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Fluorescence-Activated Cell Sorting of Human T and B Lymphocytes

I. Direct Evidence that Lymphocytes with a High Density of Membrane-Bound Immunoglobulin are Precursors of Plasmacytes[1]

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Human peripheral lymphocytes bearing either a high or a low amount of membrane-bound immunoglobulin were studied. Cells were "tagged" with fluorescein-labeled antiimmunoglobulin reagents and separated by means of a new electronic instrument, a fluorescence-activated cell sorter (FACS), into populations with either $> 10^5$ or $< 5 \times 10^3$ immunoglobulin molecules per cell. Fractions of high purities were obtained. (>80% and >99.9%, respectively). *In vitro*, different functional properties were observed: lymphocytes with high densities of membrane-Ig gave a late proliferative response after stimulation with Pokeweed mitogen (PWM). A considerable proportion of stimulated cells developed into mature plasmacytes as detected by cytoplasmic staining. Those lymphocytes with a low density or complete absence of membrane-Ig could be stimulated by both Phytohemagglutinin (PHA) and Pokeweed mitogen, but no differentiation into plasmacytes occurred. The functions are similar to those of bone marrow-derived (B) and thymus-derived (T) lymphocytes in mice. Thus, the designation as B lymphocytes for human lymphocytes with a large quantity of membrane-bound immunoglobulin seems justified.

INTRODUCTION

In recent years much attention has been paid to lymphocytes with surface-attached immunoglobulins in animals and man. In the mouse and chicken it has been convincingly shown that these cells are bone marrow or bursa-derived lymphocytes and represent precursors of antibody-forming cells (1–4). The same classification has been suggested for lymphocytes in man and is already widely used (5). There is considerable evidence from ultrastructural studies with human peripheral lymphocytes after stimulation with Pokeweed mitogen (PWM)[4] that a small subpopulation of cells are indeed plasma cell precursors (6, 7). Furthermore, a relationship

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[4] Abbreviations used in this paper: PWM, Pokeweed mitogen; FACS, fluorescence activated cell sorter; DPBS, Dulbecco's phosphate buffered saline; FCS, fetal calf serum; T cell, thymus-derived lymphocyte; B cell, bone marrow-derived lymphocyte; PHA, phytohemagglutinin; F_b anti-sheep γ, fluorescein-conjugated burro-anti-sheep IgG; and FITC, fluorescein isothiocyanate.

between lymphocytes with surface immunoglobulins and immunoglobulin-secreting cells has been demonstrated in cases of primary immunodeficiency disease (8–10). The evidence so far is strong but circumstantial. It cannot be excluded that some lymphocytes are positive by virtue of passive uptake of serum immunoglobulins on their membranes and that these plasmacytes found in cultures of PWM-stimulated lymphocytes developed from cells with no membrane-associated immunoglobulin. In the presence of an exponentially increasing literature about human lymphocytes it would be desirable to have highly purified subpopulations of cells, the function of which could be directly studied *in vitro*.

We have used a recently developed fluorescence-activated cell sorter (FACS) (11, 12). This machine selects viable cells out of heterogeneous mixtures by virtue of fluorescence and volume. Appropriate markers are introduced on membranes of desired cells by reaction with fluoresceinated antigens and antibodies. In animal models highly enriched preparations were obtained even when wanted cells constituted only a minute fraction in a heterogeneous population. The function of separated cells was not abrogated by labeling and separation procedures (13). The FACS has now been set up for work under sterile conditions. Thus, cell fractions can be put into tissue culture and examined after different intervals.

In the present investigation human peripheral lymphocytes from normal donors were separated according to the quantity of membrane-bound immunoglobulins. The functions of the two populations will be compared with the known properties of bone marrow-derived (B) and thymus-derived (T) lymphocytes in animals. This paper deals with some characteristics in general and the responses after stimulation with unspecific mitogens whereas the accompanying paper (14) deals with interferon production by the two separated cell types.

MATERIALS AND METHODS

Purification of lymphocytes. Sixty milliliters of heparinized blood from normal donors ($n = 12$) was mixed with 15 ml prewarmed 5% Dextran in saline (Dextran T 250, Pharmacia Fine Chemicals), and the red cells allowed to sediment for 30 min at 37°C. The supernatant was withdrawn. Three parts of it were carefully layered onto one part of Ficoll–Hypaque solution ($\rho = 1.076$) and centrifuged at 400*g* for 35 min at 20°C (15). The ring layer containing lymphocytes and monocytes was removed and washed twice with DPBS + 5% FCS at 200*g* for 10 min at 20°C. The final pellet was taken up in 10 ml DPBS + 20% FCS and incubated in plastic petri dishes (Falcon, 8 cm) for 30 min at 37°C. This step was found necessary because of the high contamination by monocytes in Ficoll–Hypaque purified lymphocytes [10–28% monocytes as demonstrated by naphtylacetate-esterase (16)]. Since lymphocytes, and preferentially, B cells tend to adhere to plastic surfaces but less firmly than monocytes, the cell layer was washed several times until only big mononuclear cells were left when monitored by means of an inverted microscope. Thus, it was possible to remove about 70% of monocytes without significant and preferential loss of lymphocytes. The monocyte monolayer was kept under culture conditions until the separation experiment was finished. The total yield of lymphocytes was in the range of 60–75%.

Antisera. Burro anti-sheep gammaglobulin serum (Burro 127, 12-6-71) and unabsorbed antisera, raised in goats against human IgG, IgA, and IgM were kindly provided by Dr. Roy Woods, National Immunoglobulin Reference Center, Spring-

field, VA. Class-specific antisera were purified as follows. After removal of anti-light chain activities, specific antibodies were absorbed onto Sepharose-bound immunoglobulins or myeloma proteins of the appropriate class and recovered by acid elution. The antibody fractions were free of complexes and monospecific by a sensitive radioimmunoprecipitation assay (17). A mixture of equal parts of purified antibodies was used as a polyvalent reagent. Rabbit anti-human Cohn II serum was purchased from Antibodies Incorporated, Davis, CA.

Conjugation of antisera. The immunoglobulin fractions of antisera were labeled with fluorescein-isothiocyanate (BBL, Baltimore) as described by Wood *et al.* (18). Twenty-five micrograms of FITC were added per mg protein in carbonate–bicarbonate-buffer pH 9.6, and the mixture stirred for 2–3 hr at room temperature. Free dye was then removed by passage through Sephadex G25 Pharmacia) whereupon the conjugated protein was fractionated by chromatography on DE 52 (Whatman) with continuous gradient elution: starting buffer: 0.01 *M* Tris/HCl + 0.01 *M* NaCl pH 7.6; gradient buffer: 0.01 *M* Tris/HCl + 0.5 *M* NaCl pH 7.6. The F/P ratios of labeled compounds used in these experiments was 2–3. Prior to use, antisera were absorbed with human erythrocytes and thymocytes. They were then sterilized by Millipore filtration and stored in small aliquots at about 4°C. With the labeled F^{ab} antisheep γ serum, absorption steps were finally omitted since no unspecific staining was found with this antiserum.

Fluorescent staining of lymphocytes. Lymphocytes in suspension were stained by the "sandwich" technique using purified goat antibodies against the major human immunoglobulin classes as the first layer and fluoresceinated F^{ab} antisheep γ as the second reagent. (The burro antiserum reacted equally well with gammaglobulins of either goat or sheep origin). When preparing cells for cell separation all procedures were done under sterile conditions. Lymphocytes ($5–6 \times 10^7$) were resuspended in 2.0 ml DPBS + 5% FCS. Three microliters of polyvalent anti-Ig were added per 10^6 lymphocytes and the cell suspension incubated at ice-bath temperature for 15 min. Cells were then centrifuged twice through discontinuous FCS gradients (2 ml 100% FCS, 2 ml 50% FCS, 290*g* for 5 min at 4°C). The pellet was resuspended in F^{ab} anti-sheep γ (1 mg/ml per 4×10^7 cells), left in an ice-bucket for 15 min, and washed twice more through FCS gradients. A control was run with a small aliquot using normal goat serum instead of specific antibodies. Finally, the cell concentration was adjusted to $5–6 \times 10^6$/ml in DPBS + 5% FCS. The viability as measured by trypan blue exclusion was >95%. Before separation a smear was prepared, and the number of Ig-positive small lymphocytes determined by fluorescence microscopy.

Cell separation. Our present instrument is an improved version of the recently published model (11, 12) and enables us to separate cells according to two different signals: fluorescent light, elicited by the 488 nm line of an argon-ion laser, and low-angle light scattering of a helium–neon laser which gives a signal proportional to cell size. Both signals can be combined in many ways. When preset threshold conditions are fulfilled a droplet containing the desired cell will either be charged positively or negatively and then deflected into left or right collectors while empty droplets or droplets with ambiguous or unwanted cells remain uncharged and pass down into the center container.

In these experiments thresholds were set in such a way as to deflect lymphocytes with fluorescent signals of highest amplitude corresponding in numbers to the fre-

quency of positive lymphocytes seen by fluorescent light microscopy (15–20%). A second threshold was set for lymphocytes with practically no or rather dim fluorescence (60–65%). In order to avoid contamination by ambiguous cells, lymphocytes with signals between the two thresholds were discarded (15–25%). All "wet" parts of the cell separator were steam-autoclaved before use. The deflected fractions were kept in ice during the whole length of separation. In order not to bias interpretations, cells with or without membrane-bound immunoglobulin will be designated as Ig^+ and Ig^- lymphocytes.

Culture conditions. Unseparated and separated lymphocytes were cultured in microtitration plates with flat bottoms (Falcon) as described by Janossy and Greaves (19). The culture medium was RPMI 1640 (Gibco), supplemented with 2 mmole L-glutamine, 20% FCS (Rehatuin, Armour Pharmaceutical, Inc., Kankakee, IL),[5] and 50 μg/ml Gentamicin (Microbiological Associates, Inc., Bethesda, MD). The volume of the titration wells is about 250 μl. Usually 10^5–2.5×10^5 cells were cultured per well. All cultures were set up in, at least, duplicates. Populations of Ig^- lymphocytes were totally devoid of monocytes and had to be reconstituted with a small number of these cells [cultures of Ig^+ lymphocytes were contaminated by about 5% monocytes as demonstrated by naphthyl-acetate-esterase (16)]. In order to get viable lymphocytes off plastic surfaces the Petri dishes were quickly chilled to near 0°C and vigorously shaken. By this procedure enough monocytes could be recovered from the supernatant. At least 75% were viable (by trypan blue exclusion) and could form new monolayers. The microtitration plates were placed into an air-tight box that was gassed with a mixture of 7% CO_2, 10% O_2, and 83% N_2, sealed, and left at 37°C.

Mitogens. Phytohemagglutinin (PHA-P) was purchased from Difco laboratories. Pokeweed mitogen (PWM) from Grand Island Biological Company. The vials were reconstituted with distilled water as indicated by the manufacturer. The mitogens were then aliquoted into small amounts and stored away at −20°C. Aliquots were always used once and never frozen again.

Determination of (^3H)-thymidine incorporation. Twenty-four hours prior to termination of cultures 0.1 μCi tritiated thymidine in 10 μl saline was added per well [^{3}H-thymidine (Methyl-^{3}H) Cat. No. 2533-96, spec. activity: 6.0 Ci/mmole, Schwartz-Mann, Orangeburg, NY]. Cells were harvested according to the method of Janossy and Greaves (19). In short: cells were removed from the well with a Pasteur pipette and collected on Glass Fibre Paper GF/P (Whatman) in a "manifold" multiple sample collector (Millipore). They were then washed in succession with 2 ml ice-cold PBS, 2 × 1 ml ice-cold 5% trichloroacetic acid, and 2 × 1 ml methanol. When dry, the membranes were transferred into counting vials, and left with 0.3 ml Hyamine 10× for about 12 hr at 37°C. Five milliliter scintillation fluid was then added (5.0 gm PPO, 0.1 gm POPOP, Toluol to 1000.0 ml), and the vials counted in a Packard Tri-carb Liquid Scintillation Counter. Results were expressed as cpm per culture.

Staining of cytoplasmic immunoglobulin. Well spread cell smears were prepared by using a cytocentrifuge (Shandon). Smears were air-dried and fixed immediately in 95% ethanol for at least 20 min. A drop of antiimmunoglobulin serum was placed on the cell pellet and covered with a cover slip to spread the drop. After 45 min incubation at room temperature in a wet chamber, slides were rinsed twice in PBS

[5] A selected batch which was also used for Mishell-Dutton culture.

for 10 min each, fixed in 95% ethanol for 20 min., and mounted in buffered glycerol (9 parts glycerol, 1 part PBS). Fluorescein-labeled rabbit anti-human Cohn II serum at a concentration of 1 mg/ml was used for detection of intracytoplasmic immunoglobulin. This antiserum precipitated myeloma proteins of all classes and light chain types by Ouchterlony tests and gave brilliant staining with human plasmacytes in tissue sections.

Fluorescence microscopy. A Zeiss microscope with an HBO, 22 mercury arc (Osram) light source and a combination of Zeiss BG 3 excitation and No. 47 barrier filters was used. Cells were examined under darkfield illumination using blue-violet light first and ordinary light next. By this procedure, the morphology of fluorescent cells could also be studied.

RESULTS

Class distribution of membrane-bound immunoglobulin. The percentages of lymphocytes carrying IgG, IgA and IgM on their membranes are given in Table 1. These data are derived from optimum staining conditions where a plateau value is reached when the same number of lymphocytes is incubated with different amounts of anti-class antibodies with a constant amount of the second layer and vice versa. As these studies were done on Ficoll-Hypaque purified lymphocytes without depletion of monocytes, care was taken to score only brilliantly staining small lymphocytes. The predominant molecular class was IgG which agrees (9, 10) but also disagrees (8) with results found by others. The sum of the single percentages is not so much different from the total percentage of positive lymphocytes when a mixture of equal parts of class-specific antibodies is used for staining. This

TABLE 1

PERCENTAGES OF LYMPHOCYTES WITH MEMBRANE-BOUND IMMUNOGLOBULINS

	(A) Normal adults			
Expt no.	G[a]	A[a]	M[a]	G + A + M[b]
1	10.6	3.2	8.6	20.7
2	14.0	1.6	6.8	20.8
3	16.6	1.6	7.8	25.7
4	12.7	2.0	3.2	n.d.
5	9.5	1.8	10.0	20.0
6	18.0	1.4	7.7	26.0
7	11.5	1.3	7.5	19.7
Avg	13.3	1.8	7.4	22.2
	(B) Selective IgA deficiency			
	G[a]	A[a]	M[a]	G + A + M[b]
B	14.7	0	7.0	21.8
C	20.0	0	8.7	31.4
E	13.0	0	11.0	18.3

[a] Stained with "sandwich" technique (see Methods).

[b] Stained with a mixture of equal parts of anti-IgG, anti-IgA, and anti-IgM. (Background with normal goat serum instead of antibodies was <1%.)

TABLE 2

REAPPEARANCE OF NEW IMMUNOGLOBULINS ON SEPARATED CELLS IN CULTURE

Donor	Cell type	Purity	Time in culture[a]	Percent positives[b]
1 (HT)	Ig^+	95%	24 hr	75
	Ig^-	>99.9%	24 hr	0
2 (WJ)	Ig^+	91%	48 hr	80
	Ig^-	>99.9%	48 hr	0
3 (JT)	Ig^+	92%	72 hr	78
	Ig^-	>99.9%	72 hr	0.8

[a] Without mitogens.

[b] Lymphocytes with membrane-associated immunoglobulin. No. 1 and 2 restained with original "sandwich" technique, no. 3 restained with Frabbit anti-human Cohn II.

indicates that the proportion of cells carrying immunoglobulins of two different classes on their membranes must be small.

In the lower part of Table 1 are the proportions of Ig positive lymphocytes from three members of a family with selective IgA deficiency.[6] All these individuals had anti-IgA antibodies. In contrast to findings by others (9, 10, 20), no lymphocytes with membrane-bound IgA were found. In this case, it cannot be excluded that membrane determinants were blocked by specific antibodies.

Characteristics of separated cells. It should be noted that lymphocytes were only stained for the three major classes of immunoglobulins, IgG, IgA and IgM and that a smaller proportion of cells bearing delta and epsilon chains were neglected.

After separation, purities were 90–97% for the Ig^+ and 99.9% for the Ig^- fraction with a viability of about 98% for both cell types as measured by trypan blue exclusion. The overall recovery was around 40%. This is probably due to formation of small aggregates of cells. Since the machine will only process single cells per microdroplet, doublets or triplets of cells are discarded. When smears of freshly separated Ig^+ lymphocytes were examined by fluorescent light microscopy a heterogeneous distribution of fluorescence was observed. Some cells possessed numerous fine spots (>20) while others showed broad patches or typical caps. It is possible that some cells might quickly shake off antigen–antibody complexes from their membranes. Thus, the purity of Ig^+ lymphocytes might well be underestimated.

Reappearance of new surface markers. Immunoglobulin positive lymphocytes in culture retained residues of the original "tag" for rather a long time. Even after 48 hr a small highly fluorescent spot could still be observed on one pole of the majority of cells, but was gone by Day 3. When both cell types were restained with polyvalent anti-Ig reagents, new membrane-bound immunoglobulins could readily be demonstrated on one of them whereas the other remained negative (Table 2). The new immunoglobulin was distributed in numerous tiny spots over the entire membrane. Since the culture medium contained fetal calf serum the new surface antigen must have been produced by the cells themselves.

Response to mitogens. In order to exclude the possibility that the labeling process might interfere with the stimulation by PWM or PHA, unseparated lymphocytes

[6] Fathman, C. G., and H. W. Kreth, unpublished results.

were labeled either by the direct or indirect method and cultured for three days with or without mitogens. Regardless of the technique employed, the dose–response profiles for labeled or unlabeled cells were virtually the same. (Fig. 1a). Purified cell populations, however, showed marked differences in response when stimulated for 3 days. Immunoglobulin negative lymphocytes were readily stimulated by PHA and PWM to almost the same extent as unseparated cells while hardly any ^{3}H-thymidine incorporation was induced in Ig$^+$ cultures. (Figs. 1b and c). The uptake of label above background as caused by PHA was 30–100-fold for unseparated, 33–95-fold for Ig$^-$, and 0–8-fold for Ig$^+$ lymphocytes. The corresponding values after PWM stimulation were 20–30-fold, 25–95-fold, and 3–4-fold. As a rule the maximum incorporation in Ig$^+$ cultures was only 10% of the value observed in Ig$^-$ cultures. The low- or non-responsiveness of Ig$^+$ lymphocytes could not be explained by poor survival of these cells. The viabilities were 70–80% and 50–60% of original input after 24 and 72 hr, respectively.

The pattern of response could not be changed either by preincubation of cells (15–20 hr) prior to addition of mitogens or by culturing Ig$^+$ cells in "conditioned" medium from Ig$^-$ cells. (Medium separated from mitogen stimulated cultures of Ig$^-$ cells after 24 hr and used to sustain Ig$^+$ lymphocytes for 3 days). These results seemed not to be due to poor culture conditions since similar results were obtained

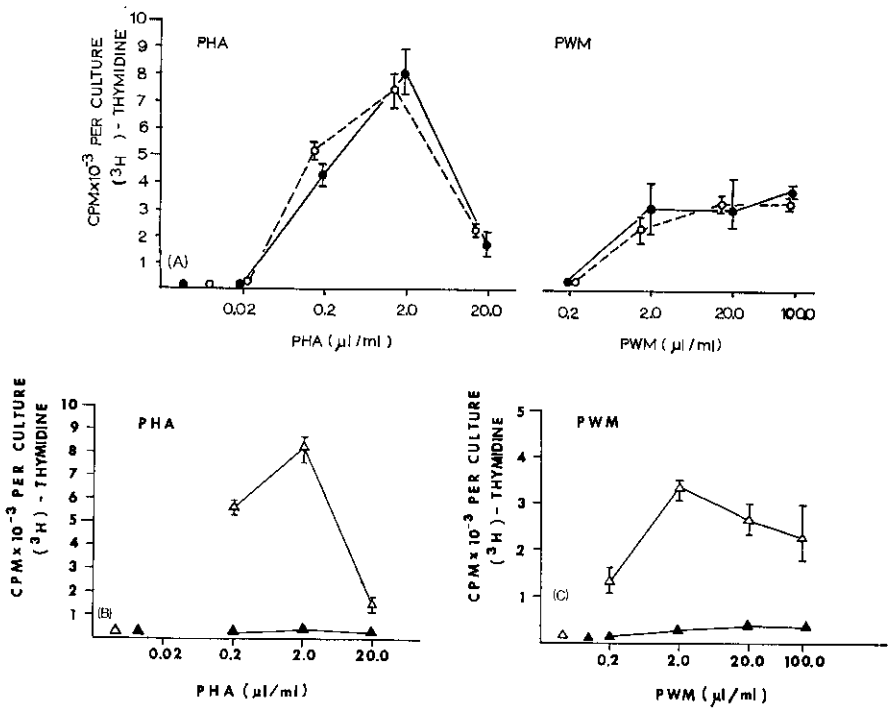

FIG. 1 A, B, C. Uptake of ^{3}H-thymidine in cultures of separated and unseparated lymphocytes after PHA and PWM stimulation (3 days). Each figure represents a different experiment. Points are arithmetic means of duplicate or triplicate cultures ± range. (A): Effect of anti-immunoglobulins on unseparated lymphocytes. ●———● stained with ^{F}rabbit anti-human Cohn II (19% Ig$^+$); ○------○ unstained. (B): Separated lymphocytes, response to PHA. ▲———▲ Ig$^+$ (95%); △———△ Ig$^-$ (> 99.9%) (C): Separated lymphocytes, response to PWM. ▲———▲ Ig$^+$ (92%); △———△ Ig$^-$ (> 99.9%)

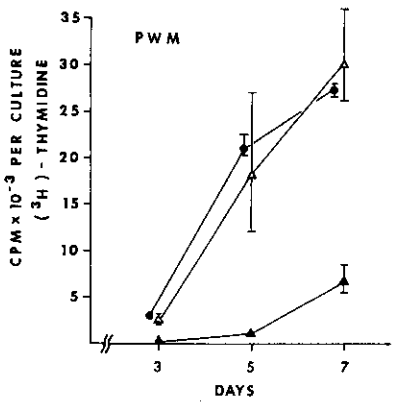

FIG. 2. PWM-induced stimulation after 2–3, 4–5 and 6–7 days of unseparated (●———●), Ig^- (△———△), and Ig^+ lymphocytes (▲———▲). Purities. Ig^-: >99.9%; Ig^+: 97%. Arithmetic means of duplicate and triplicate cultures ± range. Background subtracted. PWM concentration: 4μ/ml medium.

with a completely different culture system in a collaborating laboratory (14). Thus, there is good reason to believe that Ig^- lymphocytes are the predominantly responding cell type when unseparated human peripheral lymphocytes are stimulated for 3 days by either PHA or PWM.

When the culture period with PWM was extended to 7 days a different picture emerged (Fig. 2). Uptake of ^{3}H-thymidine was markedly increased between Days 5 and 7 in cultures of Ig^+ cells. There was also a considerable increase in Ig^- cultures but the increment of incorporation from Days 3–7 was only 10-fold whereas it was up to 60-fold for Ig^+ lymphocytes (Fig. 2). The late response of Ig^+ cells was always consistent. Studies with PHA for more than 3 days were not performed.

Alcohol fixed cytocentrifuge slides were prepared from PWM-stimulated cultures after 7 days and stained with a potent polyvalent antiimmunoglobulin conjugate, the adequacy of which for staining human plasmacytes had been proved. Cells with a strongly positive reaction only were scored. The percentage of cytoplasmically staining cells was 4.5–7.8% in Ig^+, 2–3% in unseparated and 0% in Ig^- cell cultures. Immunoglobulin negative cultures were always negative even when the total cytocentrifuge pellet was scored. Since samples available were small, no attempts were made to differentate the cytoplasmic immunoglobulin with monospecific antisera.

It should be noted that spontaneous blast formation never occurred in unstimulated cultures of Ig^+ lymphocytes. The reaction between membrane-bound immunoglobulin and antibodies is not sufficient by itself to induce transformation and subsequent proliferation of labeled lymphocytes.

DISCUSSION

The results presented here, show that human lymphocytes with a high density of membrane-bound immunoglobulin are indeed the precursors of immunoglobulin-producing cells. Thus, the classification as B lymphocytes in analogy to animal models seems justified. Cells designated as Ig^+ in this study comprise about 20% of human peripheral lymphocytes. These cells were separated by a direct approach

using a FACS (11, 12) after labeling desired cells with fluorescent antibodies. From the pulse height distributions on the FACS, it can be calculated that most Ig^+ lymphocytes carry 10^5 or more immunoglobulin molecules on their surface.

The evidence for the B cell nature of this type of lymphocyte is as follows.

(A) It could be demonstrated that the marker is a genuine membrane component and not a passively absorbed protein as observed for many rabbit lymphocytes (21). Enriched Ig^+ lymphocytes show in culture the typical sequence of events with spot and cap formation and gradual disappearance of immunoglobulin antiimmunoglobulin complexes as has been described for other cells (22–24). The dynamics, however, are much slower than those reported for mouse B cells after labeling with anti-Ig conjugates (24). Even 48 hr after separation, a single spot of intense fluorescent brightness was still present on one cell pole. It is conceivable that this is due to a difference in metabolic activity, e.g. endocytosis, between mouse spleen and human peripheral lymphocytes. If we assume that complexes are irreversibly lost and that no"recycling" takes place then reappearance of new antigenic sites can only mean resynthesis of new immunoglobulin molecules. When cells were restained with the original technique after different intervals of culture, cells with numerous spots diffusively distributed over the entire membrane were observed only in Ig^+ cultures but never in Ig^- cultures.

Restaining of cultured cells gives some information about the real purity of the original samples. As seen in Table 2 the purity of Ig^+ lymphocytes is at least 80%. There is, however, a gap of 10–15% between original input and the restaining data. Part of this deficit is probably due to monocytes (on average 5%) which were originally positive by virtue of receptors for human IgG (25) but remain negative after culture in FCS-containing medium. The rest might be B cells that failed to resynthesize enough new receptor immunoglobulins for detection by fluorescence microscopy or contaminating lymphocytes of a different line, e.g., thymus-derived. Another and more attractive possibility is that these cells are K cells (cytotoxic non-T lymphoid cells) which have Fc receptors but do not synthesize Ig. These would have enough bound Ig to be detected by the FACS, which is considerably more sensitive than the usual fluorescent microscope observations, but have not retained enough Ig to be seen by fluorescence microscopy. A precise analysis can only be done when monospecific antisera against other markers on human T and B cells are available.

(B) Immunoglobulin positive lymphoctes responded with ^{3}H-thymidine incorporation (as a measure of proliferation) when stimulated by PWM as has been reported for mouse B lymphocytes (19). The time course of stimulation of mouse and human Ig^+ cells by PWM is different. In the mouse, stimulation occurs as early as 3 days but in the human the response is delayed to 5 or 7 days (Fig. 2). Is this late onset of proliferation an artifact due to disturbed membrane function after labeling or an inherent property of human B cells? If we assume that PWM binds to immunoglobulin receptors in a fashion similar to specific antigens then it might be anticipated that the activation process is blocked by anti-Ig antibodies. This seems not to be the case. It has been reported that purified PWM will not react with radio-labeled immunoglobulin (26). Binding to membrane sites distinct from immunoglobulin receptors seems therefore rather likely. Evidence against transitory inhibition comes also from control experiments. First, preincubation of Ig^+ cells for various intervals and addition of PWM at a time when new Ig molecules

could be demonstrated again did not change the time course. Secondly, the response profiles of unseparated peripheral lymphocytes either treated with antibodies or left untreated are distinguishable (Fig. 1a).

(C) It could further be demonstrated that a substantial number of Ig^+ cells will differentiate into plasmacytes (cytoplasmically Ig staining cells) when stimulated by PWM whereas no such cells were found in cultures of Ig^- lymphocytes. Pokeweed mitogen was absolutely necessary since no blastogenesis with concomitant differentiation occurred in unstimulated cultures. Cells unequivocally positive after cytoplasmic staining were scored. This might be the reason why the incidence of plasmacytes appears low especially in enriched fractions when compared with figures of ultrastructural studies on unseparated human peripheral lymphocytes. Douglas (6) reports 20–30% lymphocytes with a well developed rough endoplasmic reticulum after PWM stimulation. The discrepancy is probably due to the different sensitivity of the two methods. Electron microscopy reveals early structural changes of intracellular organisation whereas rather late stages of differentiation are detected by immunofluorescence. Moreover, it is possible that only a minority of peripheral B cells is capable of developing into mature immunoglobulin-secreting plasmacytes under *in vitro* conditions.

During the same separation procedure another type of lymphocyte was isolated. A low threshold was chosen which allowed simultaneous deflection of cells bearing less than about 5×10^3 immunoglobulin molecules on their membranes. The finding of rather low quantities of either genuine or passively absorbed immunoglobulin on 60–65% of human peripheral lymphocytes is in good agreement with results with a sandwich radioimmunolabeling technique (27). These cells, designated here as Ig^-, are not detectable by conventional fluorescent light microscopy and display some characteristics resembling those of thymus-derived lymphocytes in mice.

1. Presence of low amounts or complete absence of membrane-bound immunoglobulins (28).
2. Excellent stimulation by Phytohemagglutinin or Pokeweed mitogen (19).
3. Lack of plasmacytes in PWM stimulated cultures (29).

In addition, mitogens induce Interferon production (14), a mediator of cellular immunity (30–32). We favor the view that these cells are a rather homogeneous T cell population through contamination by other lymphocyte-like cells (stem cells?) is possible. It should be taken into account that under present conditions not all lymphocytes were processed, but only those with fluorescent signals above or below certain thresholds. (The high threshold was chosen to correspond approximately to the sensitivity of conventional fluorescent light microscopy whereas the lower one was close to the minimum threshold of detection for the cell separator). Cells with ambiguous signals, sometimes as many as 15–25% of the total, were discarded. This puts some limitations on the present investigation: a special subpopulation of either B or T lymphocyte lineage might have been neglected.

It must be the aim of a further investigation to find out whether this fraction consists of B lymphocytes with a lower quantity of membrane-bound immunoglobulin (not detectable by fluorescence microscopy) or T cells with a high amount of true immunoglobulin receptors or with just "sticky" membranes and unspecific uptake of antibodies.

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