*Clin. exp. Immunol.* (1972) **10,** 537–554.

# LYMPHOCYTE ACTIVATION

## III. BINDING SITES FOR PHYTOMITOGENS ON LYMPHOCYTE SUBPOPULATIONS

M. F. GREAVES, S. BAUMINGER* AND G. JANOSSY†

*National Institute for Medical Research, Mill Hill, London*

(*Received* 4 *October* 1971)

SUMMARY

The presence of binding sites for concanavalin A, phytohaemagglutinin and lentil mitogen on the surface of mouse B and T lymphocytes has been investigated in relation to the previously described mitogenic selectivity of these stimulants for T cells. Purified T or B or mixed T/B suspensions of cells have been analysed both for proportions of cells with affinity for phytomitogens and for the relative density of binding sites. In addition, the specificity of mitogens for simple saccharides has been investigated in relation to binding of the former to T and B cells.

Microscopic agglutination and immunofluorescent studies show that virtually 100% of T and B lymphocytes have binding sites for the three phytomitogens. Quantitative absorption experiments have demonstrated that there is virtually no difference in the relative average density of binding sites on T and B cells.

Immunofluorescent studies with Con-A suggested that binding sites are uniformly present over the entire cell surface. However, when lymphocytes are metabolically active an interesting altered localization of bound Con-A molecules to one pole of the cell takes place.

It is suggested that binding sites for the various phytomitogens are qualitatively and topographically distinct on the cell surface but that with respect to any one mitogen T and B cells have qualitatively and quantitatively similar binding sites.

The relevance of these observations to lymphocyte activation is considered.

## INTRODUCTION

In the previous two papers (Janossy & Greaves, 1971, 1972) we have described the selectivity of different phytomitogens towards T (thymus derived) and B ('bursa-equivalent

* Present address: The Weizmann Institute of Science, Rehovot, Israel.

† Present address: M.R.C. Clinical Research Centre, Watford Road, Harrow, Middlesex.

Correspondence: Dr M. F. Greaves, National Institute for Medical Research, Mill Hill, London NW7 1AA.

derived) lymphocytes from mice. Phytohaemagglutinin (PHA), concanavalin-A (con-A) and lentil mitogen (LM) activate T but not B cells, whereas pokeweed mitogen (PWM) stimulates both T and B cells. Since the initial event in triggering of cells by activating ligands is presumably the binding of the latter by receptors it seemed logical to enquire into the presence of binding sites for phytomitogens on T and B cells as a possible basis for the observed specificity. We have assumed that the relevent sites are located on the lymphocyte surface. The best direct evidence for this view comes from recent studies on solubilization of mitogen receptors from purified plasma membranes of pig thymic lymphocytes (Allan, Auger & Crumpton, 1971). In the experiments described below we have investigated the qualitative and quantitative aspects of binding sites for mitogens on the lymphocyte surface by a variety of techniques. Immunofluorescence has been used to study the gross distribution of receptors on individual cells and the proportion of cells bearing such sites in different populations. The latter question was also approached by microscopical analysis of agglutination titrations with mitogens. Quantitative absorption of either $I^{125}$-PHA or biological activity of PHA (mitogenicity) has been used to determine the approximate mean relative densities of binding sites for PHA on T and B lymphocytes. Finally, inhibition studies with simple saccharides and cross-inhibition studies with various mitogens have been used in preliminary experiments to determine whether binding sites for an individual mitogen are qualitatively similar on T and B cells but distinct from those for other mitogens.

## MATERIALS AND METHODS

### *Mice*

*Normal (control) mice.* 6–12-week-old inbred CBA ($H\text{-}2^k$) mice were used in all experiments.

*T-cell deprived mice.* Spleens from these animals were used as *a source of B cells.* 4-week-old CBA mice were thymectomized. After 2 weeks they were irradiated with 850 r and reconstituted 24 hr later with $5\text{–}10 \times 10^6$ viable syngeneic bone marrow mononuclear cells which had been pretreated with anti-$\theta$ serum plus guinea-pig complement (Janossy & Greaves, 1971). Recipients were used as a source of B cells 4–10 weeks after reconstitution.

*Cortisone injected mice.* 4-week-old CBA mice were injected intraperitoneally with 2·5 mg of hydrocortisone acetate (Hydrocortisyl, Roussel, U.K.). Three days later their thymuses were removed and used as *a source of pure T cells* (Blomgren & Anderson, 1971; Raff & Cantor, 1971).

### *Malignant cells*

Gross lymphoma, Parrott C3H lymphomas, MOPC 104E and PC 315 myelomas were obtained from Dr Negroni (Imperial Cancer Research Fund) or from passaged (BALB/c) lines at Mill Hill.

### *Cell suspensions and manipulations*

The preparation of cell suspensions was carried out as previously described (Janossy & Greaves, 1971). All non-malignant cell suspensions were filtered through cotton wool to eliminate dead cells and macrophages (Janossy & Greaves, 1971). This was not possible

with malignant cells which adhered very readily to cotton. Viable and dead cells in these suspensions could, however, be separated very effectively by a simple Ficol/Triosil gradient density centrifugation system as described by Harris & Okaejiofo (1969).

*Lymphoagglutination*

$20 \times 10^6$ viable cotton filtered cells in 50 $\lambda$ 10% foetal calf serum in Eagles medium were mixed with 50 $\lambda$ of phytomitogen diluted in the same medium in plastic Luckham tubes. The tubes were gassed with a mixture of 7% $CO_2$, 10% $O_2$ and 83% $N_2$, stoppered and incubated at 37°C for 30 min. After this period tubes were placed in an ice bath and results evaluated. Cell aggregation induced by mitogen was assessed by placing a sample of each test in a haemocytometer and counting single non-agglutinated lymphocytes. Previous studies with embryonic cell re-aggregation systems had shown this to be a valid and accurate method for assessed cell aggregation (Curtis & Greaves, 1965).

*Immunofluorescence*

Staining of lymphocytes with Con-A-FITC was carried out using the suspension technique of Möller (1961). 25 $\lambda$ of Con-A-FITC (100 $\mu$g) was added to 100 $\lambda$ 0·1% bovine serum albumin in veronal buffered saline containing $2 \times 10^6$ viable cotton filtered lymphocytes. In some experiments 0·2% sodium azide was also included. This mixture was incubated at either 4°C or room temperature for 30 min after which time the cells were washed twice in 20 volumes of VBS containing 0·2% azide and finally suspended in 50 $\lambda$ of the same solution. A wet preparation of this material was made by placing approximately 25 $\lambda$ of cell suspension on a thoroughly cleaned glass microscope slide, placing a glass coverslip on to the drop and sealing with wax. The slides were kept at 4°C and results assessed within 3 hr using a Vickers M41 photoplan microscope with incident ultra-violet illumination.

*Binding of $I^{125}$-PHA to lymphocytes*

All experiments were carried out using viable (> 95%) cotton filtered cells in 20% foetal calf serum/Eagles medium. Plastic Luckham tubes pre-incubated for 30 min in culture medium were used throughout. In most experiments the final volume of cells plus PHA was 100 $\lambda$. Experiments were terminated by addition of 10× concentration of cold PHA and subsequent washing at 4°C in PBS.

*Absorption of PHA (mitogenicity)*

200 $\lambda$ volumes of cells and PHA in 20% foetal calf serum/Eagles medium were incubated in Luckham tubes at 4°C overnight on a rotatory shaker. Cells were spun down at 150 *g* for 10 min at 4°C and the supernatant tested for residual mitogenicity. Control samples of PHA were similarly treated in the absence of cells.

*Cell cultures*

These were set up and [$^3$H]thymidine uptake assayed as described in the previous paper (Janossy & Greaves, 1972).

*Phytomitogens*

*Phytohaemagglutinin (PHA)*. Highly purified PHA was supplied by Dr S. Yachnin (cf.

Yachnin *et al.*, 1971) and used in most of these studies. The Burroughs Wellcome purified PHA gave similar results, however. $I^{125}$-labelled PHA (purified) was also supplied by Dr S. Yachnin. Prior to absorption studies all PHA was absorbed with mouse erythrocytes.

*Concanavalin A* (*Con-A*). The 2× crystallized Miles-Yeda (Israel) product was used throughout. Con-A was labelled overnight at 4°C with fluorescein isothiocyanate (FITC) at a 1:0·05 weight ratio in bicarbonate buffer, pH 9·0. Free fluorescein was separated by dialysis.

*Lentil mitogen* (*LM*) was purified from lentils (*Lens culinaris*) as described by Howard & Sage (1969).

*Pokeweed mitogen* (*PWM*) was purified from *Phytolacea americana* roots as previously described by Börjeson *et al.* (1966).

All mitogen dilutions were made in either normal saline or culture medium. The mitogenic activity of each sample was assayed as described in the previous paper (Janossy & Greaves, 1972).

*Sugars*

N-Acetyl-D-galactosamine, N-acetyl-D-glucosamine and α methyl-D-glucoside were obtained from Sigma Laboratories.

*Anti-theta* (*θ*) *serum*

Serum used was a gift from Dr M. Raff. Details of the specificity and method of production of this anti-T cell reagent have been published (Raff, 1969, 1971). Proportions of *θ*-positive cells in various cell suspensions were calculated using the cytotoxic assay system with trypan blue dye exclusion as described by Gorer & O'Gorman (1956).

## RESULTS

*Characterization of cell suspension used*

In Table 1 the proportion of *θ*-positive cells in the various cell suspensions has been listed.

*Lymphoagglutination*

The capacity of the various mitogens to agglutinate lymphocytes from different tissues was assessed as described above, by enumerating unagglutinated single cells over a wide range of doubling dilutions of mitogen. With PHA, Con-A and LM effectively 100% of cells from all sources were agglutinated and in Table 2 the 50% end points are given. PWM caused less than 10% agglutination in this test system. The slope of the agglutination titration by the mitogens was relatively steep for lymph node, bone marrow and spleen (unselected and B) suggesting that no clear heterogeneity existed in these populations. This was not, however, the case with thymocytes. As shown in Fig. 1, the latter population appeared to be heterogeneous with respect to agglutinatability by all three mitogens, with a clear suggestion of two subpopulations. When cortisone-resistant thymocytes (CR-T) were tested these behaved much more homogeneously as shown, and appeared to correspond to a distinct subpopulation within the unselected thymocytes.

The capacity of various simple saccharides to inhibit phytomitogen induced agglutination of lymphocytes was also tested. As shown in Fig. 2, considerable inhibition of the thymocyte agglutination could be obtained. The specificity of this effect was similar to

TABLE 1. Cell suspensions used in assays for mitogen binding sites

| | θ-positive cells (%) | T or B* cells |
|---|---|---|
| 1. Normal | | |
| i. Thymocytes | 100 | $T_{(o?)}$ |
| ii. Thymocytes from cortisone-injected mice (cortisone-resistant, CR-T cells) | 100 | T |
| iii. Unselected 'normal' spleen cells | 35 | T < B |
| iv. Lymph node cells† | 71 | T > B |
| v. Bone marrow cells | < 2 | B |
| vi. B spleen cells (T-deprived mice) | 4–6 | B |
| 2. Malignant | | |
| i. Gross lymphoma | 100 | T |
| ii. C3H Parrott lymphoma | < 2 | B(?) |
| iii. MOPC 104E myeloma | < 2 | B |
| iv. PC-315 myeloma | < 2 | B |

* All θ-positive cells (cytotoxicity assay) are presumed here to be T cells (Raff, 1971). The majority of θ-negative cells are presumed to be B cells and were positive for the B cell specific MBLA marker (Raff, 1971 and personal communication).

† Mesenteric, axillary and epitrochlear.

TABLE 2. Phytomitogen agglutination of lymphocytes from different sources

| Cells | Lympho-agglutination titre* | | |
|---|---|---|---|
| | PHA | LM | Con-A† |
| Thymocytes | 5+ | 4 | 10 |
| CR-T | 7+ | 6 | 7+ |
| Lymph node | 5 | 4 | 9 |
| Unselected spleen | 5+ | 6 | 7 |
| B-spleen | 4+ | 5 | 7 |
| Bone marrow | 5 | NT | NT |
| Lymphoma‡ | 5+ | 5 | 5+ |
| Myeloma‡ | < 1 | 4+ | 5 |

* $\log_2$ highest dilution giving > 50% agglutination.

† Undiluted mitogen protein concentrations: PHA 2·0 mg/ml, LM 1·0 mg/ml, Con-A 0·12 mg/ml.

‡ There was no distinction between the two lymphomas (Parrott's C3H and Gross) or between the two myelomas (MOPC 104E and PC 315).

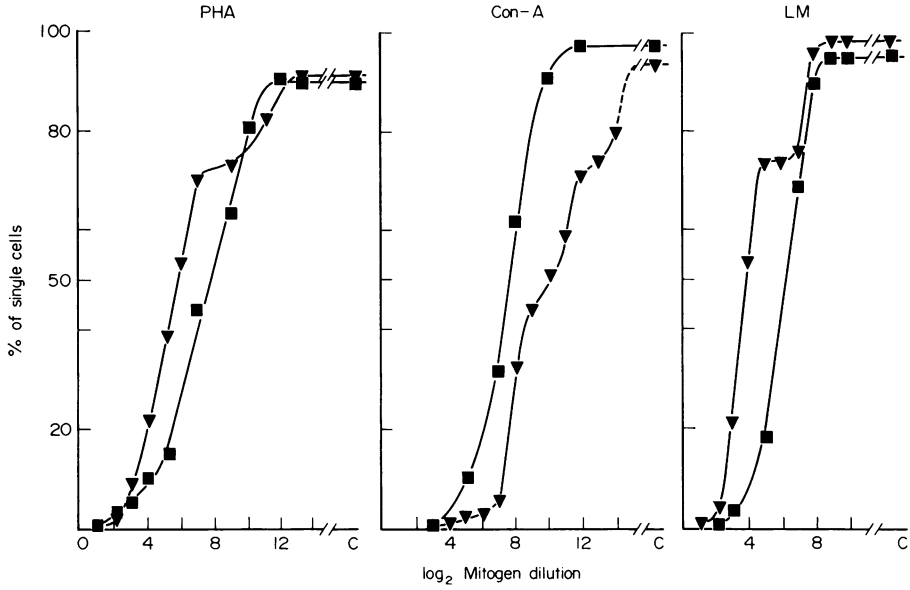


FIG. 1. Agglutination of thymocytes by phytomitogens. Unselected thymocytes; cortisone resistant thymocytes (CR-T). Protein concentrations of undiluted mitogens were; PHA, 2 mg/ml; Con-A, 0·12 mg/ml; LM 1 mg/ml.

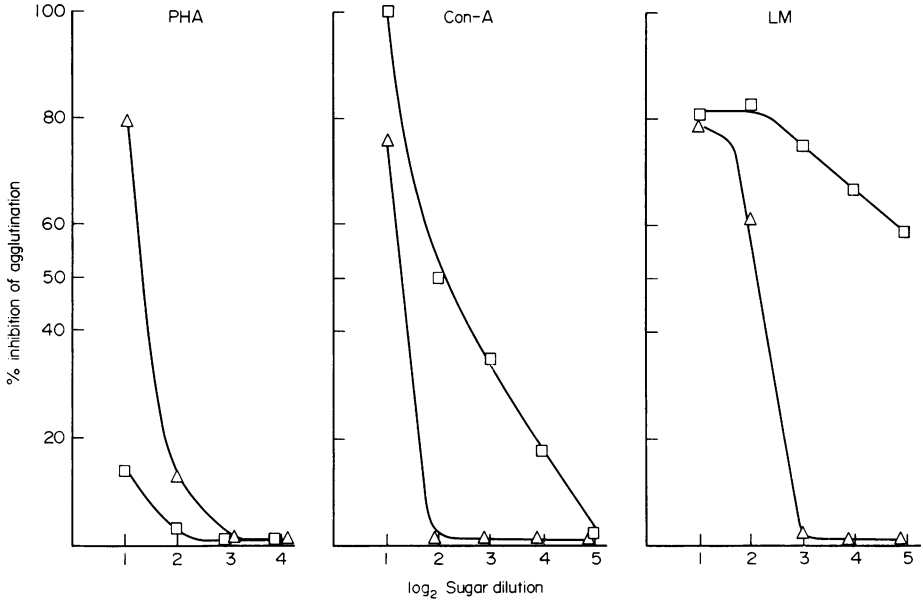


FIG. 2. Inhibition of phytomitogen induced thymocyte agglutination by simple saccharides. △, N-Acetyl-D-galactosamine; □, α-methyl-D-glucoside. Mitogens incubated for 15 min with sugars at 37°C before addition of cells. Undiluted sugar concentration: 100 mg/ml.

TABLE 3. Inhibition of phytomitogen induced B lymphocyte agglutination by saccharides

| | Sugar dilution* | % Inhibition of agglutination | | |
|---|---|---|---|---|
| | | PHA | Con-A | LM |
| N-Acetyl-D-galactosamine | 1 | 88 | | |
| | 2 | | 5 | |
| | 3 | | | 7 |
| α-Methyl-D-glucoside | 1 | 17 | | |
| | 2 | | 59 | |
| | 3 | | | 87 |

* 100 mg/ml $\log_2$ dilutions.

TABLE 4. Cell surface fluorescence with Con-A-FITC

| Technique | Cell source (% θ-positive) | Fluorescence | | |
|---|---|---|---|---|
| | | % cells positive | Staining pattern* | |
| | | | Rings (%) | Caps (%) |
| 4°C No azide | Thymocytes (100) | > 95 | 88 | 12 |
| | Gross lymphoma (100) | > 95 | 95 | 5 |
| | B spleen (3) | > 95 | 85 | 15 |
| RT No azide | CR-T (100) | > 95 | 16 | 84 |
| | Gross lymphoma | > 95 | 32 | 68 |
| | Parrott C3H lymphoma (0) | > 95 | 43 | 57 |
| | B spleen | > 95 | 19 | 81 |
| | Unselected spleen (32) | > 95 | 14 | 86 |
| RT Plus azide | CR-T | > 95 | 97 | 3 |
| | B spleen | > 95 | 96 | 4 |
| | Gross lymphoma | > 95 | 95 | 5 |
| RT Plus azide Pretreatment with: | | | | |
| i. Unlabelled Con-A (5 mg/ml) | CR-T | 3 | | |
| | B spleen | 8 | | |
| ii. PHA (5 mg/ml) | CR-T | 81 | | |
| | B spleen | 72 | | |
| iii. α-Methyl-D-glucoside (50 mg/ml) | CR-T | 7 | | |
| | B spleen | 10 | | |
| iv. N-Acetyl-D-galactosamine (50 mg/ml) | CR-T | 84 | | |
| | B spleen | 70 | | |

All reactions were carried out for 30 min and terminated with washing (4°C) and mounting in azide containing medium (see Methods).

* Rings: staining around whole cell; caps: staining restricted and polarized to less than two-thirds of cell surface.

that previously described (Powell & Leon, 1970), but was not absolute. These inhibition experiments gave very similar results when repeated with B cells implying a chemical similarity of binding sites exists in different cell populations. (Table 3.)

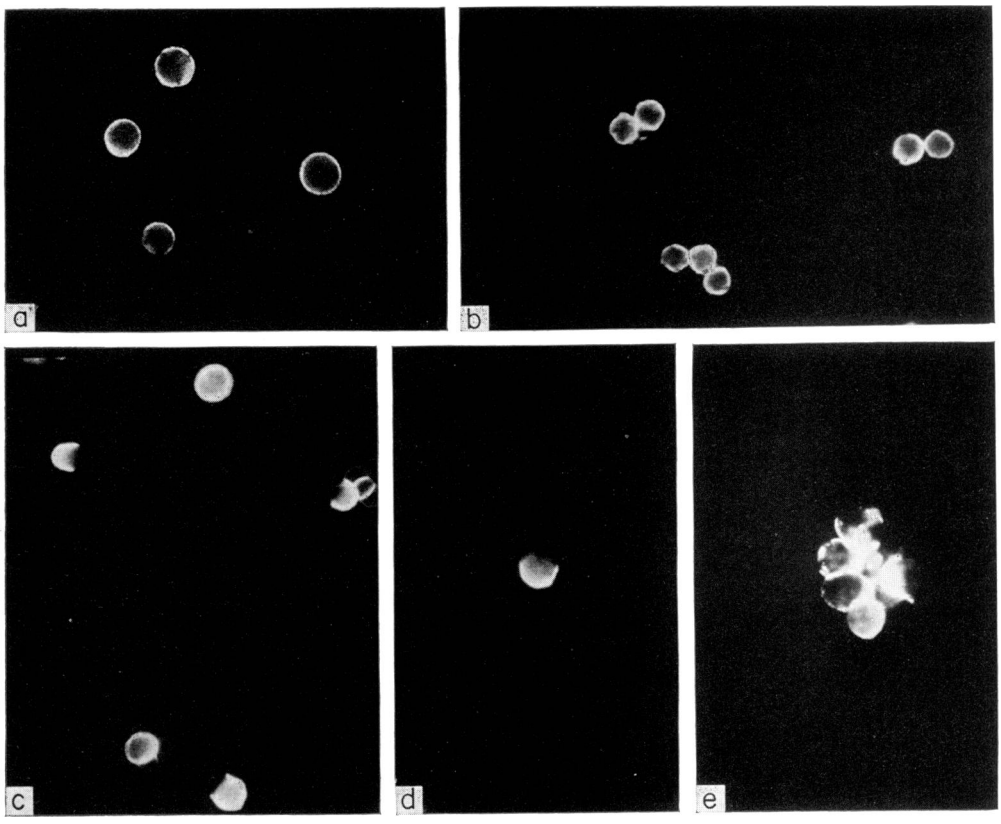


FIG. 3. Immunofluorescence of Con-A binding thymocytes. (a) Ring staining at 4°C. (b) Ring staining at room temperature in the presence of 0·2% sodium azide. (c) and (d) Staining at room temperature in the absence of azide. Note cells with sectorial fluorescence. (e) restricted localization of Con-A on cells in agglutinate (room temperature, without azide).

*Immunofluorescence*

We were unsuccessful in our attempts to fluorescein label PHA and LM and therefore our studies with this technique are limited to Con-A which labelled well. The staining reaction was performed as described under Methods. The results of a series of experiments have been collated in Table 4. As indicated, virtually 100% of cells from all sources gave positive fluorescent staining. The distribution of bound Con-A-FITC was very much dependent on the precise conditions of the reaction. Thus when the interaction of cells and Con-A was at 4°C or alternatively at room temperature in the presence of sodium azide as a metabolic inhibitor the great majority of cells exhibited a clear and fairly uniform ring fluorescence over the whole cell surface (circumference) (Fig. 3). However, when azide was omitted (at room temperature) the pattern of staining was very different and

appeared to be localized to a restricted area of the cell surface as previously described by Osunkoya *et al.* (1970) and Smith & Hollers (1970). A similar restricted or 'cap' fluorescence was noted on mouse B lymphocytes stained with anti-immunoglobulin (Raff, 1970). Observations on the same individual cells by phase microscopy suggested that staining in these situations was very often although not invariably associated with a cytoplasmic extension or 'uropod' (cf. McFarland, Heilman & Moorhead, 1966). Although no systematic titration of Con-A binding was carried out we observed that a 5-fold increase in mitogen concentration considerably reduced the proportion of stained cells which had 'cap' fluorescence without a drop in the total number of stained cells (cf. Osunkoya *et al.*, 1970).

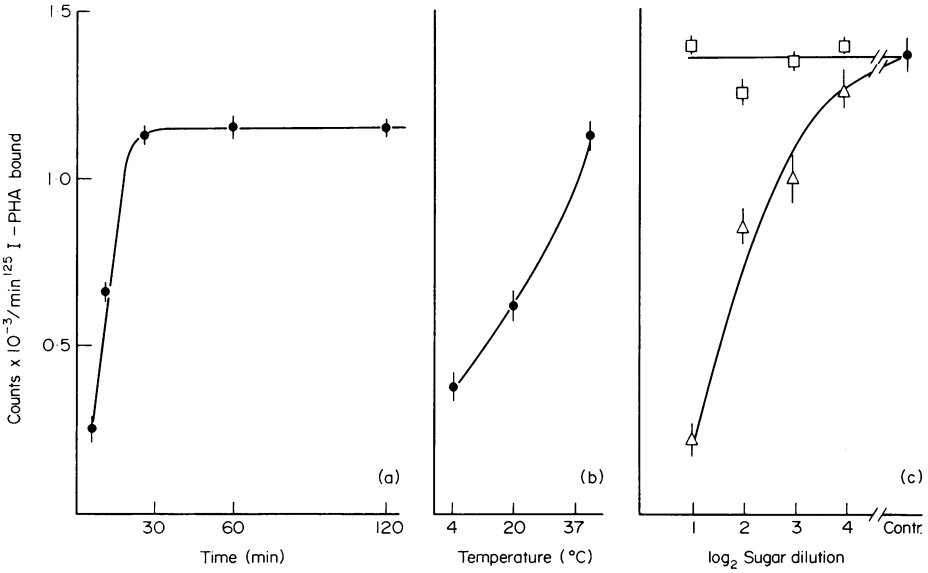


FIG. 4. Binding of $^{125}$I-labelled PHA to thymocytes. (a) Time course of binding at 37°C; (b) temperature dependence of binding (of 30 min period); (c) inhibition of binding by saccharides. □, α-Methyl-D-glucoside; △, N-Acetyl-D-galactosamine. Vertical bars represent one standard error.

The pattern of fluorescent staining was also time dependent and the proportion of 'capped' cells tended to increase even during a short observation period if the cells were not treated with azide after the experimental procedure. This was particularly evident with cells treated at 4°C but could also be observed when cells were stained at room temperature without azide for only 5 min and then inspected (again without azide). These results therefore suggest that ring fluorescence can be converted into sectorial or cap staining by an active metabolic process of the cell involving changes in the plasma membrane (cf. Taylor *et al.*, 1972).

Sectorial fluorescence was evident on both single cells and cells in aggregates (Fig. 3). In the latter situation, Con-A appeared to be localized at the region of maximal cell contact.

The staining of both CR-T and B cells by Con-A FITC could be almost completely inhibited by either unlabelled Con-A or α-methyl-D-glucoside. Only slight inhibition was obtained with PHA or N-acetyl-D-galactosamine at comparable concentrations (Table 4).

*Binding of $^{125}$I-PHA*

Initial experiments (Fig. 4) showed that the uptake of $^{125}$I-PHA by thymocytes was both time and temperature dependent, as previously reported by Lindahl-Kiessling & Mattson (1971). In all subsequent experiments cells were incubated for 30 min at 37°C with PHA. Binding of the radio-labelled mitogen was effectively inhibited by N-acetyl-D-galactosamine but not by α-methyl-D-glucoside. Virtually complete inhibition of binding was obtained also with an excess (10×) of unlabelled PHA. Only a marginal degree (17%) of inhibition was produced by unlabelled Con-A at twice the protein concentration of $^{125}$I-PHA. The absorptive capacity of increasing numbers of lymphocytes from different sources was tested and the results are plotted in Fig. 5. A consideration of the relative

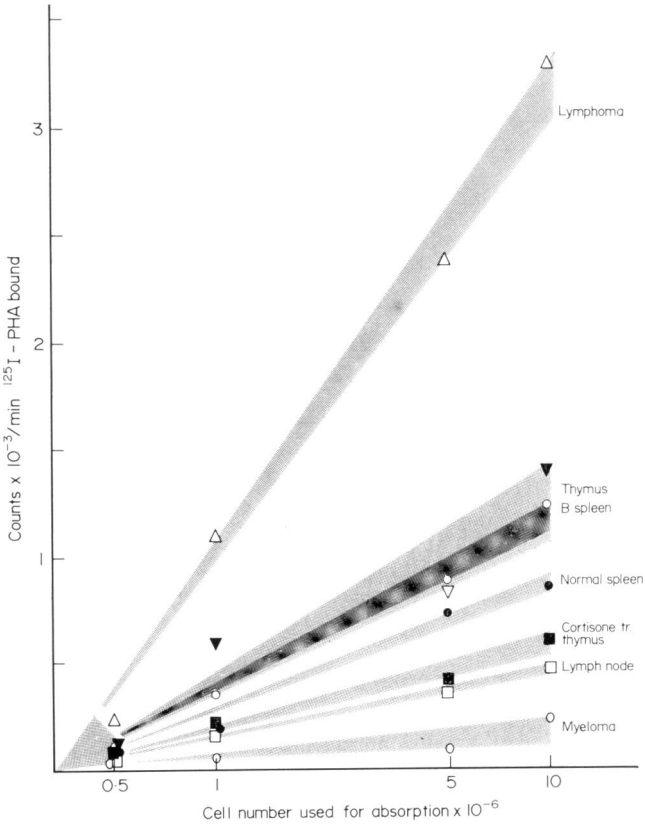


FIG. 5. Binding of $^{125}$I PHA by varying numbers of cells from different sources. Each point is the mean of three determinations.

numbers of T and B cells in the various suspensions (cf. Table 1) suggests that B cells absorb more PHA than T cells; however, the difference was not great. The malignant cells behaved distinctly from normal cells in that Gross lymphoma cells had a very high binding capacity whereas myeloma cells had practically none.

*Absorption of mitogenicity*

Since not all $^{125}$I-labelled PHA molecules may be mitogenically active we decided to also assay the capacity of lymphocytes from different sources to absorb the mitogenic activity of unlabelled PHA. In the experiments shown in Fig. 6 the cell number used was varied. The results paralleled those described above for binding of $^{125}$I-PHA in that most populations behaved very similarly in relation to each other. However, Gross lymphoma cells had a considerably greater absorptive capacity. In a repeat experiment a fixed cell number ($2{\cdot}5 \times 10^7$) was used for absorption and the residual activity titrated and compared with a control preparation of PHA incubated at 4°C without cells. The dose response of the latter PHA is given in Fig. 7(a), and the mitogenic activity of absorbed PHA expressed (in Fig. 7b) as *effective* PHA concentration (calculated by comparison with response in Fig. 7a).

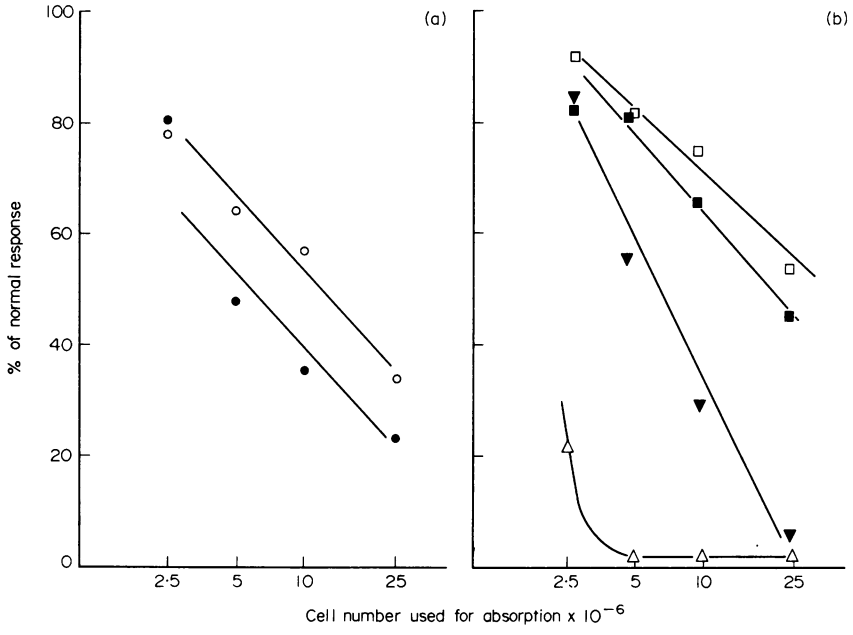


FIG. 6. Absorption of mitogenicity of PHA by cells from different sources. I. With varying cell numbers. (a) ○, B-spleen; ●, normal spleen. (b) □, lymph node; ■, CR-T; ▲, thymus; △, Gross lymphoma.

The percentage absorption of mitogenicity again varied slightly between different normal cell populations but was not considerable. The order of absorptive capacity of different cell populations, however, was identical to that found with binding of $^{125}$I-PHA (cf. Fig. 5).

*Calculation of approximate relative mitogen binding site density on T and B cells*

In order to make an approximate estimate of relative (average) densities of surface binding sites for PHA on T and B lymphocytes the absorptive capacity ($^{125}$I-PHA binding

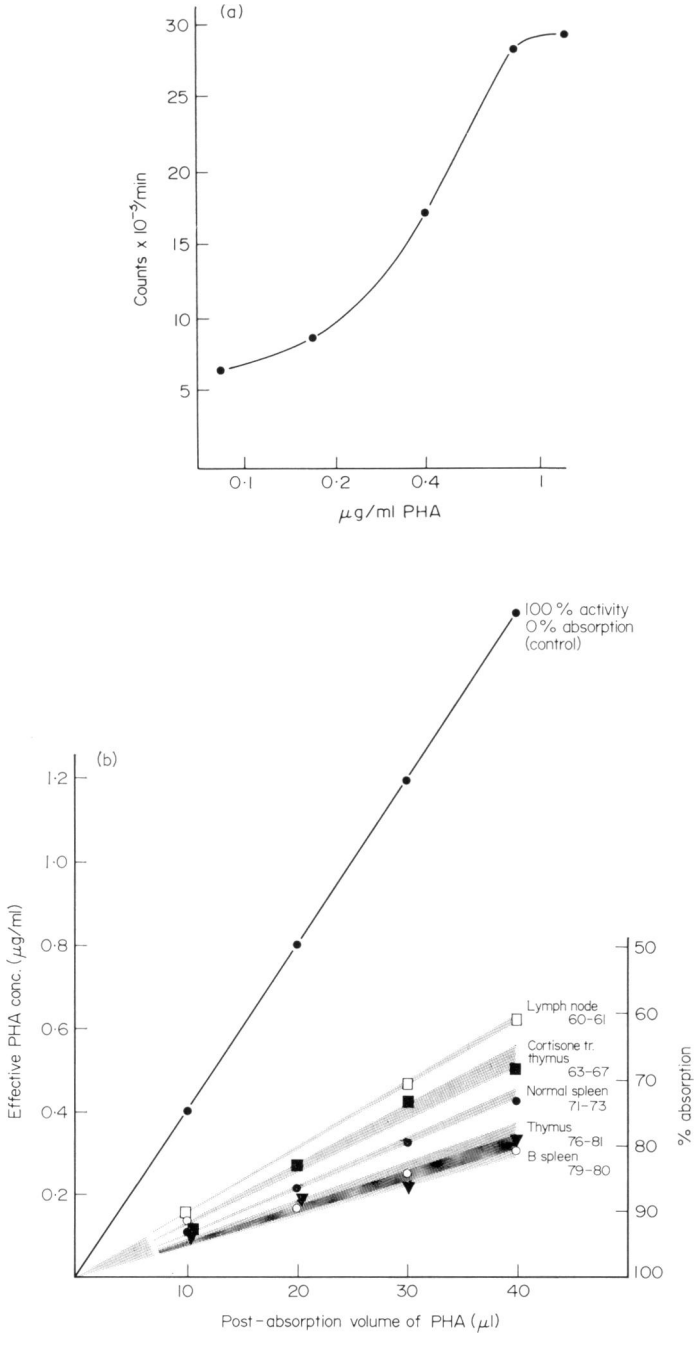


FIG. 7. Absorption of mitogenicity of PHA by cells from different sources. II. With a fixed cell number and titration of residual mitogenicity. (a) Dose–response curve of PHA control, i.e. incubated at 4°C without cells. Each point represents the mean of three cultures. (b) Relationship of residual mitogenicity (post-absorption) to activity in the same volume of control PHA.

and mitogenicity absorption) of CR-T cells and B spleen cells has been compared in relation to mean surface area of these cells. Graticule measurements of diameters of 100 cells in each population were made and used to calculate mean surface area. Relative total PHA absorptive capacity of these two cell populations was deduced from the data in Figs 5 and 7(b).

(i) *$^{125}$I-PHA binding*

$$\text{Relative absorption } \frac{B}{T} = \frac{1{\cdot}2}{0{\cdot}5}$$

$$\text{Relative surface areas (mean) } \frac{B}{T} = 1{\cdot}48$$

$$\text{Relative density of binding sites} = \frac{B}{T} = 1{\cdot}62$$

and similarly

(ii) *Mitogen absorption*

$$\text{Relative absorption } \frac{B}{T} = \frac{80}{65}$$

$$\text{Relative surface areas (mean) } \frac{B}{T} = 1{\cdot}48$$

$$\text{Relative density of binding sites} = \frac{B}{T} = 0{\cdot}83.$$

Although method (ii) is more relevant to the question at hand, both approaches suggest that the differences in PHA binding site density between B and T is less than 2-fold and within the experimental error of the techniques used.

## DISCUSSION

A primary objective of this study was to investigate the presence of binding sites for phytomitogens on lymphocytes in relation to the previously described selectivity of these stimulants for T and B cells (Janossy & Greaves, 1971, 1972). The relative proportion of T and B cells in the suspensions tested and their responsiveness to phytohaemagglutinin (PHA), concanavalin-A (Con-A), lentil mitogen (LM) and pokeweed mitogen (PWM) have been collated in Table 1 of this paper and in Table 2 of the previous paper.

The results in general confirm previous data (Borberg *et al.*, 1968; Landy & Chessin, 1969; Powell & Leon, 1970; Allan *et al.*, 1971) suggesting that the initial interaction of phytomitogens with lymphocytes is likely to occur via plasma membrane binding sites. High concentrations of all mitogens invariably agglutinated over 95% of cells from all sources implying that virtually all lymphocytes do carry binding sites for these ligands. In agglutination titration experiments cells from lymph node, spleen and bone marrow had approximately the same 50% end point as each other and the slope of the titration curve suggested little or no heterogeneity within these populations. This was not the case, however, with thymocytes which demonstrated clear heterogeneity with Con-A, PHA and LM. Part of this variation could be mapped into cortisone sensitive/resistant lymphocytes which are known to differ significantly in their immunological properties (Blomgren &

Anderson, 1971). Cortisone resistant thymocytes (CR-T) agglutinated more uniformly and readily with PHA and LM than did unselected thymocytes and appeared to correspond with the population within unselected thymocytes agglutinated by high dilutions of mitogens. It is clear, however, from quantitative considerations that since CR-T constitute only 2–5% of thymocytes, other cortisone-sensitive cells have a similar agglutinability by mitogens. The converse situation appeared to be true for agglutination of thymocytes and CR-T by Con-A. This latter observation might relate to the finding that Con-A shows a considerable activation of cortisone sensitive cells (Janossy & Greaves, 1972).

Both of the lymphomas investigated, namely the Gross $\theta$-positive (T?) and Parrott's C3H $\theta$-negative (B?) lymphoma agglutinated strongly with PHA, Con-A and LM. However, the two myelomas (B cells ?) invesitigated agglutinated very weakly with PHA but well with Con-A suggesting that these cells might be deficient in binding sites for the former mitogen. A similar result has recently been reported by Dent (1971). PWM had no agglutinating effect in this test system.

Fluorescein isothiocyanate conjugated Con-A bound to the surface of almost 100% of lymphocytes from all sources. Binding sites for this mitogen appeared to be uniformly distributed over the entire surface of T cells, B cells and the malignant cells investigated. However, in order to establish this point it was essential to perform the test either at 4°C or at room temperature in the presence of 0·2% sodium azide (as a metabolic inhibitor). At room temperature or 37°C in the absence of azide, Con-A staining was localized as a crescent or 'cap' often on the uropod as previously described by Smith & Hollers (1970). Taylor *et al.* (1972) have recently described a similar surface localization of anti-immunoglobulin antibodies and conclude that the pattern of fluorescent staining depends upon an active plasma membrane process during which divalent ligands initially bound to sites uniformly distributed on the lymphocyte surface become localized to a restricted area, usually corresponding with the uropod. These authors speculate that this may be related to initial cell activation events. Our results concord with their interpretation, rather than with the alternative of a restricted distribution of binding sites prior to addition of the mitogen as suggested by Osunkoya *et al.* (1970) and Smith & Hollers (1970). In particular, the observation of a conversion of ring staining (carried out at 4°C for 25 min or room temperature for 5 min) to 'cap' localization of fluorescence during a period of observation at room temperature in the absence of azide provides evidence for a Con-A induced, metabolically active alteration of binding site distribution. Taylor *et al.* (1972) have reported previously the same effects with anti-immunoglobulin reagents, their result having motivated us to perform the above study on the temperature dependence of staining patterns with Con-A. Osunkoya *et al.* (1970) noted that 'cap' staining was not evident unless the mitogen (PHA) was diluted, only ring staining being evident at high concentrations. They again interpreted their result as being indicative of a greater density of binding sites on the uropod. We also observed that high concentrations of Con-A-FITC gave predominantly ring staining. However, we would prefer an alternative explanation, which is that this represents either a prozone effect (univalent binding) as discussed by Taylor *et al.* (1972) or a result of excessive polyvalent binding. If one assumes that cell aggregation induced by Con-A is mediated by physical cross-linkage then the presence of aggregated but non-capped cells with high concentrations may perhaps be more readily interpreted in terms of the second alternative above. We are particularly intrigued by this possibility as such a 'rigidification' of the plasma membrane might well be relevant to the mechanism of toler-

ance induction by antigen (Feldman, 1971). Since both cap staining (Taylor *et al.*, 1972) and lymphocyte activation (Fanger *et al.*, 1970) by anti-immunoglobulin antibodies depends on cross-linkage (i.e. univalent fab monomers of antibody are ineffective in both situations) it would be of considerable interest to determine the surface localization of monomers of phytomitogens. We have been unable, however, to repeat the observations of Burger & Noonan (1970) on the preparation of monomeric fragments of Con-A by trypsin treatment and are currently seeking alternative methods.

Uropod or 'cap' staining with Con-A-FITC occurred readily not only on T cells which are activated by this mitogen, but also with B cells which do not respond (with nucleic acid synthesis) and lymphoma cells (T and B type) whose spontaneous proliferation is actually suppressed by phytomitogens (Dent, 1971; Bauminger & Greaves, unpublished). This result suggests that despite the absence of a proliferative response cells may nevertheless undergo some initial response to ligand binding. These binding experiments are, however, not strictly analogous to the culture experiments since higher concentrations of mitogens were used (cf. Janossy & Greaves, 1972).

Quantitative absorption of mitogenicity and $^{125}$I-PHA binding assays were carried out to determine the relative number of binding sites for PHA on different lymphoid cell suspensions. The results of these two approaches were mutually consistent suggesting that cells of different origin did not have a differential absorption of non-mitogenic/mitogenic PHA fractions. Differences between T and B cells were minimal and when considered in relation to surface area (see Results) it was clear that the average density of PHA binding sites on T cells was indistinguishable from that on B cells. The unresponsiveness of B cells to PHA is therefore not due to a lack of binding sites. In contrast to the results of the agglutination studies, cortisone-resistant thymocytes (CR-T) and lymph node cells (70% T cells) had less absorption capacity than unselected thymocytes. Gross lymphoma cells displayed a much greater binding activity than was evident from the agglutination study. We suspect that the latter approach may be misleading in that cell aggregation is likely to be affected by factors other than binding of ligands (e.g. cell surface charge). A similar discrepancy between agglutination and absorption (of Con-A) by tumour cells versus normal cells was reported recently by Cline & Livingstone (1971) and Ozanne & Sambrook (1971).

Recent studies have defined the chemical nature of the receptor sites for PHA (Kornfeld & Kornfeld, 1969; Allan *et al.*, 1971). These appear to be of a glycopeptide nature and may well represent heterogeneous but common plasma membrane constituents. Allan *et al.* (1971) have demonstrated that solubilized plasma membrane preparations have a high PHA-absorption capacity thus showing unequivocally that the initial reaction of PHA with lymphocytes can be equated with binding sites on the plasma membrane. The previously defined specificity/affinity of phytomitogens for simple saccharides (Borberg *et al.*, 1968; Powell & Leon, 1970) has been confirmed in this study. Relatively high concentrations of sugars were able to differentially inhibit the binding of iodinated PHA and fluoresceinated Con-A to lymphocytes and also prevented cell agglutination by PHA, Con-A and LM. It cannot be concluded that these sugars represent the reactive determinants on the actual lymphocyte receptor. Thus, partially purified lymphocyte membrane glycopeptides have an affinity for PHA which is of the order of 3 $\log_{10}$ higher than that of sugars. In addition, the determinant sugar residues may not be those commonly used for inhibition studies (Kornfeld & Kornfeld, 1969, 1970).

A qualitative distinction and topographical separation (on the cell surface) of binding sites for different phytomitogens is also implied in our experiments in which the relative ineffectiveness of Con-A and PHA to reciprocally inhibit binding was demonstrated. These results suggest therefore that binding sites for different mitogens may be distinct (see also Powell & Leon, 1970; Rogers, Gregory & Kornfeld, 1972) but qualitatively and quantitatively similar for any one phytomitogen on T and B cells.

We see no reason to suppose that phytomitogens trigger lymphocytes by binding with, or in close proximity to, immunoglobulin-like antigen receptors (Möller, 1970). Although Con-A, PHA and LM react with carbohydrate-containing immunoglobulins, principally IgM (Leon, 1967; Morse, 1968; Young *et al.*, 1971), this activity is probably unrelated to mitogenicity since heat treatment of mitogen or absorption with erythrocytes removes serum protein precipitating activity without affecting appreciably mitogenicity (Holland & Holland, 1965). Furthermore, high concentrations of IgM do not inhibit PHA induced lymphocyte activation (Greaves, 1968). It is also of relevance to this issue that anti-immunoglobulin light chain antibodies which inhibit antigen-induced proliferation of human lymphocytes do not affect the PHA response (Greaves, Torrigiani & Roitt, 1971). Since B lymphocytes have considerably more cell surface immunoglobulin determinants than T cells (Raff, 1970; Jones, Torrigiani & Roitt, 1971) it might be anticipated that the capacity of PWM to stimulate these cells would be related to its affinity for either immunoglobulin itself or for cell surface binding sites in close proximity to immunoglobulin. Our purified pokeweed preparation did not precipitate radio-labelled immunoglobulin. Similarly, a B cell stimulating anti-lymphocyte serum had no demonstrable affinity for immunoglobulin (Janossy & Greaves, 1972). We favour the view that a specific activation of both T and B lymphocytes can be induced by ligand binding at sites on the plasma membrane distinct from those receptors for specific antigens. It is, however, a possibility that di- or multi-valent binding of mitogens and subsequent receptor ligand movement (i.e. 'capping') could distort the distribution of immunoglobulin molecules or otherwise affect adjacent membrane regions bearing immunoglobulins and that this is a prerequisite for activation.

## ACKNOWLEDGMENTS

This work was supported by the Medical Research Council of Great Britain, the Wellcome Trust (Fellowship to G.J.) and E.M.B.O. (Fellowship to S.B.). We are grateful to Dr S. Yachnin for supplying purified phytohaemagglutinin. We thank Miss Frances Rose and Miss Prafulla Haria for excellent technical assistance.

## REFERENCES

ALLAN, D., AUGER, J. & CRUMPTON, M.J. (1971) Interaction of the plasma membranes of pig lymphocytes and thymocytes with phytohaemagglutinin. *Exp. Cell Res.* **66,** 362.

BLOMGREN, H. & ANDERSON, B. (1971) Characteristics of the immunocompetent cells in the mouse thymus: cell population changes during cortisone-induced atrophy and subsequent regeneration. *Cell. Immunol.* **1,** 545.

BORBERG, H., YESNER, I., GESNER, B. & SILBER, R. (1968) The effect of N acetyl D-galactosamine and other sugars on the mitogenic activity and attachment of PHA to tonsil cells. *Blood,* **31,** 747.

BÖRJESON, J., REISFIELD, R., CHESSIN, L.N., WELSH, P. & DOUGLAS, S.D. (1966) Studies on human peripheral blood lymphocytes *in vitro*. I. Biological and physiochemical properties of Pokeweed mitogen. *J. exp. Med.* **124,** 859.

BURGER, M. & NOONAN, K.D. (1970) Restoration of normal growth by covering of agglutinin sites on tumour cell surface. *Nature (Lond.)*, **228,** 512.

CLINE, M.J. & LIVINGSTON, D.C. (1971) Binding of $^{3}$H-Concanavalin A by normal and transformed cells. *Nature (New Biology)*, **232,** 155.

CURTIS, A.S.G. & GREAVES, M.F. (1965) The inhibition of cell aggregation by a pure serum protein. *J. Embryol. exp. Morph.* **13,** 309.

DENT, P.B. (1971) Inhibition by phytohaemagglutinin of DNA synthesis in cultured mouse lymphomas. *J. nat. Cancer Inst.* **46,** 763.

FANGER, M.W., HART, D.A., WELLS, J.V. & NISONOFF, A. (1970) Requirement for cross-linkage in the stimulation of transformation of rabbit peripheral lymphocytes by antiglobulin reagents. *J. Immunol.* **105,** 1484.

FELDMAN, F. (1971) Induction of immunity and tolerance to the dinitrophenyl determinant *in vitro*. *Nature (New Biology)*, **231,** 21.

GORER, P.A. & O'GORMAN, P. (1956) The cytotoxic activity of iso-antibodies in mice. *Transpl. Bull.* **3,** 142.

GREAVES, M.F. (1968) A study of cellular reactions involved in immune responses. Ph.D. thesis, London University.

GREAVES, M.F., TORRIGIANI, G. & ROITT, I.M. (1971) Inhibition of human mixed lymphocyte reaction by antibodies to immunoglobulin light chain determinants. *Clin. exp. Immunol.* **9,** 303.

HARRIS, R. & UKAEJOFO, E.O. (1969) Rapid preparation of lymphocytes for tissue typing. *Lancet*, **ii,** 327.

HOLLAND, N.H. & HOLLAND, P. (1965) Haemagglutinating, precipitating and lymphocyte-stimulating factors of phytohaemagglutinins. *Nature (Lond.)*, **207,** 1307.

HOWARD, I.K. & SAGE, H.J. (1969) Isolation and characterization of a phytohaemagglutinin from the lentil. *Biochemistry*, **8,** 1436.

JANOSSY, G. & GREAVES, M.F. (1971) Lymphocyte activation. I. Response of 'T' and 'B' lymphocytes to phytomitogens. *Clin. exp. Immunol.* **9,** 483.

JANOSSY, G. & GREAVES, M.F. (1972) Lymphocyte activation. II. Discriminating stimulation of lymphocyte subpopulations by phytomitogens and heterologous antilymphocyte sera. *Clin. exp. Immunol.* **10,** this issue.

JONES, G., TORRIGIANI, G. & ROITT, I.M. (1971) Immunoglobulin determinants on mouse lymphocytes. *J. Immunol.* **106,** 1425.

KORNFELD, S. & KORNFELD, R. (1969) Solubilization and partial characterization of a phytohaemagglutinin receptor site from human erythrocytes. *Proc. Nat. Acad. Sci. (Wash.)*, **63,** 1439.

KORNFELD, R. & KORNFELD, S. (1970) The structure of a phytohaemagglutinin receptor site from human erythrocytes. *J. Biol. Chem.* **245,** 2536.

LANDY, M. & CHESSIN, L.C. (1969) The effect of plant mitogens on humoral and cellular immune responses. *Antibiot. Chemother.* **15,** 199.

LEON, M.A. (1967) Concanavalin A reaction with normal immunoglobulin G and myeloma immunoglobulin G. *Science*, **158,** 1325.

LINDAHL-KIESSLING, K. & MATTSON, A. (1971) Mechanism of PHA action. IV. Effect of some metabolic inhibition on binding of PHA to lymphocytes and to stimulatory potential of PHA-treated cells. *Exp. Cell Res.* **65,** 307.

MCFARLAND, W., HEILMAN, D.H. & MOORHEAD, J.F. (1966) Functional anatomy of the lymphocyte in immunological reactions *in vitro*. *J. exp. Med.* **124,** 851.

MÖLLER, G. (1961) Demonstration of mouse isoantigens at the cellular level by the fluorescent antibody technique. *J. exp. Med.* **114,** 415.

MÖLLER, G. (1970) Antigen binding lymphocyte receptors. *Transpl. Rev.* **5.**

MORSE, J.H. (1968) Immunological studies of phytohaemagglutinin. I. Reaction between phytohaemagglutinin and normal sera. *Immunology*, **14,** 713.

OSUNKOYA, B.O., WILLIAMS, A.I.O., ADLER, W.H. & SMITH, R.T. (1970) Studies on the interaction of phytomitogens with lymphoid cells. I. Binding of phytohaemagglutinin to cell membrane receptors of cultured Burkitts lymphoma and infectious mononucleosus cells. *Afr. J. med. Sci.* **1,** 1.

OZANNE, B. & SAMBROOK, J. (1971) Binding of radioactively-labelled Concanavalin A and wheat germ agglutinin to normal and transformed cells. *Nature (New Biology)*, **232,** 156.

POWELL, A.E. & LEON, M.A. (1970) Reversible interaction of human lymphocytes with the mitogen Concanavalin A. *Exp. Cell Res.* **62,** 315.

RAFF, M.C. (1969) Theta isoantigen as a marker of thymus-derived lymphocytes in mice. *Nature (Lond.)*, **224,** 378.

RAFF, M.C. (1970) Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. *Immunology*, **19,** 637.

RAFF, M.C. (1971) Surface antigenic markers for distinguishing T and B lymphocytes in mice. *Transpl. Rev.* **6,** 52.

RAFF, M.C. & CANTOR, H. (1971) Subpopulations of thymus cells and thymus-derived lymphocytes. *Progr. Immunol.* (in press).

ROGERS, J., GREGORY, W. & KORNFELD, S. (1972) The nature of the lentil phytohaemagglutinin (PHA) receptor site. In: *Proceedings of the 6th Leucocyte Culture Conference.* (Ed. by R. Scharz). Academic Press, New York.

SMITH, C.W. & HOLLERS, J.C. (1970) The pattern of binding of fluorescein-labelled Concanavalin A to the motile lymphocyte. *J. Reticulo-endoth. Soc.* **8,** 458.

TAYLOR, R.B., DUFFUS, W.P.H., RAFF, M.C. & DEPETRIS, S. (1972) Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nature (New Biology)* **233,** 225.

YACHNIN, S., ALLEN, L.W., BARON, J.M. & SVENSON, R. (1971) Potentiation of lymphocyte transformation by membrane–membrane interaction. *Proceedings of 4th Annual Leucocyte Culture Conference* (Ed. by O. Ross McIntyre), p. 37. New York. Appleton-Century-Crofts.

YOUNG, M.N., LEON, M.A., TAKAHASHI, T., HOWARD, I.K. & SAGE, H.J. (1971) Studies on a phytohaemagglutinin with polysaccharides, glyco-proteins and lymphocytes. *J. Biol. Chem.* **246,** 1596.

## NOTE ADDED IN PROOF

We have recently found (Greaves & Bauminger, 1972, *Nature (Lond.)*, in press) that B cells do proliferate in response to insoluble PHA covalently linked onto Sepharose particles. This result demonstrates that both T and B cells have the capacity to respond to phytomitogens but that the physical/configurational form of the stimulant plays a critical role in the triggering event.