. J. Immunol.* **2**, 99, 1972.
29. Feldmann, M., *Nature (New Biol.)* **242**, 82, 1973.
30. Wybran, J., and Fudenberg, H. H., *N. Engl. J. Med.* **288**, 1072, 1973.
31. Eisen, S. A., Wedner, H. J., and Parker, C. W., *Immunol. Commun.* **1**, 571, 1972.
32. Julius, M. H., Simpson, E., and Herzenberg, L. A., *Eur. J. Immunol.* **3**, 645, 1973.
33. Epstein, L. B., and Ammann, A. J., *J. Immunol.* **112**, 617, 1974.