

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

II. EVIDENCE FOR SEPARATE STIMULATORY AND INHIBITORY CELLS

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The addition of concanavalin A (Con A)¹ to spleen cell cultures can give rise to both stimulatory and inhibitory effects on the humoral response of mouse spleen cell suspensions to erythrocyte antigens. These mitogen-triggered effects may represent the mitogen-stimulated activity of cells having a physiological role in the regulating of the immune response.

Evidence is presented in support of the hypothesis that these effects are mediated by two separate subpopulations of thymus-derived cells and against the concept that inhibition is caused by supraoptimal stimulation. Some other hypotheses are also discussed.

We have shown previously that the presence of Con A inhibits the response of mouse spleen cell suspensions to erythrocyte antigens, stimulates the incorporation of tritiated thymidine and increases cell recovery (1). Similar observations have been made by Sjöberg et al. (2), Watson et al. (3), and by Rich and Pierce (4, 5). Con A also restores the depressed response of cell preparations treated to remove thymus derived cells (1, 2). In our hands the dose-response curve for all four effects shows peak activity at 2 μ g/ml although other authors have observed minor departures from this.

The depressed in vitro response of spleen cell suspensions from adult thymectomized irradiated, bone marrow-restored mice (ATXBM) is also restored by Con A (1, 2). 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 (1).

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 h before the addition of Con A, the response to antigen is no longer inhibited but is stimulated instead (1).

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; PFC, plaque-forming cells; PHA, phytohemagglutinin; SRBC, sheep red blood cells; T cells, thymus-derived lymphocytes.

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 stimulatory effect has not been unequivocally identified.

Materials and Methods

Animals and Reagents.—BDF₁ mice (C57Bl/6 female × DBA/2 male) and mice homozygous for the mutation nude (6) were bred in our own colony. The nude mice were bred from stock kindly donated by Dr. J. Watson of the Salk Institute (see Watson et al. [3] for further details). In one experiment, see text, heterozygous nudes were used. ATXBM BDF₁ mice were used in some experiments. Con 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. Bacto Phytohemagglutinin-P (PHA) was obtained from Difco Laboratories, Detroit, Mich.

Immunization.—In vitro cultures received 3×10^6 SRBC culture on day 0.

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

RESULTS

Restoration of the Immune Response of Spleen Cell Suspensions from Nude Mice by the Addition of Graded Numbers of Normal and Irradiated Normal Spleen Cells in the Presence of Con A.—The data in Table I confirm the findings of Watson et al. (3) that the addition of Con A to spleen cell suspensions from nude mice does not restore their ability to respond to antigen. A variety of Con A concentrations and times of addition were tested. The response could be restored, however, by the addition of irradiated allogeneic spleen cells as a source of T-cell activity in the presence of Con A at 2 μg/ml. In these experiments the allogeneic spleen cell suspensions (in most cases from BDF₁ mice) were irradiated

TABLE I
Effect of Con A on the Response of Spleen Cell Suspensions from Nude Mice

Cell suspensions	Nude	Nude	Nude + <i>N</i> Irrad.
Additions:			
None	140	140	125
Con A (μg/ml) 8	0	156*	140
4	0	145*	655
2	70	65*	1120
1	63	65*	960
½	50	45*	840

The figures represent the number of anti-SRBC PFC per culture measured at day 4. All cultures contained 8×10^6 nude spleen cells. *N* irradi. = 2×10^6 irradiated BDF₁ spleen. The irradiation dose was 800 R.

* These cultures received Con A at 24 h. All other Con A additions were at time zero.

to reduce the inhibitory activity previously demonstrated in unirradiated spleen cells (1).

In further experiments a test was made to distinguish between the hypothesis that Con A acted directly on B cells in the nude spleen cell suspension but that the response still required the presence of T cells or, alternatively, that Con A acted directly on a cell present in the irradiated normal population and thus indirectly stimulated the B cells.

The cell suspensions from the nude and normal mice were incubated separately from the first 24 h. They were then washed and the two suspensions combined and incubated together for a further 3 days. Con A was added either to the nude spleen or the BDF₁-irradiated spleen during the first 24 h only. No added Con A was present after the suspensions were combined. It can be seen from Table II that only when the irradiated BDF₁ were exposed to Con A was there a significant response (line B versus line A).

In a second experimental design varying numbers of normal or irradiated cells were added to a constant number of spleen cells from nude mice in the presence of 2 $\mu\text{g}/\text{ml}$ Con A (Fig. 1). Very low numbers of unirradiated cells produced some restoration of the response but this restoration reaches a plateau at approximately 10^6 added cells. Small numbers of irradiated cells were less effective but no plateau was seen with increasing cell numbers. It is important to note that the restoration seen with optimal numbers of irradiated cells was five times that seen with optimal numbers of unirradiated cells.

Fig. 2 shows the effect of irradiation dose on the restoration of the response of nude spleens by cells from the spleens of (in this experiment) heterozygous nude mice. Increasing doses of irradiation shift the curve of stimulation seen

TABLE II
Preincubation of Con A with Spleen Cells from Nude Mice or with Irradiated Spleen Cells from BDF₁ Mice

Exp.	Anti-SRBC PFC/culture (day 4)			
	110	161	162	167
(A) Con A preincubated with nudes	15	20	0	25
(B) Con A preincubated with irradiated BDF ₁	175	95	80	370
(C) Con A present entire time nude + BDF ₁ irradiated	770	685	295	255
(D) Nude alone	15	95	20	30
(E) BDF ₁ irradiated alone	0	0	0	0

In (A) 5×10^6 spleen cells from nude mice were incubated with 2 μg in 1 ml culture medium for 24 h. The cells were then washed twice in 50 ml sterile BSS and then incubated for a further 3 days with 5×10^6 irradiated spleen cells from BDF₁ mice. In (B) the same procedure was followed except that it was the irradiated BDF₁ spleen cells that were exposed to Con A during the first 24 h. In (C) 2 $\mu\text{g}/\text{ml}$ Con A was incubated with 5×10^6 nude spleen and 5×10^6 irradiated BDF₁ spleen for the entire 4 days. (D) and (E) are the controls, 1×10^7 nude spleen or 1×10^7 irradiated BDF₁ spleen incubated alone.

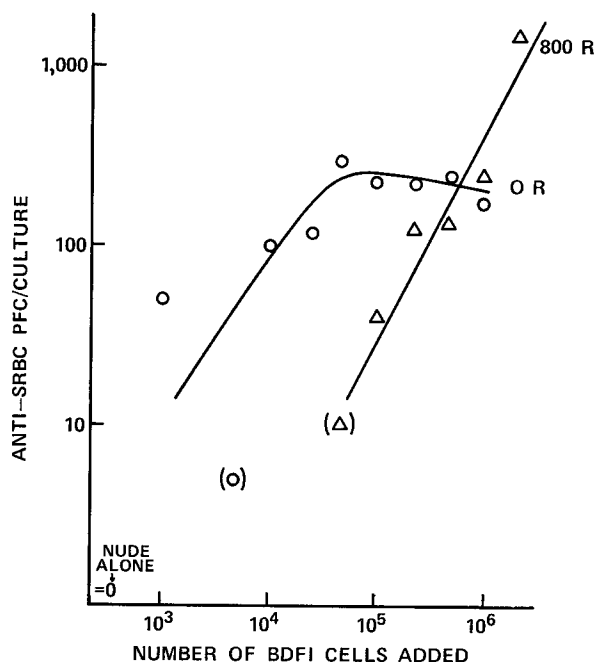


FIG. 1. Restoration of the response of nude spleen with Con A-stimulated cells. All cultures contained 8×10^6 spleen cells from nude mice, SRBC, and $2 \mu\text{g/ml}$ Con A. To these were added graded numbers of irradiated (Δ) or unirradiated (\circ) spleen cells from BDF₁ mice. The irradiation dose was 800 R. The numbers of anti-SRBC PFC per culture were assayed on day 4. Control cultures of 8×10^6 nude spleen cells in the absence of Con A developed 65 PFC/culture by day 4 (Exp. 171).

with small numbers of cells to the right and eliminates the plateau seen with higher numbers of cells. It would appear that both the stimulatory effects, seen at low added cell numbers and the inhibitory effects seen at high added cell numbers are radiosensitive.

Effect of Con A and PHA on the Response Spleen Cell Suspensions from Normal and ATXBM Mice.—We have previously shown that the addition of Con A at zero time to spleen cell suspensions from ATXBM mice stimulated rather than inhibited the response (1). The effect was in general more striking if the Con A was added 24 h after the start of incubation (Table III). Similar stimulatory effects had also been seen when Con A was added at 24 h to spleen cells from normal mice (1). Thus, the ability to inhibit the response is lost by 24 h.

PHA will inhibit the response of normal cells if added at zero time (Fig. 3). It is less inhibitory if added at 24 h. PHA fails to restore the response of spleen cells from ATXBM added at either 0 or 24 h (Table III and Fig. 3). On the other hand, it does not inhibit the residual response of ATXBM mice. In addition, and in contrast to Con A, there is no concentration of normal cells which

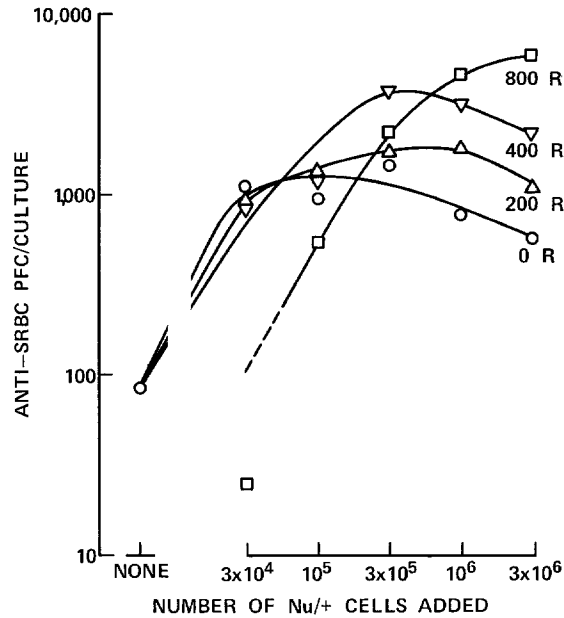


FIG. 2. Effect of irradiation dose on the restoration of the response of nude spleen. The details of this experiment are the same as those described for Fig. 1, except that in this experiment the added spleen cells were from heterozygous nude mice instead of BDF₁. These added cells received zero R (○), 200 R (△), 400 R (▽), or 800 R (□). All cultures contained 8×10^6 nude spleen cells + 2 $\mu\text{g/ml}$ Con A (Exp. 184).

TABLE III
Effect of Con A and PHA Addition at 0 or 24 h on the Immune Response of Spleens
from ATXBM Mice

Expt.	120	122	124	223	232	234b
No addition	345	130	390	1120	6560	1680
+ Con A at 0 h	1000	2160	2220	7200	5860	3420
+ Con A at 24 h	—	—	—	4840	10620	10080
+ PHA at 0 h	30	145	305	110	2040	615
+ PHA at 24 h	—	—	—	2000	9140	2360

In these experiments spleen cell suspensions were incubated at 1×10^7 cells/ml and Con A at 2 $\mu\text{g/ml}$ and PHA at a final dilution of 1,500 (see Materials and Methods) were added at the times indicated. The numbers represent the anti-SRBC PFC per culture measured at day 4.

in the presence of PHA will restore the response of spleen cells from nude mice (Fig. 4).

DISCUSSION

Our previous studies (1) and those of other workers (2-5) have shown that Con A can exert both inhibitory and stimulatory effects on the immune re-

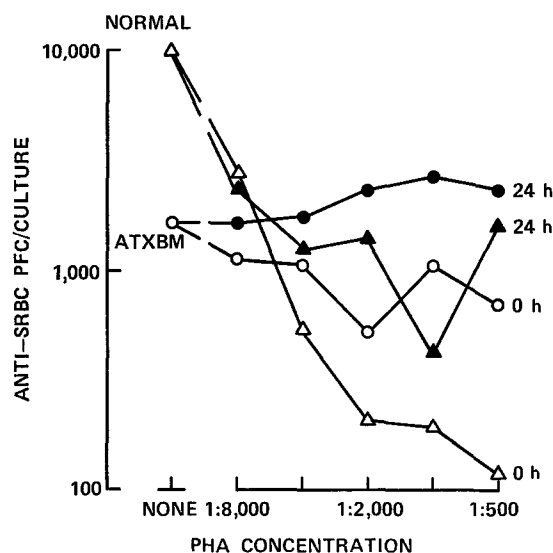


FIG. 3. Effect of PHA on the response of normal and ATXBM spleen. 10^7 spleen cells in 1 ml from normal (Δ) or ATXBM (\circ) mice were incubated with SRBC and the indicated concentrations of PHA. One vial of Bacto Phytohemagglutinin P (Difco) was dissolved in 5 ml of sterile BSS. The concentration of PHA is expressed as the final dilution of this stock solution in the culture medium. PHA was added at time zero (open symbols) or at 24 h (closed symbols). The number of anti-SRBC PFC were determined on day 4. 200 PFC per culture were obtained when Con A was added to the cells from normal mice at zero time and 3,660 at 24 h. The corresponding figures for ATXBM mice were 3,420 and 10,080 (Exp. 234).

sponse. Inhibition occurs when mitogenic doses of Con A are added to whole spleen cell suspensions. In the case of Rich and Pierce, stimulation was achieved by the use of submitogenic doses of Con A (in the range of 0.02–0.1 $\mu\text{g}/\text{ml}$) while the other investigatory demonstrated stimulation using variously T-cell-depleted cultures.

Stimulatory effects could be shown in irradiated or anti- θ -treated, spleen cell suspensions or in cell suspensions that were incubated for 24 h before the addition of mitogenic doses of Con A.

These observations raise the possibility that there are two cell targets of Con A stimulation—one that is activated to inhibit the immune response and a second which is activated to stimulate the response. On this hypothesis the inhibitory cell can be described as a thymus-derived radiosensitive cell, short lived in culture, which in some way inhibits the response of other cells to antigen. The stimulatory cell would also appear to be thymus derived since Con A does not restore the response of spleen cells from nude mice (reference 3 and Table I).

It was possible, however, that Con A acted on some other cell target present in the spleen cell population from nude mice but that T cells were still required to complete the system for a response to occur. The experiment described in

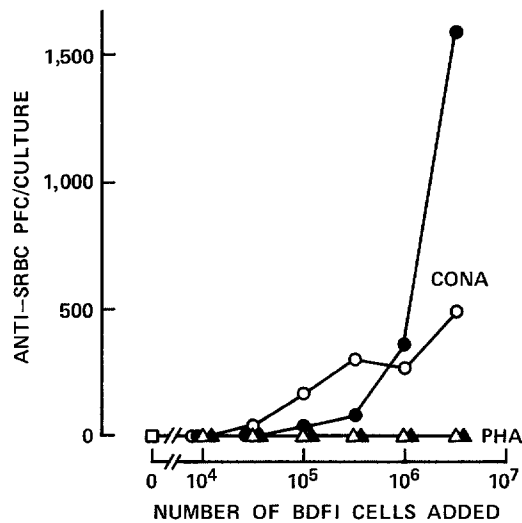


FIG. 4. Restoration of the response of nude spleen with Con A or PHA stimulated cells. The details of this experiment are similar to those described in Fig. 1. Graded numbers of unirradiated (open symbols) or irradiated (closed symbols). BDF₁ cells were added to 8×10^6 spleen cells from nude mice and SRBC in the presence of $2 \mu\text{g}/\text{ml}$ Con A (O) or 1:500 PHA (Δ) added at zero time. The irradiation dose was 800 R. The number of anti-SRBC PFC were determined on day 4 (Exp. 187).

Table II would seem to exclude this possibility. In this experiment, the two cell suspensions, nude spleen or irradiated BDF₁ spleen are incubated separately in the presence or absence of Con A for the first 24 h before being washed and combined to complete the responding system. Only when the irradiated BDF₁ cells are preincubated with Con A does a response occur.

A number of alternatives to the two cell hypothesis can be proposed. The simplest of these is that there is a single cell which is stimulated by Con A to make a single product. This product is hypothesized to stimulate the response to antigen when present in low concentrations and inhibit the response when present in higher concentrations. Much of the evidence presented above is consistent with the two cell hypothesis is also compatible with the single cell-single product hypothesis. In short all the procedures that convert inhibition to stimulation (irradiation, treatment with anti- θ serum, taking the spleens from ATXBM mice, incubation for 24 h before addition of Con A) can be argued to work because they reduce the numbers of a single cell from an inhibitory to a stimulatory number. Two lines of evidence presented here argue against this interpretation.

(a) The data illustrated in Figs. 1 and 2 show the effect of titrating the stimulation when unirradiated or irradiated BDF₁ spleen cells are added to spleen cell suspensions from Nude mice in the presence $2 \mu\text{g}/\text{ml}$ Con A. Although

stimulation is obtained with smaller numbers of unirradiated cells than with irradiated, the magnitude of this stimulation is always less than 20% of that which can be obtained with irradiated cells. If irradiation were merely reducing the numbers of a single responding cell than the maximal stimulation obtained with unirradiated cells should have been at least as high as that seen with larger numbers of irradiated cells. The data are compatible with the hypothesis of two cells with antagonistic effects (stimulation and inhibition) and that both are radiosensitive. At low cell numbers where the stimulatory effect is stronger than the inhibitory effect, irradiation of the added cells reduces the response. At higher cell numbers where the inhibitory effect is reducing the size of the stimulatory effect irradiation causes an increase in the net response. This effect is illustrated in Fig. 2.

Because we can only measure the net effect rather than the two components it is not possible to obtain the information necessary to calculate the irradiation dose-response curve for the hypothetical stimulatory and inhibitory cell.

A similar conclusion has been reached by Rich and Pierce (8). They showed that the inhibitory activity of Con A-stimulated lymphocytes could be mediated by a supernatant fraction. Low concentrations of this inhibitory material did not stimulate the response.

(b) The second line of evidence comes from the differential effect of Con A and PHA on the stimulatory and inhibitory effects. Stobo and Paul (*see* reference 9 for a review) have shown that the T-cell population can be divided into subsets on the basis of the proliferative response to those two mitogens. Several other properties have been correlated with the PHA and Con A reactivity but will not be discussed here. In essence it would appear that there is one subset which responds to Con A only and another that responds to PHA and/(or?) Con A. In the experiments described in this paper it was shown that PHA was equally as effective in inhibiting the immune response as Con A, when added to spleen cell suspensions from normal mice (Fig. 3). It was also much less inhibitory under the same experimental conditions where inhibitory activity was no longer elicited by Con A. Thus, it failed to inhibit the response of spleen cells from ATXBM mice when added at 24 h and was only slightly inhibitory when added at zero time (Table III) it was less inhibitory to spleen cells from normal mice when added at 24 h compared to the effect it had when added at zero time (Fig. 3).

On the other hand, there was only at the most a very marginal stimulation of the response of spleens from ATXBM mice and there was no concentration of irradiated BDF₁ spleen cells which would restore the response of spleen cells from Nude mice in the presence of PHA. The data are thus compatible with the hypothesis that there is a stimulator cell which responds to Con A but not PHA and inhibitor cell that responds to both Con A and PHA. They are incompatible with a one cell-one product hypothesis providing that the assumption is made that PHA and Con A both activate cells in the same way. It is not

clear whether this assumption is valid. It should also be noted that although the difference in PHA and Con A effects parallels the heterogeneity observed by Stobo and Paul, it does not mean that these two sets of properties can necessarily be "mapped" on to the same pair of cells. Stobo and Paul measured the mitogen-stimulated increase in thymidine uptake while we have measured the mitogen-induced stimulatory and inhibitory effects on the immune response to antigen. These may be mediated by soluble factors that could be synthesized in the absence of DNA synthesis and cell division.

Although not completely conclusive, the weight of evidence would seem to be in favor of two cells, one inhibitory and one stimulatory.

A number of additional points remain to be made. Although there would seem no good evidence for a thymus-derived cell capable of being stimulated to inhibit other cells in a "trans" manner, the evidence for the existence of stimulatory cells capable of a similar stimulatory effect is less conclusive. It is possible to replace the requirement for T cells of nude spleens with allogeneic supernates (10) or for T-depleted spleens with supernates of of antigen-stimulated activated T cells (11). By analogy one might suppose that similar effects could be obtained by activating these same T cells that produce these supernatants with Con A. So far, however, we have been unable to obtain such supernatants from activated T cells.

Alternative explanations of the stimulatory effect must therefore be considered. Andersson et al. (12) and Greaves and Bauminger (13) have shown that locally concentrated Con A will stimulate B cells directly to increased thymidine uptake and immunoglobulin synthesis. It is therefore possible that Con A becomes bound to a cell surface and stimulates the B cell directly for similar reasons. The cell that binds Con A in this fashion however would have to be absent from the spleen cells of Nude mice.

Gery and Waksman (14) have presented evidence in support of the concept that Con A stimulates macrophages to make a factor that allows T cells to be stimulated by Con A. It is possible that the stimulatory activity that we observe is mediated by such a mechanism. The Con A-stimulated macrophage would then provide a factor that would in some way potentiate the normal stimulatory activity of helper T cells.

SUMMARY

The addition of concanavalin A to mouse spleen cell suspensions can either inhibit or stimulate the immune response to heterologous erythrocytes according to the experimental conditions. Evidence is presented which is incompatible with the hypothesis that these two effects are mediated by a single cell and which favors the hypothesis of separate inhibitor and stimulatory cells.

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