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# Immunoglobulin Light Chain Determinants on Unstimulated and Stimulated Human Blood Lymphocytes, Assayed by Indirect Immunofluorescence

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**Summary.** The occurrence of immunoglobulin determinants on the surface of lymphocytes from human blood was assessed by indirect immunofluorescent staining of living cells after cultivation with phytohaemagglutinin or other stimulants. While antisera to $\gamma$, $\mu$ or $\alpha$-determinants only stained a few cells, antisera to light chain determinants stained a larger proportion of the cells. Positive staining was recognized as 'ring' staining comprising smaller or larger parts of the cell surface. The specificity of staining was ascertained by several types of controls.

After 48 hours of cultivation, anti-$\kappa$ serum, applied at dilutions of 1:10–1:16 stained about 35–50 per cent and anti-$\lambda$ serum about 15–20 per cent of the cells in the PHA cultures but only 3–5 per cent in the cultures incubated without PHA. When the antisera were applied at higher concentrations, positive light chain staining was also seen in the unstimulated cultures. At the highest concentrations, which could be used without increasing the non-specific background, the maximum number of $\kappa$-positive cells in the unstimulated cultures was approximately 25 per cent. Antiserum titrations showed that about 5–10 times less antiserum was needed to stain the optimal fraction of PHA treated cells. No increased staining of heavy chain determinants was achieved by increasing antiserum concentrations under the present conditions.

Similar results were obtained with lymphocytes stimulated by 3 days of incubation with concanavalin A, or by 6–7 days of incubation under mixed culture conditions. Lymphocytes of a tuberculin positive donor also gave increased staining for light chain determinants after incubation for 6–7 days with antigen (PPD).

The results indicate that lymphocyte stimulation is accompanied by increased amounts of surface bound immunoglobulins. At the present stage of knowledge, several explanations may account for the fact that light chain determinants are primarily accessible for staining.

The above results were obtained under conditions in which no protein was present in the washings performed during processing for immunofluorescence. In the presence of low concentrations of protein more than 60 per cent of both unstimulated and stimulated cells stained for light chain determinants, while staining for heavy chain determinants remained unchanged and at a low level. It is possible

that protein-free washing removed a more loosely adsorbed immunoglobulin fraction passed on from producing to neighbouring non-producing cells.

## INTRODUCTION

Antigen reactive lymphocytes possess surface receptors which combine with antigen by an immunologically specific reaction similar to that of humoral antibodies. Evidence for the existence of these antibody like receptors comes from experiments employing rosette formation between lymphoid cells and antigenic erythrocytes (for references see Coombs and Franks, 1969), binding of radioactively labelled antigen (Sulitzeanu and Naor, 1969; Byrt and Ada, 1969), antigen specific stimulation of lymphocytes from sensitized donors to blast transformation and mitosis (Ling, 1968) and the specific cytotoxicity of such cells towards antigenic target cells (for references see Perlmann and Holm, 1969). Antigen reactive lymphocytes have also been separated out of a larger population by passing them through columns containing unsolubilized antigen (Wigzell and Andersson, 1969).

It is usually postulated that the antigen reactive receptors have the chemical structure of immunoglobulins. Evidence for surface bound immunoglobulin has come from experiments making use of procedures similar to those outlined above (Sell and Gell, 1965; Sell, 1967; Coombs, Feinstein and Wilson, 1969; Coombs, Gurner, Janeway, Wilson, Gell and Kelus, 1970). Other workers have used cell electrophoresis (Bert, Massaro, Di Cossano and Maja, 1969), or opsonic adherence to macrophages of lymphocytes treated with anti-immunoglobulin (Greaves, Torrigiani and Roitt, 1969). Direct immunofluorescence staining techniques and autoradiography have also been applied to demonstrate immunoglobulin determinants on mouse lymphocytes (Raff, Sternberg and Taylor, 1970).

While such studies indicate that immunoglobulins or subunits thereof are present on lymphocytes, they do not prove that the antigen reactive receptors also are immunoglobulins. However, it has been possible to inhibit antigen specific rosette formation between mouse, guinea-pig or human lymphoid cells and antigenic erythrocytes by anti-immunoglobulin serum (McConnell, Munro, Gurner and Coombs, 1969; Biozzi, Binaghi, Stiffel and Mouton, 1969; Greaves, 1970). Greaves *et al.* (1969) have recently reported that antiserum to human immunoglobulin light chains efficiently blocked blast transformation of lymphocytes from sensitized donors by antigen (PPD), or that of normal donors in the mixed lymphocyte reaction. Mason and Warner (1970) successfully prevented graft vs. host reaction and transfer of delayed hypersensitivity by pretreating mouse lymphoid cells with anti-light chain serum.

Stimulation to blast transformation by antigen, allogeneic lymphocytes or plant mitogens such as phytohaemagglutinin (PHA) is assumed to be a property of the thymus derived lymphocytes among which at least part of the antigen reactive cells are found. Although stimulation is followed by a strong increase of protein synthesis, most authors agree that only a small portion of the newly synthesized protein during blast transformation is immunoglobulin (Greaves and Roitt, 1968). However, available data do not exclude that the immunoglobulin synthesis which does occur could affect the amounts of available immunoglobulin receptors on the surface of the stimulated cells. Alternatively, alterations in surface structure could give rise to changes in concentration of surface bound immunoglobulin (Greaves and Roitt, 1968).

The question of a possible increase of surface bound immunoglobulin after lymphocyte

stimulation has important biological implications. In this work, this question has been studied in more detail by making use of indirect immunofluorescence staining of living human lymphocytes exposed to PHA or other stimulants. The stimulated cells were compared with lymphocytes in the resting stage. Special attention was directed to the occurrence of determinants typical for immunoglobulin light chains. The reason for this was the independent finding that the cytotoxicity to target cells of 'non-specifically' stimulated human lymphocytes is most easily inhibited by antibodies to $\kappa$- or $\lambda$-determinants, while antisera to heavy chain determinants seemed to be less efficient inhibitors or were inactive (Holm, Perlmann and Perlmann, 1970).

## MATERIAL AND METHODS

### PREPARATION OF LYMPHOCYTES

Healthy volunteers served as blood donors. The blood was defibrinated by shaking with glass beads. Lymphocytes were isolated by flotation in 3 per cent gelatin (Coulson and Chalmers, 1964) and subsequent filtration through a column packed with 'Scrubbed nylon fibre', Fenwal Laboratories, Morton Grove, Ill., U.S.A. (Greenwald, Gajewski and McKenna, 1962). 98–99·5 per cent of the white cells were lymphocytes. In some experiments, the remaining red blood cells were removed from the cell suspension by centrifugation at 500 *g* for 40 minutes through a layer consisting of 10 parts of 33·9 per cent Isopaque (Nyegaard & Co, Oslo, Norway) and 24 parts of 9 per cent Ficoll (Pharmacia AB, Uppsala, Sweden) (Böyum, 1968). After washing with Hanks's balanced salt solution, buffered with 0·15 M tris buffer, pH 7·4 (TH), and containing 5 per cent of heat inactivated (56°, 90 minutes) foetal bovine serum (FBS), Microbiological Assoc., Bethesda, Md, U.S.A.), the cells were counted in a Bürker chamber. Viability was controlled by staining with trypan blue. All details have been given elsewhere (Perlmann and Perlmann, 1970).

### CULTURE CONDITIONS

The culture medium was Parker's medium 199 (TC 199, State Bacteriological Laboratory, Stockholm) supplemented with 15 per cent heat inactivated foetal bovine serum, 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin and 2 mM glutamin. In those experiments in which the lymphocytes were stimulated with PPD or by mixed culture conditions, they were used directly after gelatin flotation and 15 per cent heat inactivated human AB-serum instead of FBS were incorporated into the medium. One ml aliquots of medium containing $2{\cdot}5 \times 10^6$ viable lymphocytes were added to conical centrifuge tubes. To half of the tubes were added 0·5 ml aliquots of TC 199 only. Phytohaemagglutinin (PHA-P, Difco Laboratories, Detroit, Mich., U.S.A.) was added to a final concentration of 25 $\mu$g/tube and PPD (Purified protein derivative from *Mycobact. tuberculosis*, Parke & Davis, Detroit, Mich., U.S.A.) to a final concentration of 2·5 $\mu$g/tube. Concanavalin A (Con A) was extracted from commercial jackbean meal (*Canavalia ensiformis*) and purified as described (Agrawal and Goldstein, 1965). In these experiments, the lymphocytes were first washed with TH and were then suspended in the incubation tubes in 0·5 ml of TC 199 without serum ($5 \times 10^5$ lymphocytes/tube). 0·5 ml of TC 199 containing 20 $\mu$g Con A was added to each tube. In the controls Con A was omitted. After incubation for 30 minutes at 37°, the cells were washed and were then suspended in 0·5 ml of supplemented TC 199. In the mixed culture experiments, equal numbers of lymphocytes ($1{\cdot}0 \times 10^6$) from two

donors were mixed and incubated in 2 ml of supplemented TC 199. In the controls, $2{\cdot}0 \times 10^6$ lymphocytes from each donor were incubated in separate tubes.

Tubes containing lymphocytes with or without stimulating agents were incubated under tissue culture conditions for various periods of time in an atmosphere of 95 per cent air and 5 per cent $CO_2$.

### ANTISERA AND CONJUGATES

Two different batches of rabbit antisera specific for heavy chains of human immunoglobulin (Ig) G, M or A, respectively, were from Behringwerke AG, Marburg, Western Germany. Rabbit antisera specific for either $\kappa$- or $\lambda$-light chains were purchased from Behringwerke and from Brostex A/S, Copenhagen, Denmark. At least two batches of each serum were used during the investigation. One rabbit anti-$\kappa$ and one anti-$\lambda$ serum were also supplied by Dr G. Johansson, Uppsala. All antisera precipitated human immunoglobulins and were specific for various light or heavy chains as indicated by double diffusion tests in agar and by immunoelectrophoresis. Sera from three individual rabbits served as controls. A rabbit anti-human lymphocyte serum (ALS), prepared by repeated injections into rabbits of human blood lymphocytes in Freund's complete adjuvant was used for standardization of the conjugate. All sera were heat inactivated for 60 minutes at 56°.

The indirect staining method was used throughout, except for a few experiments in which the lymphocytes were stained directly with a commercial preparation of fluorescein conjugated goat anti-$\kappa$ and anti-$\lambda$ serum, respectively (Hyland Laboratories, Costa Mesa, Calif., U.S.A.). Anti-rabbit Ig was prepared in a sheep by three injections of purified human IgG in Freund's complete adjuvant. The conjugation of $(NH_4)_2SO_4$ precipitated sheep globulin with fluorescein isothiocyanate was carried out according to Clark and Shepard (1963). Conjugate of normal sheep globulin was prepared in the same way. The conjugates were passed through Sephadex G-25 (Pharmacia AB, Uppsala, Sweden). In the initial experiments the conjugates were absorbed with mouse liver powder (100 mg/ml). This procedure was later omitted when it was found that it did not affect the results. All conjugates were applied at fluorescein:protein weight ratios varying from $3{\cdot}5$ to $6{\cdot}0 \times 10^{-3}$ (Nairn, 1969).

### IMMUNOFLUORESCENT STAINING

The cells were stained in the living state for membrane fluorescence as originally described by Möller (1961). After incubation with or without stimulant, the lymphocytes were washed three times by centrifugation for 5 minutes at 100 *g* with 1 ml serum free TH each time. The cell pellets were suspended in 5 ml of TH ($5 \times 10^5$ lymphocytes/ml) and distributed to five tubes in 1 ml aliquots. To each tube was added 0·1 ml of antiserum or normal rabbit serum, respectively. The serum dilutions applied ranged from 1 : 4 to 1 : 40. The cells were resuspended by gentle shaking and were incubated for 20 minutes at room temperature.

After three washes as described above, 0·1 ml of the properly diluted conjugate was added to each tube. Optimal dilutions of conjugate were established in pilot experiments in which the lymphocytes were stained after treatment with ALS. The conjugate dilutions chosen for subsequent experiments were those giving specific 'ring' staining of lymphocytes treated with ALS at four two-fold dilution steps below the last dilution resulting in visible staining. The optimal dilutions of conjugate were in the range of 1:16–1:32, corresponding to 1–2 mg protein/ml.

The cells were incubated with conjugate for 20 minutes at room temperature. They were then washed three times with protein free TH as described. The cell pellets were finally

suspended in a drop of 50 per cent glycerol in TH on microscope slides and covered with a cover slip. The slides were examined in a Reichert Zetopan fluorescent microscope (Reichert, Vienna, Austria), using barrier filter BG 12/3, absorption filter OG1/GG9 and a dark field condenser. The source of the ultraviolet light was an Osram HBO 200 high pressure mercury burner.

For assessment of the percentage of cells showing ring staining, all lymphocytes in a microscopic field were first counted in tungsten lamp illumination, using planachromatic dry objectives (63 × /0·80 and 40 × /0·65). Illumination was then changed to ultraviolet and all cells showing ring type fluorescence were recounted. 100–300 lymphocytes and several high power microscopic fields were examined for each specimen.

In all experiments, cell smears for light-microscopic examination were prepared by centrifuging 0·15 ml of the cell suspensions in TC 199 directly onto microscopic slides by means of a cytocentrifuge (600 ***g***, 15 minutes) (Doré and Balfour, 1965). The cells were stained with May-Grünwald-Giemsa.

## RESULTS

### SURFACE STAINING OF LYMPHOCYTES TREATED WITH PHA

When living lymphocytes are investigated for surface antigens, a positive reaction is recognized as typical 'ring' staining of the cell surface (Möller, 1961). This ring may be

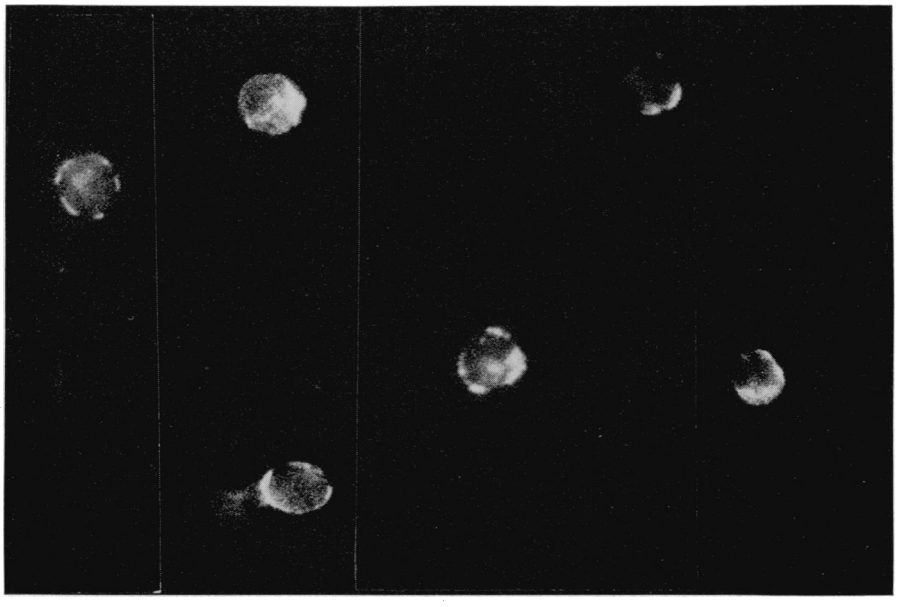

FIG. 1. Surface staining of lymphocytes incubated with PHA for 48 hours. Treated with anti-$\kappa$ reagent, diluted 1:10, and stained with conjugate of sheep anti-rabbit IgG, diluted 1:320 × 20.

complete. Mostly it comprises only part of the surface, leading to the appearance of fluorescent crescents, caps or occasionally dots. Typical examples of positive staining are shown in Fig. 1. In the present investigation, optimal results were obtained by restricting the period of incubation to 20 minutes at room temperature. Longer staining periods (30–40 minutes) or elevated temperatures (37°) resulted in pronounced pinocytosis of

fluorescent material. The surface staining of living cells can easily be distinguished from the diffuse cytoplasmic staining of dead or injured cells (Greaves and Roitt, 1968).

In a first series of experiments, PHA treated and control lymphocytes were studied for the occurrence of immunoglobulin determinants after different times of incubation. When incubation was ended after 2 hours, only few cells were stained with anti-light chain sera and antiserum to $\gamma$, $\mu$ or $\alpha$-chains applied at dilutions of 1/10. There was no difference between PHA treated cultures and controls. However, marked differences were seen after 24 or 48 hours of incubation. In this case a much larger fraction of the lymphocytes in the PHA cultures stained with the anti-$\kappa$ and the anti-$\lambda$ reagent. Staining with the antisera against heavy chain determinants was much less consistent. Staining with anti-$\gamma$, anti-$\mu$ and anti-$\alpha$ serum at dilutions of 1/16–1/10 was observed occasionally but only rarely comprised more than 5 per cent of the lymphocytes, in spite of the fact that the same reagents gave strongly positive results when used under identical conditions to establish the immunoglobin class of human autoantibodies in a different investigation (Zeromski, Perlmann, Lagercrantz, Hammerström and Gustafsson, 1970). The anti-$\mu$ serum

TABLE 1

SURFACE STAINING OF LYMPHOCYTES TREATED WITH PHA*

| Time of incubation (hr) | Antiserum to: $\kappa$† | $\lambda$† | $\gamma$ | $\mu$ | $\alpha$ |
|---|---|---|---|---|---|
| 2 | +(3) | +(1)<br>−(2) | +(1)<br>−(4) | +(1)<br>−(2) | −(2) |
| 24 | ++(4)<br>−(1) | ++(4)<br>−(1) | +(1)<br>−(3) | +(1)<br>−(3) | +(1)<br>−(2) |
| 48 | +++(5) | ++(4)<br>−(1) | +(1)<br>−(3) | −(3) | −(2) |

* − = No fluorescence; + = ⩽5 per cent; ++ = 5–30 per cent and +++ = 30–50 per cent of the lymphocytes exhibiting ring staining. The numbers in parentheses give the number of experiments. Several batches of anti-immunoglobulin sera and of conjugate were tested. Antiserum dilutions 1:10. Conjugates were applied at dilutions of 1/16 or 1/32 (see Material and Methods). Of three normal rabbit sera included for control only one gave weak staining (< 1 per cent cells stained) at one occasion after 24 hours of incubation.

† In one experiment, occurrence of $\kappa$- and $\lambda$-determinants was also demonstrated by direct staining with fluorescein conjugated goat anti-$\kappa$ and anti-$\lambda$-reagents.

also gave strongly positive results when added under identical conditions to cells from a Burkitt lymphoma cell line known to have surface bound $\mu$-determinants (Klein, Klein, Nadkarni, Nadkarni, Wigzell and Clifford, 1967; the cells were kindly provided by Dr E. Klein, Institute for Tumor Biology, Stockholm). Under these experimental conditions, lymphocytes in the control cultures incubated for the same length of time without PHA were stained much less regardless of the anti-immunoglobulin reagent used. A summary of the results is shown in Table 1.

The specificity of this staining was ascertained in several ways. In all experiments, tests with normal rabbit serum or TH instead of anti-immunoglobulin sera were included as controls. The results were invariably negative. No staining was seen when the conjugated sheep anti-rabbit globulin was replaced by a conjugated globulin from normal sheep serum. Positive staining could further be blocked by adding unconjugated sheep anti-rabbit immunoglobulin to the antiserum treated cells before addition of the conjugate.

In additional experiments, staining with the anti-light chain reagents was studied as a function of time of incubation with PHA. In order to facilitate quantitative assessment,

red blood cells were lysed in some experiments by a brief treatment with hypotonic medium (Ling, 1968) before antisera and conjugates were added. Fig. 2 shows the results of a typical experiment. Very similar results were obtained in two additional experiments in which the red cells were removed before incubation by gradient centrifugation. It may be seen that the fraction of cells positive for $\kappa$-determinants increased up to 48 hours but thereafter fell to a lower level. The time course of $\lambda$-staining showed a similar behaviour but at a lower level. Staining for $\gamma$-determinants was not significant. Usually, the fraction of $\lambda$-positive cells was half or even less than that of the $\kappa$-positive cells. This $\kappa/\lambda$

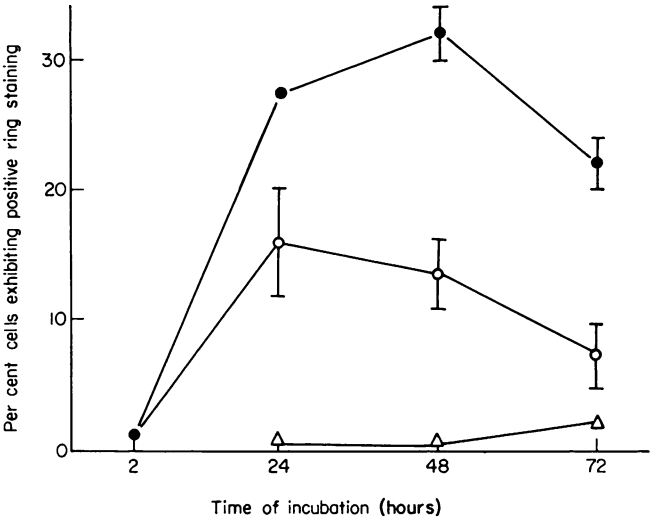


FIG. 2. Surface staining for immunoglobulin determinants of lymphocytes incubated with PHA. ●, Staining with anti-$\kappa$ reagent; ○, with anti-$\lambda$-reagent; △, with anti-$\gamma$-reagent. Each point ($\kappa$ and $\lambda$) represents the mean and the vertical lines the range of two tests made with two antisera of different origin. All antisera were applied at dilution of 1 : 10. 2 × 100 lymphocytes were counted for each antiserum. In the controls incubated without PHA, the maximal number of cells staining for $\kappa$-determinants was 4 per cent (48 hours). All other reagents and normal rabbit serum gave negative results.

ratio and the drop after longer incubations was observed in all three experiments. During the first 48 hours the appearance of stained cells was correlated with that of blast transformed cells seen in cytocentrifuge preparations after staining with May-Grünwald Giemsa stain. In the fluorescent microscope, both small and medium sized lymphocytes and lymphocytes undergoing transformation were stained. While the lymphocytes exhibited distinct ring staining, the transforming cells often had a fluorescent cap, probably corresponding to their uropod (McFarland and Heilman, 1965).

### THE SURFACE STAINING OF LYMPHOCYTES EXPOSED TO OTHER STIMULANTS

In some experiments, the effect of stimulants other than PHA was studied. The plant protein Concanavalin A is known to provoke blast transformation and mitosis of human peripheral blood lymphocytes to almost the same extent as PHA (Powell and Leon, 1970). Table 2 shows the results of one experiment in which the lymphocytes were stained for $\kappa$-, $\lambda$- and $\gamma$-determinants after 30 minutes' exposure to the stimulant in the absence of serum and subsequent cultivation for 3 days in supplemented TC 199. It will be seen that

κ-staining increased with time and was more pronounced than λ-staining. As in the case of PHA, staining for γ-determinants was hardly significant. Similar results were obtained in a second experiment.

In two experiments, lymphocytes from a tuberculin positive donor were exposed to tuberculoprotein (PPD) as described in the Material and Methods section. In contrast to PHA and ConA which exhibit no immunological specificity in lymphocyte stimulation,

TABLE 2

SURFACE STAINING OF LYMPHOCYTES TREATED WITH CONCANAVALIN A (CONA), OR UNTREATED (−)*

| Time of incubation (hr) | Antiserum to: κ ConA | − | λ ConA | − | γ ConA | − |
|---|---|---|---|---|---|---|
| 2 | 5 | 4 | 0 | 0 | 0 | 0 |
| 24 | 21 | 4 | 5 | 0 | 4 | 0 |
| 46 | 20 | 3 | 7 | 0 | 2 | 1 |
| 70 | 28 | 4 | 3 | 0 | 0 | 0 |

* For details of treatment see Material and Methods. The numbers are the percentage of lymphocytes showing positive fluorescence. All antisera were applied at dilutions of 1:10. Dilution of conjugate 1:32. Normal rabbit serum controls (1:10) were included at each time and were negative in all cases.

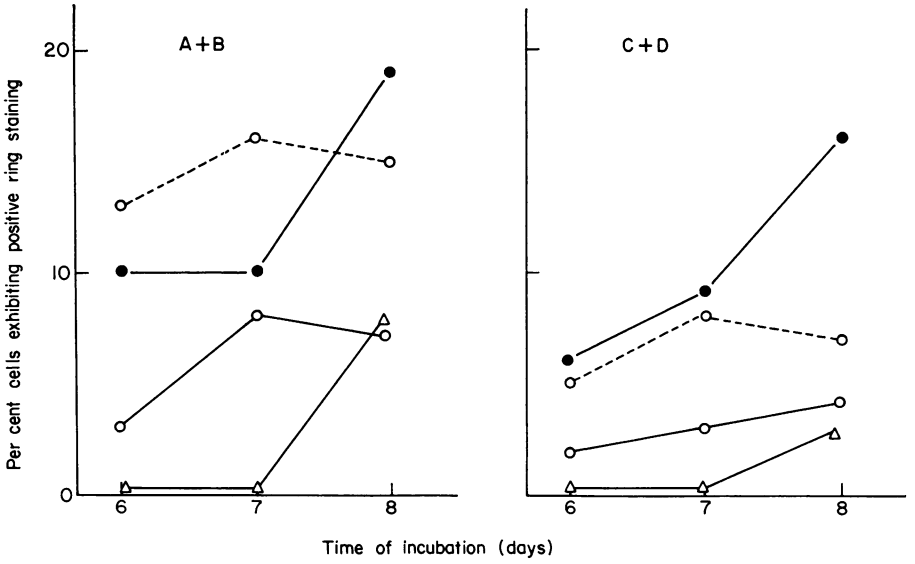


FIG. 3. Surface staining for immunoglobulin determinants of lymphocytes from four different donors (A, B, C, D) incubated in mixed culture. Broken lines: per cent blast transformed cells; for other symbols see legend to Fig. 2. Antiserum dilutions 1:16.

PPD acts as antigen and has no stimulatory effect on lymphocytes from donors who are tuberculin negative (Ling, 1968). In the present case, PPD treatment of the lymphocytes for 6–7 days gave 14–18 per cent blast cells. At the same time, approximately 40 per cent of the cells stained positively for κ-determinants. Approximately 20 per cent were positive for λ-determinants (antisera diluted 1:16). Normal rabbit sera gave negative results. In control cultures incubated for the same time without PPD, <2 per cent of the cells were positive in the test for κ-determinants while none stained for λ-determinants.

When lymphocytes from two donors which differ in their major histocompatibility antigens are mixed, a certain percentage of the cells are also stimulated to blast transformation (mixed lymphocyte culture reaction). Fig. 3 shows the results of two experiments with lymphocytes from four different donors (A, B, C, D). In both experiments, cultivation in mixed culture for 6–8 days led to a pronounced increase of the fraction of cells stained for $\kappa$-determinants. The number of $\lambda$-positive cells was again lower. It will also be noted that $\gamma$-determinants appeared first after 8 days of incubation. The significance of this latter finding is uncertain, however. When the cells of each donor were incubated separately the only measurable immunoglobulin structures were $\kappa$-determinants (1–3 per cent positive cells).

## IMMUNOGLOBULIN STAINING OF UNSTIMULATED LYMPHOCYTES

Although incubation of lymphocytes with PHA or other stimulants markedly increased stainability with the anti-light chain reagents, the data indicated that light chain determinants as well as other immunoglobulin determinants also were present on lymphocytes

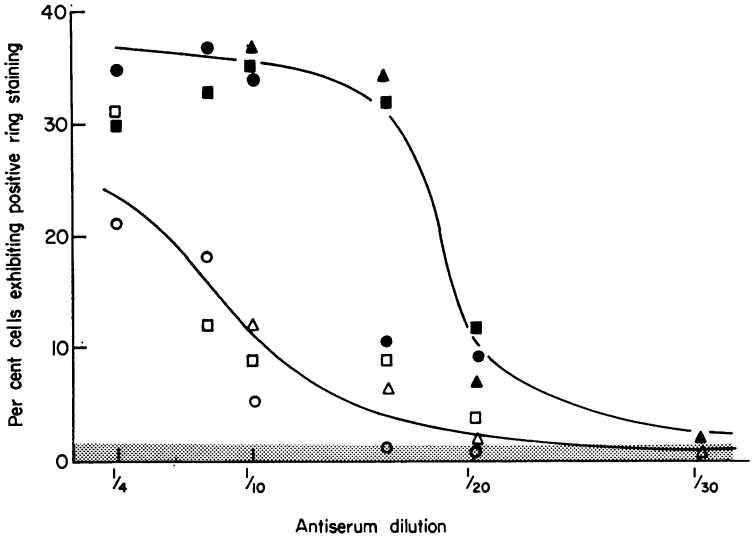


FIG. 4. Surface staining for immunoglobulin determinants of lymphocytes incubated for 20 hours with PHA (filled symbols) or without stimulant (open symbols). Circles, rectangles and triangles represent the data for three independent experiments, respectively. The shaded area at the bottom shows the background staining obtained with anti-$\gamma$, anti-$\mu$ or normal rabbit serum, respectively. The conjugate was applied at a dilution of 1:32 in all cases.

incubated without stimulants. Since the antiserum concentration in the experiments described above were chosen to give optimal staining of stimulated cells against a low background, additional experiments were performed in which antiserum concentrations were increased. Fig. 4 shows the typical results of antiserum titrations from three independent experiments. In these experiments, the lymphocytes were first incubated for 20 hours in medium with or without PHA and were then exposed to dilutions of anti-$\kappa$, anti-$\gamma$, anti-$\mu$ or normal rabbit serum, respectively. It will be seen that PHA treated cells reached optimal staining (approx. 35 per cent cells stained) for $\kappa$-determinants at an antiserum dilution of 1/16. However, the fraction of $\kappa$-positive cells in the control cultures also

increased significantly at dilutions of the anti-$\kappa$ reagent lower than 1/10. Occasionally up to 30 per cent of the cells showed clear-cut ring staining at antiserum dilutions of 1/4 but in most experiments the fraction of positive cells was approximately 25 per cent. Staining was specific since no reaction ($\leqslant$ 1 per cent cells stained) appeared with normal rabbit serum. Staining with anti-$\gamma$ and anti-$\mu$ reagent was low in both stimulated and unstimulated cultures (in the experiments of Fig. 4 $\leqslant$2 per cent stained cells). The anti-$\lambda$ reagent gave staining similar to the anti-$\kappa$ reagent but at a lower level (not shown in Fig. 4).

Fig. 4 shows the results after 20 hours of incubation which is before the PHA reaction has reached its optimum (see Fig. 2). When similar titrations were performed after 48

TABLE 3

SURFACE STAINING OF LYMPHOCYTES INCUBATED FOR 2 HR WITH PHA OR WITHOUT STIMULANT*

| Antiserum dilutions | PHA | | No stimulant | |
|---|---|---|---|---|
| | $\kappa$ | $\gamma$ | $\kappa$ | $\gamma$ |
| 1:2 | | 2 | | 5 |
| 1:4 | 26 | | 27 | |
| 1:8 | 17 | | 24 | |
| 1:10 | 8 | 0 | 6 | 0 |
| 1:16 | 8 | | 10 | |
| 1:20 | 5 | 0 | 3 | 0 |
| 1:32 | 4 | | 1 | |
| 1:40 | 0 | | 0 | |

* The numbers are the percentage of stained cells at various dilutions of anti-$\kappa$ or anti-$\gamma$ reagent. Controls treated with normal rabbit serum (diluted 1:2) contained 2 per cent stained cells in both cultures. Normal rabbit serum diluted 1:10 gave no staining. Dilution of conjugate 1:32.

TABLE 4

THE EFFECT OF PROTEIN IN THE WASH FLUID ON SURFACE STAINING OF LYMPHOCYTES INCUBATED WITH PHA OR WITHOUT STIMULANT (—)*

| Serum | Protein free | | 0·2 per cent gelatin | |
|---|---|---|---|---|
| | PHA | — | PHA | — |
| Anti-$\kappa$ 1:10 | 36 | 12 | 58 | 53 |
| Anti-$\kappa$ 1:16 | 34 | 6 | 48 | 43 |
| Anti-$\kappa$ 1:20 | 7 | 2 | 37 | 6 |
| Anti-$\kappa$ 1:30 | 2 | 0 | 16 | 4 |
| Anti-$\mu$ 1:10 | 3 | 2 | 1 | 1 |
| Normal rabbit 1:10 | 1 | 1 | 1 | 3 |

* The numbers are the percentage of stained cells appearing after treatment with anti-$\kappa$, anti-$\mu$ or normal rabbit serum as indicated. After incubation for 20 hours with or without PHA, the cells were washed three times with protein free TH or with TH containing 0·2 per cent gelatin. After treatment with conjugate 1:32 the cells were washed again three times in the same way (see Material and Methods).

hours of incubation, optimal $\kappa$-staining of the PHA cells reached a plateau which was often at a higher level (40–50 per cent of the cells stained at antiserum dilutions $\leqslant$1/20). In contrast, staining of the unstimulated cells at 48 hours was the same as in Fig. 4. As was to be expected, staining was similar to both PHA treated and control cultures when the cells were harvested after only 2 hours of incubation (Table 3).

THE EFFECT OF PROTEIN IN THE WASH FLUID DURING THE STAINING PROCEDURE

An important technical detail in the experiments described above was the effect of protein in the medium used for washing of the cells after incubation with or without stimulant. Several experiments were performed in which the wash medium (TH) was supplemented with 2·5 per cent of heat inactivated foetal bovine serum, 0·25 per cent bovine serum albumin or 0·2 per cent gelatin, respectively. In these experiments stainability of the lymphocytes treated with PHA or other stimulants was increased when anti-$\kappa$ serum or anti-$\lambda$ serum was used for staining. Usually $\geqslant$60 per cent of the cells were positive for $\kappa$-determinants or for $\lambda$-determinants. Moreover, in the controls not exposed to stimulants, stainability was also strong and the differences between stimulated and control cultures were not always apparent (Table 4). In contrast, stainability for heavy chain determinants ($\gamma$, $\alpha$, $\mu$) remained at a low level in both stimulated cultures and controls (Table 5).

TABLE 5

THE EFFECT OF PROTEIN IN THE WASH FLUID ON SURFACE STAINING OF LYMPHOCYTES INCUBATED WITH PHA OR WITHOUT STIMULANT (−)*

| Exp. No. | Serum | Protein free | | 0·2 per cent gelatin | |
|---|---|---|---|---|---|
| | | PHA | − | PHA | − |
| 1 | Anti-$\kappa$ 1:10 | 47 | 9 | 60 | 70 |
| | Anti-$\gamma$ 1:5 | 2 | 1 | 2 | 1 |
| | Anti-$\alpha$ 1:10 | 1 | 0 | 2 | 1 |
| | Normal rabbit 1:10 | 1 | 0 | 2 | 0 |
| 2 | Anti-$\kappa$ 1:10 | 44 | 14 | 55 | 63 |
| | Anti-$\mu$ 1:10 | 1 | 1 | 1 | 1 |
| | Normal rabbit 1:10 | 0 | 0 | 0 | 1 |

* The numbers are the percentage of stained cells obtained in two independent experiments with various antisera or normal rabbit serum as indicated. Washing after 48 hours incubation with or without PHA as described in legend to Table 4. Dilution of conjugate 1:32.

## DISCUSSION

It has been shown that immunoglobulin determinants can be demonstrated on the surface of human blood lymphocytes by means of indirect immunofluorescence staining under carefully standardized conditions. Staining of $\kappa$-, and to a lesser extent, $\lambda$-chain determinants predominated, whilst the incidence of positive staining with antisera to $\gamma$-, $\mu$- and $\alpha$-chain determinants was much lower. Controls with normal rabbit serum, conjugated globulin from normal sheep, and blocking experiments all showed that the staining described in this paper was specific.

Stimulation of lymphocytes to blast transformation led to a marked increase of stainability with the anti-$\kappa$ and anti-$\lambda$ reagents. Since no increase was observed with the other antisera, it can be concluded that staining was specific and not due to non-specific alterations (e.g. pinocytosis) of the surface properties of activated cells. Similar findings were made both with 'non-specific' mitogens such as PHA or Concanavalin A, and with immunologically specific stimulants such as PPD (antigen) and alloantigens (mixed culture conditions). Titration of antisera (anti-$\kappa$ and anti-$\lambda$) indicated that approximately 5–10 times less antiserum was needed to give visible staining of an optimal fraction of cells in the stimulated cultures as compared to the unstimulated ones. While these results suggest that the amounts of accessible light chain determinants increases upon stimulation, they give no

information as to the magnitude of this increase. No attempt has been made to estimate the intensity of surface fluorescence on individual cells.

Within 20 hours of stimulation with PHA approximately 30–35 per cent of the lymphocytes gave visible $\kappa$-staining and this fraction was not increased by using antisera at higher concentrations than 1/16. Prolonged incubation with PHA for 48 hours, i.e. before cell proliferation occurs, sometimes raised the fraction of $\kappa$-stained cells to 50 per cent of the population. In contrast, incubation without PHA gave 20–30 per cent $\kappa$-positive cells in all experiments, regardless of the length of incubation ($\lambda$-positive cells approximately 10 per cent). However, these latter numbers were obtained with antiserum added at the highest concentrations which could be used without raising the non-specific background (dilution 1/4) and it can be assumed that the proportion of blood lymphocytes equipped with surface bound immunoglobulin was higher. Thus far, no experiments have been performed to establish whether the two types of light chains appeared on the same or on different cells.

In contrast to the strong reactions obtained in stimulated cultures with the anti-light chain reagents, no consistent change in stainability was observed with antisera to heavy chain determinants. Less than 5 per cent of the cells were stained with antisera to $\gamma$, $\mu$ or $\alpha$-determinants, regardless of the culture conditions. (The same applied to $\varepsilon$-determinants, in unpublished experiments.) The reduced staining with antisera to heavy chain determinants was not due to lesser strength of the antisera used since the same reagents gave excellent staining under similar conditions in another investigation (Zeromski *et al.*, 1970). The results are not informative as to the possible occurrence of a surface bound immunoglobulin with heavy chains of unknown structure. If increased staining of stimulated lymphocytes with anti-immunoglobulin is an expression of biosynthesis, the results may reflect a synthetic imbalance, characterized by synthesis of light chains in excess over heavy chains (Askonas and Williamson, 1967). However, at the present stage of the investigation it cannot be ruled out that the heavy chain determinants against which our reagents were directed were present but were not accessible for staining. In any event, the findings are compatible with those reported for human lymphocytes in other experimental situations (Greaves *et al.*, 1969; Bert *et al.*, 1969). They are also in concord with our findings that the cytotoxicity of non-specifically activated lymphocytes is easily inhibited by anti-$\kappa$ or anti-$\lambda$ serum but not by antiserum to $\gamma$, $\mu$ or $\alpha$-chains (Holm, Perlmann and Perlmann, 1970).

It was observed that the presence of unrelated protein in the medium during processing for immunofluorescence resulted in the appearance of more cells which were stained with the anti-light chain reagents while staining with anti-heavy chain reagents did not change. Under these conditions more cells were also stained in unstimulated cultures. The reasons for this phenomenon, are not yet clear. It may be speculated that immunoglobulins (or subunits) are passed on to neighbouring cells by the cells which produce them. Washing with protein-free medium would remove this globulin fraction, while that which remained would consist of polypeptide chains more firmly attached to the surface of the cells in which they are produced. If such loosely adsorbed immunoglobulins possess antibody activity this might provide a mechanism by which lymphocyte stimulation by antigen may be amplified. However, further studies are needed to establish the significance of these findings.

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