INHIBITORY AND STIMULATORY EFFECTS OF CONCANAVALIN A ON THE RESPONSE OF MOUSE SPLEEN CELL SUSPENSIONS TO ANTIGEN

I. CHARACTERIZATION OF THE INHIBITORY CELL ACTIVITY

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A variety of plant lectins have been shown to bind to receptors on the surface of cells and in the case of some lymphocytes trigger these cells into new patterns of activity. The earliest of these to be studied was phytohemagglutinin (PHA)\(^1\) (1). This material, which is obtained as an extract of beans (*Phaseolus vulgaris*), has affinity for the sugar residue N-acetyl D-galactosamine or the galactose penultimate to N-acetylneuraminic acid (2). Concanavalin A (Con A) (3), which is obtained from the jack bean (*Canavalia ensiformis*), binds to \(\alpha\)-D-mannopyranosyl, \(\alpha\)- and \(\beta\)-D-glucopyranosyl, and \(\beta\)-D-fructofuranosyl groups (4–9). It has been shown to have a powerful mitogenic activity (10, 11). Con A exists as a tetramer with four subunits of a molecular weight of 27,000 with one saccharide binding site per monomer. Other forms may exist and it is not clear which forms have biological activity (12). Other plant mitogens include ricin, from castor beans, which binds \(\beta\)-D-galactopyranosyl residues (13) and pokeweed mitogen (PWM), from *Phytolacca americana*, whose specificity is unknown.

It has been shown that viral-transformed cells are more readily agglutinated by various plant mitogens (14, 15). The change in agglutinability appears to be due to a change in the distribution or accessibility of the mitogen binding site rather than to an increase in the number of sites (16–18). The increased agglutinability correlates with a loss of contact inhibition (15). Similar changes are observed when normal cells are treated with proteolytic enzymes (19, 20) or during mitosis (21). The release from contact inhibition in transformed cells can be reversed by treatment with trypsin-treated (monovalent) Con A (22).

Treatment of normal lymphoid cells with plant mitogens produces a variety of effects. Although all these mitogens appear to bind to the surface of all lymphoid cells in approximately equal amounts, their patterns of activity differ quite markedly (23, 24). Thymus-derived lymphocytes (T cells) of the mouse (23, 25, 26) and cells of the thymus medulla (27) are stimulated to blast transformation, increased thymidine

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\(^1\)Abbreviations used in this paper: ATXBM, adult, thymectomized, irradiated, bone marrow-protected; B cells, bone marrow- or bursa-derived lymphocytes; BSS, balanced salt solution; Con A, concanavalin A; goat anti-BA thymus antigen, goat anti-brain-associated thymus antigen; PFC, plaque-forming cells; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SRBC, sheep red blood cells; T cells, thymus-derived lymphocytes; TdR\(^{\text{3H}},\) tritiated thymidine; TNP, trinitrophenyl.
uptake, and cell division by PHA. Cells in the thymus cortex and bone marrow-derived cells do not respond although they have been shown to bind approximately the same amount of mitogen. There are on the order of 10^6 Con A binding sites on all lymphoid cells and thymidine uptake is maximally stimulated when 16–25% of the Con A sites are bound (23). It has been claimed that PWM (25, 26), lipopolysaccharide from Gram-negative bacteria (28), and insoluble preparations of Con A (29, 30) stimulate bone marrow-derived cells to blast transformation and increased thymidine uptake directly in the absence of thymus-derived cells. Mitogen-stimulated lymphocytes have also been shown to be stimulated to make a variety of products characteristic of thymus-derived lymphocytes. For example, it has been claimed that PHA-stimulated lymphocytes produce lymphotoxin (31–33), and that Con A-stimulated lymphocytes produce migration inhibition factor (34, 35). PWM-, Con A-, or PHA-stimulated lymphocytes have all been shown to be cytotoxic to labeled mouse fibroblast cultures (36–38) although the mechanism of action of PWM may be different from that of the other two (38).

The literature on the effect of plant mitogens on the immune response in vivo or in vitro is extensive but conflicting. The bulk of the evidence suggests that the administration of mitogen is inhibitory to the response to antigen. Rich and Pierce (39) have recently demonstrated that Con A inhibits the immune response in the mitogenic concentration range but produces some stimulation at lower concentrations. Yahara and Edelman (40) have shown that higher concentrations of Con A inhibit the “cap” formation induced in bone marrow-derived lymphoid cells (B cells) incubated with anti-immunoglobulin sera.

We recently received a number of unpublished manuscripts from Doctors Andersson, Sjöberg, and Möller which described a variety of experiments dealing with the effects of plant mitogens and bacterial endotoxins on the immune response. In one of these, now published, they have shown that Con A restored the immune response of mouse spleen cell suspensions from the thymus-deficient nude mice (41). We, therefore, decided to embark on a systematic study of the effect of plant mitogens on the response of mouse spleen cell suspensions to antigen in vitro in the hope that it would lead to some greater understanding of the mechanism of the triggering of B cells to proliferation and antibody synthesis.

We have been interested in the mechanism of T cell-B cell interaction and the possibility that the helper effect of T cells is mediated by a non-antigen-specific stimulatory factor that acts on B cells (42, 43). We argued that the plant mitogens must either act directly on the B cell or must trigger the T cell to synthesize and/or release the hypothetical thymus-derived mediator, which then acts on the B cell. In this paper we have clarified the nature of the conflicting inhibitory and stimulatory effects of Con A and have made a partial identification of the cell targets of Con A action.

Materials and Methods

**Animals.** BDF1 mice (C57BL/6 female × DBA/2 male) were bred in our own colony. Adult thymectomized, irradiated, bone marrow-restored (ATXBM) mice were also used.

**Antigens.** Sheep erythrocytes (SRBC) (Colorado Serum Company, Denver, Colo.) and
trinitrophenylated SRBC (TNP-SRBC) were prepared by the method of Rittenberg and Pratt (44) as modified by Kettman and Dutton (45). Concanavalin A, twice crystallized (code 79-001, lot No. 41), was obtained from Miles-Yeda Ltd., Miles Laboratories, Inc., Kankakee, Ill. It was stored at 4°C. Substocks were thawed for use and refrozen. There was no indication of any loss or change in activity over 3-4 wk. Tritiated thymidine (TdR-3H) was obtained from Schwarz Bio Research, Inc., Orangeburg, N.Y., at specific activity 11.3 Ci/m mole.

**Immunization.**—Immunization for T cell priming was by injection of 0.2 ml of a 0.1% v/v SRBC suspension in the tail vein 3 days before sacrifice (46). In vitro cultures received 3 × 10^6 SRBC or 3 × 10^6 TNP-SRBC/1 ml culture on day zero.

**Cultures.**—Mouse spleen cell suspensions were cultured at 1 × 10^7 cells/ml (unless otherwise indicated) by the method of Mishell and Dutton (47). Fetal bovine serum lot No. 722 obtained from Reheis Co., Inc., Berkeley Heights, N.J., was used throughout.

**Cell Recoveries.**—Cell suspensions were harvested at the end of the incubation period, centrifuged, and resuspended in balanced salt solution (BSS) for assay. Cell counts were expressed as per cent of the cells present at the start of incubation. No correction was made for cell viability. Cell viabilities were determined in some cases by trypan blue dye exclusion.

Thymidine incorporation was measured by the addition of 0.1 ml of BSS containing 1 μCi of TdR-3H in 0.5 μg of TdR.

**Goat Anti-Brain-Associated Thymus Antigen Serum (Goat Anti-BA Thymus Antigen).**—Spleen cells were treated with anti-BA thymus antigen serum and guinea pig complement sequentially to remove T cells (46). The antiserum was prepared in goats as described by Golub (48). 10^6 spleen cells in 2 ml of BSS (47) were incubated with 1.0 ml of a 1:18 dilution of antiserum in BSS for 30 min at 37°C. The cells were then washed twice and resuspended to the original volume in BSS. 1 ml of guinea pig serum (six times absorbed with spleen and thymus and once with heterologous erythrocytes) diluted 1:4 was added and incubated for 30 min at 37°C. The cells were then washed three times in BSS and resuspended in complete medium at 10^7/ml. This treatment killed 95% of thymus cell suspensions at antiserum dilutions down to 1:30 and 30-40% spleen cells between 1:10 and 1:60 dilution of antiserum.

**RESULTS**

### The Effect of Concanavalin A on Normal Spleen Cells.

Cell suspensions from the spleens of normal mice were cultured at 1 × 10^7 cells/ml in the presence of 3 × 10^6 SRBC. Varying amounts of concanavalin A (Con A) were added at zero time and the number of direct anti-SRBC plaque-forming cells (PFC’s) was determined at day 4. The following points are illustrated in Fig. 1. There was a marked depression of the response at concentrations of 1-4 μg/ml of Con A with maximum inhibition at 2 μg/ml. At 8 μg/ml there was less inhibition. At concentrations above 8 μg/ml Con A again inhibited the response. This biphasic curve was seen in all of many experiments. It can be seen in Fig. 2 that the response to the hapten TNP coupled to SRBC as carrier was similarly affected by Con A. This was also true for the anti-TNP and anti-SRBC responses of carrier-primed mice (injected with low doses of SRBC 3 days before sacrifice).

Con A also has a marked effect on cell recovery (Figs. 1 and 2). In the control cultures the cell recovery represents 50% of cells present at the start of incubation. This is increased in the presence of Con A over the range of 1/4-4 μg/ml and the cell recoveries have ranged from 60 to 150% of the initial number. The position of the curve of increased cell recovery corresponds with the
inhibitory effect on the PFC response, but is a somewhat broader, flatter curve. The increased cell recovery is presumably the consequence of the mitogenic properties of Con A and the uptake of thymidine-$^3$H is also increased in the same concentration range (Fig. 1). At higher Con A concentrations, corresponding to the second range of inhibition of the PFC response there is a marked fall in cell recovery to levels below that seen in the control cultures and an inhibition of thymidine-$^3$H uptake. Cell viabilities were measured in three consecutive experiments and the percentage of dye-excluding cells was significantly lower in the Con A-stimulated cultures (Table I) and the total number of viable cells was no different from that in the control. Qualitative examination of stained preparations showed a marked increase in the number of blast cells in the Con A-stimulated cultures.

Kinetics of PFC Response in the Presence of Con A.—The inhibitory effect of Con A added at time zero becomes more marked with time and the increase in cell recovery becomes dramatic only on day 4. The effect of the time of addition

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**Fig. 1.** The effect of Con A on spleen cell suspensions from normal mice. The Con A was added at the concentrations indicated at zero time ($T = 0$). All assays were made at day 4. $\bigcirc - \bigcirc$, the response to SRBC in PFC/culture; $\triangle - \triangle$, the cell recovery expressed as per cent of cells present initially; $\bullet - \bullet$, the incorporation of thymidine-$^3$H present during the last 16 hr of culture (80-96 hr) expressed as cpm per culture.
The effect of Con A on the response to hapten and carrier antigen. Spleen cell suspensions from normal mice were incubated with the indicated concentrations of Con A added to T = 0. All assays were made at day 4. ○—○, anti-SRBC PFC/culture; ■—■, anti-TNP PFC/culture; △—△, cell recovery as per cent of cells present initially.

**TABLE I**

| Expt. No. | Control cultures | Con A stimulated (2 μg/ml) |
|-----------|------------------|-----------------------------|
|           | Recovery | Viability | Total | Recovery | Viability | Total |
| 94        | 57      | 69        | 39    | 77       | 55        | 42    |
| 95        | 52      | 62        | 32    | 73       | 43        | 32    |
| 96        | 57      | 68        | 39    | 74       | 52        | 38    |

of Con A is shown in Fig. 3. The ability of Con A to inhibit the response was lost after only 5 hr of culture. Con A was stimulatory when added at 24 hr.

**Reversal of Con A Effects with α-Methyl d-Mannopyranoside.**—The addition of α-methyl d-mannopyranoside alone to cultures had little effect on the anti-SRBC measured at day 4 to concentrations of 10 mg/ml. There was a 50–75% inhibition at 20 mg/ml (see Fig. 4). Increasing concentrations of the sugar increasingly suppressed the inhibitory effect of 2 μg of Con A. There was almost
complete suppression at 10 and 20 mg/ml. The sugar also blocked the stimulatory effect of Con A on cell recovery.

The Effect of Con A on the Response of Goat-Anti-BA Thymus Antigen Serum-Treated Spleen Cell Suspensions.—The response of goat antiserum-treated spleen cell suspensions of normal mice is depressed. The addition of Con A at zero time restores the response, Fig. 5. The restoration of the response was achieved by the same concentrations of Con A that inhibited the normal response. In other experiments higher concentrations of antisera were used in the preparation of the treated cell suspensions (Fig. 6). It can be seen that T cell “helper” activity was lost at concentrations of antisera that did not prevent the restoration seen on adding Con A.

The Effect of Con A on the Responses of ATXBM Mice.—The in vitro responses of a number of ATXBM mice were depressed to varying degrees, ranging from 90% to no depression at all. The depressed responses were completely restored by Con A. In addition, those ATXBM mice that had no depression in their response nevertheless responded differently from normal mice in the presence of Con A in that they showed no inhibition at 2 \( \mu g/ml \), Fig. 7.

In contrast to all previous effects of Con A, the dose-response curve did not
show a marked peak of activity at 2 μg/ml. Con A seemed to be equally effective at restoring the ATXBM-depressed responses over a wide concentration range from 1/4 up to 4 μg/ml (see Fig. 7). The cell recovery was increased by Con A in three out of four cases.

The Effect of Adding Con A-Treated Cells to Normal Cells.—We have seen above that the same concentration of Con A that restores the response of goat antiserum-treated cells inhibits the response of normal cells. One possible explanation of this finding was that Con A stimulated T cells to produce a factor that in low concentrations stimulated the B cells but in higher concentrations was inhibitory. In order to test this, normal spleen cell suspensions were incubated with inhibitory concentrations of Con A for 48 hr. They were then washed twice in BSS and graded numbers of these cells were added to fresh normal cells in the absence of Con A. It can be seen from Fig. 8 that even small numbers of Con A-stimulated cells inhibited the response of normal cells. Also shown is the effect of the addition of cells incubated in the absence of Con A as a control.

The Effect of Irradiation on the Inhibitory Effects of Con A.—An alternative
The effect of Con A on ATXBM and the loss of the inhibitory effect on pre-incubation noted above lends some support to the hypothesis that such mice lacked a short-lived, radiosensitive T cell, mediator of the inhibitory effect, but retained a second cell, mediator of the stimulatory effect on the B cell response. We therefore tested the effect of irradiation on the Con A-mediated effect in the following manner. Goat antisera-treateed cell suspensions were incubated with varying numbers of normal or irradiated normal spleen cells in the presence of Con A at 2 µg/ml (Fig. 9). The response of the goat antisera-treated cells was restored by the addition of Con A as seen in previous experi-
ments. The addition of increasing numbers of normal cells resulted in increasing inhibition of the cultures. When irradiated normal cells were added, however, the response of the goat antiserum-treated cells was unaffected. Cell recoveries were also measured. In either case there was a marked increase in the cell recovery which was only slightly greater in the presence of the normal cells. Irradiation thus blocked the inhibitory effect of Con A-treated cells. This experiment does not reveal whether the stimulatory effect is radioinsensitive since the goat antiserum-treated cells are restored by Con A alone without the addition of irradiated normal cells.

**DISCUSSION**

The addition of Con A to spleen cell cultures of normal mice inhibited the response to erythrocyte antigens, stimulated the incorporation of tritiated thymidine, and led to an increased cell recovery. The dose-response curve for each of these parameters was the same with a narrow peak of activity at 2 μg of Con A/ml. Fourfold higher concentrations had less effect while still higher concentrations appeared to be nonspecifically toxic. It is of interest that responses to antigen are not inhibited by concentrations of Con A that are high enough to give some inhibition of capping of cell surface immunoglobulins (40). The mitogenic effect and the inhibition of the response to antigen could both be blocked by the presence of sufficient α-methyl D-mannopyranoside. A vast
FIG. 7. Effect of Con A on the response of four ATXBM mice spleens to SRBC. Spleen cell suspensions from four individual ATXBM mice were incubated with SRBC and various concentrations of Con A added at $T = 0$. The number of PFC/culture and the cell recoveries were assayed at day 4. The results obtained with four separate mice are illustrated $\bigcirc$, $\triangle$, $\blacktriangle$, $\diamond$. A molecular excess of sugar was needed to achieve a 50% block (approximately $2 \times 10^{22}$ molecules of sugar, $5 \times 10^{16}$ molecules of Con A monomer, and $10^{14}$ Con A binding sites). The inhibitory effect of Con A was equally apparent for the response to hapten and for the response of spleens from carrier-primed mice.

A number of procedures appeared to remove a cell or cell activity that Con A stimulated to produce the inhibitory effect. In the absence of the inhibitory cell effect Con A was seen to stimulate the response to antigen. Thus, if Con A was added to the spleens of normal mice after 24 hr preincubation, a twofold stimulation of the response was routinely observed. The addition of Con A to spleen cells suspensions treated with goat anti-BA thymus antigen restored the response to antigen. The dose-response curve again showed a sharp peak of activity at 2 $\mu$g/ml. The response of spleen cell suspensions from ATXBM mice was restored to above normal values by Con A. It was curious that the dose-response curve for this last effect was markedly different in that it did not show a narrow peak of activity at 2 $\mu$g/ml but rather a broad range of activity from 0.25 to 4 $\mu$g/ml (Fig. 8).

The radiosensitivity of the cell mediating the inhibitory effect was demon-
Fig. 8. Effect of adding Con A-treated cells to normal mouse spleen cell suspensions. Spleen cell suspensions from the spleens of normal mice were incubated with or without 2 μg/ml Con A for 48 hr. They were then washed two times in 10 ml of sterile BSS and resuspended in complete medium at 1 × 10⁷/ml. Graded amounts of the resulting suspensions were added to suspensions of fresh normal mouse spleens keeping the total cell number constant at 1 × 10⁷/ml. SRBC were added at T = 0 and the anti-SRBC PFC response was measured at day 4. Cells preincubated with Con A (O—O) and without (△—△).

strated in the experiment where normal or normal, irradiated cells were added to goat anti-BA thymus antigen-treated cells in the presence of Con A. The results are compatible with the hypothesis that at least two cells respond to the presence of Con A. The first cell, which mediates the inhibitory effect is rapidly lost in culture or at least changes in its response to Con A. It is radiosensitive and is eliminated by treatment with goat anti-BA thymus antigen. Its properties are therefore similar to those of T1 cells described by Raff and Cantor (49).

It may also be the same cell that mediates the Con A-stimulated cytotoxic effects in the experiments of Stavy et al. (37) and of Ginsburg et al. (38). However, Perlmann et al. (50) have shown that Con A-treated human lymphocytes are no longer stimulated to cytotoxic activity by incubation with PHA. It is possible that the inhibitory effect of Con A on the response to antigen is not mediated by a cytotoxicity effect but by some other mechanism.

The inhibitory effect does seem to be mediated by some active process of one cell on another since small numbers of Con A-treated cells inhibit the response of fresh normal cells (Fig. 8). There is also evidence that the cytotoxic activity that is expressed after treatment with mitogens is the property of a different cell
Fig. 9. Effect of irradiation on the inhibitory effect of Con A on the response to antigen. Spleen cell suspensions from normal mice were treated with goat antisera to remove most T cells. The response of the treated cells was restored by the addition of 2 μg/ml Con A. Graded numbers of either normal (△--△) or irradiated normal cells (○--○) were added keeping the total cell number constant at 1 × 10^7/ml. SRBC were added at T = 0 and the anti-SRBC response was assayed at day 4.

from the one that responds by blast transformation and proliferation. Thus, in the experiments referred to above (50) Con A does not block the proliferative response to PHA and Stites et al. (51) have shown that thymus cells of the human fetus respond to PHA by proliferation but exhibit no cytotoxic activity against xenogeneic cells while the reverse pattern of response was observed in cells from fetal bone marrow. In the face of evidence for such extensive heterogeneity in lymphocyte populations it would be unwise to draw any firm conclusions as to the nature of the inhibitor cell.

The second cell cannot be identified on the basis of the data provided in this paper. It would appear to replace the function of the helper T cell in the B cell response. Con A does not appear to act directly on B cells (41). It is therefore possible that it is another T cell which is triggered to produce a factor that allows the B cell to respond to antigen but other possibilities cannot be excluded at this time.

*Watson,* J., R. Epstein, I. Nakoinz, and P. Ralph. The role of humoral factors in the initiation of *in vivo* primary immune responses. II. Effects of lymphocyte mitogens. *J. Immunol.* In press.
SUMMARY

The presence of concanavalin A (Con A) inhibits the immune response of mouse spleen cell suspensions to erythrocyte antigens, stimulates the incorporation of tritiated thymidine, and increases cell recovery. Con A also restores the depressed response of cell preparations treated to remove thymus-derived cells. The dose-response curve for all four effects shows peak activity at 2 µg/ml. The depressed in vitro response of spleen cell suspensions from adult thymectomized, irradiated, bone marrow-restored mice is also restored by Con A. Here the dose-response curve is quite different with activity over a much wider range of concentration. The restoration of thymus-derived cell-depleted cultures by Con A is inhibited by the addition of untreated, unirradiated, mouse spleen cell suspensions, but is not inhibited by untreated, irradiated cells.

Small numbers of spleen cells that have been preincubated with Con A and washed will inhibit the response of fresh, untreated cells to antigen. If the mouse spleen cell suspensions are incubated for 24 hr before the addition of Con A, the response to antigen is no longer inhibited but is stimulated instead.

The data are compatible with the hypothesis that there are at least two cell targets for the action of Con A. One cell, that mediates the inhibitor effect, is a short-lived, radiosensitive, thymus-derived cell. The other cell, that mediates the stimulating effect, cannot be identified from the data presented here but may also be of thymus origin on the basis of studies by other investigators.

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