Surface Immunoglobulin on Activated Human Peripheral Blood Thymus-Derived Cells

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ABSTRACT Human peripheral blood lymphocytes grown in vitro were stimulated with nonspecific mitogens and in mixed lymphocyte culture. The presence of IgM and thymus (T) surface markers on large and small lymphocytes was investigated by immunofluorescence and correlated with spontaneous rosette formation. All stimulated large lymphocytes formed spontaneous rosettes and all except pokeweed mitogen (PWM)-stimulated large lymphocytes had IgM and T markers. IgG, IgA, and light chain determinants were only detected on PWM-induced large lymphocytes. Thus, surface markers expressed on activated human lymphocytes may differ for different mitogens. IgM was always present on large cells which had the T markers, and it cannot be used to identify a lymphocyte as a bone marrow-derived (B) cell. Due to the overlap of surface markers, the classification into B and thymus-derived (T) cells ought to be restricted to functional phenomena of antibody-production or cell-mediated immunity.

INTRODUCTION

Although thymocytes capable of combining with an antigen have been demonstrated in in vitro systems (1), the actual mechanism of the interaction has not been resolved. Immunoglobulin, due to its established specificity, would be an obvious candidate for the surface receptor involved in a reaction of thymocytes with an antigen.

The presence of immunoglobulins on thymus-derived (T) cells has been a subject of much controversy for some time. The controversy exists because similar techniques used to identify T-cell immunoglobulins have produced contradictory findings in the hands of different investigators. Some were unable to identify any immunoglobulin by immunofluorescence (2, 3), radioimmunolabeling (4, 5), surface iodination with lactoperoxidase (6, 7), or radioiodination and ultrastructural examination (8). On the other hand, many others using the same techniques (9-17) have detected immunoglobulins on T-cell membranes. It has been suggested that small amounts of surface IgM on T-cell membranes are responsible for these conflicting results. However, Marchalonis et al. (18) reported similar amounts of IgM on both bone marrow-derived (B) and T cells. Their data indicated that immunoglobulins on T cells may be buried in the membrane and thus may be poorly accessible to surface labels. In addition, there is recent evidence indicating that the immunoglobulin found on T-cell surfaces may be passively absorbed (19, 20).

Antigenic or mitogenic activation of T lymphocytes grown in vitro has been found to increase the concentration of detectable surface immunoglobulins in both rats and mice (12, 21). However, in vivo T-cell activation has not produced detectable immunoglobulin on rat lymphocytes (22). Mitogenic stimulation of human T cells has been reported to lead to the presence of easily detectable concentrations of a light chain (23), and a small proportion of cells which also contain γ-heavy chain (24). Another approach used for determining the presence of immunoglobulins on T lymphocytes is the suppression of T-cell function by antiimmunoglobulin serum. Mitogenic responses (25, 26), lymphokine production (27), and antigenic recognition (1) have all been shown to be susceptible to such antisera.

The purpose of this communication is to show that IgM and T-surface markers are present on human peripheral blood lymphocytes activated in vitro. The ability of these IgM-bearing cells to form spontaneous rosettes serves as an indicator of T-cell activity. The simultaneous presence of IgM, T marker, and spontaneous rosetting ability of stimulated lymphocytes in vitro is discussed in relation to the division of lymphocytes into T and B classes.

METHODS

Mitogens and reagents. Phytohemagglutinin (PHA) [1] (Difco Laboratories, Detroit, Mich.) and pokeweed mitogen

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[1] *Abbreviations used in this paper:* PHA, phytohemagglutinin; PWM, pokeweed mitogen; Con A, concanavalin A; HBSS, Hank's balanced salt solution.

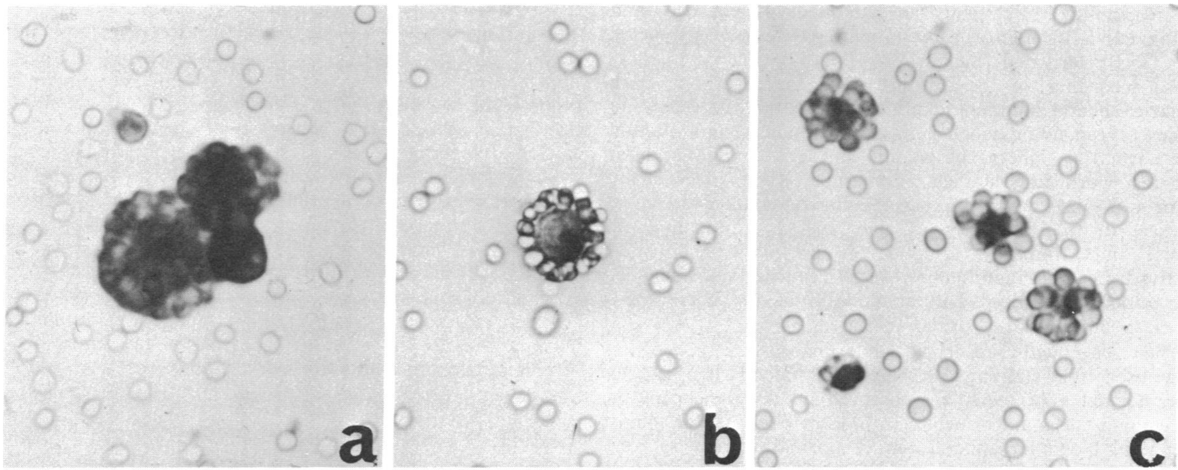

FIGURE 1 Sheep-erythrocyte rosettes formed by large (*a*, *b*) and small (*c*) lymphocytes cultured with mitogens for 72 h. Initial magnification × 400.

(PWM) (Grand Island Biological Co., Grand Island, N. Y.) were used at 1:10 and 1:100 dilutions of the stock solution, and concanavalin A (Con A) (Pharmacia Fine Chemicals, Piscataway, N. J.) at a concentration of 4 μg/ml. To determine the optimal stimulatory concentrations, dose-response curves were run for all three mitogens with cells of all individuals studied.

D-[*α-methyl*]Mannoside (Sigma Chemical Co., St. Louis, Mo.) and *N*-acetyl-D-galactosamine (Pfanstiehl Labs., Inc., Waukegan, Ill.) solutions in Hank's balanced salt solution (HBSS) at a concentration of 50 mM were used for washing of lymphocytes cultured with Con A and PHA, respectively.

Human lymphocyte suspensions. Blood was collected from normal volunteers in 35-ml syringes containing 2 ml of preservative-free heparin (200 U per ml). The blood was allowed to settle for 1 h at 37°C, and the leukocyte-rich plasma was removed aseptically and centrifuged. The cells were washed three times in HBSS (Grand Island Biological Co.), and cultured in autologous plasma.

Culturing of lymphocytes. Washed lymphocytes were cultured at a concentration of 5×10^5 cells per ml (2 ml/tube) in medium 199 buffered with Hepes (Grand Island Biological Co.) to the final concentration of 40 mM and containing streptomycin and penicillin. The medium was supplemented with 15–20% autologous plasma. Cultures were grown in triplicate, using screw-cap plastic culture tubes, and incubated at 37°C in the absence of a CO_2-containing air mixture. The mitogens were added at the start of culture, and all cultures were harvested after 72 h. Radioactive thymidine at a concentration of 2 μCi per tube was added 24 h before termination of the cultures.

Stimulated cultures were sampled at different times after the addition of a given mitogen to study the kinetics of lymphocyte blastogenesis, the presence of immunoglobulins, and the ability to form spontaneous rosettes. The degree of lymphocyte stimulation expressed as uptake of tritiated thymidine at each sampling time after mitogenic stimulation was correlated with the number of large and small rosette-forming lymphocytes and with the presence of immunoglobulin and thymic markers on the surface of transformed cells. The number of small and large lymphocytes in each culture was determined microscopically by observation (see Fig. 1 *a*, *b*, and *c*). Lymphocytes were considered large when their diameter was at least 1.5 times that of a human erythrocyte.

To dissociate Con A and PHA from the surfaces of stimulated lymphocytes, D-[*α-methyl*]mannoside and *N*-acetyl-D-galactosamine solutions were added, respectively, to the cultures for the last hour of incubation. Cells were then harvested and washed three times in the appropriate carbohydrate solutions and twice in HBSS in preparation for staining and rosette formation.

Immunofluorescent staining. Aggregate-free monospecific fluorescein-labeled antihuman α-, μ-, γ-, κ-, and λ-antisera (Meloy Laboratories, Inc., Springfield, Va.) were used to detect surface immunoglobulins. The concentration of cultured lymphocytes was adjusted to 2×10^6 per ml in HBSS, and 0.1 ml of this suspension was mixed with an equal volume of undiluted fluorescein-labeled antiserum. All conjugated sera were titrated against normal peripheral blood lymphocytes and titers of two, four, and eight all gave comparable percentages of positive cells. After 20–30 min incubation with the antiserum at 4°C without sodium azide, cells were centrifuged, washed three times with HBSS, mounted on slides in glycerol phosphate buffer, and examined in a Zeiss Photoscope II (Carl Zeiss, Inc., New York). The number of cells exhibiting membrane fluorescence was counted and expressed as a percentage of the total number of cells in several fields (at least 200 cells were counted). Blocking controls in which cells were reacted with unlabeled antihuman immunoglobulins (Meloy Laboratories, Inc.), washed, then treated with labeled sera and nonspecific controls with fluorescein-labeled human albumin were run simultaneously with the specimens. Lymphocytes were also treated with labeled goat antirabbit globulin as a control for nonspecific staining in the indirect method. All controls were negative.

Specificity of fluorescein-labeled antisera. Monospecificity of the conjugates was tested in immunoelectrophoresis with normal human serum. In addition, the conjugates were absorbed with Sepharose-bound human immunoglobulins. Sera of multiple myeloma patients were a source of IgG and IgA, while IgM was obtained from serum of a patient with Waldenström's macroglobulinemia. These sera had marked hypogammaglobulinemia of the nonhomogeneous immunoglobulins. The immunoglobulins were purified by chromatography on Ultrogel AcA-34 (LKB Instruments,

Inc., Rockville, Md.), tested in immunoelectrophoresis, and lyophilized. 5–10 mg of each immunoglobulin was reacted with CNBr-activated Sepharose 4B (Pharmacia Laboratories) according to specifications of the manufacturer. Each Sepharose-bound immunoglobulin absorbed out the reactivity of the corresponding labeled antiheavy chain serum, so that both normal peripheral blood and mitogen-stimulated lymphocytes were not stained. However, when anti-μ-chain serum was absorbed with, e.g. Sepharose-coupled IgA, no decrease in the reactivity of the labeled antiserum with normal and stimulated lymphocytes was observed. Thus, reactivity of each immunofluorescent reagent was abolished only with insolubilized immunoglobulin of the corresponding class but not of a different class.

When specifically-absorbed and unabsorbed conjugates were used for staining of peripheral blood lymphocytes from normal volunteers, we observed no differences in the percentages of stained cells. In the course of this study, peripheral blood lymphocytes in 17 normal volunteers were enumerated using the same lots of antisera. The following ranges and mean percentages of each immunoglobulin class were found: IgG-bearing lymphocytes, 0–22% (mean 9.15 ±6.0); IgA-bearing lymphocytes, 2–14.6% (mean 6.14 ±4.0); IgM-bearing lymphocytes, 3–16% (mean 8.6±4.4). The range and mean of the total immunoglobulin-bearing lymphocytes were 12–41.6 and 23.8±8.1%, respectively. The mean percentage of total immunoglobulin-bearing cells corresponded reasonably closely, but not completely, to the mean percentage of complement receptor lymphocytes of 21.9±4.6 identified by human-erythrocyte-antibody-complement mouse rosette assay (28). This is in agreement with data reported by Jondal et al. (29). In these experiments phagocytic cells were removed by incubating blood with polylysine-coated iron particles (Technicon Instruments Corp., Tarrytown, N. Y.) before centrifugation on Ficoll-Hypaque gradients and passing the separated leukocytes through tubing wound around a magnet.

The specificity of the anti-immunoglobulin sera was also confirmed when bone marrows of more than 20 multiple myeloma patients were examined by immunofluorescence. In all cases the immunofluorescent staining of plasma cells in bone marrow smears corresponded to the homogenous heavy and light chains identified in serum and/or urine by immunoelectrophoresis.

Human anti-T-marker serum was prepared by injecting rabbits with homogenized human brain (a slice of cerebrum with both gray and white matter). 300 mg (wet weight) of brain homogenate emulsified with complete Freunds' adjuvant (4 mg *M. tuberculosis* H37RA/ml) was injected into multiple sites subcutaneously at biweekly intervals. Each rabbit received a total of seven injections. The specificity of antiserum for the T marker was amplified by absorptions with human type 0 erythrocytes, lyophilized human liver, and lymphocytes from two different chronic lymphocytic leukemia patients (30). Indirect immunofluorescence with fluorescein-conjugated goat antirabbit immunoglobulin serum (Meloy Laboratories, Inc.) was used to detect the T marker. The fluorescein-conjugated antiserum neither reacted with purified human immunoglobulins in immunoelectrophoresis nor stained human B or T cells in a direct assay. The reactivity and selectivity for T cells of our antibrain serum is described in the Results section.

Spontaneous rosette formation on blasts and small lymphocytes. Rosette formation with sheep erythrocytes was studied using either lymphocytes isolated from peripheral blood by Ficoll-Hypaque centrifugation (31) or cultured lymphocytes by a modification of the method of Jondal et al. (32) as follows. Washed lymphocytes were resuspended in a solution of 0.1% gelatin in RPMI-1640 medium (Grand Island Biological Co.) at a concentration of 4×10^6 cells/ml. 0.25 ml of the cell suspension was mixed with an equal volume of washed sheep erythrocytes (1.0%), centrifuged for 5 min at 200 *g* and placed at 4°C for 12–18 h. A total of 200 lymphocytes were counted in each preparation, and the proportion of rosettes formed on large vs. small lymphocytes was determined (Fig. 1). Lymphocytes with three or more adherent erythrocytes were considered positive. Viability of cultured or peripheral blood lymphocytes was routinely tested by the trypan blue exclusion test so that a correction could be made for any nonviable cells. For enumeration of rosettes on cultured lymphocytes, toluidine blue was added to the resuspended cells before counting. Our mean laboratory value for spontaneous rosettes of 16 normals was 61.8–8.8.

The effect of antihuman IgM, IgA, and IgG antisera and anti-T-antiserum on the ability of cultured lymphocytes to form spontaneous rosettes was studied by incubating the cells with the unlabeled antisera for 30 min at room temperature, washing twice, and then reacting the lymphocytes with sheep erythrocytes as described. To see if distinct membrane markers are involved in reactions with anti-immunoglobulin sera and rosette formation, cultured lymphocytes were stained with fluorescein-conjugated anti-IgM antiserum, washed, resuspended in gelatin-RPMI solution, and allowed to form spontaneous rosettes.

Mixed lymphocyte cultures. Peripheral blood lymphocytes from unrelated normal individuals were used in two-way mixed lymphocyte cultures. Cell suspensions were cultured at a final concentration of 1×10^6 cells per ml in medium 199 containing 20% human plasma. Each tube contained 1 ml of cells from one person plus 1 ml of cells from the second person. All cultures were set up in triplicate and incubated for 5 days at 37°C. The degree of allogenic stimulation was measured by incorporation of tritiated thymidine (2 μCi per tube) and only cultures with stimulation indices greater than five were used. The background level of thymidine incorporation in controls varied between 200 and 700 cpm. A typical two-way mixed lymphocyte culture had an average control count of 500 cpm, stimulated count of 10,330 cpm, and the stimulation index of 20. Lymphocytes from the mixed lymphocyte cultures were assessed for the presence of surface immunoglobulins and the extent of spontaneous-rosette formation as described above.

RESULTS

Spontaneous rosette formation by cultured lymphocytes. The effect of PHA, Con A, and PWM on spontaneous-rosette formation by cultured lymphocytes was investigated. Human peripheral blood lymphocytes produced varying percentages of large lymphocytes when exposed to different mitogens (Table I). The amount of [^{3}H]thymidine taken up by lymphocytes cultured with mitogens corresponded to the percent increase in large lymphocytes, PHA giving the highest values for each. Both small and large lymphocytes (defined morphologically as described in Methods) were capable of forming spontaneous rosettes (Fig. 1). While large lymphocytes present in nonstimulated control cultures did not form spontaneous rosettes, all the large lymphocytes in PHA, Con A, and PWM cultures did so (Table I, column 4).

Table I

*Spontaneous Rosette Formation by Large and Small Lymphocytes Cultured in the Presence of Various Mitogens for 72 h**

Mitogen	[³H]Thymidine uptake	Total lymphocyte population		Large lymphocytes which form rosettes	Small lymphocytes which form rosettes
		Large lymphocytes	Small lymphocytes		
	cpm	%		%	
None	680±145	20±11	80±12	0	41±17
PHA	156,350±51,753	62±21	38±19	100	45±18
Con A	74,710±50,051	35±14	65±14	100	54±13
PWM	79,547±44,882	27±10	73±10	100	41±8

* The results represent average percentages and SDs obtained on 12 normal individuals. The mitogens were used at the concentrations described in Methods.

In contrast, only about 50% of the small lymphocytes present after 72 h of incubation were rosette-forming cells.

The question of nonspecific adherence of stimulated lymphocytes as a basis for their ability to form rosettes was investigated. Lymphocytes exposed for 1 h to either PHA, Con A, or PWM, then washed and placed in culture medium for 72 h formed the same percentage of rosetting large lymphocytes as that seen in cultures grown in the continuous presence of each mitogen. In some cases, particularly with PHA, clumping of transformed lymphocytes made determinations of spontaneous rosettes difficult. It was found that using 30–60-min incubations with a mitogen minimized the clumping without affecting the number of spontaneous rosettes after 72 h in culture. All experiments in which clumping occurred were repeated using a short-term mitogen treatment. Incubation and washing of PHA-stimulated cells with *N*-acetyl-D-galactosamine before rosetting abolished clumping and facilitated counting of rosettes. The results obtained were identical to those given in Table I. Similarly, treatment of Con A cultures with D-[*α-methyl*]mannoside did not alter the results. In addition, allogeneic stimulation of lymphocytes in the two-way mixed lymphocyte cultures was used as a further control. From 50 to 70% of lymphocytes in our mixed lymphocyte cultures were large, depending on the stimulation index of each culture. All large lymphocytes pro-

Table II

Properties of Antihuman Brain Serum Made in Rabbits and Absorbed with Human Erythrocytes, Lyophilized Human Liver and Chronic Lymphocytic Leukemia Cells

1. Stained by indirect immunofluorescence:
   70–80% of lymphocytes in circulation of normal individuals (30)*
   95% of thymocytes in suspensions of human thymus (5)
   27–35% of lymphocytes in suspensions of human spleen (3)
   5–10% of lymphocytes in suspensions of human tonsils (3)
2. Did not stain lymphocytes from patients with C.L.L.‡
3. Stained only T-dependent areas of cryostat-sectioned normal human lymph nodes
4. Does not stain erythrocytes, plasma cells in marrow of MM patients or macrophages (cells adherent to plastic)
5. Does not stain circulating lymphocytes which form HEAC mouse rosettes. Polyvalent anti-Ig serum stains 80–90% of CRLs forming HEAC mouse rosettes
6. After Ficoll-Hypaque sedimentation of HEAC mouse rosettes, 75% of cells at the interface formed T rosettes and 65–75% were stained with the anti-brain serum

* Number of individual preparations examined is given in parentheses.
‡ Abbreviations; CLL, chronic lymphocytic leukemia; CRL, complement receptor lymphocytes; HEAC, human erythrocyte antibody complement; MM, multiple myeloma.

Table III

*Surface Markers Detectable on Large Lymphocytes Cultured in the Presence of Different Mitogens**

Surface determinants	Mitogen					
	PHA		Con A		PWM	
	24 h	72 h	24 h	72 h	24 h	72 h
T	100	100	100	100	0	0
IgM	100	100	30±15	90±10	0	0
IgG	0	0	0	0	±	50±25
IgA	0	0	0	0	±	20±11
κ	25±5	0	±	0	20±5	30±15
λ	30±10	0	±	0	10±8	20±10

* Average percentages with SDs of stained lymphocytes from cultures of ten different normal individuals. No markers were detected on large lymphocytes in control cultures.

Table IV

Distribution of Surface Markers on Small and Large Lymphocytes in 72-h Cultures Grown in the Presence of Various Mitogens

Surface determinant	Control		PHA		Con A		PWM	
	Small	Large	Small	Large	Small	Large	Small	Large
				% Stained*				
T	5±3	0	100	100	25±10	100	5±1	0
IgM	5±1	0	0	100	0	90±10	5±2	0

* Average percentages with SDs from cultures of 10 different normal individuals.

duced by allogeneic stimulation formed sheep erythrocyte rosettes.

Specificity of antibrain serum. Antiserum to human brain prepared as described in Methods was tested for its selectivity for the T-dependent lymphocyte population. Table II summarizes the properties of this antiserum. It appears that the antiserum is selective for T lymphocytes and does not react with B cells.

Antigenic markers on lymphocyte membranes. The presence of immunoglobulin markers on the surface of lymphocytes stimulated in vitro with different mitogens was studied by immunofluorescence. Table III shows that in cultures stimulated with PHA all large lymphocytes had surface markers detectable with anti-T and anti-IgM antisera. Cells staining with anti-IgG and anti-IgA sera were not identified after 1–3 days in culture. With Con A, 100% of the large lymphocytes stained with anti-T-cell serum at both 24 and 72 h. However, not all cells stained with anti-IgM serum. The number of IgM-bearing large lymphocytes increased gradually from 30% in 24-h cultures to about 90% in 72-h cultures. In contrast, no IgM or T-cell markers could be detected on lymphocytes stimulated with PWM. Instead, lymphocytes bearing IgG and IgA were identified in PWM-stimulated cultures. All of these large lymphocytes formed spontaneous rosettes (Table I).

Dissociation of lectins by washing the PHA-stimulated cells with *N*-acetyl-D-galactosamine and Con A-stimulated cells with D[α-methyl]mannoside eliminated a possibility that the observed staining was due to non-

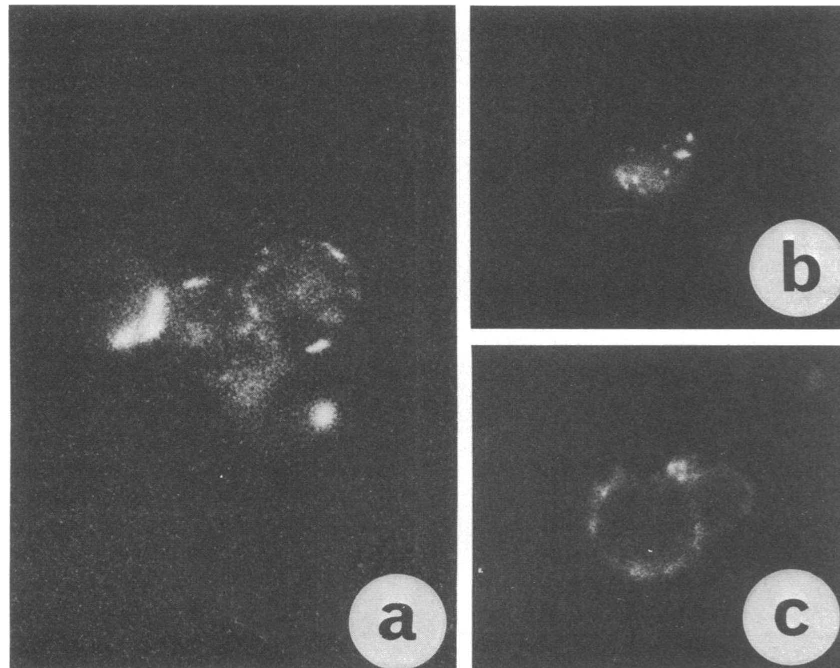

Figure 2 Lymphocytes from PWM-stimulated cultures stained with fluorescein-conjugated anti-IgA (*a*), anti-IgG (*b*) and anti-κ-sera (*c*). Initial magnification in all immunofluorescence photographs is × 400.

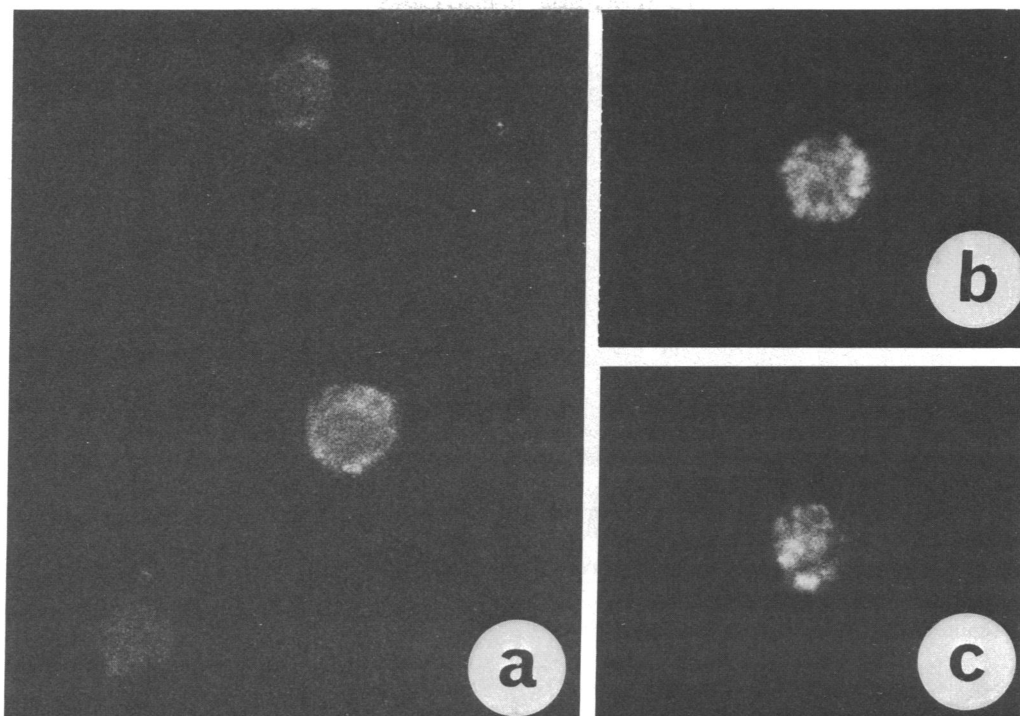

FIGURE 3 Lymphocytes cultured in the presence of PHA (*a*), in mixed lymphocyte culture (*b*) and with Con A (*c*) stained with fluorescein-conjugated anti-IgM.

selective binding of immunoglobulins by lectins. After the dissociation, the staining of large lymphocytes with anti-M and anti-T sera remained unaltered.

All cell cultures were stained for the presence of κ- and λ-determinants (Table III). These were present on some lymphocytes in the 24-h PHA and Con A-stimulated cultures, but could no longer be found in 48- and 72-h cultures. In contrast, 25–30% of lymphocytes in PWM-stimulated cultures had κ-determinants and 10% had λ-determinants on their surfaces throughout the entire culture period.

While the surface markers were seen mostly on large, activated lymphocytes, a certain percentage of small cells with surface immunoglobulins was identified as well (Table IV). In unstimulated controls and PWM cultures, there was a variable population of small cells staining for IgM and for the T marker. In the PHA cultures, the small lymphocytes which reacted with anti-T antiserum did not have detectable IgM on their surfaces. However, all large lymphocytes stained for both IgM, and the T marker. With Con A, only 25% of small lymphocytes stained with anti-T, but none stained with anti-IgM. Neither IgM nor the T-cell marker was detected on large lymphocytes in PWM cultures.

The types and intensities of staining obtained with lymphocytes cultured in the presence of different mitogens are demonstrated in Figs. 2–4. Fig. 2 shows PWM-stimulated cells stained for IgA and IgG and κ-determinants. The granular type of staining is similar to that seen with peripheral blood lymphocytes (20, 21). In Fig. 3, the PHA- and Con A-stimulated lymphocytes which reacted with anti-IgM serum are characterized by patchy, weak membrane staining. Finally, Fig. 4 illustrates the strong uniform membrane staining obtained with anti-T serum.

To ascertain that the staining seen with cultured lymphocytes was not due to passive adsorption of immunoglobulins from sera used in the culture media, cultures were set up in medium 199 in the absence of any serum. Stimulation in serum-free medium was poor and only a few large lymphocytes were present. All these stained with anti-IgM and anti-T sera. All the large lymphocytes in mixed lymphocyte cultures stained for IgM and the T marker. This indicated that the staining could not be attributed merely to the presence of mitogens.

Anti-immunoglobulin sera and spontaneous rosette formation. The markers for spontaneous rosette formation appear to be different from immunoglobulin receptors on surfaces of stimulated lymphocytes. Large lymphocytes from mitogen-stimulated cultures simultaneously stained with fluorescein-conjugated anti-IgM and formed spontaneous rosettes with sheep erythrocytes,

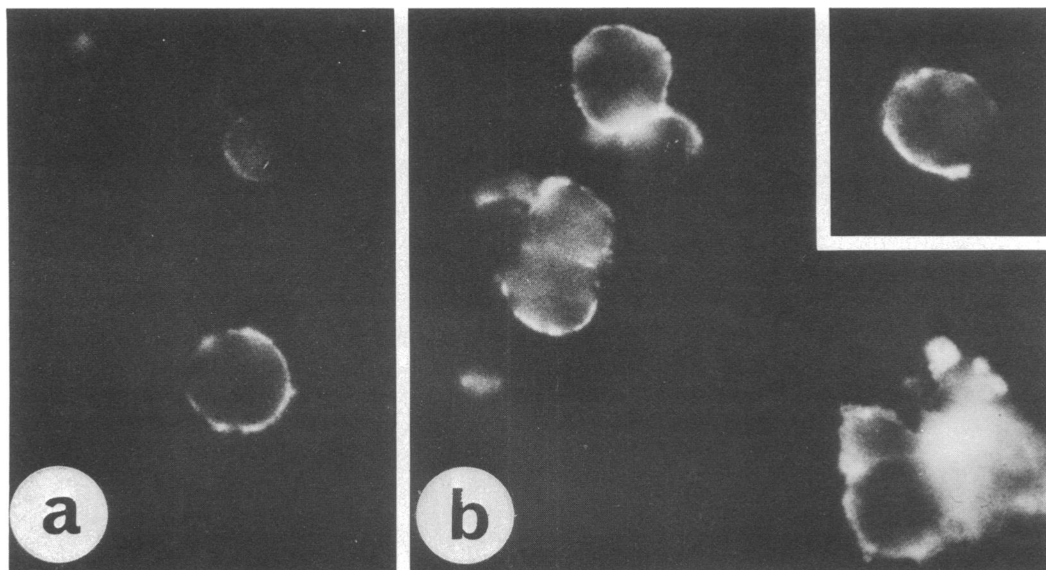

FIGURE 4 Lymphocytes cultured in the presence of Con A (*a* and insert) and of PHA (*b*) stained for the T marker.

so that rosettes with a labeled lymphocyte could be identified in the fluorescence microscope. When unstimulated control cells and mitogen-stimulated lymphocytes were treated with antihuman IgA, IgM, or anti-T serum before rosette formation, no inhibition of spontaneous rosette formation occurred. For example, in PHA-stimulated cultures, total percent of spontaneous rosettes was 86.4% without preincubation with an antiserum and 93.0, 89.0, 82.0%, respectively, after preincubation with anti-IgM, anti-T, and anti-IgA sera.

DISCUSSION

Evidence for the presence of surface immunoglobulin that may function as an antigen recognition unit on T cells has been rapidly accumulating (11, 12, 17, 33–35). The use of highly sensitive radioimmunolabeling techniques (18) and of anti-immunoglobulin reagents (10, 12, 35) demonstrated that mouse and rat T cells possess immunoglobulin receptors (12, 13, 17, 18) which are comparable in number to those found on B cells (36), and that T lymphocytes actively synthesized these receptors (10). Nevertheless, the nature of the specific receptors on membranes of T cells remains controversial in view of the fact that many investigators using equally reliable and sensitive methods have been unable to detect IgM on thymocytes or T lymphocytes (5, 7, 37).

The status of human T lymphocytes as bearers of surface immunoglobulins is still unresolved, although Marchalonis et al. (13) isolated monomeric IgM molecules from surface of human thymocytes. The early experiments of Hellström et al. (23) and Biberfeld et al. (24) indicated that mitogen-activated human lymphocytes have surface Ig molecules (light chain determinants) which are more easily detectable by anti-Ig reagents in transformed than in nontransformed cells. In contrast to these findings, we find that mitogen- and/or alloantigen-stimulated human lymphocytes predominantly bear IgM and T marker. In addition to looking for surface immunoglobulins on the stimulated cells, we also sought to identify the cells as T cells by their ability to form spontaneous rosettes with sheep erythrocytes (32). This was necessary in view of the recent reports that mitogens such as PHA, Con A, and PWM do not preferentially stimulate either B or T cells in humans (38, 39).

The present experiments show that all mitogen-induced and allogeneically stimulated large lymphocytes form spontaneous rosettes and all except PWM-stimulated cells have surface IgM and T markers. Some PWM-induced blasts bear IgG, IgA, and the light chain determinants. It appears that in the presence of either PHA, Con A, or PWM all large lymphocytes acquire the capability to express the T-rosette receptor. However, some mitogen-stimulated large lymphocytes, although capable of forming spontaneous rosettes lack IgM and T markers. These results seem to indicate that the response of human lymphocytes in vitro to various mitogens cannot be unequivocally defined in terms of T- or B-cell activation. While it may appear that the mitogens used were primarily selective for T cells, there is a possibility that at least some transformed

lymphocytes express mixed T and B markers. Indeed, it has been recently proposed that the term D-lymphocytes be used to designate such double marker cells (40).

In all cases where IgM is present on large lymphocytes the T-cell marker is also detectable. These results, as well as recent reports describing the similar responses of human T and B cells to mitogens (38, 39), the presence in human peripheral blood of lymphocytes with both T- and B-surface markers (41, 42), and the conflicting data regarding the appearance of human T and B cells in the scanning electron microscope (43, 44), clearly indicate that the prevailing view of B or T cells as distinctly different lymphocyte populations may be an oversimplification.

If IgM is present on both T and B cells, as this study indicates, then its usefulness as a B-cell marker is limited. This IgM may be synthesized by the stimulated cells or may be an adsorbed product of B cells present in the culture. Recent reports present evidence that some T lymphocytes can adsorb cytophilic antibodies (19, 20, 45) and that antigen-activated T cells can bind antigen-antibody complexes (46) and thus bear easily detectable surface immunoglobulins. We have made no attempt to study the synthesis of IgM by peripheral blood lymphocytes activated in vitro. However, several lines of indirect evidence seem to indicate that adsorption may not account for the observed phenomenon. Small lymphocytes in PHA- and Con A-stimulated cultures do not express surface IgM, although they have T markers (Table IV). A small proportion of small lymphocytes, but none of the large ones, bear both IgM and T markers in control and PWM-stimulated cultures. This finding suggests that nonspecific adherence of the Ig to blasts cannot account for presence of Ig on cell surface. Finally, large lymphocytes in PWM-stimulated cultures, presumably "enriched" in B cells, bear neither IgM nor T markers.

Another potential source of error in the study of surface markers on in vitro stimulated lymphocytes is the presence of lectins. Such lectins as Con A and PHA which are known to bind to glycoproteins may influence the results of immunofluorescence as well as T-rosette assays. For example, the presence of an increased quantity of lectins on the large lymphocytes could facilitate selective T-rosette formation. Alternatively, increased T-rosette numbers could be due to the reaction of lectins bound to surfaces of large lymphocytes with glycoproteins in erythrocyte membranes. Our experiments included the necessary controls, and, thus, the presence of IgM-, T-marker, and sheep erythrocyte- reactive sites on the stimulated lymphocytes cannot be due to nonspecific effects of lectins.

The problem in detecting the presence of IgM on the surface of lymphocytes by fluorescent antiimmunoglobulin sera may not be a purely technical one. The difficulty might be due to a particular location of the IgM receptors in the membrane of human peripheral blood lymphocytes (16). The receptors may be partially hidden in unstimulated cells and become increasingly uncovered in stimulated and/or dividing lymphocytes. This apparently happens with receptors for spontaneous rosettes, since all mitogen-induced blasts formed T rosettes as opposed to small lymphocytes, only half of which underwent spontaneous rosetting. The loss of light chain reactivity on the cell surfaces of the PHA- and Con A-stimulated cells may also be accounted for by the orientation of the immunoglobulin in the membranes.

We would like to suggest that, in view of the overlap in markers on human peripheral blood lymphocytes, the classification into T- and B-cell populations should be restricted to the functional phenomena of cell-mediated immunity and antibody production, respectively, and that operational characteristics such as responsiveness to PHA or ability to bind sheep erythrocytes be used to define individual lymphocyte populations.

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