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LYMPHOCYTE ACTIVATION

II. DISCRIMINATING STIMULATION OF LYMPHOCYTE SUBPOPULATIONS BY PHYTOMITOGENS AND HETEROLOGOUS ANTILYMPHOCYTE SERA

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SUMMARY

The mitogenic selectivity of four phytomitogens, phytohaemagglutinin (PHA), concanavalin (ConA), lentil mitogen (LM) and pokeweed mitogen (PWM), and a series of heterologous antilymphocyte sera (ALS) for mouse T and B lymphocytes has been investigated using thymidine uptake as a measure of proliferative activity.

The results show that PHA, ConA and LM stimulate T but not B cells whereas PWM activates both T and B cells. Of six mitogenically active ALS, five were T cell specific and one B cell specific. Cortisone resistant (T) cells within the thymus showed a much greater response to all mitogens than unselected thymocytes. However, ConA and LM activated a considerable response in the latter population.

Selective activation of T and B cells by aspecific means may be useful both as a clinical tool and as an approach to gaining an understanding of antigen induction of immune responses.

INTRODUCTION

In the previous paper we have shown that phytohaemagglutinin (PHA) activated RNA and DNA synthesis in T (thymus-derived) but not in B (bursa-equivalent derived) lymphocytes, whereas pokeweed mitogen (PWM) stimulated B lymphocytes (Janossy & Greaves, 1971). The selectivity of PHA for T cells was based on experiments involving the culture of purified B lymphocytes and comparing the response with that of T/B cell mixtures from normal spleens. In this study we have examined two additional phytomitogens—concanavalin-A (Con-A) from the Jack bean, *Canavalia ensiformis* (Powell & Leon, 1970), and lentil mitogen (LM) from *Lens culinaris* (Young *et al.*, 1971)—and also fourteen heterologous anti-mouse antilymphocyte sera, for their capacity to stimulate T and B lymphocytes. Reactivity of B cells has been compared with both normal spleen cells (i.e. T plus B cells) and thymocytes.

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In addition the responsiveness of the small immunocompetent T lymphocyte population within the thymus has been studied following *in vivo* selection of these cells by injection of cortisone acetate (Blomgren & Anderson, 1970).

MATERIALS AND METHODS

Mice

Normal (control) mice. 6–12-week-old inbred (CBA H-2^k) mice were used in the majority of experiments. Thymuses were from 4-week-old mice.

T-cell deprived mice (source of B spleen cells). 4-week-old CBA mice were thymectomized, X-irradiated with 850 r and reconstituted with $5–10 \times 10^6$ viable bone marrow cells which had been pretreated with anti-θ serum plus complement (Janossy & Greaves, 1971).

Cortisone-treated mice (source of T cells). 4-week-old mice were injected intraperitoneally with 2·5 mg hydrocortisone acetate (Hydrocortisyl—Roussel, U.K.) 3 days before removal of the thymus.

Lymphocyte cultures

Cell sources and manipulations. The preparation of cell suspensions was carried out aseptically as described previously (Janossy & Greaves, 1971). The spleen cells from T lymphocyte-deprived animals (B spleen) were filtered through a cotton wool column to eliminate macrophages (Janossy & Greaves, 1971). This was necessary since the presence of macrophages permits a small but significant response of B lymphocytes to PHA (Janossy & Greaves, 1971) and might also be expected to result in phagocytosis (via opsonization) of lymphocytes in the experiments with anti-lymphocyte serum (Greaves *et al.*, 1969). All cell suspensions were adjusted to contain 2×10^6 viable leucocytes/ml.

Culture media. RPMI-1640 medium (Flow Labs.) was supplemented with 10% heat inactivated foetal calf serum (Flow Labs.). The medium was further supplemented with freshly prepared glutamine solution (400 mM/2 ml per 100 ml medium) and antibiotics (penicillin 200 U/ml and streptomycin 100 μg/ml).

Culture conditions. A microplate method was used in this study. The microplates (Falcon MicroTest II, Gateway Int., U.S.A.) contained ninety-six wells with flat bottoms, each of 0·36 ml volume and 0·28 cm^2 surface area. Optimal cell density for maximum thymidine incorporation was found to be 5×10^5 leucocytes per 0·25 ml medium. The cell suspension was distributed by a Repette syringe of 10 ml capacity (Jencons, U.K.) and the stimulants were added with a Hamilton microsyringe (100 μl) adjusted into a modified repeating dispenser (Hamilton). The plates were covered with a lid (Falcon) and placed into a desiccator gassed with a mixture containing 7% CO_2 and 10% O_2 in N_2. This vessel was put into a thermostat at 37°C. All spleen and thymus cell cultures were set up in triplicate and cortisone-treated thymocyte cultures in duplicate.

Measurement of responses. After a 40 hr cultivation period 0·1 μCi [^{3}H]thymidine was added to cultures with a Hamilton repeating dispenser. Twenty-four hours later the microplate was put on ice and the cell suspension from each well transferred with a pasteur pipette onto a wet membrane (GF/C 2·5 cm, Whatman) placed in a Manifold multiplex sample collector (Millipore). Each culture well was washed with approximately 0·5 ml cold phosphate buffered saline (lacking calcium and magnesium ions). This washing fluid was poured onto the same membrane and the latter subsequently washed twice with PBS, twice with TCA,

twice with methanol (approximately 1 ml each). The membranes were left to dry and placed into scintillation vials. The radioactive material on membranes was solubilized and counted as described previously (Janossy & Greaves, 1971).

Cytological examinations. The cultures were regularly inspected under an inverted microscope. The advantage of this simple morphological approach is that it can be repeated during the cultivation period and it allows, therefore, an empirical monitoring of the response in terms of blastogenesis.

Cytotoxic tests. The cytotoxic effects of ALS were titred using trypan blue dye exclusion as described by Gorer & O'Gorman (1956).

Phytomitogens and antilymphocytic sera

Phytomitogens. Phytohaemagglutinin (PHA) (reagent grade, Wellcome, U.K.) and Concanavalin-A (Con-A) (2 × crystallized, Miles-Yeda, Israel) have been used. Pokeweed mitogen (PWM) was purified from the plant stems (*Phytolacca americana*) using the method of Borjeson *et al.* (1966). Lentil mitogen (LM) was purified from lentil seeds (*Lens culinaris*) according to the method described by Howard and Sage (1969). Stimulants were stored at −20°C in small aliquots.

Antilymphocytic sera. These sera were provided by Dr Sandra Nehlsen and Dr Elizabeth Simpson. We have tested ten rabbit anti-mouse lymphocyte sera (R-1, R-2, R-3, R-4, R-5, R-6, R-7, R-8); two of them were purified anti-lymphocytic globulin (ALG-1, ALG-2). In addition, two calf anti-mouse lymphocyte sera (C-1, C-2), one pig anti-mouse lymphocyte serum (P-1) and one rat anti-mouse lymphocyte serum (Rat-1) were tested. Rabbit ALS were produced by the method of Levey & Medawar (1966). Other ALS were produced as described by Binns *et al.* (1971). All sera had previously been shown to be effective as *in vivo* immuno-suppressants (skin graft rejection).

RESULTS

Response of thymocytes, T and B lymphocytes, to phytomitogens

Phytohaemagglutinin (PHA). PHA stimulated both normal spleen and cortisone-resistant thymocyte (CR-T) cell suspensions (Fig. 1). The rise of the PHA dose/thymidine incorporation curve was steep with a peak at 50–100 μg/ml and with sharp decrease owing to PHA toxicity above 200 μg/ml.

Only marginal or no activation of thymocyte and B spleen cell suspensions could be observed, the increments in thymidine incorporation being less than 1/25 of the response of normal spleen or CR-T. In absolute terms, however, PHA induced a seven-fold increase in thymidine uptake of thymocytes, when the non-stimulated control value was as low as 150 cpm.

Concanavalin-A (Con-A). The response of normal spleen suspensions to Con-A was regularly 30–40% higher than that of PHA-stimulated spleen cell suspensions (Fig. 2). Extremely strong stimulation took place in CR-T cultures. Even in thymocyte suspensions the magnitude of thymidine incorporation was only slightly less than in PHA-stimulated spleen cultures. The elevating slopes of dose/response curves were rather steep both in Con-A stimulated normal spleen and thymocyte cultures (optimal doses at 2·5 μg/ml and 8 μg/ml, respectively).

Con-A was a mild leukoagglutinin at optimal stimulating doses, and most blast cells did

not appear in aggregates. In CR-T suspensions virtually all surviving cells could be judged to be lymphoblasts.

In contrast, in B spleen cell suspensions only a very marginal increase in thymidine uptake could be seen. *In situ* inspection of cultures revealed that in spite of the excellent cell survival the number of proliferating lymphoblast foci was less than 2% of the total cell number, which might possibly represent the fraction of residual T lymphocytes.

Lentil mitogen (LM). LM stimulated essentially the same cell suspensions (normal spleen,

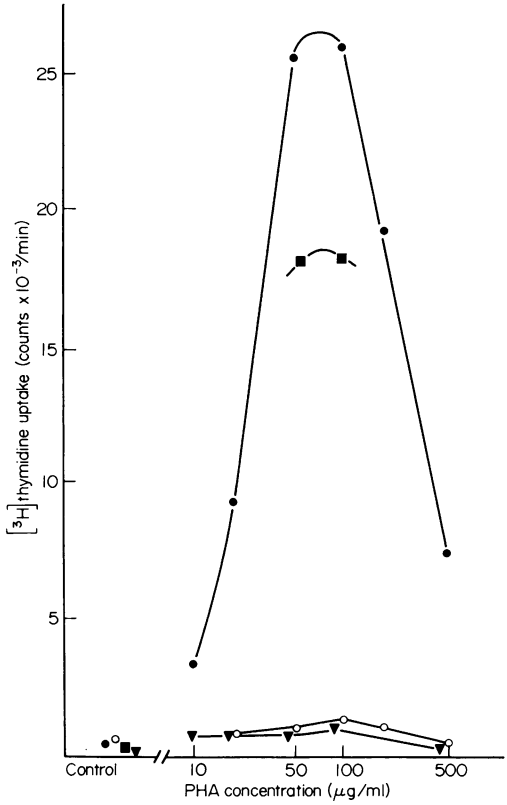

FIG. 1. [^{3}H]thymidine incorporation in mouse lymphocyte cultures stimulated by phytohaemagglutinin (PHA-W). ●, Normal spleen cells; ○, B spleen cells; ■, thymocytes from cortisone-treated mice (T cells); ▼, thymocytes.

CR-T, and thymocyte suspensions) as Con-A (Fig. 3). The LM dose/thymidine incorporation curve with unselected spleen cells differed slightly from that of PHA and Con-A, showing a plateau effect at high concentrations in the absence of toxicity (optimal response at 10–100 μg/ml). Although LM markedly stimulated thymocytes, this effect was only seen with very high doses (200 μl/ml). At this dose, after 3 days' incubation, approximately 25–40+ of thymocytes could be classified as enlarged cells (lymphoblasts—Fig. 4). LM did not activate B cells.

Pokeweed mitogen (PWM). In marked contrast to the previous mitogens, PWM stimulated B spleen cell suspensions in the same magnitude as normal spleen cultures. The slopes of

dose/response curves were relatively shallow and the peak of thymidine incorporation was lower than in PHA stimulated cultures (Fig. 5). PWM also stimulated CR-T and to a lesser extent thymocytes. Although PWM is a very weak lympho-agglutinin (Greaves, Bauminger & Janossy, 1972), effectively all PWM-stimulated blast cells were found initially in aggregates.

Not all B cells responded to PWM in terms of morphological transformation, i.e. percentage blast cells on day 3 of cultures was 40–50%, and it is quite possible that this mitogen

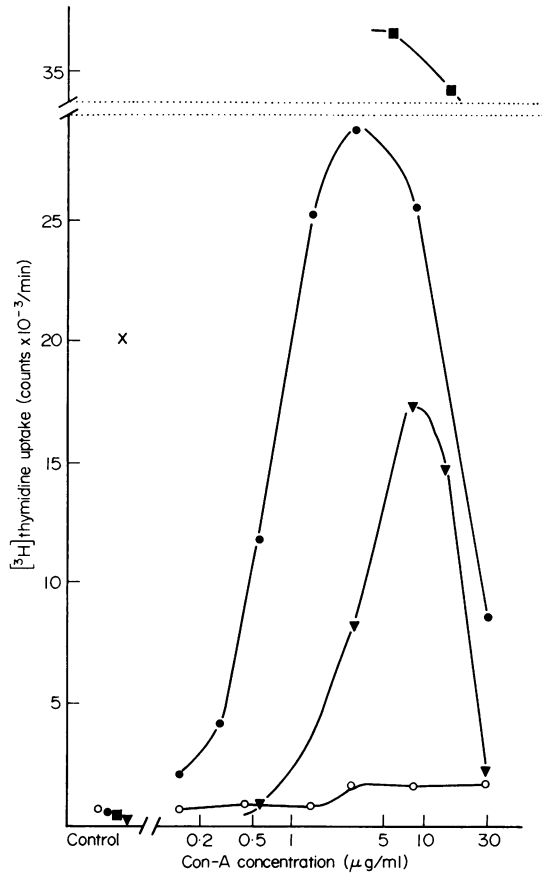

FIG. 2. [^{3}H]thymidine incorporation in mouse lymphocyte cultures stimulated by concanavalin-A (Con-A). Symbols as in Fig. 1. ×, 100 μg/ml PHA-W.

selectively activates a distinct subpopulation of B cells which might represent only a small fraction of the initial cell population set up in culture.

Responses of thymocytes, T and B lymphocytes, to various ALS

Eight of the fourteen heterologous ALS (R-2, R-4, R-5, R-6, R-8, ALG-1, P-1, Rat-1) proved to be ineffective in stimulating mouse lymphocytes; treated cultures exhibiting the same or less counts than controls incubated with 30–100 μl/ml normal rabbit serum (NRS). Three of the remaining six ALS were only moderately stimulating, the observed maximal

thymidine incorporation values with normal spleen cells were as follows: control NRS, 50 μl/ml 1200 cpm; PHA 100 μg/ml 25300 cpm; ALS R-3, 100 μl/ml 3150 cpm; ALG-2, 100 μl/ml 5800 cpm; ALS C-2, 10 μl/ml 2600 cpm. These three ALS did not stimulate B-spleen cell suspensions. We have analysed in detail the effects of the remaining 3 ALS (ALS C-1, R-1 and R-7).

ALS C-1. This ALS stimulated both normal spleen cell and CR-T suspensions, the latter at slightly higher dose (Fig. 6). The magnitude of the response was similar to that of PHA-stimulated cultures. In thymocyte suspensions only a moderate stimulation was observed

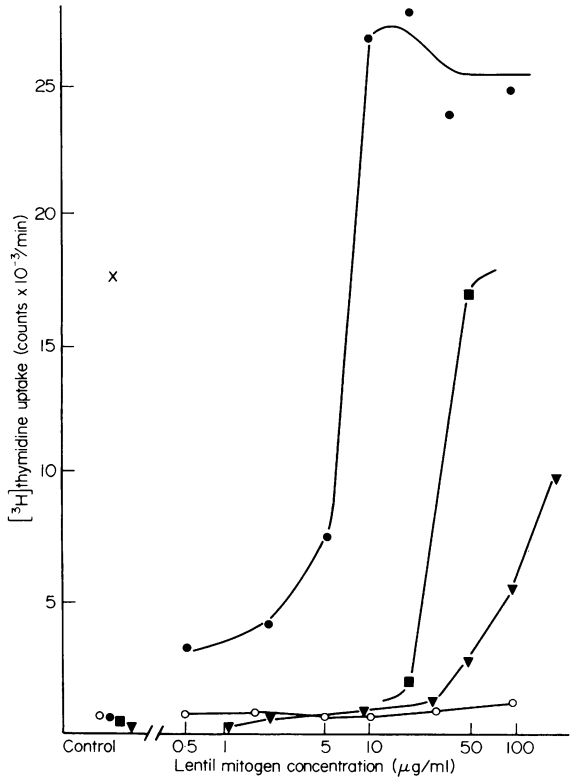

FIG. 3. [^{3}H]thymidine incorporation in mouse lymphocyte cultures stimulated by lentil mitogen (LM). Symbols as in Fig. 1. ×, 100 μg/ml PHA-W.

and a minor subpopulation (approximately 3–4% of total thymocyte number at the 40th hour of cultivation) developed into enlarged cell forms. In B spleen cultures the counts were regularly lower with ALS C-1 than in NRS incubated controls.

ALS R-1. This serum also stimulated thymidine incorporation into normal spleen and CR-T suspensions (Fig. 7). The magnitude of the response was slightly less than in PHA stimulated cultures. This ALS had no significant stimulating effect on thymocyte and B spleen cell suspensions.

ALS R-7. In both normal and B spleen cell suspensions the stimulating effects of ALS R-7 were similar in magnitude and dose/response characteristics as observed in PWM stimulated

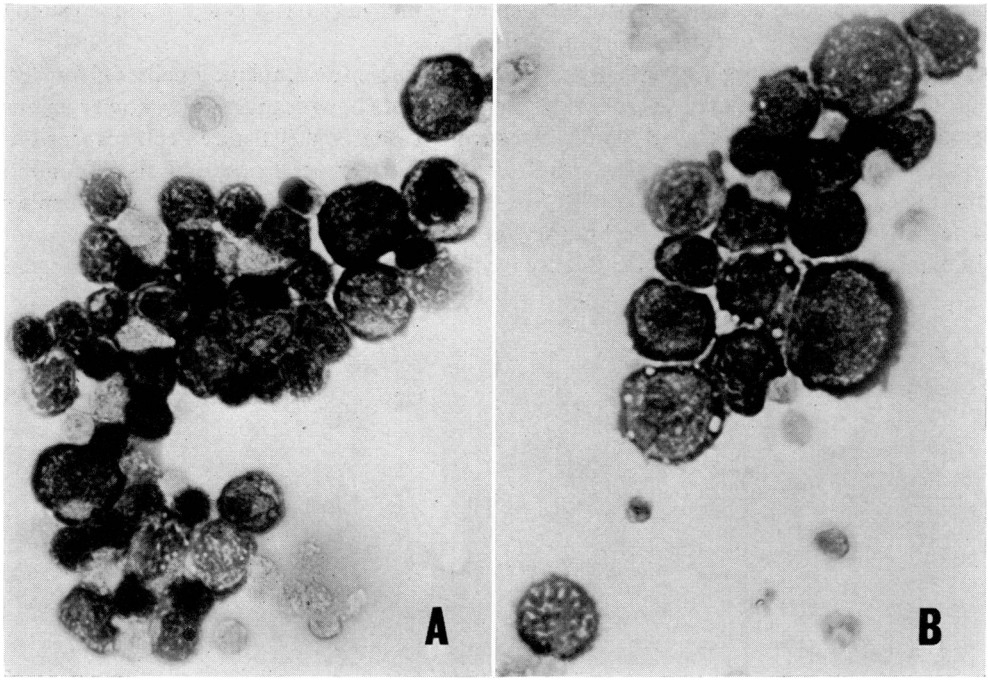

FIG. 4. Thymocyte cultures stimulated with lentil mitogen (LM), after 66 hr, stained with May-Grünwald-Giemsa. (A) Typical picture; (B) Blasts of different maturity.

cultures (Fig. 8). However, this stimulant was distinct from PWM in that no activation of CR-T or thymocytes could be observed. This ALS was not cytotoxic for CR-T in complement-free culture medium (42% survival of CR-T at 44 hr of incubation with 10 μl/ml R-7).

Cytotoxicity of ALS for T and B cells

The cytotoxic activity of the above three sera in the presence of guinea-pig complement was tested against B and CR-T cells using trypan blue dye exclusion. The results as shown in Table 1 indicated that there was no clear correlation between selective mitogenicity and cytotoxicity.

DISCUSSION

We have previously described the discriminatory effects of pokeweed mitogen (PWM) and phytohaemagglutinin (PHA) on lymphocytes (Janossy & Greaves, 1971). B cells were stimulated by PWM but not by PHA.* We also concluded from this and other evidence (Doenhoff *et al.*, 1970) that the PHA responsive cells in T/B mixtures were in fact predominantly T cells. In the current study this situation has been substantiated and extended to include the responsiveness of thymocytes, as well as purified T cells from the thymus selected by

* Stockman *et al.* (1971) have also recently reported that thymectomized, irradiated, bone marrow reconstituted mice respond to PWM but not to PHA. The suggestion by these authors that PWM is selective for B cells is, however, not in accord with our observations (see above).

injection of cortisone acetate (cortisone resistant thymocytes: CR-T). The results are tabulated in a simplified summarized form in Table 2.

Of the four phytomitogens studied, only PWM was able to stimulate B cells. Of fourteen heterologous antilymphocytic sera tested only one from six with mitogenic activity against normal spleen cells stimulated B lymphocytes from T-deprived (B) spleen cell suspensions. The stimulation of B lymphocytes by ALS R-7 has been confirmed by an analysis of immunoglobulin synthesis. ALS R-7, but not ALS R-1, induced IgM production in normal spleen cell suspensions as does PWM (Parkhouse, Janossy & Greaves, 1972).

In marked contrast to B cells, CR-T cells (T lymphocytes) responded to all phytomitogens.

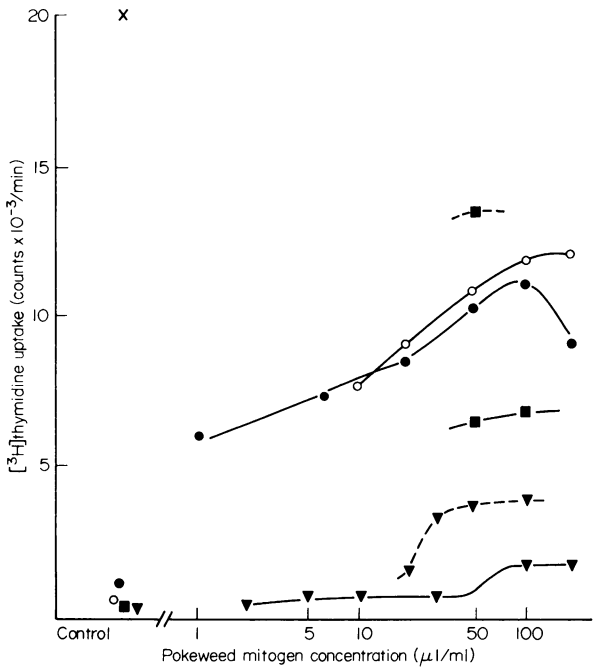

FIG. 5. [^{3}H]thymidine incorporation in mouse lymphocyte cultures stimulated by pokeweed mitogen (PWM). Symbols as in Fig. 1. ×, 100 μg/ml PHA-W. Dashed line: repeat experiment giving slightly different result.

A substantial activation of *unselected* thymocytes was inducible particularly with concanavalin-A (Con-A) and to a lesser extent with lentil mitogen (LM). Although selection for immunocompetent cells by cortisone enhances the response to all phytomitogens, simple quantitative considerations suggest that the response of unselected thymocytes to Con-A and LM cannot be mapped solely onto the CR-T population. A variable proportion of cortisone-*sensitive* cells also have the capacity to respond to phytomitogens. This heterogeneity may possibly reflect maturational changes within the thymus correlating with the acquisition of immunocompetence. The observation that higher doses of Con-A, and particularly of lentil, were necessary to activate unselected thymocytes might also be related to the relative

immaturity of these cells. It is possible that PHA would also stimulate thymocytes if it were not cytotoxic above the stimulating doses for CR-T.

Of the three mitogenic ALS tested, two (ALS R-1 and C-1) activated CR-T well and thymocytes weakly. It is of particular interest that the B lymphocyte stimulating ALS (ALS R-7) was inactive against thymocytes and CR-T. No attempt has been made in these experiments to assess the *proportion* of cells within purified T and B populations which respond to the various mitogens, and it is not excluded that the responses observed involved subpopulations of cells within the two main categories.

The basis for the selectivity of various stimulants for T and B cells is unresolved. Experiments reported in the following paper (Greaves *et al.* 1972) suggest that lack of B lymphocyte responsiveness to phytomitogens is not due to a deficiency of cell surface binding sites,

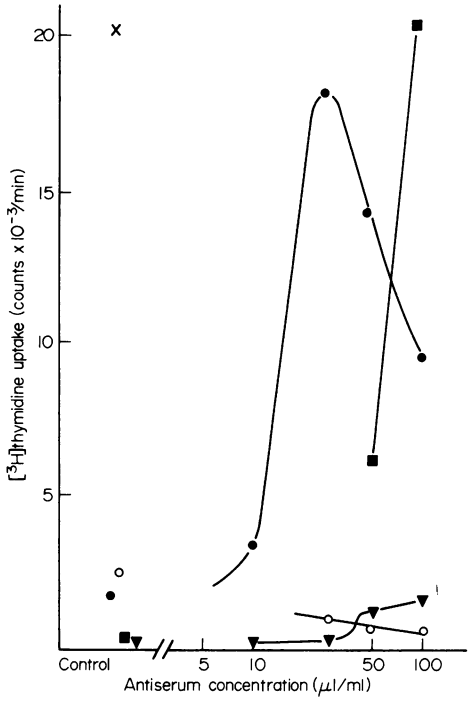

FIG. 6. [^{3}H]thymidine incorporation in mouse lymphocyte cultures stimulated by ALS C-1. Symbols as in Fig. 1. ×, 100 μg/ml PHA-W.

and the same appears to be true for the ALS investigated. The two sera with potent mitogenicity for T cells (ALS C-1 and R-1) were cytotoxic in the presence of guinea-pig complement for both T and B cells although more so against the former. The serum selective for B cells (ALS R-7) was equally cytotoxic for B and T cells. However, ALS of the kind used are heterogeneous and the mitogenically active antibodies may well constitute only a small proportion of those binding to the lymphocyte surface (Woodruff, 1968) and not correlate with the cytotoxic potency of the sera (Greaves *et al.*, 1969). Absorption studies will be necessary to determine if antibodies specific for B or T cell surface antigen (cf. Raff, 1971)

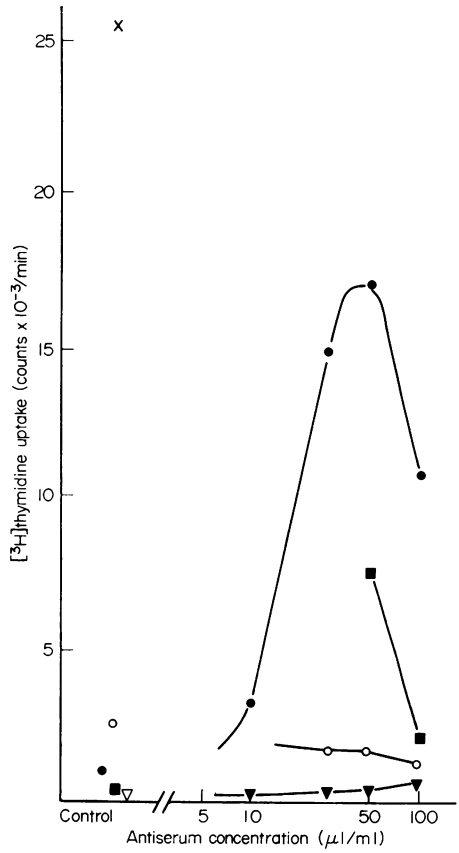

FIG. 7. [^{3}H]thymidine incorporation in mouse lymphocyte cultures stimulated by ALS R-1. Symbols as in Fig. 1. ×, 100 μg/ml PHA-W.

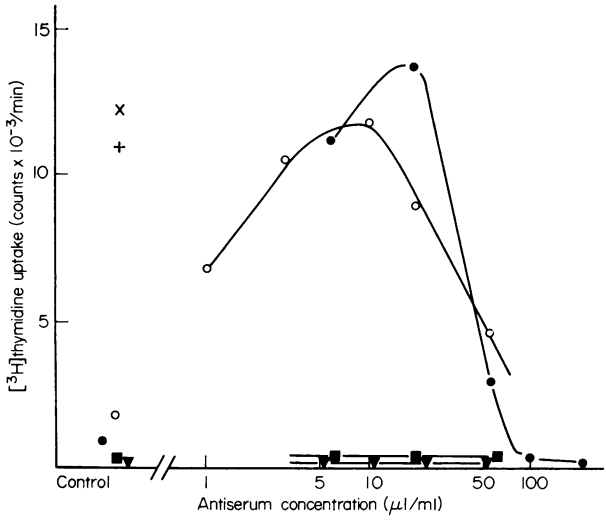

FIG. 8. [^{3}H]thymidine incorporation in mouse lymphocyte cultures stimulated by ALS R-7. Symbols as in Fig. 1. ×, normal spleen cells, 100 μl/ml PWM; +, B spleen cells, 100 μl/ml PWM.

exist in these antisera. The B lymphocyte stimulating ALS R-7 did not contain any anti-immunoglobulin antibodies demonstrable by a sensitive radio-precipitin assay (Herzenberg & Warner, 1968).

We can therefore define mitogens that are selective for T or B lymphocytes, and one, pokeweed mitogen, which stimulates both populations. Other known lymphocyte mitogens may also be selective. Only weak responses can be induced in mouse lymphocyte cultures with

TABLE 1. Specificity of ALS for T and B cells

	Mitogenic activation	Cytotoxicity*	
		CR-T	B
C-1	T	11	8
R-1	T	10	7
R-7	B	7	7

* 50% end point in trypan blue assessment of viable cells, $\log_2$ antiserum dilution. Guinea-pig complement used.

TABLE 2. Selective activation of lymphocyte subpopulations

Stimulant	T-deprived spleens (B)	Normal spleens (T+B)	Normal thymus (T_0*+T)	Cortisone-treated thymus (T)
Phytomitogens				
1. PHA	−	+++	±	+++
2. Lentil	−	++++	++	+++
3. Concanavalin A	−	++++	+++	+++++
4. Pokeweed	++	++	±	++
Heterologous antilymphocyte sera				
1. R-7	++	++	−	−
2. C-1	−	+++	±	+++
3. R-1	−	++	±	+

See Raff & Cantor (1971) for a discussion of the significance of thymocyte heterogeneity.

* Immunologically incompetent or immature T cells (cortisone sensitive).

anti-immunoglobulin sera (Greaves, 1970). It is likely, however, that such reagents, particularly directed against Fc determinants, will be B cell specific (Alm & Peterson, 1969; Ivanyi, Marvanova & Skamene, 1969). There is also evidence that B cells are preferentially activated by bacterial lipopolysaccharides (I. Gery, personal communication 1971).

The purpose of studying the responsiveness of T and B lymphocytes to mitogens *in vitro* is two-fold. Firstly, by defining the selectivity of particular stimulants we will have a means of testing proliferative capacity of T and B lymphocytes in clinical situations and, secondly, differential activation of T and B cells offers an approach to the study of the mechanisms of antigen induced activation and differentiation of these cells *in vivo*.

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