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Lymphocyte Activation

IV. THE ULTRASTRUCTURAL PATTERN OF THE RESPONSE OF MOUSE T AND B CELLS TO MITOGENIC STIMULATION *IN VITRO*

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Summary. Thymocytes from cortisone-treated mice ('T' cells), 'B' spleen cells (B lymphocytes from thymectomized, irradiated, marrow reconstituted mice) and normal spleen (T+B) cells were examined by electron microscopy after 60 hours stimulation by Concanavalin A (a T cell specific mitogen), endotoxin (B cell specific mitogen), and pokeweed mitogen (which stimulates both T and B cells). Stimulation of T cells by Con A or PWM induced the appearance of lymphoblasts (Type I) and only PWM or endotoxin stimulated B cells developed 'plasmablast' features (dilated, vesicular rough endoplasmic reticulum; Type II). A few stimulated B cells also had lymphoblast morphology. Large cells from normal (T+B) spleen stimulated by PWM were heterogeneous consisting of 55–60 per cent plasmablasts and 40–45 per cent lymphoblasts. It was concluded that the ultrastructure of stimulated lymphocytes depended on whether T or B cells were stimulated and not primarily on the mitogen used. In general, the response evoked by mitogens paralleled at the ultrastructural level that induced by antigens. It was also found that multivesicular bodies and glycogen particles occurred predominantly in the cytoplasm of stimulated T cells (lymphoblasts).

INTRODUCTION

Lymphocytes stimulated by phytohaemagglutinin (PHA) *in vitro* enlarge into blast cells and divide. These cells have a characteristic paucity of rough endoplasmic reticulum (RER) which contrasts with the prominent development of their ribosomes, mitochondria, Golgi apparatus and nucleoli (reviewed by Biberfeld, 1971a). The electron microscopic appearance of lymphocytes stimulated by pokeweed mitogen (PWM) is different from that observed when the same population is stimulated by PHA (Douglas, Hoffman, Börjeson and Chessin, 1967; Barker, Lutener, and Farnes, 1969). Two distinct types of blasts appear; one of which closely resembles those in PHA cultures and has little RER, while the other resembles antibody secreting plasmablasts with well developed RER. Two hypotheses have been suggested to account for these two cell types (Douglas *et al.*, 1967). The first suggests that the pattern of the individual cell's response depends upon the stimulus used while the alternative hypothesis suggests that there are at least two populations of lymphocytes with distinctive developmental programmes which can be elicited by phytomitogens.

The second interpretation is in line with current knowledge of lymphocyte heterogeneity. Two major populations of lymphocytes exist: T (thymus-derived) and B ('bursa equivalent'-derived) cells. These T and B cells are very similar ultrastructurally when they are unstimulated 'resting' small or medium lymphocytes (Perkins, Karnovsky and Unanue, 1972). However, when they are stimulated *in vivo* only B cells secrete readily detectable immunoglobulins (Mitchell and Miller, 1968) and develop prominent RER (Harris, Hummeler and Harris, 1966). This suggests that the two types of stimulated cells seen in PWM cultures are due to the stimulation of both T and B cells. It also raises the possibility of an important analogy between the activation of T or B cells by antigens *in vivo* and mitogens *in vitro*.

These questions are now open to experimental investigation as relatively pure populations of mouse T and B lymphocytes can be cultured *in vitro* (Janossy and Greaves, 1971, 1972). Three mitogens are used in the present study: PWM which stimulates T and B cells, Concanavalin A (Con A) which, like PHA, stimulates only T cells, and *E. coli* endotoxin which is a mouse B cell mitogen (Stobo, Rosenthal and Paul, 1972; Gery, Krugel and Speisel, 1972; Andersson, Möller and Sjöberg, 1972; reviewed by Greaves and Janossy, 1972a).

MATERIALS AND METHODS

Sources of T and B cells

Normal mice (source of T+B cells). Spleens from 12-week-old inbred CBA (H-2^k, θC3H) mice were used. According to the percentage of cells bearing θ (C3H) antigen or immunoglobulins, this suspension contains approximately 30 per cent T and 50–55 per cent B lymphocytes (Raff, 1970; Janossy and Greaves, 1972).

Cortisone-treated mice (source of 'T' cells). Four-week-old mice were injected intraperitoneally with 2·5 mg hydrocortisone acetate (Hydrocortisyl, Roussel, U.K.) 3 days before the recovery of the thymus. The residual cortisone resistant thymocyte population has many of the properties of peripheral T cells, e.g. cell surface antigenicity, immunocompetence (Blomgren and Andersson, 1971; Raff and Cantor, 1972) and responsiveness to mitogens (Blomgren and Svedmyr, 1971; Andersson *et al.*, 1972; Janossy and Greaves, 1972). They differ in their density (Shortman, Byrd, Williams, Brunner and Cerottini, 1972); in terms of response potential, however, it is likely that this population of thymus lymphocytes is essentially the same as peripheral (e.g. spleen) T cells. We refer to them as 'T' cells. One hundred per cent of 'T' cells carry θ antigen and less than 1 per cent carry detectable surface immunoglobulin.

T-cell-deprived mice (source of B lymphocytes). Four-week-old CBA mice were thymectomized, X-irradiated with 850 r and reconstituted with 9×10^6 viable bone marrow cells which had been pretreated with anti-θ serum plus complement (Janossy and Greaves, 1971). Spleen cells were harvested from these mice after 12 weeks; less than 3 per cent had the antigenic characteristics of T cells.

Mitogens

The twice crystallized Concanavalin A (Con A), Miles-Yeda, Israel, was used. Optimal doses of 3 μg/ml were applied. Pokeweed mitogen (PWM) was prepared from plant stems (*Phytolacca americana*) using the method described by Börjeson *et al.* (1966). The TCA precipitable protein was used. Optimal doses of 75 μg/ml were used. Bacterial endotoxin

(from *E. coli* 055 : B5) was prepared by the method of Westphal, Lüderite and Bister (1952). The optimal dose of 300 μg/ml was used throughout. The dose-response curves for the mitogens have been published (Janossy and Greaves, 1972).

Lymphocyte cultures

The preparation of cell suspensions from spleens and thymus was described previously (Janossy and Greaves, 1972). RPMI-1640 medium (Flow Labs) was supplemented with glutamine, antibiotics and 10 per cent heat inactivated foetal calf serum (Flow Labs) selected as optimal culturing for mouse lymphocytes. Screw cap plastic vials with flat bottoms (area 180 mm^2, 8 ml capacity, Sterilin Ltd) were used. Each vial contained $3–3{\cdot}2 \times 10^6$ living lymphocytes in 2 ml medium. All cultures were set up in 12–14 replicates gassed with a mixture of 10 per cent 0_2, 7 per cent CO_2 and 83 per cent N_2 and incubated at 37°. After a 36-hour incubation period 0·1 μCi [^{3}H]-thymidine (Radiochemical Centre, Amersham, spec. act. 6 Ci/mmol) was added to two replicates. Twenty-four hours later these two samples were harvested for scintillation counting (Janossy and Greaves, 1972). Additional smears were prepared using a cytocentrifuge, fixed in methanol and autoradiographs were prepared by stripping film technique (Kodak AR.10 film, 7 days exposure). Smears were finally stained with haematoxylin and eosin. All cultures for morphological analysis were terminated at 60 hours. Two replicates were subjected to cell counting (by dye exclusion test using 0·5 per cent trypan blue) and conventional smears were prepared and stained with May-Grünwald-Giemsa. The remaining eight to ten replicates were pooled, centrifuged and the cell pellet used for electron microscopic analysis.

Electron microscopy

Preparation of cell pellet. The cells were fixed by adding the double fixative, described by Hirsch and Fedork (1968), to cells pelleted at the bottom of a centrifuge tube. After 5 minutes fixation, the cells were resuspended as small clumps and left in fixative for another 30 minutes. After dehydration with alcohol followed by propylene oxide, the cells were embedded in Epon and sectioned on an LKB ultratome III. The sections were stained with uranyl acetate in 50 per cent alcohol for 15 minutes, then with Reynold's lead citrate for 1 minute. Following this they were stabilized by coating with a thin layer of carbon, and electron micrographs were taken with Philips EM 300 electron microscope.

Assortment of cells. The individual cells of each culture were classified according to their appearance on the viewing screen of the electron microscope. Small and medium lymphocytes were excluded from the classification and this part of the examination was focused on the blast cell population. Blast cells were distinguished from small and medium lymphocytes by their larger size, their copious cytoplasm and loosely arranged chromatin in the nucleus. If the size of a tangentially cut cell corresponded at least to the size of a medium lymphocyte with only a small part of a nucleus, the cell was considered to be a blast. Sixty blasts from a non-stimulated normal spleen culture and 100–200 blasts from each stimulated culture were scanned. Subsequently, a series of electron micrographs of lymphoblasts and plasmablasts were taken from different cell suspensions, in order to confirm the observations made by visual cell counts, and also to study differences in their fine structure. The assortment (see Results) was based on previously observed differences between the cytoplasmic structure of the lymphoblast (cf. Gudat, Harris, Harris and Hummeler, 1970; 'immunoblast', Movat and Fernando, 1965; De Petris and Karlsbad,

TABLE 1

THE NUMBER OF SMALL, MEDIUM AND LARGE CELLS IN CULTURES AND THE ISOTOPE INCORPORATION DATA AFTER 60 HOURS INCUBATION WITH AND WITHOUT STIMULANTS

Stimulant	Cortisone-resistant thymocytes* ('T' lymphocytes)				Normal spleen* (T+B lymphocytes)				'T-deprived' spleen* (B lymphocytes)			
	Number of cells†			Thymidine‡ uptake	Number of cells			Thymidine uptake	Number of cells			Thymidine uptake
	Small§	Medium	Large		Small	Medium	Large		Small	Medium	Large	
None	1·09	0·18	0·03	230	0·87	0·15	0·20	1580	0·74	0·26	0·22	1710
Con A	0·20	0·21	2·50	20120	0·75	0·32	1·81	14140	n.t.	n.t.	n.t.	n.t.
PWM	0·86	0·27	0·97	9310	0·75	0·20	1·30	9750	0·75	0·30	1·26	10020
Endotoxin	n.t.	n.t.	n.t.	n.t.	0·80	0·31	1·24	8870	n.t.	n.t.	n.t.	10830

* Cell concentration at 0 hours $1{\cdot}5$–$1{\cdot}6 \times 10^6$ living leucocytes per ml (approx. $1{\cdot}28$–$1{\cdot}36 \times 10^6$ small, 0.13×10^6 medium and $0{\cdot}10 \times 10^6$ large cells).

† $\times 10^6$ Living leucocytes/ml, counted in chamber after 60 hours incubation. The relative proportion of cells of different size was confirmed by counting three slides, 200–220 cells on each.

‡ Small : 6–8 μm; medium: 8–11 μm; large: above 11 μm diameter.

§ Cpm/0·1 μCi/cultures.

n.t. = Not tested (for details see Janossy and Greaves, 1972; Greaves and Janossy, 1972a).

1965; 'PHA blast', reviewed by Biberfeld, 1971; 'PWM blast cell type I', Douglas *et al.* 1967) *versus* that of plasmablast (Movat and Fernando, 1965; Gudat *et al.*, 1970; 'PWM intermediate sized cell type II', Douglas *et al.*, 1967). A small proportion of the blasts could not be classified exactly, and also the error of sampling in examining thin sections of whole cells has to be considered when results are interpreted.

RESULTS

THE NUMBER OF CELLS OF VARYING SIZES AND THE ISOTOPE UPTAKE IN CONTROL AND STIMULATED CULTURES

This part of the study served as quantitative control for electron microscopic examination. It was also necessary to choose a time after stimulation at which the maximal number of stimulated cells in the cultures were blasts, as assessed by size and incorporation of thymidine. Table 1 shows that 60 hours after stimulation by different mitogens many large cells are present ($1–2{\cdot}5 \times 10^6$/ml) and 90 per cent of these blasts incorporate

TABLE 2

AUTORADIOGRAPHIC ANALYSIS OF THE SIZE OF [^{3}H]THYMIDINE INCORPORATING CELLS IN STIMULATED NORMAL (T+B) SPLEEN CULTURES AT 60 HOURS

Stimulant	Percentage of cells labelled during the 36–60-hour incubation period (within each category)		
	Blasts 12 μm	Medium sized mononuclear cells 8–11 μm	Small mononuclear cells 6–8 μm
Con A	90	25	3
PWM	90	27	0
Endotoxin	89	29	1

[^{3}H]thymidine (Table 2). On the other hand, in all but one stimulated culture 50–60 per cent of the starting cell population can be recovered as small cells (Table 1) which apparently do not incorporate [^{3}H]thymidine (Table 2). In Con A stimulated 'T' cell cultures only 14 per cent of cells were small lymphocytes.

The percentage of blasts in *non-stimulated* cultures of cells from different sources varied, being considerably higher in B or T+B cultures than in 'T' cell culture. It is not known whether these spontaneously activated cells also exist in mitogen stimulated cultures; if we assume that they do, then up to 85 per cent (in both B and T+B cultures) or 99 per cent (in 'T' cell cultures) of blast cells can be thought to have responded to the mitogens used.

As shown in Table 2, 25–30 per cent of medium-sized cells in stimulated cultures also took up [^{3}H]thymidine. We assume many of these to have been descendants of blast cells. In absolute terms, however, these medium-sized cells made up only 5–9 per cent of stimulated cells in 60-hour cultures. This interval was therefore used for electron microscopic studies.

CLASSIFICATION OF BLAST CELLS BY ELECTRON MICROSCOPY

The first analysis of the morphology of large cells from cultures of either Con A stimulated 'T' cells or endotoxin stimulated B lymphocytes confirmed that two broad categories

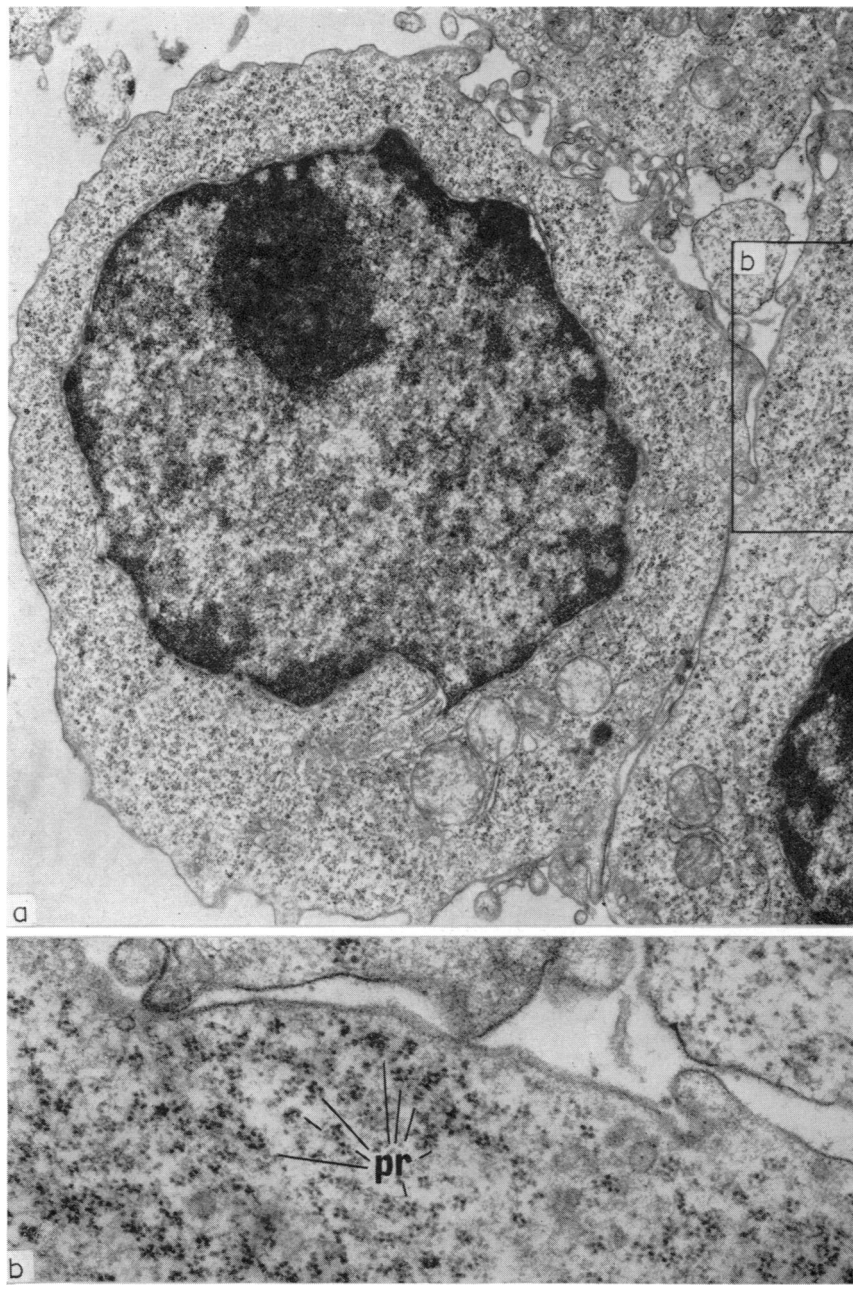


FIG. 1. Electron micrograph of lymphoblast (Type I) from culture of 'T' cells, stimulated by PWM. The cytoplasm contains a high proportion of ribosomes in clusters (polyribosomes), and swollen mitochondria with few cristae which are disorganized. The nucleus is irregular and contains mostly euchromatin, while the heterochromatin is organized in clumps located on the edge of the nucleus. Area in frame is enlarged in (b). In (b) the polyribosomic pattern (pr) is illustrated at higher magnification. (a × 10,840; b × 32,000.)

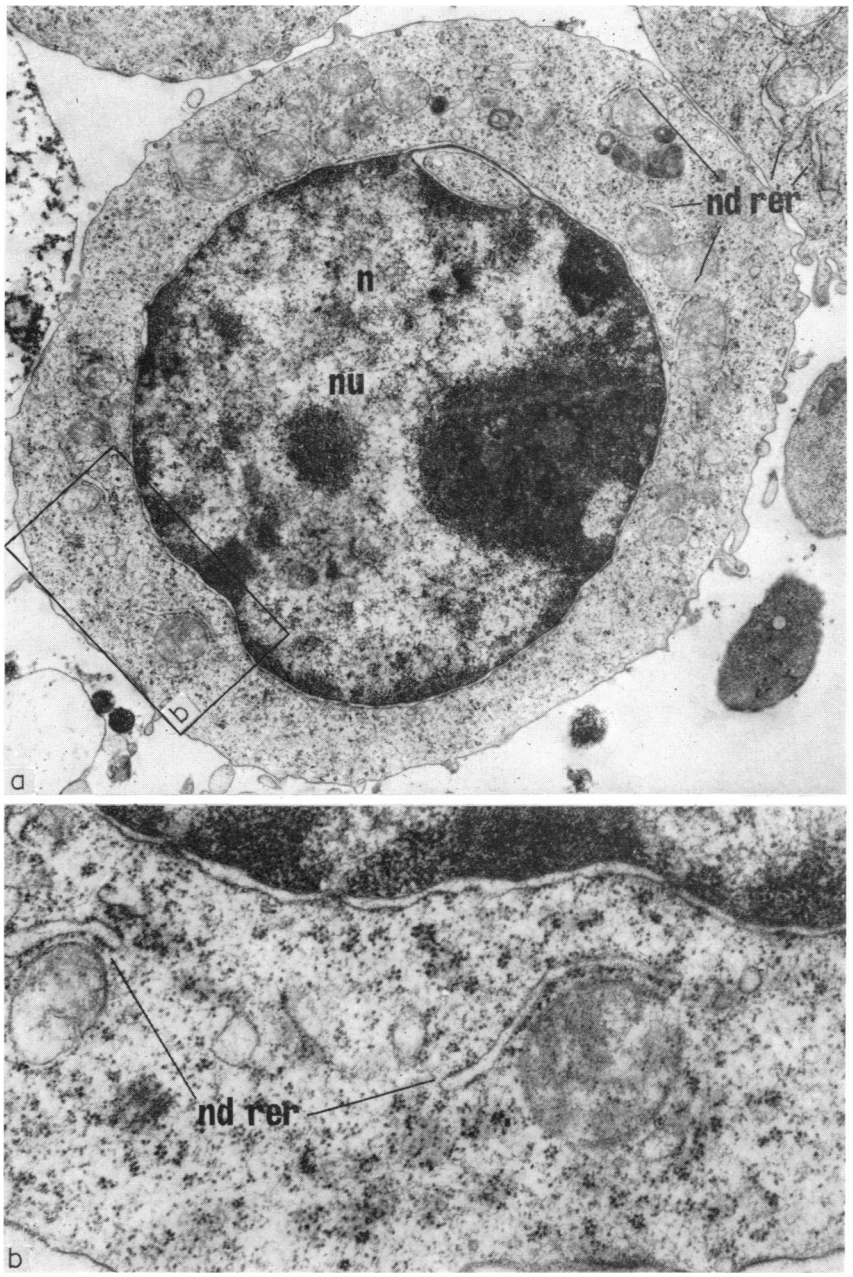
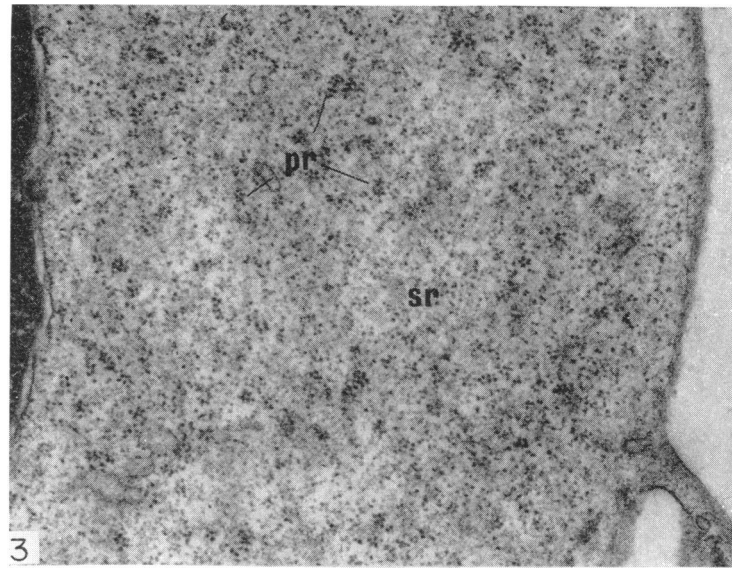


FIG. 2. Lymphoblast from normal spleen (T+B) cell culture stimulated by PWM (Type I). This cell exhibits non-dilated rough endoplasmic reticulum (nd rer), closely associated with the mitochondria (m). The cytoplasm contains many ribosomes in clusters. A portion of the cell is enlarged in (b) where the non-dilated RER is illustrated at higher magnification. (n, nucleus; nu, nucleolus.) (a × 10,840; b × 32,000.)



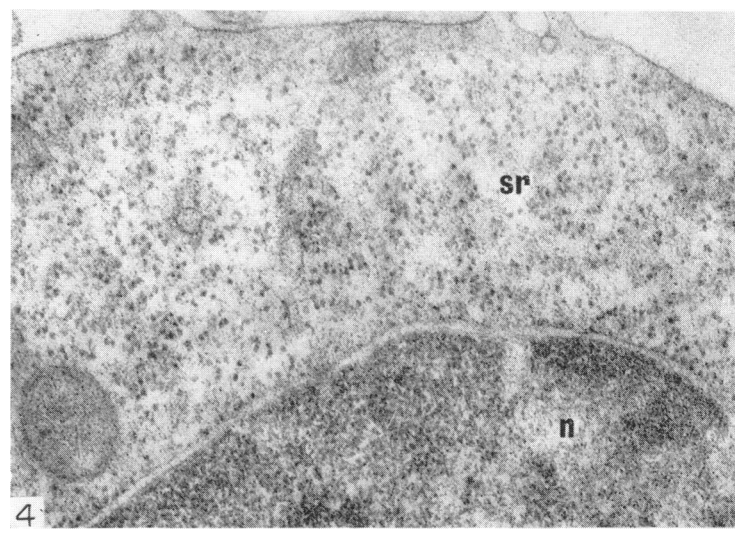


FIG. 3. Detail of a lymphoblast (Type I) from normal spleen cell suspension, stimulated by endotoxin. The cytoplasm contains single ribosomes (sr) mixed with polyribosomes (pr). (×28,000.)

FIG. 4. 'T' cell stimulated by Con A (Type I). The cytoplasm contains single ribosomes (sr). (n, nucleus). (×28,000.)

of blasts can be recognized: lymphoblasts and plasmablasts, which correspond to the Type I and Type II cells of Douglas *et al.* (1967).

Lymphoblasts (Type I): The distinctive feature of these cells of typical blastoid character was the absence of dilated vesicles of RER. Non-dilated RER was, however, frequently seen in these cells and consisted of short channels with a constant narrow distance between rows of ribosome-bearing membranes, which were characteristically adjacent to mitochondria (Fig. 2). The amount of non-dilated RER seen was variable

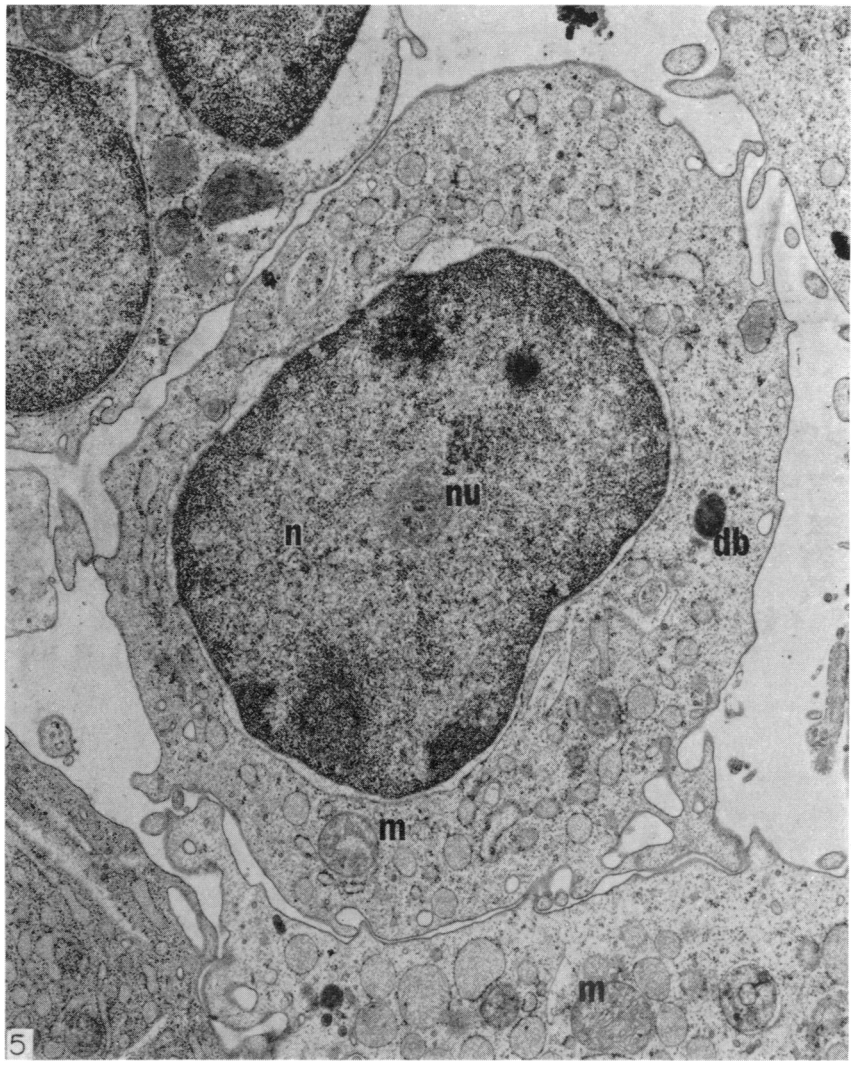


FIG. 5. Blasts from normal spleen (T+B) cell cultures stimulated by endotoxin (Type IIa). The cytoplasm contains scattered vesicles of dilated RER, swollen mitochondria (m) and both single and polyribosomes. (n, nucleus; nu, nucleolus; db, dense body). (×9,840.)

and may have depended upon the plane of section. Large numbers of ribosomes were seen filling the cytoplasm. In some cells polyribosomes were predominant (Figs 1 and 2), in others single ribosomes were more common (Figs 3 and 4).

Plasmablasts (Type II): These were characterized by the presence of 'dilated' RER. The vesicles of dilated RER were filled with a faintly staining substance; they were numerous and were scattered throughout the cytoplasm independently of the mitochondria. There were large variations in size and number of dilated RER vesicles. Variation in the number of ribosomes attached to membranes and in the ratio between single and polyribosomes were also considerable. In some cells (Type IIa, Fig. 5) the vesicles of dilated

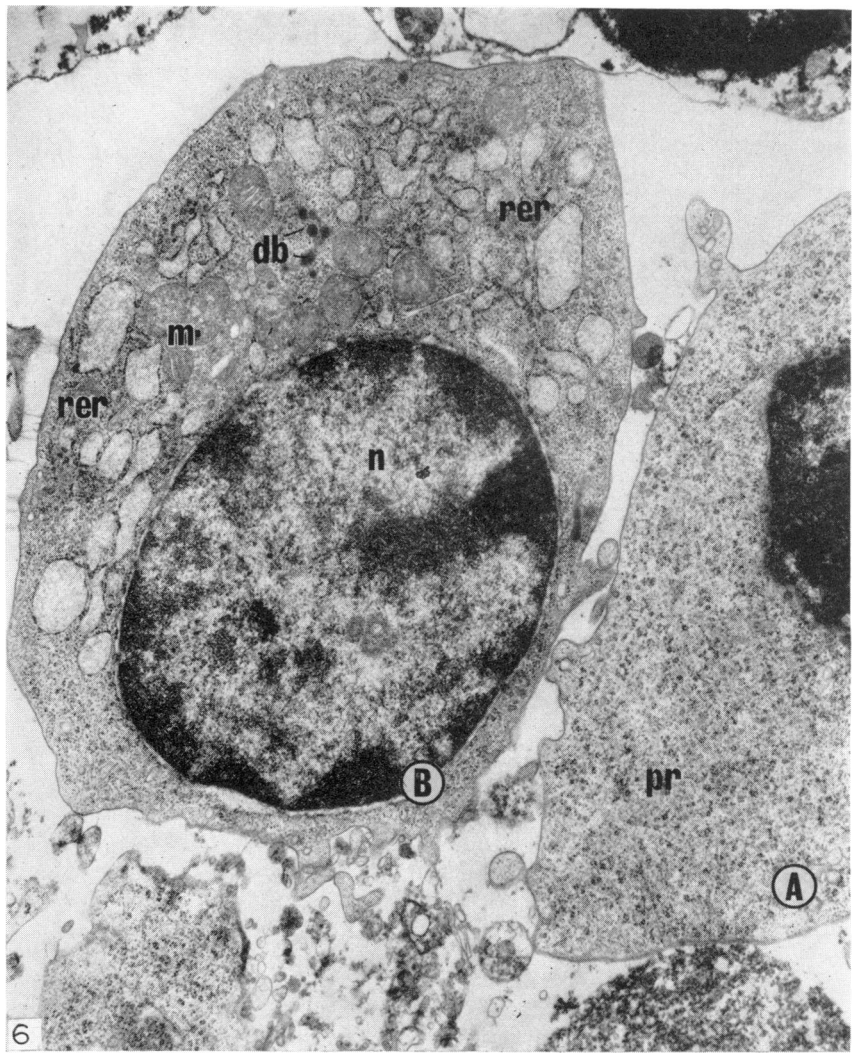


FIG. 6. Two blasts from normal spleen (T+B) cell culture stimulated by PWM. Blast 'B' (Type IIb): the cytoplasm is filled with dilated RER (n, nucleus; rer, dilated RER; m, mitochondria; db, dense bodies). Blast 'A' is a lymphoblast (Type I) containing many polyribosomes (pr). (×9,840.)

RER occupied only a relatively small proportion of the cytoplasm and both free and membrane-bound ribosomes were scanty. In others (Type IIb, Fig. 6), dilated RER was profusely covered with ribosomes and often the cytoplasm was completely filled with widely extended sacs of RER.

RATIO OF LYMPHOBLASTS AND PLASMABLASTS IN DIFFERENT CULTURES

Nearly all the blasts in cultures of stimulated T cells were lymphoblasts (Type I) with few if any profiles of non-dilated RER (Table 3). This was true whether the blasts were induced by Con A in 'T' or T+B (normal spleen) cultures or by PWM in 'T' cell culture.

A characteristic feature of the T-lymphoblast population stimulated by PWM was the presence of cells filled with typical polyribosomes (Figs 1, 2 and 6). This occurred in 80 per cent of the lymphoblasts. Cells with predominance of polyribosomes over single ribosomes were less common in Con A stimulated 'T' cell and normal (T+B) spleen cultures, where only 34–45 per cent of blasts contained mostly polyribosomes.

A completely different morphological picture—that of plasmablasts (Type II)—was observed in the PWM and endotoxin stimulated B lymphocytes as well as endotoxin stimulated normal (T+B) spleen cell suspensions (Fig. 5). In the two stimulated B

TABLE 3

DISTRIBUTION OF BLAST CELLS, CLASSIFIED ACCORDING TO THEIR CYTOPLASMIC CHARACTER, IN CULTURES OF T, T+B AND B LYMPHOCYTES STIMULATED WITH VARIOUS MITOGENS

Type of lymphocyte in culture	Stimulant	Percentage of different types of blast cells*		
		Lymphoblast (Type I) (Fig. 1–4)	Plasmablast (Type II)	
			(a) Moderate number of dilated RER (Fig. 5)	(b) Large numbers of vesicles of dilated RER (Fig. 6)
'T'‡	Con A	100 (34)†	0	0
'T'	PWM	100 (80)	0	0
T+B§	Con A	99 (45)	0	1
T+B	PWM	43 (38)	37	20
T+B	Endotoxin	26 (8)	28	46
B**	PWM	12 (4)	20	68
B	Endotoxin	21 (6)	15	68
T+B	None	63 (13)	23	14

* Determined by counting 100–200 blast cells per culture, except non-stimulated control, where sixty blasts were counted. For details of assortment see Results and Figures.

† Numbers in parentheses represent the percentage of cells where the cytoplasm is filled with polyribosomes and only very few single ribosomes can be seen (Figs 1 and 2).

‡ Cortisone resistant thymocytes.

§ Normal spleen cultures.

** T-deprived (B) spleen cell suspension.

lymphocyte cultures the majority of blasts developed extensive dilated RER, and only few lymphoblasts (10–20 per cent) could be found. In the endotoxin stimulated T+B cell culture, approximately 75 per cent of the blasts were plasmablasts, while 26 per cent were lymphoblasts or unclassifiable blastoid cells.

The PWM stimulated normal (T+B) spleen cultures were very heterogeneous and consisted of 57 per cent plasmablasts and 43 per cent lymphoblasts (Fig. 6). The cytoplasm of lymphoblasts was filled in most cases with polyribosomes.

In control *non-stimulated* cultures of normal (T+B) spleen cells, slightly more blasts could be found in the lymphoblast category (Type I: 60–65 per cent) than in the Type II group (35–40 per cent). Very few blasts could be observed, compared to cultures stimulated by mitogens (see Table 1).

ADDITIONAL MORPHOLOGICAL ASPECTS OF LYMPHOBLASTS AND PLASMABLASTS

The lymphoblasts were taken from 'T' cell suspensions stimulated by either PWM or Con A and from normal (T+B) spleen cultures stimulated by Con A. The plasmablasts were derived from normal (T+B) spleen cells stimulated by endotoxin or from B spleen

cells stimulated by PWM. This analysis was focused on comparisons between lymphoblasts and plasmablasts, although 20 per cent of the blasts in stimulated B cell populations were lymphoblasts (see Table 3).

There were a number of similarities in the two blast populations. Both lympho- and plasmablasts were of the same size (10–14 μm diameter). Most of the cells were round or oval and some of them showed 'polarization' of the nucleus and cytoplasm. Typical uropods, however, were rarely seen. The cell surfaces were irregular and both lympho- and plasmablasts developed cell processes of variable degree. The means of cytoplasmic-nuclear ratios were approximately 6:4 in both populations.

The cytoplasm of both types of blasts contained many mitochondria. All showed a pale matrix with few disorganized cristae which were frequently not clearly visualized (Figs 1, 5 and 10). Most of the mitochondria were round and swollen and only a few were long and irregular in shape. The Golgi apparatus was well developed (Figs 7 and 10) as described by Biberfeld (1971a). Homogeneous dense bodies which varied greatly in size were also frequently found in both types of blasts (Figs 5 and 7). These dense bodies may be granules derived from the Golgi apparatus, lysosomes or bodies associated with endocytosis as multivesicular bodies described below.

By definition, plasmablasts show dilated RER, while lymphoblasts lack this feature (see above). There were also a number of other differences between lymphoblasts and plasmablasts.

The cytoplasm of lymphoblasts contained multivesicular bodies that were extensively described in PHA stimulated human blood lymphocytes by Biberfield (1971b). These bodies were limited by a single membrane. They were subdivided into two groups. The first group included 'pale multivesicular bodies', with an empty appearance (Fig. 9a and b), and 'dense multivesicular bodies' which had an electron dense matrix. The second group included 'compound multivesicular bodies' which contained, in addition to internal vesicles, one or more lumps of electron-dense material or membranes in concentrically arranged lamellae (Fig. 9c and d). Both groups of multivesicular bodies were very rare in plasmablasts.

A considerable number (15 per cent) of sections from lymphoblasts possessed areas in the cytoplasm containing pale, round particles (Fig. 7). These areas varied greatly in size and shape and were randomly distributed. Frequently large, pale almost ribosome-free areas could also be seen in the cytoplasm of lymphoblasts (20 per cent of sections: Fig. 8). Both features are compatible with the presence of glycogen and in this respect the morphological appearance of areas with pale particles is characteristic. These features were never seen in plasmablasts. This finding has been confirmed by diastase sensitive PAS staining (Burns, Savage and Janossy, see Discussion).

Some lymphoblasts showed one or two bundles of cytoplasmic fibrils close to the nuclear membrane (Fig. 8). Annulate lamellae were also observed occasionally in lymphoblasts (Fig. 10). Neither of them were found in plasmablasts.

The nuclei of the plasmablasts were mostly round or oval in contour, while those of the lymphoblasts were mostly irregular. However, examination of early culture of lymphoblasts (1½ days) revealed nuclei which were round and regular (Shohat and Janossy, unpublished). There were no differences in the distribution of chromatin between the two types of blasts. In most cells, after 60 hours incubation, homogeneous pale euchromatin was bounded by a thick and irregular layer of dense heterochromatin next to the nuclear membrane. In a few cells the heterochromatin layer was thin and uniform.

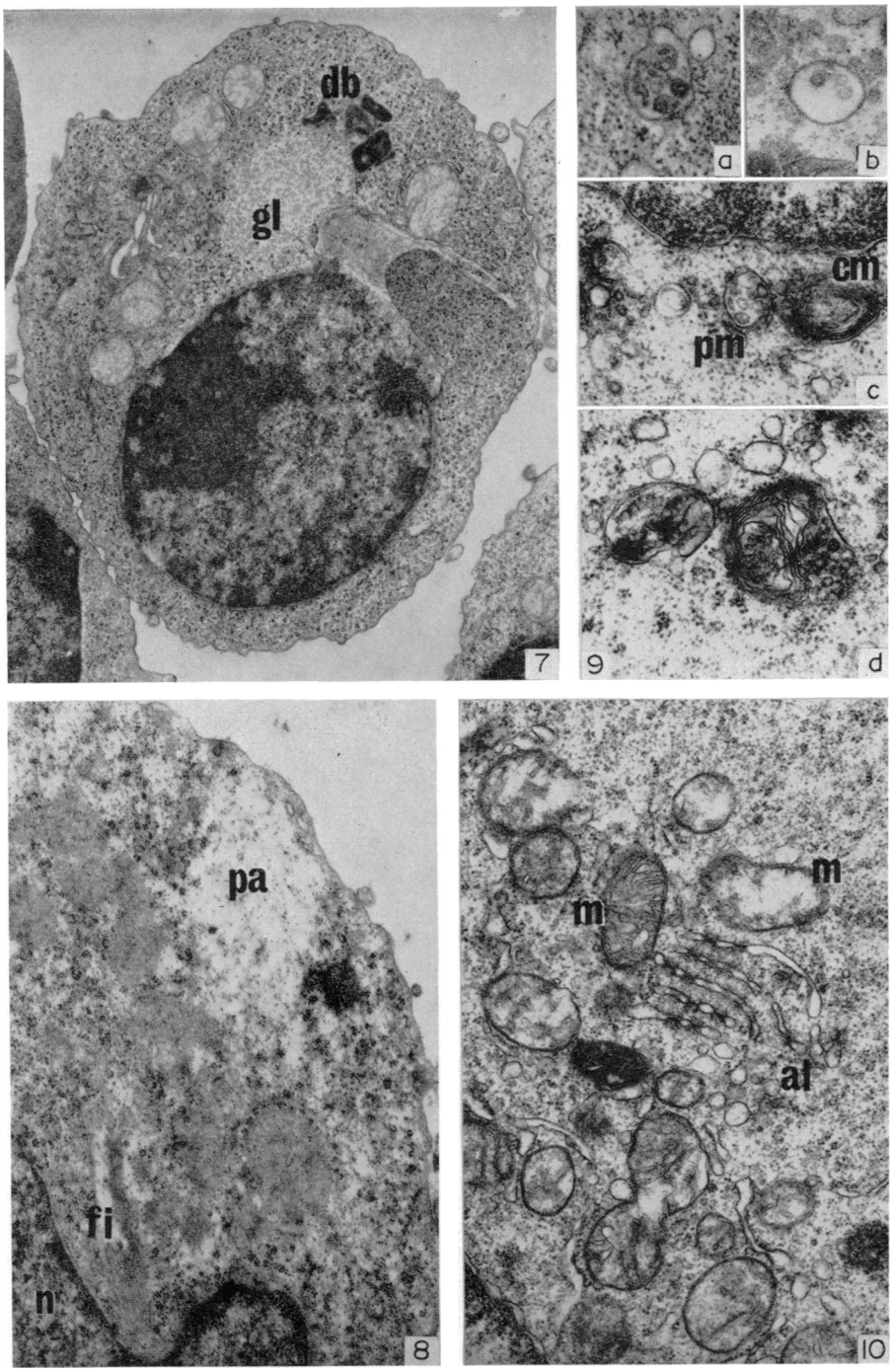


FIG. 7. Electron micrograph of lymphoblast (Type I) from culture of normal spleen (T+B) cells stimulated by PWM. There is a large area of glycogen (gl) and a well developed Golgi apparatus (db, dense bodies). (×9,000.)

FIG. 8. Part of a lymphoblast from culture of 'T' cells stimulated by Con A, with a pale, almost ribosome-free area (pa) in the cytoplasm. (n, nucleus; fi, fibrils). (×19,800.)

FIG. 9. (a and b) Pale multivesicular bodies containing internal vesicles; (c) pale (pm) and compound (cm) multivesicular bodies; (d) compound multivesicular body which contains membranes in concentrically arranged lamellae in addition to internal vesicles. a, and ×28,350; b, and ×30,600; c and d, ×28,350.)

FIG. 10. Annulate lamellae (al) in lymphoblast from culture of 'T' cells, stimulated by Con A. (m, mitochondria). (×19,800.)

DISCUSSION

Activation of lymphocytes by 'non-specific' stimulants (mitogens) gives rise to populations of blast cells with considerable structural heterogeneity. Two broad categories of responsive cells were previously described as Type I ('PHA blast' or 'PWM blast type I') and Type II ('PWM intermediate cell') (Douglas *et al.*, 1967). This classification is applicable to blast cells from stimulated mouse lymphocyte cultures.

In category 'Type I' are the lymphoblasts (Fig. 1). These cells predominate in thymus dependent areas of lymph nodes in animals rejecting skin grafts (Andre-Schwarz, 1964) or immunized with contact sensitizing agents (DePetris, Karlsbad, Petris and Turk, 1966; Turk, 1967), and are therefore likely to be mostly activated T cells. Their paucity of RER (Fig. 2) is compatible with the view that T cells do not actively secrete antibody (Davies, 1969). Some antibody secreting (i.e. B) cells, however, also have poorly developed RER (DePetris and Karlsbad, 1965; Leduc, Avrameas and Bouteille, 1968; Hummeler, Harris, Harris and Farber, 1972). Despite the morphological overlap of activated T and B cells *in vivo*, there are distinctive features: many antibody-secreting B cells develop extensive RER (plasmablasts, plasma cell). Type II blasts in our studies correspond with this type of development (Figs 5 and 6). It is generally considered that T cells do not undergo this type of differentiation and all myeloma cells so far investigated have the cell surface characteristics of B cells (Takahashi, Old, McIntyre and Boyse, 1971).

The selectivity of mitogens for T and B cells has now been established (reviewed by Greaves and Janossy, 1972a). In this study we have attempted to map the morphological heterogeneity of mitogen-activated cells into the type of responding cells. Our prediction was that the pattern of response would mimic the characteristic features of T and B cells stimulated *in vivo*.

Our results are in accord with this hypothesis since only PWM or endotoxin-activated B cells developed the 'plasmablast' (Type II) features. Stimulation of T cells with the same (i.e. PWM) or a different phytomitogen—Con A—induced a response in which effectively all of the activated cells had the appearance of lymphoblasts (Type I). Some activated B cells also fell into Type I, as might be anticipated from the behaviour of B cells *in vivo*. However, the proportion of such cells was much smaller than expected (see below).

Mixtures of T and B cells (normal spleen cultures) stimulated with Con A, PWM and endotoxin gave a result that was predictable from the response of T and B lymphocytes cultured separately, thus confirming that Con A is a T specific mitogen and endotoxin selectively stimulates B lymphocytes. PWM stimulated both T and B cells in a mixture of these two cell types (Fig. 6). According to the evidence as presented in Table 3, the majority of Type I blasts with a polyribosomic pattern in the PWM stimulated normal spleen cultures are members of the T lymphocyte line and are not precursors ('immature' forms) of plasmablasts.

We suppose that our culture system Con A stimulates the majority (80–100 per cent) of T cells (Table 1), while PWM or endotoxin stimulate only subpopulations (15–25 per cent) of lymphocytes (see also below). These numbers are sufficiently high to suggest that the mitogens used are unlikely to be functioning as antigens, i.e. they are immunologically non-specific. However, our morphological data show that mitogens evoke a response mimicking that induced by immunogens (antigens). This view is supported by functional studies.

Lymphokines (e.g. migration inhibition factor, MIF) are released by sensitized

lymphocytes on re-exposure to antigen. Lymphocytes—presumably T cells—stimulated by Con A, PHA or antilymphocyte serum also liberate MIF (reviewed by Pick and Turk, 1972). It is known that T lymphocytes from animals sensitized to allogeneic antigens exert a cytotoxic effect on the relevant target cells *in vitro* (Cerottini, Nordin and Brunner, 1971) and it was also shown that antigens (e.g. oxazolone) which induce cell mediated immunity *in vivo* give rise to activated T cells which are cytotoxic against 'irrelevant' target cells (i.e. mastocytoma cells) in the presence of binding agents (Asherson, Ferluga and Allwood, personal communication). Using the same system, Con A or PWM stimulated cortisone-resistant thymocytes ('T' cells) —but not PWM stimulated B lymphocytes—were also found to be cytotoxic against mastocytoma target cells (Asherson, Ferluga and Janossy, unpublished observations), thus confirming earlier findings on non-specific cytotoxic effects of PHA activated lymphocytes (Perlmann and Holm, 1969).

On the other hand, B cells stimulated by antigens (e.g. by sheep erythrocytes) *in vitro* or by mitogens (PWM or endotoxin) synthesize and secrete immunoglobulins (Parkhouse, Janossy and Greaves, 1972; Greaves and Janossy, 1972b). These functional analogies suggest that T and B cell populations have pre-programmed differentiation pathways which can be evoked by both antigens and mitogens. We therefore interpret the action of mitogens as a polyclonal activation, rather than an 'abortive' sterile response. The possible implications of this suggestion for the mechanism of lymphocyte triggering have been recently discussed (Greaves and Janossy, 1972a).

A few minor qualifications to our overall conclusion should be added. The particular mitogen used did exert some influence on the response pattern observed. Con A-stimulated lymphoblasts frequently contained many single ribosomes, while PWM stimulated lymphoblasts accumulated mostly polyribosomes. This may reflect a quicker rate of development in Con A blasts, as the formation of polyribosomes in these cells appears relatively early during the culture and presumptive descendants of lymphoblasts in 'late' cultures contain mostly single ribosomes (e.g. Biberfeld, 1971a; De Petris *et al.* 1966; Shohat and Janossy, unpublished observations). Alternatively, single ribosomes may be characteristic for a T cell subpopulation which is stimulated by Con A but not by PWM.

There is evidence which suggests that both PWM and endotoxin stimulate approximately 15–25 per cent of B cells carrying surface immunoglobulin (Janossy, Doenhoff and Greaves, in preparation; see also Table 1). Stimulation of B cells by mitogens occurs independently of T cells and IgM is the only immunoglobulin class produced in the system we have used (Parkhouse *et al.* 1972). It is quite possible that the mitogen-responsive B cell subpopulation in our studies is analogous to B cells participating in thymus-independent IgM responses after antigenic stimulation (i.e. by thymus-independent antigens). During primary antigenic stimulation *in vivo*, cells are able to transform into IgM-producing plasmablasts within 24 hours (Gudat, Harris, Harris and Hummeler, 1971) and this could explain why after 60 hours incubation in our stimulated B cell cultures many plasmablasts and relatively few lymphoblasts were found. A time course study is in progress to answer this question. In this respect, however, the development of IgM responses are likely to be different from the early stages of the primary IgG responses *in vivo*.

The paucity of RER contrasted with the abundant dilated RER was the primary difference between stimulated T and B cells. Furthermore, it was interesting that lymphoblasts showed more multivesicular bodies than plasmablasts. These bodies are thought to be a result of endocytosis (Biberfeld, 1971b), that follows a redistribution of membrane elements ('cap formation') to one pole of the lymphocytes incubated with anti-immuno-

globulins and mitogens (Taylor, Duffus, Raff and De Petris, 1972; Greaves, Bauminger and Janossy, 1972). One or other of these successive events could play a decisive role in lymphocyte triggering, and we suppose that the scanty occurrence of signs of endocytic activity in plasmablasts might reflect some differences in the triggering events of B and T cells (Greaves and Janossy, 1972a).

Finally, Chessin, Börjeson, Welsh, Douglas and Cooper (1966) described that PHA-blasts and PWM 'Type I' blasts showed coarse, diastase-sensitive PAS positive granules (glycogen) in the cytoplasm. These granules were absent in PWM 'Type II' blasts. Our results confirm their data. In a similar experiment it was found that 80 per cent of PWM stimulated 'T' cells contained PAS positive (diastase sensitive) granules, as opposed to less than 8 per cent of PWM-activated B cells (Burn, Savage and Janossy, unpublished data). These observations suggest that some metabolic processes may also be different in stimulated T and B cells.

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REFERENCES

ANDERSSON, J., MÖLLER, G. and SJÖBERG, O. (1972). 'Selective induction of DNA synthesis in T and B lymphocytes.' *Cell. Immunol.* (in press).

ANDRE-SCHWARTZ, J. (1964). 'The morphologic responses of the lymphoid system to homografts. III. Electron-microscopy study.' *Blood*, **24**, 113.

BARKER, B. E., LUTENER, M. A. and FARNES, P. (1969). 'Ultrastructural properties of pokeweed stimulated leukocytes *in vivo* and *in vitro*.' *Proceedings of the 3rd Annual Leucocyte Culture Conference* (Ed. by W. O. Rieke), p. 588. Appleton-Century-Crofts, New York, 1969.

BIBERFELD, P. (1971a). 'Morphogenesis in blood lymphocytes stimulated with phytohaemagglutinin (PHA).' *Acta path. microbiol. scand. A.*, **223**, 1.

BIBERFELD, P. (1971b). 'Endocytosis and lysosome formation in blood lymphocytes transformed by phytohaemagglutinin.' *J. Ultrastruct. Res.* **37**, 41.

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

BLOMGREN, H. and SVEDMYR, E. (1971). '*In vitro* stimulation of mouse thymus cells by PHA and allogeneic cells.' *Cell. Immunol.*, **2**, 285.

BÖRJESON, J., REISFIELD, R., CHESSIN, L. N., WELSH, P. and DOUGLAS, S. D. (1966). 'Studies on human peripheral blood lymphocytes *in vitro*. I. Biological and physiochemical properties of the pokeweed mitogen.' *J. exp. Med.* **124**, 873.

CEROTTINI, J. C., NORDIN, A. A. and BRUNNER, K. T. (1971). 'Cellular and humoral response to transplantation antigen. I. Development of alloantibody forming cells and cytotoxic lymphocytes in the graft versus host reaction.' *J. exp. Med.*, **134**, 553.

CHESSIN, L. N., BÖRJESON, J., WELSH, P. P., DOUGLAS, S. D. and COOPER, H. L. (1966). 'Studies on human peripheral blood lymphocytes *in vitro*. II. Morphological biochemical studies on the transformation of lymphocytes by Pokeweed mitogen.' *J. exp. Med.*, **124**, 873.

DAVIES, A. J. S. (1969). 'The thymus and the cellular basis of immunity.' *Transplant. Rev.*, **1**, 43.

DE PETRIS, S. and KARLSBAD, G. (1965). 'Localization of antibodies by electron microscopy in developing antibody-producing cells.' *J. Cell Biol.*, **26**, 759.

DE PETRIS, S., KARLSBAD, G., PETRIS, B. and TURK, J. L. (1966). 'Ultrastructure of cells present in lymph nodes during the development of contact sensitivity.' *Int. Arch. Allergy*, **29**, 112.

DOUGLAS, S. D., HOFFMAN, P. F., BÖRJESON, J. and CHESSIN, L. N. (1967). 'Studies on human peripheral blood lymphocytes *in vitro*. III. Fine structural features of lymphocyte transformation by Pokeweed mitogen.' *J. Immunol.*, **98**, 17.

GERY, I., KRUGEL, J. and SPIESEL, S. Z. (1972). 'Stimulation of B lymphocytes by endotoxin. Reactions of thymus deprived mice and karyotypic analysis of dividing cells in mice bearing T6T6 thymus grafts.' *J. Immunol.*, **108**, 1088.

GREAVES, M. F., BAUMINGER, S. and JANOSSY, G. (1972). 'Lymphocyte activation. III. Binding sites for phytomitogens on lymphocyte subpopulations.' *Clin. exp. Immunol.*, **10**, 537.

GREAVES, M. F. and JANOSSY, G. (1972a). 'Elicitation of selective T and B lymphocyte responses by cell surface binding ligands.' *Transplant. Rev.* **11**, 87.

GREAVES, M. F. and JANOSSY, G. (1972b). 'Activation of lymphocytes by phytomitogens and antibodies to cell surface components—a model for antigen

induced differentiation.' In *3rd Lepetit Colloquium on Cell Interactions*. North Holland Co., Amsterdam.

GUDAT, F. G., HARRIS, T. N., HARRIS, S. and HUMMUELER, K. (1970). 'Studies on antibody producing cells. I. Ultrastructure of 19S and 7S antibody producing cells.' *J. exp. Med.*, **132**, 448.

GUDAT, F. G., HARRIS, T. N., HARRIS, S. and HUMMELER, K. (1971). 'Studies on antibody producing cells. III. Identification of young plaque-forming cells by thymidine-H^3 labelling.' *J. exp. Med.*, **134**, 1155.

HARRIS, T. N., HUMMELER, K. and HARRIS, S. (1966). 'Electron microscopic observations on antibody-producing lymph node cells.' *J. exp. Med.*, **123**, 161.

HIRSCH, F. G. and FEDORKO, M. F. (1968). 'Ultrastructure of human leukocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and postfixation in uranyl acetate.' *J. Cell. Biol.*, **38**, 615.

HUMMELER, K., HARRIS, T. N., HARRIS, S. and FARBER, M. B. (1972). 'Studies on antibody producing cells. IV. Ultrastructure of plaque-forming cells of rabbit lymph.' *J. exp. Med.*, **135**, 491.

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

JANOSSY, G. and GREAVES, M. F. (1972). 'Lymphocyte activation II. Discriminating stimulation of lymphocyte subpopulations by phytomitogens and heterologous antilymphocyte sera.' *Clin. exp. Immunol.*, **10**, 525.

LEDUC, E. H., AVRAMEAS, S. and BOUTEILLE, M. (1968). 'Ultrastructural localization of antibody in differentiating plasma cells.' *J. exp. Med.*, **127**, 109.

MITCHELL, G. F. and MILLER, J. F. A. P. (1968). 'Cell to cell interaction in the immune response. II. The source of hemolysin forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes.' *J. exp. Med.*, **128**, 821.

MOVAT, H. Z. and FERNANDO, N. V. P. (1965). 'The fine structure of the lymphoid tissue during antibody formation.' *Exp. mol. Pathol.*, **4**, 155.

PARKHOUSE, R. M. E., JANOSSY, G. and GREAVES, M. F. (1972). 'Selective stimulation of IgM synthesis in mouse B lymphocytes by Pokeweed mitogen.' *Nature (New Biology.)*, **235**, 21.

PERKINS, W. D., KARNOVSKY, M. J. and UNANUE, E. R. (1972). 'An ultrastructural study of lymphocytes with surface bound immunoglobulin.' *J. exp. Med.*, **135**, 267.

PERLMANN, P. and HOLM, G. (1969). 'Cytotoxic effects of lymphoid cells *in vitro*.' *Advanc. Immunol.* **11**, 117.

PICK, E. and TURK, J. L. (1972). 'The biological activities of soluble lymphocyte products.' *Clin. exp. Immunol.*, **10**, 1.

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

RAFF, M. C. and CANTOR, H. (1972). 'Subpopulations of thymus cells and thymus derived lymphocytes.' *Progr. Immunol. (1st Intern. Congr. Immunol.)*, 83.

SHORTMAN, K., BYRD, W., WILLIAMS, N., BRUNNER, U. T. and CEROTTINI, J. C. (1972). 'The separation of different cell classes from lymphoid organs VIII.' *Aust. J. exp. Biol. med. Sci.* (In press).

STOBO, J. D., ROSENTHAL, A. S. and PAUL, W. E. (1972). 'Functional heterogeneity of murine lymphoid cells. I. Responsiveness to and surface binding of Concanavalin-A and phytohaemagglutinin.' *J. Immunol.*, **108**, 1.

TAKAHASHI, T., OLD, L. J., MCINTYRE, U. R. and BOYSE, E. A. (1971). 'Immunoglobulin and other surface antigens of cells of the immune system.' *J. exp. Med.*, **134**, 815.

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

TURK, J. L. (1967). *Delayed Hypersensitivity*. North Holland Publishing Co., Amsterdam.

WESTPHAL, O., LÜDERITE, O. and BISTER, F. (1952). 'Über die Extraktion von Bakterien mit Phenol/Wasser.' *Zeit. Naturf.*, **76**, 148.