CONCANAVALIN A POTENTIATES SYNGENEIC RESPONSE IN MURINE LYMPHOCYTES

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Thymus-derived lymphocyte (T-cell) responses to alloantigens that are expressed in mixed lymphocyte reactions (MLR)[1] and in the generation of cytotoxic lymphocytes (CL) are complex and incompletely understood; one need only cite the fact that only metabolically active cells are capable of inducing these reactions (1). Neither fragments of membrane nor soluble membrane components are capable of evoking a reaction despite their retention of serologically detectable antigenic determinants (2). The picture is further complicated by the report that immunocompetent allogeneic thymocytes, although they carry antigens, are unable to stimulate T cells unless syngeneic spleen cells are present (3). Although recent genetic investigations have contributed further to the analysis of cellular antigens which could not be detected by conventional serological assays (4, 5), relatively little is known about the triggering of the MLR.

In this work we have attempted to view T-cell response as a function of membrane recognition. We have investigated T-cell reactions in experiments in which we have modified membrane properties of lymphocytes with concanavalin A (Con A). This approach has been investigated in other systems. Siraganian and Siraganian (6) reported that Con A binding to human basophils effects the release of histamine. Kubota and Kanatani demonstrated that Con A activates the induction of 5-methyl adenine which induces oocyte maturation in the starfish (7). Moreover, Burger (8) reported that the interaction of SV40-transformed 3T3 cells with chymotrypsin-treated Con A restored the normal growth pattern. Very recently, Elfenbein and Gelfand (9) reported that T-cell surface-bound mitogen directly stimulates the proliferation of syngeneic B cells in vitro. The modification of T-cell immunity by another reagent was demonstrated by Shearer (10), who showed that trinitrophenyl-modified syngeneic spleen cells induced T-cell-mediated cytotoxicity.

In our experiments we have been concerned with exploring the consequences of changes in the cell membranes of both stimulating and responding components of the reaction. Thus we pretreated stimulating cells and responding cells separately with Con A, which alone is not mitogenic under these experimental conditions. We now present evidence that when cortisone resistant thymocytes

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[1] *Abbreviations used in this paper:* CL, cytotoxic lymphocytes; Con A, concanavalin A; CRT, cortisone-resistant thymocytes; [^{3}H]TdR, tritiated thymidine; MLC, mixed lymphocyte cultures; MLR, mixed lymphocyte reactions; α-MM, methyl-α-D-mannopyranoside; N-Con A, native Con A; S-Con A, succinyl Con A.

(CRT) are pretreated with Con A, they undergo significant proliferation when exposed to either syngeneic or allogeneic spleen cells. Furthermore, untreated CRT are triggered by syngeneic (or allogeneic) spleen cells which have been pretreated with Con A. Even more surprising is our further finding that proliferation by Con A pretreatment of either stimulating or responding cells ultimately suppresses the generation of CL in allogeneic combinations. This finding is discussed in relation to the suppression by Con A described in another manuscript.[2]

Materials and Methods

Mice and Cells. Male mice, congenic resistant strains C57BL/10ScSn(B10), B10.D2/nSn, and CBA/J, 4- to 8-wk old were purchased from The Jackson Laboratory, Bar Harbor, Maine. CRT and spleen cells were obtained from cortisone-treated and normal mice, respectively. Details are described in another paper.[2]

Succinyl-Concanavalin A (S-Con A). S-Con A was prepared from native Con A (N-Con A) (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) according to Gunther et al. (11). 100 mg of N-Con A was dissolved in 25 ml of saturated sodium acetate at room temperature. After centrifugation at 5,000 rpm for 20 min, the supernate was reacted with 30 mg of succinic anhydride (Sigma Chemical Co., St. Louis, Mo.) at 0°C for 1 h with gentle stirring. After dialysis against distilled water, the product was lyophilized and kept at −70°C.

Methyl-α-D-Mannopyranoside (α-MM). This specific inhibitor of Con A (12), α-MM (Calbiochem, San Diego, Calif.) was dissolved in RPMI 1640 at 5×10^{-1} and kept at −70° until use.

Pretreatment of Responding or Stimulating Lymphocytes with Con A. For the pretreatment of responding cells, B10 CRT at 5×10^6 cells in RPMI 1640 plus 10% fetal calf serum were treated with various concentrations of N-Con A or S-Con A at 37°C for 30 min. Cells were washed twice with the culture medium. They were resuspended in the same volume of fresh medium and mixed with varying numbers of syngeneic or allogeneic (B10.D2) stimulating cells which had been treated with mitomycin C (40 μg/ml at 37°C for 30 min). Stimulating cells treated with mitomycin C followed by three washings were obtained from normal spleens or thymuses. For the pretreatment of stimulating cells, cells from normal syngeneic or allogeneic spleens or thymuses were first treated with mitomycin C (40 μg/ml) at 37°C for 30 min. After three washings the stimulating cells were resuspended at $5–8 \times 10^6$ cells/ml in RPMI 1640 plus 10% fetal calf serum and treated with N-Con A or S-Con A at 37°C for 30 min. After two washings, the resuspended stimulating cells were mixed at different concentrations with normal CRT. In some experiments Con A-pretreated cells were further treated with α-MM at 10^{-1} M at 37°C for 20 min, then washed once, and mixed with the responding "partner" cells. To determine the time dependence of Con A effects, α-MM at 10^{-1} M was added to mixed lymphocyte cultures at various times. Cell suspensions were washed after 20 min incubation at 37°C and resuspended in fresh medium after which the cultures were continued.

Mixed Lymphocyte Cultures (MLC). Assays of DNA synthesis were performed on MLC maintained in 12 × 75 mm plastic tubes (Falcon Plastics, Div. of BioQuest, Los Angeles, Calif.). Each tube contained $1.25–2 \times 10^6$ responding cells and varying numbers of stimulating cells in 0.5 ml. For the determination of the generation of CL, MLC were performed in Marbrook chambers (Bioresearch Glass, Vineland, N. J.). The details of these techniques are presented in another paper.[2] The number of lymphoblasts (enlarged cells with distinctive cytoplasm) were determined by Giemsa staining.

Results

Con A Pretreatment of Responding CRT Enhances the Proliferative Response in One-Way MLC. To determine how the MLR is affected when the surfaces of

[2] Ozato, K., W. H. Adler, and J. D. Ebert. 1975. Differentiation of suppressor cell populations as revealed by studies of the effects of mitogens on the mixed lymphocyte reaction and on the generation of cytotoxic lymphocytes. *Cell. Immunol.* In press.

responding cells are modified by Con A, B10 CRT were pretreated with N-Con A or S-Con A at 50 μg/ml for 30 min. They were washed to remove unbound Con A, then mixed with congenic B10.D2 spleen cells pretreated with mitomycin C. Tritiated thymidine ([^{3}H]TdR) incorporation at day 3 is depicted in Fig. 1 *a*. CRT pretreated with N-Con A manifested highly significant enhancement in proliferation. Since Con A-pretreated CRT without allogeneic stimulation showed almost no [^{3}H]TdR incorporation, the DNA synthesis demonstrated in these combinations was not a mitogenic effect of Con A. In fact, to induce a mitogenic response by Con A alone, Con A must be present in the medium for about 20 h (13). S-Con A pretreatment at 50 μg/ml did not produce a significant effect on MLR. The enhancement in [^{3}H]TdR incorporation correlated roughly with the increase in blast cells. About 1.2×10^4 lymphoblasts per culture were found in normal MLR, whereas the number of lymphoblasts in cultures of Con A-pretreated CRT was 5.3×10^4 per culture. The culture of Con A-pretreated CRT alone did not produce any transformed lymphocytes.

Con A Pretreatment of Stimulating Spleen Cells Enhances the Proliferative Response in One-Way MLC. In this series of experiments, stimulating spleen cells were pretreated with either N-Con A or S-Con A. B10.D2 spleen cells were pretreated with Con A after they were treated with mitomycin C and then added to normal B10 CRT. Fig. 1 *b* indicates [^{3}H]TdR incorporation on day 3. MLC

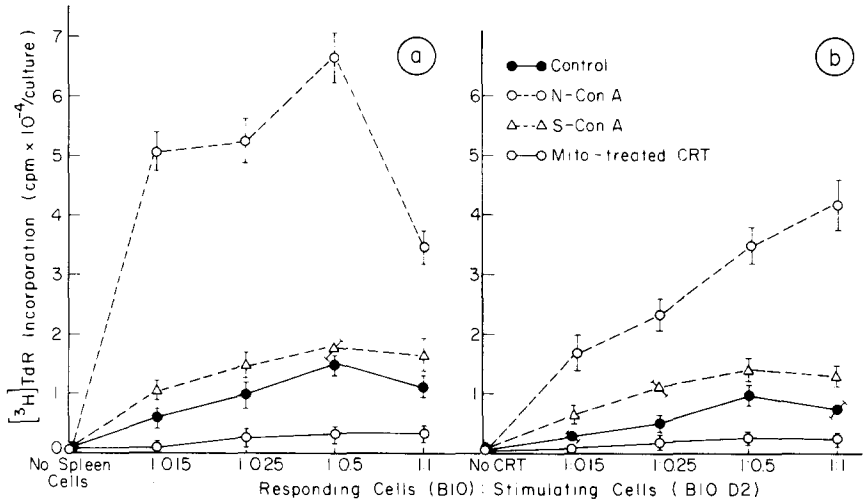

FIG. 1. (*a*) Proliferative response by Con A-pretreated CRT in one-way MLC. 1.7×10^6 normal B10 CRT or B10 CRT pretreated with 50 μg/ml of N-Con A or S-Con A were sensitized with different numbers of allogeneic spleen cells (B10.D2). CRT pretreated with N-Con A followed by treatment with mitomycin C (mito-treated) were also tested. The abscissa represents the ratio of responding to stimulating cells. Cells were labeled with [^{3}H]TdR (1 μCi/ml) on day 3. Each value indicates the average of triplicate cultures ± standard deviation (SD). (*b*) Proliferative response induced by Con A-pretreated stimulating spleen cells in one-way MLC. 1.7×10^6 normal B10 CRT were sensitized with various numbers of normal B10 CRT spleen cells or spleen cells pretreated with 50 μg/ml of N-Con A or S-Con A. CRT treated with mitomycin C were also sensitized with N-Con A-pretreated spleen cells. Cells were labeled with [^{3}H]TdR (1 μCi/ml) for 18 h on day 3. Each value indicates the average of triplicate cultures ± SD.

stimulated with allogeneic spleen cells pretreated with N-Con A showed a three- to fivefold enhancement of proliferation. The greatest enhancement was observed when equal numbers of stimulating and responding cells were mixed. The response in such cultures can be regarded as attributable to DNA synthesis by responding CRT, since spleen cells alone without CRT did not show any response, their DNA synthesis having been abrogated by mitomycin C. Moreover, when responding CRT were treated with mitomycin C the [^{3}H]TdR incorporation was abolished. S-Con A showed only a slight effect in enhancing [^{3}H]TdR incorporation.

Syngeneic Spleen Cells are Capable of Triggering Con A-Pretreated CRT. This series of experiments was performed to test whether similar effects could be obtained in syngeneic combinations. Moreover, it was of interest to determine whether thymocytes that are weak stimulators in the MLR (3, 14) can trigger DNA synthesis. Responding B10.D2 CRT were pretreated with Con A. As shown in Table I, significant [^{3}H]TdR incorporation was demonstrated in syngeneic combinations after N-Con A pretreatment. The incorporation was lower than that in allogeneic combinations (B10.D2 vs. CBA), but was nevertheless striking. The number of lymphoblasts per culture on day 3 was about 4.0×10^4 in syngeneic combinations, whereas it was 6.1×10^4 in allogeneic combina-

TABLE I
Effect of Con A Pretreatment of Responding CRT

Exp.	Responding CRT* (B10.D2) pretreatment	Stimulating cells		[^{3}H]TdR incorporation (cpm per culture)	
		Allogeneic (CBA)	Syngeneic B10.D2	Day 3	Day 5
I					
	N-Con A	Spleen Cells		30,740 ± 2,023	3,001 ± 135
	S-Con A	Spleen Cells		10,633 ± 719	4,085 ± 281
	Culture medium	Spleen Cells		8,247 ± 604	6,352 ± 601
	N-Con A	Thymus Cells		501 ± 22	403 ± 27
	S-Con A	Thymus Cells		381 ± 15	534 ± 41
	Culture medium	Thymus Cells		420 ± 11	251 ± 16
	N-Con A		Spleen	21,272 ± 1,018	102 ± 8
	S-Con A		Spleen	1,613 ± 502	348 ± 21
	Culture medium		Spleen	197 ± 10	81 ± 5
	N-Con A		Thymus	397 ± 22	14 ± 1
	S-Con A		Thymus	242 ± 15	92 ± 8
	Culture medium		Thymus	87 ± 3	43 ± 4
II	Normal CRT in the presence of Con A			35,806 ± 2,659	6,981 ± 761

* In experiment I, B10.D2 (1.7×10^6) CRT pretreated with Con A at 50 μg/ml were cultured with 1×10^6 spleen or thymus cells treated with mitomycin C either from allogeneic (CBA) or syngeneic (B10.D2) mice. In experiment II, normal CRT were cultured in the presence of mitogenic dose (5 μg/ml) of N-Con A. [^{3}H]TdR (1 μCi/ml) was added 50 or 100 h after incubation and cells were labeled for 18 h. The values represent means of duplicate cultures ± SD.

tions. On the other hand normal thymocytes, from neither allogeneic nor syngeneic mice were able to induce stimulation even though allogeneic thymus cells can induce a slight MLR. This observation clearly indicates that N-Con A-pretreated CRT can respond selectively to the stimulation provided by spleen cells. The DNA synthesis obtained in syngeneic combinations tapered off and ceased on day 5. In allogeneic combinations on day 5, DNA synthesis in normal MLC exceeded that in Con A-pretreated combinations. Similar striking proliferation in syngeneic combinations was recorded in B10 and CBA strains.

Normal CRT are Triggered by Con A-Pretreated Syngeneic Spleen Cells. Since Con A-pretreated CRT could be triggered by normal syngeneic spleen cells, tests were done to determine whether Con A-pretreated syngeneic spleen cells are capable of stimulation. The possible stimulatory role of thymocytes was also examined in the B10.D2 strain. The results are shown in Table II. Syngeneic spleen cells that cannot alone stimulate syngeneic CRT produced a remarkable stimulation when they were pretreated with N-Con A. [^{3}H]TdR

TABLE II
Effect of Con A Pretreatment of Stimulating Lymphocytes

Stimulating cells pretreatment	[^{3}H]TdR incorporation (cpm per culture)	
	Day 3	Day 5
Spleen cells:allogeneic (CBA)*		
N-Con A	32,094 ± 1,053	1,162 ± 99
S-Con A	10,950 ± 1,104	1,874 ± 63
Culture medium	3,085 ± 280	1,836 ± 73
Thymocytes:allogeneic (CBA)		
N-Con A	9,611 ± 1,521	2,224 ± 179
S-Con A	3,932 ± 325	967 ± 81
Culture medium	360 ± 15	212 ± 16
Spleen cells:syngeneic (B10.D2)		
N-Con A	24,393 ± 1,805	891 ± 79
S-Con A	2,790 ± 212	816 ± 62
Culture medium	94 ± 4	28 ± 2
Thymus cells:syngeneic (B10.D2)		
N-Con A	9,295 ± 1,765	1,078 ± 112
S-Con A	2,330 ± 183	370 ± 24
Culture medium	66 ± 3	23 ± 1
Supernate from Con A CRT‡	150 ± 2	38 ± 3
Supernate from Con A spleen	123 ± 7	32 ± 2

* Spleen cells or thymus cells from syngeneic (B10.D2) or allogeneic (CBA) mice were pretreated with Con A (50 μg/ml) at 5×10^6 cells/ml after they were treated with mitomycin C. 1.7×10^6 normal CRT were cultured with 1×10^6 stimulating cells pretreated with Con A. [^{3}H]TdR (1 μCi/ml) represent means of duplicate cultures ± SD.

‡ Supernatant fluid was obtained from 48 h cultures of B10.D2 CRT or B10.D2 spleen cells pretreated with N-Con A (50 μg/ml). Immediately after centrifugation, they were added to normal culture medium at a 1:1 dilution.

incorporation in syngeneic combinations was again slightly less than that in allogeneic combinations. While normal thymocytes showed much less ability to induce the reaction, an effect was nonetheless obvious, with kinetics similar to those in the previous experiment. Again, in syngeneic combinations, proliferation ceased by day 5. In allogeneic combinations proliferation in cultures stimulated by Con A-pretreated cells was lower than observed in the normal MLC. In order to compare the degree of responsiveness induced in this manner with the mitogenic response to soluble Con A, the same numbers of normal CRT were cultured in the presence of a mitogenic concentration of N-Con A. The response to mitogenic Con A was slightly higher than the response to allogeneic spleen cells coated with N-Con A. The response in syngeneic combinations was about 60% of the Con A mitogenic response. A similar syngeneic responsiveness was demonstrated in other combinations (CBA and B10).

Dose Response in Con A Pretreatment of Responding CRT. In order to find the optimal concentration of Con A pretreatment, varying doses of N-Con A or S-Con A were tested. Fig. 2 shows the dose response curve in allogeneic combinations (B10 vs. B10.D2). N-Con A showed the highest enhancing effect at 50 μg/ml. At concentrations over 100 μg/ml the potentiating effect was reduced, until 300 μg/ml at which DNA synthesis was no longer observed. The optimal concentration of S-Con A was substantially higher than for N-Con A. At 150–200 μg/ml, S-Con A triggers proliferation equivalent to 50 μg/ml of N-Con A. When N-Con A pretreatment was followed by treatment with α-MM (10^{-1} M) the potentiating effect was completely abrogated indicating that the effect is dependent on the binding of Con A. The CRT pretreated with N-Con A did not show appreciable DNA synthesis at any concentration in the absence of spleen cells, indicating that the effect is dependent on the presence of stimulating cells.

Dose Response in Con A Pretreatment of Stimulating Cells. Similar dose response tests were done when stimulating spleen cells were pretreated with Con A in syngeneic combinations. The results are shown in Fig. 3. The optimal concentration of N-Con A was again 50–100 μg/ml. S-Con A was again equally capable of stimulating, but also at higher concentrations than N-Con A. Stimulating thymocytes pretreated with N-Con A produced only a modest triggering effect. In addition, thymocytes pretreated with S-Con A at higher concentrations showed triggering to a slight extent.

Lack of Stimulating Activity in the Culture Fluid of the Con A-Pretreated Lymphocytes. To determine whether substances released by Con A-pretreated cells into the culture medium might be stimulatory, the supernatant fluid from syngeneic cultures in which either stimulating or responding lymphocytes were pretreated with 50 μg/ml of N-Con A was harvested at 24, 48, and 72 h, and added to normal cultures in differing amounts. Representative data are shown in Table II, indicating that culture fluids collected in this manner were unable to trigger CRT.

The Reversibility of the Effect by α-MM. As shown in Table III the augmented proliferation by CRT pretreated with Con A could be reversed by treatment with the specific inhibitor of Con A, α-MM, until 8 h of incubation. N-Con A-pretreated B10 CRT mixed with allogeneic cells were treated with α-MM

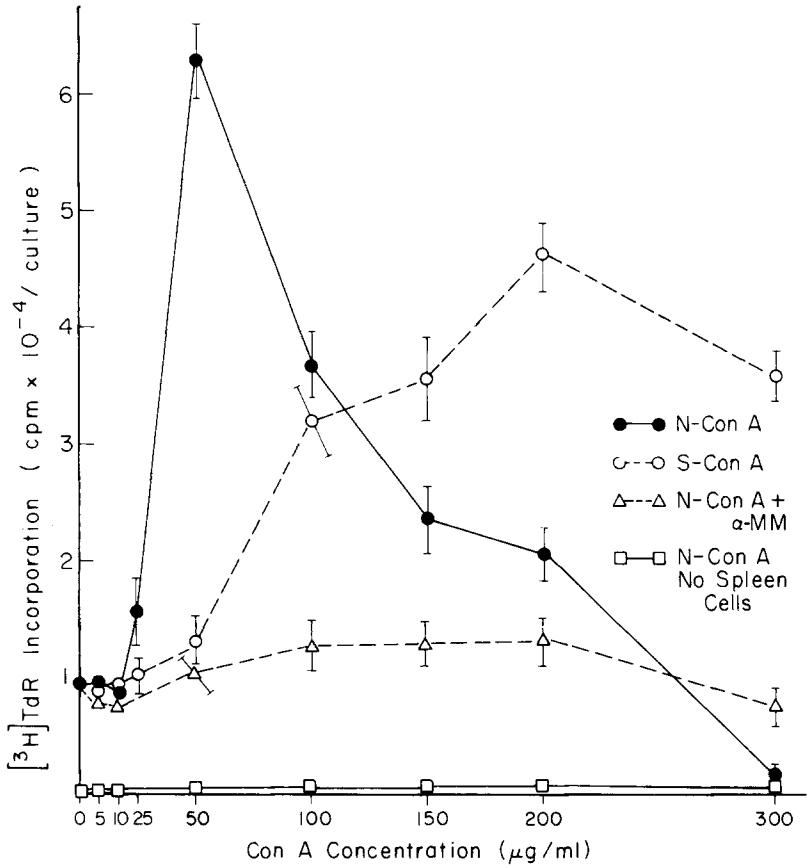

FIG. 2. Dose response in Con A pretreatment of responding CRT. 1.7×10^6 B10 CRT pretreated with various concentration of N-Con A, S-Con A, and with N-Con A followed by α-MM (10^{-1} M) were cultured together with allogeneic B10.D2 spleen cells treated with mitomycin C. CRT pretreated with N-Con A cultured alone were also tested. Each value indicates [^{3}H]TdR incorporation into the cells on day 3 (average of triplicates ± SD).

(10^{-1} M) at various times for 20 min. When α-MM was added at any time before 8 h from the onset of incubation, the response could be almost completely reversed. The same concentration of galactose was ineffective.

Con A Pretreatment of Responding CRT Suppresses the Generation of CL. To delineate the relationship between enhanced proliferation by Con A pretreatment and subsequent generation of CL, B10 CRT pretreatment with Con A were incubated with either allogeneic (B10.D2) or syngeneic spleen cells in three different doses. Cytotoxic activity of sensitized lymphocytes was tested on day 5 by the ^{51}Cr-release assay using P815 cells as targets (Fig. 4). In allogeneic combinations, the cytotoxicity produced by Con-A pretreated CRT was strongly suppressed. Inhibition (60–80%) was noted at every cell ratio. The concentration of Con A which induced suppression in the generation of CL was the dose which showed potentiation of proliferation on day 3. Pretreatment by Con A at lower concentrations which did not induce an appreciable increase in

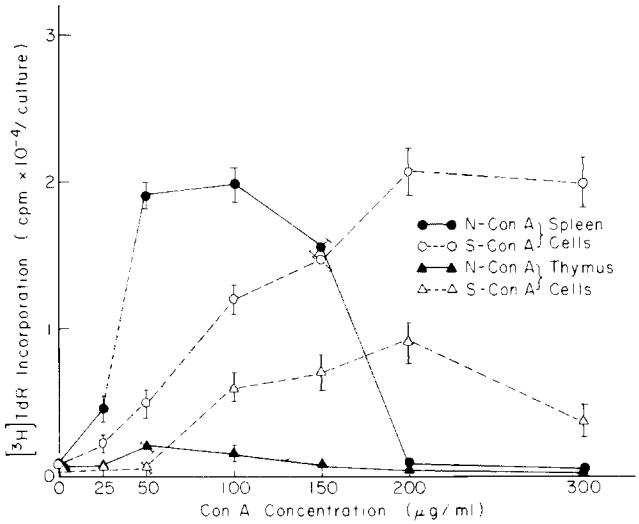

FIG. 3. Dose response in Con A pretreatment of stimulating cells. 1.7×10^6 normal B10 CRT were cultured together with syngeneic B10 spleen cells or thymus cells treated with various concentrations of N-Con A or S-Con A. Before Con A pretreatment stimulating cells were treated with mitomycin C. Each value indicates [^{3}H]TdR incorporation into the cells on day 3 (average of triplicates ± SD).

TABLE III
Reversibility of the Con A Pretreatment Effect by α-MM

Experimental group	[^{3}H]TdR incorporation (cpm × 10^{-2}/culture)*						
	α-MM treatment (h after the incubation)						No α-MM
	1/2	2	4	8	15	24	
N-Con A-pretreated CRT + α-MM	38.4	49.8	40.2	81.2	355.2	407.0	450.3
N-Con A-pretreated CRT + galactose	381.3	447.3	421.8	395.7	387.2	458.5	422.5
Normal CRT	45.7	51.5	ND	63.4	ND	60.5	57.0

Specific inhibitor of Con A, α-MM (10^{-1} M final concentration in RPMI), or galactose at the same concentration was added at the times indicated and then incubated for 20 min at 37°C. Cells were washed once and reincubated in the culture medium until harvest (72 h after incubation). Cells were labeled with 1 μCi/ml [^{3}H]TdR for 18 h before harvesting. The values represent means of duplicate cultures.

* 1.5×10^6 B10 CRT pretreated with N-Con A (50 μg/ml) were cultured together with 1×10^6 allogeneic (B10.D2) spleen cells treated with mitomycin C in 0.5 ml.

proliferation did not suppress the generation of CL; rather at 10 μg/ml of Con A, a slight enhancement in cytotoxic activity was demonstrated. S-Con A at less than 50 μg/ml did not produce suppression. On the other hand, in syngeneic combinations a significant degree of cytotoxicity against syngeneic tumor cells was not demonstrated by Con A pretreatment despite the striking proliferative

response. The suppression by N-Con A-pretreated CRT was also found on days 4 and 6 indicating that kinetic alteration is not the mechanism.

Con A Pretreatment of Stimulating Spleen Cells Suppresses the Generation of CL. The generation of CL was further studied by sensitizing normal CRT with spleen cells pretreated with Con A either in allogeneic or syngeneic combinations. Fig. 5 shows the results. Again striking suppression was noted when allogeneic stimulating cells were pretreated with N-Con A at 50 μg/ml. Lower concentrations of N-Con A or S-Con A did not reduce cytotoxic activity. Even at 10 μg/ml of N-Con A a slight enhancement which was also demonstrated in Con A-pretreated CRT was found. Thus in allogeneic sensitization, proliferative response antagonized the generation of CL. The syngeneic combination did not exhibit cytotoxic activity to syngeneic target cells. The suppression was seen throughout the cultures even on day 6.

Discussion

In attempting to better understand the recognition mechanism in the MLC, the surfaces of responding and stimulating lymphocytes were modified by N-Con

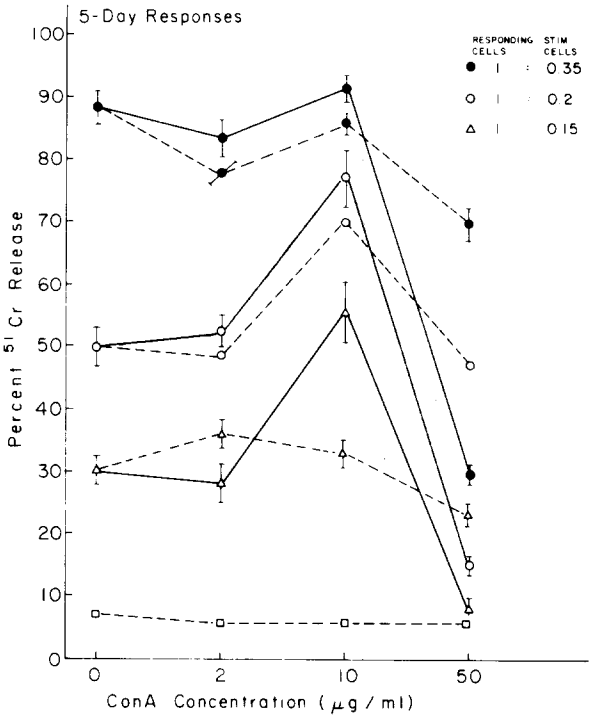

FIG. 4. Generation of CL by CRT pretreated with Con A. 1×10^7 B10 CRT pretreated with various concentrations of N-Con A (solid lines) or S-Con A (dashed lines) were sensitized with different number of B10.D2 spleen cells. Cytotoxic activity per chamber on day 5 is expressed as percent ^{51}Cr release from target P815 cells (mean of duplicates ± SD). B10 CRT pretreated with N-Con A were also cultured with 5×10^6 syngeneic spleen cells. Cytotoxic activity from EL-4 target cells (□) was measured in the same manner. Stim., stimulating cells.

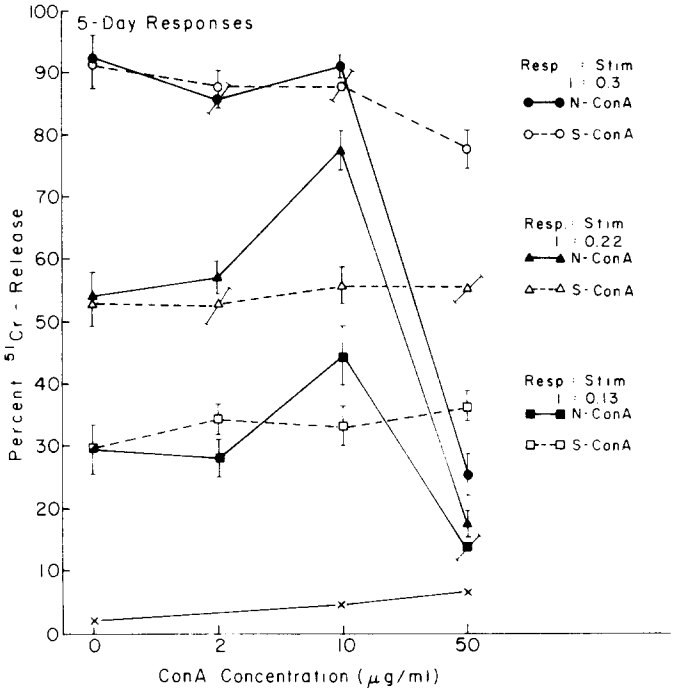

FIG. 5. Generation of CL by sensitization with Con A-pretreated spleen cells. B10.D2 spleen cells were pretreated with N-Con A (solid lines) or S-Con A (dashed lines) before they were added to 1 × 10^7 B10 CRT in different cell ratios. B10 spleen cells (X) were also pretreated with N-Con A for syngeneic combinations. Resp., responding cells; stim., stimulating cells.

A or S-Con A, with almost pure T-cell populations, CRT, being used as responding cells. This study started with the observation that in allogeneic combinations pretreatment of either responding or stimulating cells with N-Con A enhanced MLR more than fivefold, although Con A pretreatment alone was never mitogenic. We further discovered that pretreatment of lymphocytes with N-Con A triggered a highly significant proliferation even in syngeneic combinations. The triggering of proliferation in both cases was dependent on two factors: (*a*) The presence of Con A molecules on one "partner" in the MLC (since a specific Con A inhibitor abolished the response). (*b*) The presence of viable spleen cells as the stimulating cells, culture fluids from Con A-pretreated cells being unable to evoke the effect. In addition, thymocytes were very weak in producing the effect. Furthermore, we found that despite the remarkable proliferative response effected by Con A pretreatment of either partner, the subsequent generation of CL was strongly suppressed.

Neither the potentiation of proliferation nor the suppression of cytotoxicity appear to be related to any modification of antigenicity or appearance of new antigens on the stimulating cell surface because normal spleen cells manifest the effect when responding cells are treated with Con A. Moreover, the effect cannot be regarded simply as an alteration of the receptors on responding cells, since Con A-pretreated spleen cells were equally able to trigger the response.

Therefore it seems to be logical to discuss the nature of the two factors separately. We begin with the discussion of stimulating cells.

In the present work we described the extraordinary triggering ability of syngeneic spleen cells. Proliferation on day 3 in syngeneic combinations was more than twice that in the normal MLR. Taking into account recent reports by others, syngeneic spleen cells appear to play an ill-defined stimulatory role in normal MLC. Dyminski and Smith (3) found that immunocompetent thymus cells cannot be triggered by allogeneic immunocompetent thymocytes, but can be triggered if spleen cells syngeneic to responding cells treated with mitomycin C are present. Moreover the proliferative response in syngeneic combinations of thymocytes stimulated by spleen cells in certain strains of mice were reported by Howe et al. (14) and von Boehmer and Byrd (15). Shearer (10) showed that syngeneic spleen cells could generate CL when they were modified by the hapten trinitrophenyl. In the latter work, syngeneic stimulation by spleen cells might be playing a role since cytotoxicity was not directed toward the hapten molecules but toward the modified cell surface. These several reports imply that T cells can be stimulated by syngeneic spleen cells under certain conditions.

Thus the profound stimulation observed in our syngeneic combinations by spleen cells under the influence of Con A pretreatment might well be a reflection of a natural activation process in T lymphocytes. The inability of syngeneic cells to stimulate in conventional MLR might be explained on the basis that they lack a necessary component which in the work of Dyminski and Smith (3) is provided by allogeneic thymocytes, and in our experiments is provided by Con A. In fact, the activation of T cells by stimulating cells might commonly be dependent on two distinct factors. In this context the triggering in our system is somewhat reminiscent of the hypothesis that two signals are crucial for the induction of immune response proposed by Bretscher and Cohn (16) although their hypothesis is basically concerned with B-cell activation. At this moment it remains to be resolved whether both factors are functioning as "discrete" signals or whether one of them is facilitating the other (17).

The character of spleen cells as "stimulators" is especially interesting in relation to the weak ability of thymus cells to perform similar roles. In some MLR, using populations in which T cells have been depleted, B cells are highly capable of stimulating T cells (18). von Boehmer and Sprent (19) indicated that the differences in the *M* locus which control MLR but are different from the major histocompatibility complex are expressed predominantly on B cells. Recently it has been reported that *Ir* genes controlling MLR in the major histocompatibility complex are also predominantly expressed as Ia antigens on B cells (20). Dyminski and Smith demonstrated that in their system the Ig-bearing cells in the spleen population were responsible for stimulation (3). Thus the stimulatory function of spleen cells in our system might be attributable to a B-cell role. At this moment, however, alternative possibilities, such as subpopulations of T cells which are located preferentially in the spleen cannot be ignored. In fact, recently Piguet et al. (21) reported that in an F_1 vs. parent system, parental spleen T cells are powerful stimulators whereas CRT are not competent. Further, in a similar F_1 vs. parent system Harrison and Paul (22) reported that the response of F_1 T cells is dependent on T cells in the parental population. These facts imply a stimulatory role of spleen T cells.

In their recent study of B-cell stimulation by T-cell mitogens, Elfenbein and Gelfand (9) found that in order to stimulate B-cell proliferation, thymocytes bearing Con A or phytohemagglutin and treated with mitomycin C and cycloheximide are more effective than B cells bearing the same mitogens. Although we dealt with similar phenomena in the sense that the lymphocytes bearing mitogens can trigger normal cells, there is an interesting difference. In our system where T cells are the responding population, normal thymocytes were rather weak in stimulatory activity. Especially when responding CRT were pretreated with Con A, we found virtually no activation by normal thymocytes. This difference might be explained by invoking different triggering mechansims for T and B cells, which await to be determined.

We may next examine the possible role of Con A. In normal triggering the binding of antigenic determinants to receptors would make necessary cell to cell contact between responding and stimulating cells. It is conceivable that in normal MLC, syngeneic combinations cannot trigger T cells because specific antigen-receptor sites are missing. Con A bound to lymphocytes might act as a substitute for either antigenic determinants or receptors. Since N-Con A is tetravalent, while S-Con A is most likely divalent (12), bound Con A on either partner might be able to facilitate close cell to cell contact via residual binding sites. In our system, therefore, cell-bound Con A might be somehow facilitating firm contact between responding and stimulating cells. If so the difference in dose effectiveness between N-Con A and S-Con A could well be explained. The observation that agglutination can be obtained when one of two cells in a pair is coated with Con A, reported by Rutishauser and Sachs (23), supports the idea.

The extraordinary enhancement in proliferation however ultimately resulted in suppression of the generation of CL in allogeneic combinations. Since suppression occurred when either one of the partner lymphocytes was pretreated with Con A, it is not possible to attribute suppression to obstruction of antigenic sites or hindrance of receptors. Rather Con A pretreatment seems to interfere with the differentiation process in some way, yet unknown, which must be related to the proliferation pattern because only at concentrations of Con A that produced the proliferative response could suppression be seen. The finding that syngeneic combinations did not produce cytotoxic activity against syngeneic target cells implies that proliferation may have nothing to do with the subsequent generation of CL. It is interesting that in the trinitrophenyl modification system reported by Shearer et al. (24), the proliferative response by responding cells against modified syngeneic cells was much lower than normal MLR despite their comparable extent of cytotoxic activity. In some way the triggering of proliferation antagonizes the generation of CL. Suppression by Con A in the generation of CL which cannot be regarded as suppressor cells described in another paper[2] might be a direct consequence of this phenomenon.

Summary

In an attempt to modulate the recognition processes that occur on lymphocyte membranes in mixed lymphocyte culture, responding cortisone resistant thymocytes or stimulating spleen cells (treated with mitomycin C) were pretreated with native concanavalin A (N-Con A) or succinyl-Con A (S-Con A). Highly

significant cell proliferation was observed in syngeneic combinations when either the responding cells or the stimulating cells were so treated with Con A, although Con A pretreatment alone was never mitogenic. In allogeneic combinations the proliferative response with Con A pretreatment of either partner on day 3 was five to seven times higher than in the normal mixed lymphocyte reactions. The triggering of proliferation was dependent on two factors: (*a*) The presence of spleen cells as the stimulating cells (thymocytes were much less effective). (*b*) The binding of Con A molecules to either one of the partners, the effect being abrogated by the specific inhibitor of Con A, α-mannopyranoside. The optimal concentration of S-Con A was about twice that of N-Con A. Even more striking was the observation that cultures in which either one of the partners was pretreated with Con A in allogeneic combinations showed a strong suppression (60–80% inhibition) in the subsequent generation of the cytotoxic lymphocytes (CL). The Con A concentration required to trigger a proliferative response corresponded to that for suppressing the generation of CL. Con A pretreatment did not result in a cytotoxic activity toward syngeneic tumor cells.

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