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Selective Induction of DNA Synthesis in T and B Lymphocytes

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The ability of concanavalin A (Con A) and a lipopolysaccharide from *E. coli* bacteria (LPS) to induce DNA synthesis in various types of mouse lymphocytes cultivated *in vitro* was investigated. The results demonstrate that Con A is selectively active on T cells, being capable of activating DNA synthesis in thymocytes, cortisone-treated thymocytes, peripheral "educated" T cells, spleen cells and to a smaller degree bone marrow cells. When T cells are removed from spleen cell suspensions by treating them with antitheta serum, the effect of Con A is markedly reduced. Spleen cells from thymectomized and lethally irradiated mice repopulated with antitheta serum treated bone marrow (T × B mice) or spleen cells from congenitally athymic (nude) mice do not respond to Con A.

Cortisone-treated thymocytes and spleen cells responded equally well to Con A; whereas untreated thymocytes respond 10 to 20 times less, suggesting that the cortisone-resistant thymocytes are the responsive cells both in the thymus and in the spleen. Pure T cells exhibited a very narrow dose response profile to Con A, 5 μg being optimal and 1 or 10 μg giving a small, or no, response. Contrarywise, a mixed T and B cell population, such as the spleen, showed a broad dose response profile to Con A. It is suggested that Con A-activated T cells can influence B cells to respond to Con A; whereas B cells by themselves cannot be activated by Con A.

LPS cannot activate thymocytes, cortisone-treated thymocytes, or educated peripheral T cells, but stimulates DNA synthesis to an equal degree in normal spleen cells, antitheta-treated spleen cells, spleen cells from T × B or nude mice and normal bone marrow cells. LPS can induce DNA synthesis in spleen cells from animals tolerant to LPS to the same degree as in normal spleen cells. Another thymus-independent antigen (PVP) cannot activate DNA synthesis in normal spleen cells, suggesting that LPS exerts a nonspecific stimulatory effect on B cells.

INTRODUCTION

Both thymus-derived (T) and bone marrow-derived (B) lymphocytes are necessary for induction of antibody synthesis to certain antigens (1, 2). Other antigens (thymus independent) appear to be competent to induce an immune response without the participation of T cells (3, 4). The mechanism underlying the dependence of the T cells for activation of B cells in the immune response is not fully understood.

When lymphocytes from antigen-pretreated animals are confronted with the

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same antigen *in vitro,* they are stimulated to morphological transformation and division. Analogous reactions can be induced in lymphocytes by different mitogens. Most of these mitogens such as phytohaemagglutinin (PHA) and certain antilymphocyte sera have been shown to activate T cells exclusively (5–7); whereas others are claimed to activate B cells (pokeweed mitogen and endotoxin) (7–9). Therefore, mitogens may serve as a model for studying the selective activation of different types of lymphocytes, as well as the mechanism by which a certain class of cells become triggered.

As a first step to achieve this, the selectivity of activation with regard to T and B lymphocytes has been studied with two mitogens, namely cancanavalin A (Con A) and a lipopolysaccharide from *E. Coli* bacteria (LPS). In addition, the optimal stimulation conditions, the kinetics and the dose–response curves of these mitogens for different types of lymphocytes have been investigated.

MATERIALS AND METHODS

Animals. Mice of the inbred strains C57BL × DBA and C57BL × CBA were used at 4–8 weeks of age. Congenitally athymic mice ("nude") were kindly supplied by Dr. I. Lefkovits and used at 4–8 weeks of age.

Cell sources. 1. T cells: Three sources of cells were used: (a) normal thymus cells; (b) thymus cells from animals treated with 125 mg of hydrocortisone acetate (Fluka A.G., Switzerland)/kg of body weight 3 days before use (10); (c) "educated" thymocytes obtained from spleens of X-irradiated F_1 hybrids injected 1 week before use with 100×10^6 parental thymus cells.

2. B cells: The three main sources of these cells were: (1) spleen cells of mice that had been thymectomized as adults, irradiated (Philips RT 305 at 300 kV and 10 mA with a 2,7 mm Cu filter, giving a dose of 650 R as determined with a PTW-Duplex dosimeter) and reconstituted with 5×10^6 syngeneic bone marrow cells pretreated with anti-θ serum. The mice were used 2 to 3 weeks after reconstitution. (2) Spleen or bone marrow cells from congenitally athymic mice; and (3) spleen cells from normal mice treated with anti-θ serum and complement prior to use in culture.

Cell suspensions. The thymus glands and/or the spleens were removed aseptically and gently pressed through a 60-mesh stainless-steel screen into sterile phosphate buffered saline (PBS) (11). Clumps of cells were removed by gravity sedimentation and the single cell suspension thus obtained was washed twice in PBS. The cells were resuspended in Eagle's medium in Earle's solution containing 10% heat-inactivated foetal bovine serum (Flow Laboratories, Irvine, Scotland), 50 IU of penicillin, and 100 μg of streptomycin/ml. Cell counting was performed in a haematocytometer and cellular viability was assayed by the trypan blue exclusion test (12). Cell suspensions showing less than 90% viability were excluded.

Cell cultures. Cultures containing 1 ml with 10^6 living lymphocytes were placed in 12 × 75 mm plastic tissue culture tubes (No. 2058, Falcon Plastics, Los Angeles, CA). Triplicate cultures were incubated at 37° in an atmosphere of 10% CO_2 in air.

Mitogens. Concanavalin A (Con A) (Miles-Yeda Ltd., Rehovot, Israel, Lot No. 36) was purified according to the method of Agrawal and Goldstein (13), dialyzed

extensively against water, lyophilized, and stored desiccated at −20°. Immediately before use, 1 mg/ml of Con A was dissolved in PBS and sterilized by filtration (Millipore filter, 0.45-m μ); 0.1 ml of various dilutions of Con A was added to the cultures at the onset of incubation.

Lipopolysaccharide (LPS) of *E. coli* O55:B5 was obtained by phenol–water extraction according to the method of Westphal, Lüderlitz, and Bister, (14), with some modifications (15). The preparation was dialyzed against distilled water to remove material with molecular weight less than 10^4. The LPS was lyophilized and stored desiccated. Prior to use in culture, 1 mg/ml was dissolved in PBS (pH 8.0) and boiled for 2 hr; 0.1 ml aliquots of various dilutions of LPS solution was added to the cultures.

Tolerance induction. Tolerance was induced by five injections of 3 mg of alkali-detoxified LPS during 14 days and maintained by weekly injections of 3 mg of LPS. Routinely the tolerant mice were given a test dose of 10 μg of LPS 7 days after the last tolerance-maintaining dose and assayed 5 days later for antibody-forming cells against LPS-coated red cells in the local haemolysis in gel assay according to methods described earlier (16).

Determination of DNA synthesis. Twenty-four hours before harvest 1 μCi/ml of ^{3}H-thymidine (^{3}H-TdR, The Radiochemical Centre, Amersham, Buckinghamshire, England, sp act, 5 Ci/mmole) was added to the cultures.

At 72 hr, the cultures were harvested by pouring them onto glassfiber filters (Whatman GF/A) in a "Manifold" multiple sample collector (Millipore Corp., Bedford, MA). The cells were washed successively by 10 ml of PBS, 10 ml of ice-cold 10% trichloroacetic acid (TCA) and 2 ml of ethanol. The filters were placed into scintillation vials and left to dry overnight. Ten milliliters of a toluene scintillation fluid (5 g of PPO and 0.2 g of POPOP in 1000 ml of toluene and 858 ml of Triton X) were added to each vial; and the samples were immediately counted in a liquid scintillation spectrometer (Mark I, Nuclear Chicago Corp., Des Plains, IL). The data are presented as counts per minute per 10^6 living lymphocytes originally placed in culture.

RESULTS

Characteristics of the Test System

The number of lymphocytes giving an optimal *in vitro* response to Con A with regard to induction of DNA synthesis studied 2–3 days after addition was found to be 10^6 lymphocytes/ml (Fig. 1). Therefore, this cell concentration was used in all subsequent experiments.

The kinetics of the response to Con A and LPS was studied by adding ^{3}H-thymidine to cultures at various time periods. As shown in Fig. 2, optimal DNA synthesis occurred between days 2 and 3 for both Con A (2a) and LPS (2b).

Response of Thymocytes and Spleen Cells to Con A and LPS

As a first attempt to investigate the selectivity of Con A and LPS on T and B cells their effect was studied on thymocytes, containing only T cells, and spleen cells, being a mixture of T and B cells. Various concentrations of Con A were added to suspensions of thymocytes and spleen cells, respectively. As shown in Fig. 3,

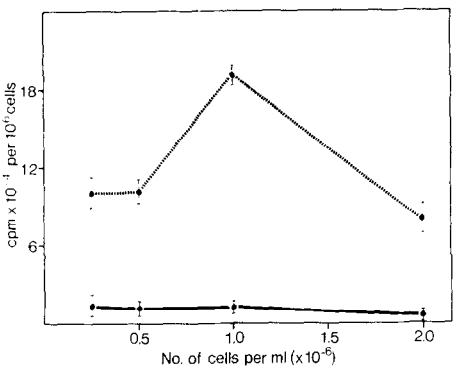

FIG. 1. Effect of spleen cell concentration on the response to 5 μg/ml of Con A (●••), unstimulated cultures (●—).

both cell populations respond with DNA synthesis to certain concentrations of Con A. The response of the thymocytes was markedly dose dependent. Thus, 5 μg/ml gave an optimal response; whereas 1 or 10 μg/ml failed to stimulate the cells or gave a very weak response. Spleen cells treated with Con A were also stimulated, the magnitude of the response being 2–10 times greater than that of thymocytes. The dose-response curve was also different, even though the optimal stimulating dose was always the same in both cases, the spleen cells responding to a broader range of Con A concentrations. The results were similar whether the experiments were performed in tubes or in tissue culture dishes.

Analogous experiments were performed with LPS. As shown in Fig. 4, thymus cells failed to respond with DNA synthesis to any of the doses of LPS added. The range of doses tested varied from 10^{-5} μg/ml to 200 μg/ml, but thymocytes did not become activated at any concentration. In contrast, spleen cells responded with a marked increase in DNA synthesis, optimal doses being around 10 μg/ml of LPS, both lower and higher doses stimulating less well. The spleen cells responded to a 10 times broader range of LPS concentration than Con A concentrations.

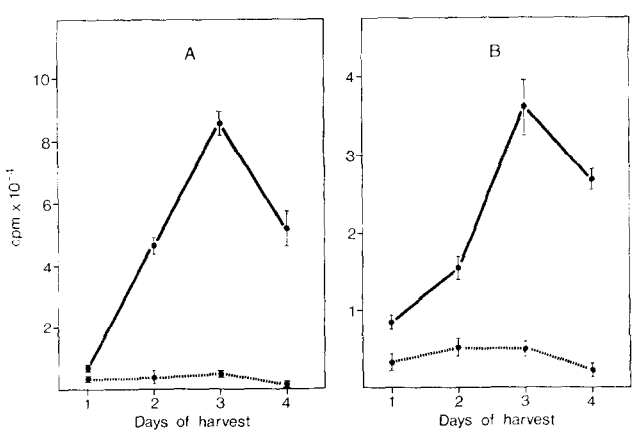

FIG. 2. ^{3}H-thymidine incorporation into spleen lymphocytes at various times of culture: (A) Con A, 5 μg/ml (●—); controls (●--); (B) LPS 10, μg/ml (●—); controls (●--).

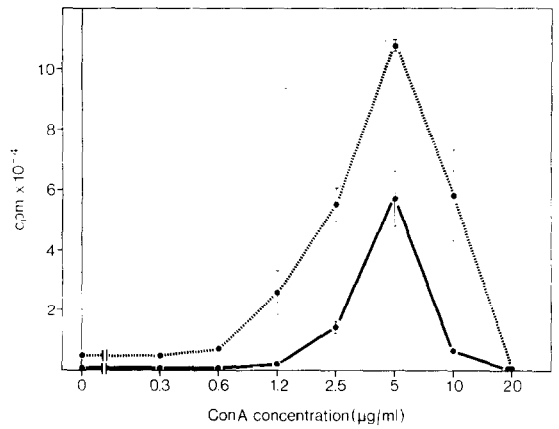

FIG. 3. Response of spleen cells (●– –); and thymocytes (●—) to various concentrations of Con A.

Thus, Con A stimulated pure T cells whereas LPS did not. Both stimulated spleen cells, being a mixture of T and B cells. In analogy with previous findings using other mitogens (17), it was found that low doses failed to stimulate; and there was an optimally stimulating dose. Superoptimal doses suppressed the response to background levels.

Effect of Con A and LPS on T Cells

As shown above, the thymocytes responded well to certain concentrations of Con A but not to LPS. However, it is known that only a small proportion of the thymocytes appear to have properties in common with peripheral T cells (18). In order to test whether peripheral T lymphoid cells, as well as T cells present in thymus respond in the same way as thymocytes, two types of experiments were performed. Peripheral T cells were prepared as "educated" T cells by inoculating le-

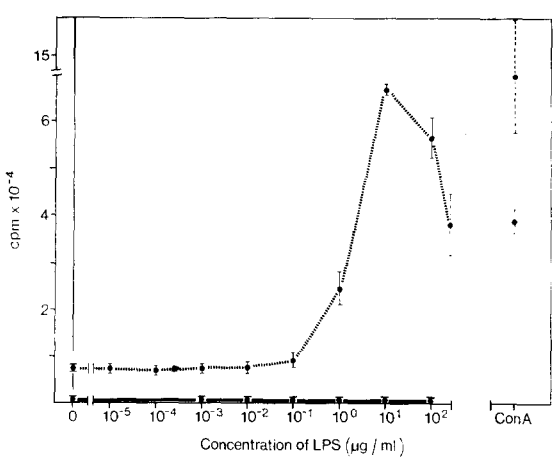

FIG. 4. Response of spleen cells (●– –); and thymocytes (●—) to various concentrations of LPS: The response to 5 μg/ml of Con A is indicated to the right.

thally irradiated F_1 hybrids with parental thymocytes in order to initiate a graft-versus-host reaction in the total absence of B cells. Seven days after inoculation the spleen cells were taken out and treated with various concentrations of Con A and LPS, respectively. As shown in Fig. 5, the educated T cells in the spleen responded to Con A, but not to LPS. The magnitude of the response was lower than that found with normal thymocytes, but the dose–response profile was similar. Thus, peripheral (and) activated T cells respond generally as thymocytes.

It has been demonstrated previously (10, 18) that cortisone eliminates about 90% of the thymus cells but the remaining 10% of the cells have all the immunological capacities of the untreated population. Therefore, it has been assumed that the cortisone resistant cells are the biologically functioning T cells; whereas the cortisone sensitive cells are immature precursors or cells destined to die. Thymocytes from cortisone-treated animals were treated in culture with Con A and LPS, respectively, and as shown in Fig. 6; Con A stimulated DNA synthesis in these cells, whereas LPS did not. The degree of response in the Con A-stimulated cultures were of the same order of magnitude as that found with spleen cells and about 10–20 times higher than that obtained with untreated thymocytes (Fig. 7). The dose–response profile to Con A in cortisone-resistant thymocytes was similar to that of normal thymocytes, but clearly distinguishable from the profile obtained with spleen cells. Thus, it is likely that the responding cells in the thymus are cor-

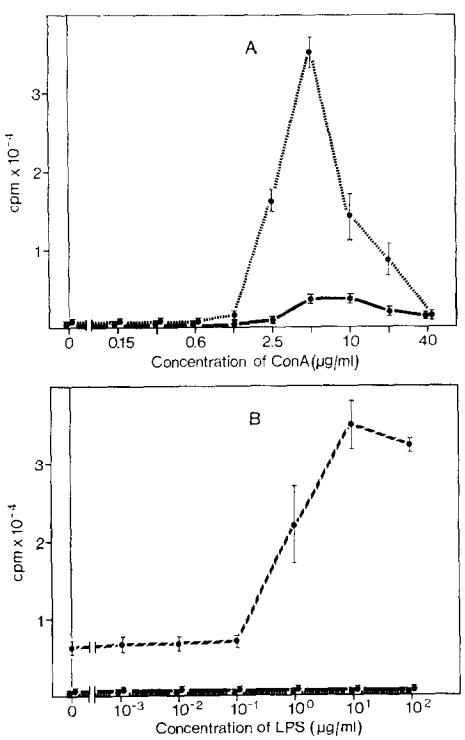

FIG. 5. Response of spleen cells (●--); educated T cells (●—); and thymocytes (●••); to Con A (A) and LPS (B).

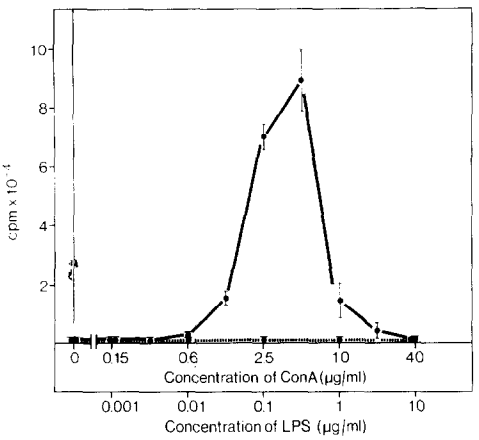

FIG. 6. Response of cortisone-resistant thymus lymphocytes to various concentrations of Con A (●—); and LPS (●--).

tisone resistant; whereas the responding cells in the spleen may be different, even though educated T cells in the absence of B cells respond to Con A.

Effect of Con A and LPS on B Cells

The findings reported clearly indicate that Con A is competent to activate T cells both in the thymus and in the periphery; whereas LPS cannot achieve this. In order to investigate the effect on B cells of these two mitogens in the absence of T cells, three types of experimental systems were used. In the first, spleen cells from normal animals were used and T cells eliminated by treating them with antitheta serum and complement, which is known to selectively kill T cells. In the second, adult mice were thymectomized and 2–3 weeks later lethally X-irradiated and repopulated with bone marrow cells, which had been pretreated with antitheta serum and complement to eliminate residual T cells present in the bone marrow. These animals will be referred to as T × B mice. Thirdly, congenitally athymic (nude) mice were used; and spleens from these animals were treated in culture

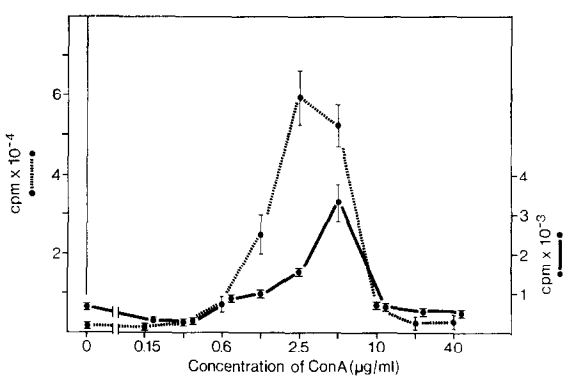

FIG. 7. Response of normal thymocytes (●—); and cortisone-resistant thymus lymphocytes (●--) to Con A.

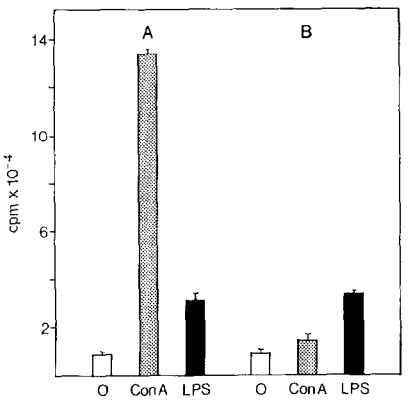

FIG. 8. Response of normal spleen cells (A); and anti-θ serum-pretreated spleen cells (B); to 5 μg/ml of Con A (shaded); and 10 μg/ml of LPS (filled); (0) control cultures (open).

with the two mitogens. These mice do not contain functional T cells. Antitheta-treated spleen cells responded poorly with regard to induction of DNA synthesis by Con A compared to normal serum-treated spleen cells. As a rule DNA synthesis was suppressed between 50 to 100% (Fig. 8). In contrast, LPS induced a marked degree of DNA synthsis in antitheta-treated spleen cells, the dose–response curve being analogous to that of a normal spleen (Fig. 9). The failure of Con A to stimulate antitheta-treated spleen cells was not due to a nonspecific cytotoxic effect also on the B cells, because such spleen cells responded normally to LPS.

Analogous experiments were performed with T × B mice; and the results were similar to those found with antitheta serum-treated spleen cells (Fig. 10). Thus, Con A failed to induce DNA synthesis in T cell-deprived spleen cells; whereas LPS activated the cells to the same degree as normal spleen cells. Spleen cells from

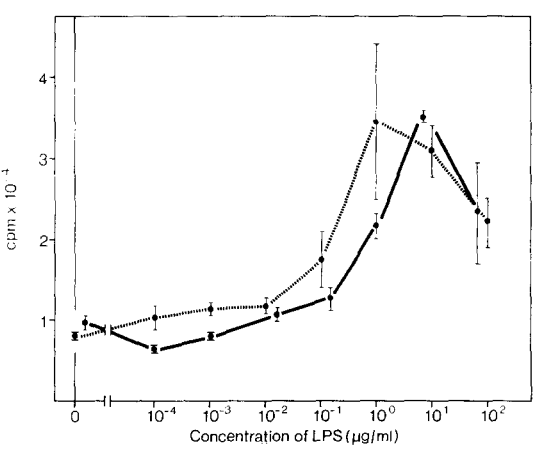

FIG. 9. Response of normal spleen cells (●—); and anti-θ serum-pretreated spleen cells (●--); to LPS.

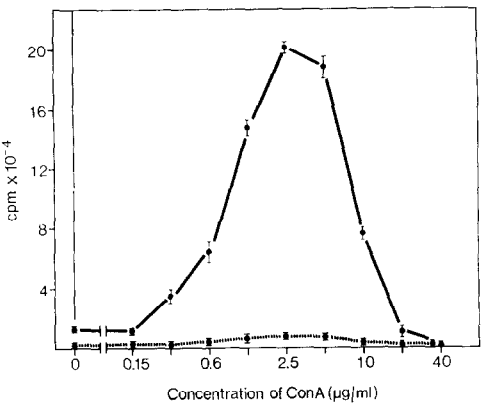

FIG. 10. Response of normal spleen cells (●—); and T × B spleen cells (● • •); to Con A.

nude mice were not activated by Con A; but LPS induced a normal response, the dose–response profile being similar to that found with normal spleen cells (Fig. 11).

Bone marrow cells from normal animals were also tested with Con A and LPS. Both mitogens induced DNA synthesis; but the total response of Con A-stimulated cultures were only $\frac{1}{10}$ of that found with spleen cells, suggesting that only a minor proportion of the bone marrow cells consists of T cells which can be stimulated by Con A. LPS induced a response as shown in Fig. 12.

Thus, it can be concluded that Con A fails to stimulate B cells obtained by three different procedures and only give a weak response in bone marrow cells, presumably because the latter contain contaminating T cells. Contrarywise, LPS stimulates B but not T cells. The response of the B cells to LPS was equally strong in T cells depleted, as in a mixed T and B population. The results are summarized schematically in Table 1.

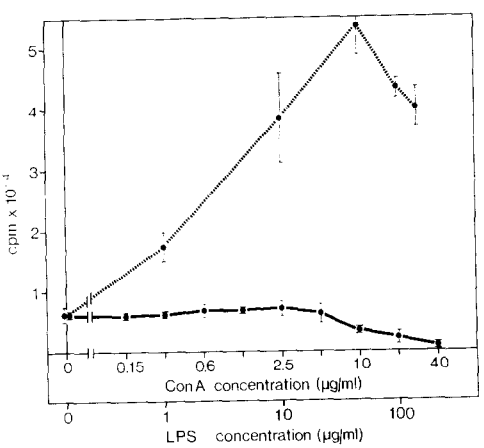

FIG. 11. Response of nude spleen cells to various concentrations of Con A (●—); and LPS (●- -).

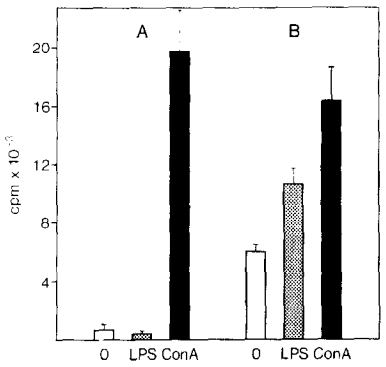

FIG. 12. Histogram showing the response of thymocytes (A); and bone marrow (B); to 5 μg/ml of Con A (filled); and 10 μg/ml of LPS (shaded); (0) control cultures (open).

Effect of LPS on Tolerant Cells

Because LPS is a T-independent antigen capable of activating immunocompetent B cells in the absence of T cells, it seemed possible that LPS activates B cells by combining with immunoglobulin receptors on B cells directed against the antigenic determinants of LPS. Therefore, tolerance was induced to LPS; and spleen lymphocytes from mice tolerant to LPS were treated with LPS *in vitro*. These cells exhibited a dose–response to LPS analogous to that obtained with normal spleen cells (Fig. 13). Thus, in this system, the proliferative response in spleen cell cultures to LPS is nonspecific in nature and independent of the immunologic specificities of the responding cells. This conclusion is further strengthened by the finding that polyvinylpyrrolidone (PVP), another T-independent antigen, failed to stimulate DNA synthesis in B cells or normal spleen cells in any dose.

TABLE 1

SUMMARY OF MITOGENIC EFFECTS OF CON A AND LPS ON DIFFERENT CELL TYPES

Cell source	Cell type	Stimulation by		Dose–response profile	
		Con A	LPS	Con A	LPS
Thymus	T	++	−	Narrow	−
Cortisone thymus [a]	T	++++	−	Narrow	−
Educated peripheral T cells [b]	T	+	−	Narrow	−
T × B spleen [c]	B	−	++++	−	Broad
Anti-θ-treated spleen	B	− (+)	++++	−	Broad
Nude spleen [d]	B	−	++++	−	Broad
Spleen	Mixed	++++	++++	Broad	Broad
Bone marrow	Mixed	+++	++++	Broad	Broad

[a] Thymocytes from cortisone treated mice.

[b] Spleen cells from irradiated F_1 mice inoculated 7 days previously with parental thymocytes.

[c] Spleen cells from thymectomized, irradiated mice repopulated with anti-θ-treated bone marrow cells.

[d] Spleen cells from congenitally athymic mice.

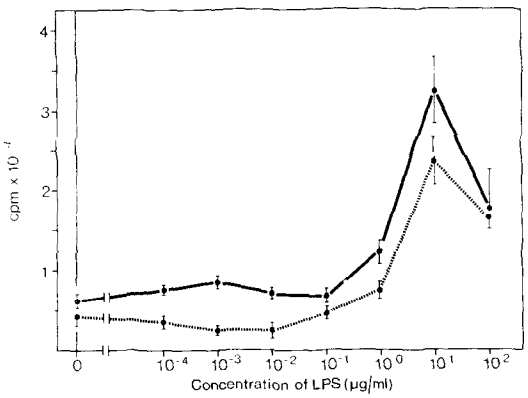

FIG. 13. Response of normal spleen cells (●--); and LPS-tolerant spleen cells (●—); to LPS at various concentrations.

DISCUSSION

The above results clearly demonstrate that Con A and LPS can be used as functional markers for T and B cells both in the central lymphoid organs (thymus and bone marrow) and in the periphery (spleen). Therefore, it is possible to detect T and B cells selectively in a mixed population and probably also quantitate the proportion of each type using Con A and LPS. Whereas the dose–response profile of B cells to LPS was identical in all experimental situations and organs studied, there appeared to be differences between T cells in the peripheral and central lymphoid organs with regard to their response to Con A. The difference was particularly marked between thymocytes and spleen cells. This need not necessarily indicate a difference between central and peripheral T cells. The fact that educated T cells in the graft-versus-host reaction responded as thymocytes tends to argue against this possibility. Alternatively, a mixed T and B population in which the T cells are activated by Con A may secondarily result in activation of B cells. Experimental findings supporting this possibility have been obtained (19) by the demonstration that T cells secrete factors that make B cells competent to respond to Con A, although the factors themselves have no effect on B cells.

The majority of the T cells in the thymus do not respond to Con A as judged by the quantitatively higher response obtained with cortisone-resistant thymocytes making up only about 10% of the total population. It seems likely, that only the cortisone-resistant cells respond to Con A.

The basis for the selective activation of T and B cells by different mitogens is poorly understood. In the case of Con A, it has been clearly demonstrated that both T and B cells have an equal number of receptors for Con A on the membrane and both are competent to bind Con A to the same extent (20). It follows that B cell activation by Con A requires more than binding of Con A to the surface. The mechanism of activation of B cell in the immune response has received particular attention, because of the T and B cell interaction necessary for triggering of B cells by some antigens. Three different models have been suggested for activation of B cells by antigen. In one, it is suggested that the inducing substance (antigen) by it-

self only can induce tolerance when it binds to the surface of the reactive B cells. To activate the cell, the antigen must interact with free antibody or receptors on T cells (21). This model explains the inability of Con A to activate B cells; but then, one must also assume that T cells can be activated in the absence of additional inducers. In the other model, activation of B cells by antigen is entirely quantitative (22–24). It requires a certain number of molecules bound for a certain time to achieve triggering (23). This may not be easily satisfied when the receptors on the cells have a low affinity for the inducer. Therefore, a variety of helper mechanisms may exist to decrease dissociation of inducer from the cell. Multiple bonding of the inducer to the cell would achieve this and T–B cell interaction can be viewed as resulting in better binding on the inducer to the B cell. Also antibody to the inducer would cause better binding. In a special alternative of this quantitative scheme it is suggested that cross-linking of the receptors are needed for activation to occur (25). In terms of this model, it can be assumed that T cells have a lower threshold for activation than B cells. However, this cannot be the explanation, because no level of Con A caused B cell activation. Cross-linking is unlikely for the same reason. It is possible though that the inducer must be presented to the B cell in a local high concentration which cannot be achieved by having soluble Con A in the medium. Actually recent findings suggest that high local concentration of Con A causes B cell activation (26). Finally, a third concept is that B cells require humoral factors secreted from the T cells to be activated. In terms of this hypothesis, B cells could never be activated by Con A in the absence of T cell. Strong support for this concept has been obtained recently by the demonstration that humoral factors from T cells make B cells respond to Con A (19). This latter hypothesis can also explain the differences in response to Con A by a mixed T and B cell population (spleen) and a pure T cell population (thymus).

A similar discussion could be made for the inability of LPS to stimulate T cells. However, presently, several basic facts are unknown with regard to LPS, such as the number of receptors on T and B cells; and, therefore, such a discussion would be premature.

It seems established that LPS can bind to lymphoid cells by two different mechanisms. Due to its antigenic characteristics it can attach to lymphocytes having specific immunoglobulin receptors directed against the antigenic determinants. Secondly, it can bind to other receptors on cells, which are not well characterized, but apparently are present on a variety of cells, including red cells. The latter binding property constitutes the basis for using this antigen in the Jerne agar plaque assay. It has been demonstrated previously that B cells contain immunoglobulin receptors for the antigenic determinants of LPS; whereas T cells lack them, even if the mice are preimmunized to the antigen (27). However, the immunoglobulin receptors are not likely to be responsible for the activation of DNA synthesis in the B cells for several reasons. The major part of the B cells appear to respond to LPS *in vitro;* whereas the frequency of antigen-sensitive cells to this antigen is 1 cell in 100,000 (3). Furthermore, induction of immunological tolerance to LPS, which results in suppressed ability to produce antibodies, did not alter the response to LPS *in vitro.* It has also been shown that LPS added to normal spleen cells in culture causes the development of antibody-producing cells against noncross-reacting heter-

ologous red cells (unpublished data). Finally, another antigen (PVP), which is also thymus independent and capable, therefore, of stimulating an immune response in B cells in the absence of T cells, was totally incapable of activating DNA synthesis in normal B cells.

ACKNOWLEDGMENT

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