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Suppression of the *in Vitro* Secondary Antibody Response of Rabbit Lymphoid Cells by Concanavalin A[1] (38817)

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Plant lectins such as concanavalin A (Con A) and phytohemagglutinin (PHA) have been widely used to study lymphocyte activation and function. These mitogens transform nonspecifically a large percentage of lymphocytes in a population in contrast to the limited number of lymphocytes activated by a specific antigen. Exposure of lymphocytes to mitogenic concentrations of Con A and PHA, furthermore, can induce these cells to produce lymphokines (1–5). Moreover, addition of either Con A or PHA to cultures of murine lymphocytes undergoing a primary *in vitro* antibody response to sheep red blood cells (SRBC) can either enhance or suppress the response depending on the conditions employed (6–9). Suppression of the humoral immune response *in vivo* by Con A and PHA has been reported as well (10–13). Evidence derived from *in vitro* experiments indicates that thymus-derived cells (T-cells) are responsible for this suppression (6, 9).

Studies concerned with the *in vitro* effects of Con A on the humoral immune response have focused mainly on the murine primary antibody response employing dissociated spleen cells (6–9). The purpose of this investigation is to extend these studies by examining the effects of Con A on rabbit spleen and lymph node cells undergoing an *in vitro* secondary antibody response to SRBC as well as to investigate the mechanism of Con A-induced suppression. The results demonstrate that suppression is mediated by a soluble substance(s) which is produced by lymphoid cells after a short exposure to suppressive concentrations of Con A and that neither prolonged exposure to Con A nor cell division is a prerequisite for its production.

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Materials and Methods. Animals and immunization. Adult New Zealand white rabbits were immunized intravenously with 1.0 ml of a 20% suspension of SRBC (Animal Blood Center, Syracuse, NY) and in each hind footpad with 0.5 ml of a 20% suspension of SRBC. Rabbits were sacrificed 10 or 38 days after immunization, and the spleen and popliteal lymph nodes removed.

Preparation of cell cultures. Single cell suspensions were prepared from the spleens and lymph nodes according to the method of Roszman *et al.* (14). Nucleated cells were counted in a hemocytometer and the suspensions adjusted to 2×10^7 nucleated cells/ml in Eagle's minimal essential medium (MEM), supplemented with glutamine, vitamins, nonessential amino acids, penicillin, streptomycin, and 20% fresh heat-inactivated, normal rabbit serum. One and a half milliliters of cell suspension was placed in 35-mm plastic petri plates. Spleen cultures were stimulated with 3×10^7 SRBC and lymph node cultures with 3×10^6 SRBC. Duplicate cultures were incubated at 37°C in 5% CO_2 in air and rocked on a platform at 7–10 cycles/min.

Plaque assay. Aliquots of the cell suspensions were removed from the cultures on various days of the response and the cells washed once in 2 ml of MEM. Direct (IgM) PFC were assayed by a modification of the Cunningham liquid plaque assay (15).

Enumeration and viability of cells. On various days of the response the number of nucleated cells in the cultures was determined by counting in a hemocytometer and the percentage of viable cells determined by the trypan blue method.

Concanavalin A. A 1-mg/ml solution of twice-recrystallized Con A (Sigma Chem. Co., St. Louis, MO) in MEM was sterilized by passage through a 0.45-μm Millipore

filter (Millipore Corp., Bedford, MA) and stored at −20°C in 1-ml aliquots. A competitive inhibitor of Con A binding α-methyl-D-mannoside (MDM) was obtained from the Sigma Chem. Co., St. Louis, MO, and prepared in MEM at 300 mg/ml and sterilized as described above. Tritiated thymidine was obtained from New England Nuclear, Boston, MA, at a specific activity of 6.7 Ci/mmole and added to appropriate Con A-stimulated cultures at a concentration of 1 μCi/ml in a 0.1 ml of MEM. Radioactivity incorporated into DNA was determined by precipitating TCA-insoluble material on glass filters and counting in a Packard liquid scintillation spectrometer.

Results. In Table I is shown the effect of various concentrations of Con A on the IgM response of spleen and lymph node cells undergoing the secondary antibody response to SRBC. Complete suppression of the response was obtained with 10 μg/ml Con A and partial suppression with 1 μg/ml Con A. The suppression of the antibody response by Con A was not a result of cell death since Con A-treated cultures had viabilities similar to those of control cultures. In addition, the effect of adding 10 μg/ml of Con A either to

TABLE I. EFFECT OF VARIOUS CONCENTRATIONS OF CON A ON THE *in Vitro* IGM SECONDARY ANTIBODY RESPONSE OF SPLEEN AND LYMPH NODE CULTURES[a]

Experiment number	Concentration of Con A (μg/ml)[b]				
	0	0.01	0.1	1.0	10
	PFC/10⁶ nucleated cells recovered[c]				
Spleen					
297	515	378	327	65	0
301	360	343	310	241	6
314	328	284	218	72	0
321	950	696	729	617	0
Lymph node					
297	1587	1241	1102	758	5
301	1058	1133	1170	204	17
314	1670	2255	1450	1230	0
	2620	370	358	468	0

[a] Lymphoid cells obtained from animals primarily immunized 10 days previously.

[b] Various concentrations of Con A added at the time of initiation of the cultures.

[c] Assayed on day 3 of the response.

TABLE II. EFFECT OF ADDING 10 μG/ML OF CON A AT DIFFERENT TIMES AFTER ANTIGENIC CHALLENGE ON THE IGM *in Vitro* SECONDARY ANTIBODY RESPONSE OF SPLEEN AND LYMPH NODE CULTURES.[a]

Experiment number	No Con A	Time of addition of 10 μg/ml Con A after antigenic stimulation	
		24 hr	48 hr
	IgM PFC/10⁶ nucleated cells recovered[b]		
Spleen			
310	2040	815	1205
325	1450	230	470
466	332	24	100
Lymph node			
310	2310	995	1900
323	455	16	584
325	1393	713	795

[a] Lymphoid cells obtained from animals primarily immunized 10 days previously.

[b] Assayed on day 3 of the response.

spleen or lymph node cultures at either 24 hr or 48 hr after antigenic stimulation was determined and the results are shown in Table II. Considerably greater suppression of the response resulted when the Con A was added at 24 hr than at 48 hr after antigenic stimulation.

Experiments were performed to determine how long spleen cells must be exposed to 10 μg/ml Con A in order to inhibit the IgM response. Spleen cells were exposed to 10 μg/ml of Con A for various lengths of time, washed to remove the Con A, and resuspended in fresh MEM. The results presented in Table III demonstrate that inhibition is observed with a 2-hr exposure to Con A and that maximal inhibition is observed at 10 hr. Addition of ^{3}H-thymidine to cultures treated in a similar manner indicates that significant blast transformation does not occur unless cells are exposed to Con A for at least 24 hr (Fig. 1).

To determine whether MDM could reverse the effect of Con A, various concentrations were added to spleen cultures followed by the addition of 10 μg/ml of Con A and SRBC. The results presented in Table IV demonstrate that MDM can reverse the suppressive effects of 10 μg/ml of Con A. There is, however, some inhibition of the

TABLE III. EFFECT OF THE LENGTH OF EXPOSURE TO 10 μG/ML OF CON A ON THE IGM *in Vitro* SECONDARY ANTIBODY RESPONSE OF SPLEEN CULTURES[a]

Experiment number	Con A (10 μg/ml)	Length of exposure (hrs)[b] 0	2	4	6	10	24
		IgM PFC/10^6 nucleated cells recovered[c]					
318	+	—	138	126	60	32	28
	−	845	673	479	581	614	472
324	+	—	595	575	540	—	310
	−	790	1815	1810	1700	—	1720
327	+	—	837	455	530	300	377
	−	1775	2160	1780	1875	1337	1597

[a] Lymphoid cells obtained from animals primarily immunized 10 days previously.
[b] Cells washed twice in 20 ml of MEM at times indicated and resuspended in fresh MEM.
[c] Assayed on day 3 of the response.

IgM response with MDM alone particularly with 30 mg/ml.

In order to determine whether or not the suppression observed was mediated by a soluble substance(s) the following experiments were performed. Spleen cultures were treated with 10 μg/ml of Con A for 4 hr then washed to remove the unbound Con A and resuspended in fresh medium. Untreated control cultures were processed in a parallel fashion. After an additional 20 hr of incubation the cells of each group were removed by centrifugation and the supernatant fluids employed as follows. Spleen cultures prepared from the same rabbit and stimulated with SRBC 24 hr previously were centrifuged and the supernatant fluids discarded. These cells were resuspended in 1.5 ml of supernatant fluids obtained from either the Con A-stimulated or the nonstimulated cells in the presence or absence of 20 mg/ml MDM. The results in Fig. 2 demonstrate that suppression of the IgM response by Con A can be mediated by soluble substance(s) released into the medium by spleen cells. The results, furthermore, indicate that residual Con A which might be released by cells into the supernatant fluids with or without MDM give essentially similar results.

Discussion. In the present study the effect of various concentrations of Con A on the *in vitro* secondary antibody response of spleen and lymph node cells to SRBC was determined. The results from this study revealed the following. First, that 10 μg/ml of Con A completely suppressed the antibody response of both spleen and lymph node cultures when added to the culture at the time of initiation, whereas 1 μg/ml of Con A induced partial suppression (Table I). Second, suppression of the immune response occurred when cells were exposed to 10 μg/ml of Con A for as little as 2 hr after antigenic challenge. Finally, the suppression observed in this study was mediated by a

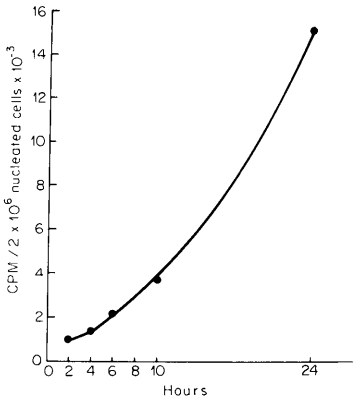

FIG. 1. Effect of the length of exposure to 10 μg/ml of Con A on the incorporation of ^{3}H-thymidine by spleen cultures. Con A was added at time 0 and the cultures washed at the times indicated to remove Con A. One μCi/ml of ^{3}H-thymidine was added to the cultures at 24 hr and the incorporation of the label into DNA determined 16 hr later. Control cultures not stimulated with Con A averaged 804 cpm/2 × 10^6 nucleated cells.

soluble substance(s). These results suggest that there is a population of cells found in both lymph nodes and spleen that can interact with Con A and induce the suppression of the *in vitro* secondary antibody response. Others (6–9) have observed that Con A can suppress the *in vitro* primary antibody response of murine spleen cells to SRBC.

The mechanism whereby Con A induces suppression of the humoral response is unknown. Our results do, however, have a bearing on several salient features of this suppression. Thus, exposure of spleen cells to 10 μg/ml of Con A for 2 hr after antigen can result in suppression of the immune response. However, cells must be in contact with Con A for at least 24 hr in order to obtain substantial incorporation of ^{3}H-thymidine, an observation in agreement with others (16–19). These data indicate that cell division is not a prerequisite for suppression. This, together with the observation that suppression becomes progressively less effective when 10 μg/ml of Con A is added to cultures at 24 hr and 48 hr after antigenic challenge, suggests Con A is in some manner interfering with the early cellular events involved in the initiation of the immune response.

Since soluble Con A can bind to but not stimulate bone marrow-derived cells (B-cells) to undergo transformation (20, 21) and inhibit antigen and anti-immunoglobulin-induced cap formation of receptors on B-cells (22), it is possible that the suppression noted is a result of Con A binding to antigen-specific B-cells and inhibiting their differentiation into antibody-forming cells. There is evidence, however, which does not favor B-cell involvement but which suggests that T-cells are responsible for the Con A-induced suppression. Thus, it has been observed that *in vivo* administration of Con A suppresses the antibody response of a thymus-dependent antigen SRBC but not that of lipopolysaccharide, a thymus-independent antigen (11). While it has been reported that the *in vitro* primary antibody response to polymerized flagellin, also a thymus-independent antigen, is inhibited by Con A, spleen cell populations markedly depleted of T-cells yield an enhanced response to the antigen in the presence of Con A (23). Finally, others have clearly demonstrated that suppression of the *in vitro* primary antibody response of murine spleen cells is mediated by T-cells (6, 9).

On the basis of the data provided in the

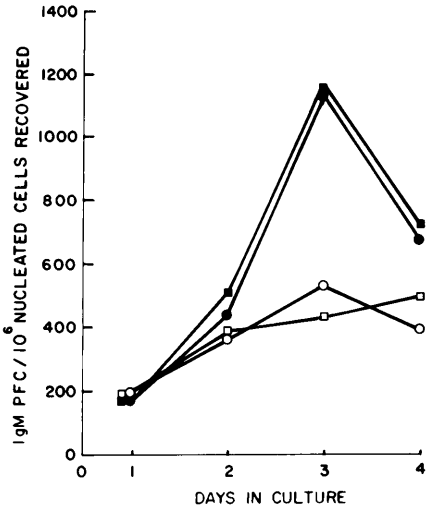

FIG. 2. Effect of supernatant fluids obtained from spleen cultures which were either treated or not treated with 10 μg/ml of Con A (see text for description). ■——■ supernatant fluids from Con A-nonstimulated cultures; ●——● supernatant fluids from Con A-nonstimulated cultures + 20 mg/ml MDM; □——□ supernatant fluids from Con A-stimulated cultures; ○——○ supernatant fluids from Con A-stimulated cultures + 20 mg/ml MDM.

TABLE IV. REVERSAL OF CON A INHIBITION OF THE IgM *in Vitro* SECONDARY ANTIBODY RESPONSE OF SPLEEN CULTURES BY MDM[a]

Con A (10 μg/ml)	MDM[b] (mg)	PFC/10^6 nucleated cells recovered[c]
−	—	644
+	—	0
+	10	360
−	10	396
+	20	348
−	20	364
+	30	196
−	30	188

[a] Lymphoid cells obtained from animals primarily immunized 10 days previously.
[b] MDM = α-methyl-D-mannoside.
[c] Assayed on day 4 of the response.

present study, the cell type responsible for the observed suppression cannot be identified. It is known, however, that Con A transforms rabbit T-cells but not B-cells (24). In addition, our results indicate that the suppression is mediated by a soluble substance(s), a finding more compatible with a T-cell function. Thus, supernatant fluids obtained from spleen cell cultures 20 hr after a brief exposure to 10 μg/ml of Con A could suppress the *in vitro* secondary antibody response to SRBC when added as replacement medium to 24-hr-old antigen-stimulated cultures. Residual Con A which may have been released from the cells during the 20-hr incubation period was not responsible for the inhibitory action of the supernatant fluids since the addition of MDM did not reverse the suppression. Moreover, the suppression was not due to cell death since the cell viability of cultures treated wilh Con A supernatant fluids was similar to those treated with control supernatant fluids. Attempts to isolate the suppressor from supernatant fluids of Con A-treated cultures by various procedures have proved unsuccessful indicating that it is labile. While this work was in progress Rich and Pierce (25) reported that suppression of the *in vitro* primary antibody response to SRBC by Con A is mediated by a soluble substance. Their suppressor was not, however, labile. This indicates that there may be fundamental differences between these suppressor substances, since we were unsuccessful in isolating the suppressor from supernatant fluids of Con A-treated cultures using procedures similar to those of Rich and Pierce. This may, in part, be due to differences between the animal species used (mouse vs rabbit) or between the humoral immune responses studies (primary vs secondary). Our results suggest, then, that Con A is acting on a population of T-cells resulting in the rapid release or synthesis of a labile suppressor substance.

Summary. The effect of various concentrations of concanavalin A (Con A) on the *in vitro* secondary antibody response of rabbit lymph node and spleen cells to sheep red blood cells (SRBC) was studied. Complete suppression of the IgM plaque-forming cell (PFC) response of both lymph node and spleen cultures was observed when 10 μg/ml of Con A was added at the time of initiation of the cultures whereas only partial suppression was observed when 1 μg/ml of Con A was added. Moreover, marked suppression of the immune responses of both spleen and lymph node cultures was observed when 10 μg/ml of Con A was added at 24 hr after antigenic challenge and to a lesser extent when added at 48 hr. Suppression of the IgM PFC response was also detected when spleen cultures were exposed to 10 μg/ml of Con A for as little as 2 hr after antigenic challenge. However, substantial increases in DNA synthesis were observed only in those cultures which were in contact with Con A for at least 24 hr. Finally evidence is presented that the Con A-induced suppression is mediated by a soluble substance(s).

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