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RECONSTITUTION OF IMMUNOCOMPETENCE IN B CELLS BY ADDITION OF CONCANAVALIN A OR CONCANAVALIN A-TREATED THYMUS CELLS

O. SJÖBERG,* G. MÖLLER† AND J. ANDERSSON*

*Basel Institute for Immunology,** 487 *Grenzacherstrasse, CH*-4058 *Basel, Switzerland and Division of Immunobiology,† Karolinska Institutet, Wallenberglaboratory,* 104 05 *Stockholm* 50, *Sweden*

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SUMMARY

The effect of soluble Concanavalin A (Con A) on the primary antibody response *in vitro* against sheep red cells (SRC) by mouse spleen cells was studied. Stimulation of normal spleen cells with Con A caused a slight increase of the background number of plaque-forming cells (PFC). Cultures stimulated with SRC did not show an increased PFC response after Con A treatment, except in experiments where the PFC response against SRC was rather low in the absence of Con A. Concentrations of Con A higher than 0·5 μg/ml were inhibitory to the immune response of SRC-treated cultures also early during the culture period. In contrast, T cell depleted spleen cultures, incapable by themselves to respond to SRC, were reconstituted by addition of Con A at concentrations of 0·5 μg/ml or more. Presumably, residual T cells were activated by Con A. Con A activated T cells could reconstitute the PFC response in T cell-deficient cultures. In contrast, it was not possible to obtain more than a marginal stimulation of the antibody response in cultures of spleen cells depleted of adherent cells by addition of soluble Con A or Con A-activated thymocytes.

The results suggest that Con A may stimulate the antibody response by activation of T cells, and support the concept that activated T cells can non-specifically stimulate the antibody response of B cells. Large numbers of activated T cells were inhibitory to the immune response and it is suggested that this phenomenon is analagous to antigenic competition. Furthermore, activated T cells do not seem capable of substituting for adherent cells in the primary immune response *in vitro*, suggesting that adherent cells are important for the functions of B cells.

INTRODUCTION

Three cell types are involved in the induction of an immune response *in vitro*. An adherent cell type, generally considered to be macrophage-like (Mosier, 1967) and thymus-derived

Correspondence: Dr Olof Sjöberg, Division of Immunobiology, Karolinska Institutet, Wallenberg-laboratory, 104 05 Stockholm 50, Sweden.

lymphocytes (T cells) (Mosier *et al.*, 1970; Munro & Hunter, 1970) act as helper cells for the stimulation of bone marrow-derived lymphocytes (B cells), which are the direct precursors of antibody-producing cells (Miller & Mitchell, 1969). The mechanism by which the helper cells stimulate the B cells into division and differentiation is still unknown. In general the effect of the adherent cells is considered to be non-specific, although different opinions have been expressed also on this point (Fischman & Adler, 1967). T cells are needed for antibody production carried out by B cells against certain antigens (thymus-dependant antigen) (Transplantation Reviews, 1969) both *in vivo* and *in vitro*.

The low frequency of specific cells involved in the immune response to any particular antigen makes detailed studies on the mechanisms of T and B cell co-operation difficult. This can be overcome by the use of non-specific mitogens, which have the ability to selectively stimulate T or B cell populations. Examples of such mitogens are Concanavalin A (Con A), which only stimulates T cells (Janossy & Greaves, 1971; Andersson *et al.*, 1972a), and lipopolysaccharide (LPS) from *Escherichia coli*, which selectively activates B cells (Peavy *et al.*, 1970; Andersson *et al.*, 1972a). Previous studies have shown that LPS causes an increased synthesis of 19S proteins *in vitro* by spleen cells and also an increased number of specific antibody-forming cells to heterologous red cells (Andersson *et al.*, 1972b). Furthermore, LPS could substitute for T cells in the antibody response against heterologous red cells *in vivo* and *in vitro*, and it was also possible to reconstitute the antibody response of spleen cells depleted of adherent cells (Sjöberg *et al.*, 1972a; Möller *et al.*, 1972).

In this investigation we studied the effect of Con A on the primary antibody response to heterologous red cells *in vitro*. Furthermore, the ability of Con A to substitute for different helper cells, such as adherent cells and T cells in the PFC response was analysed.

MATERIALS AND METHODS

Animals

Mice of the F_1 hybrids (A/J × C57Bl/10) or (C57Bl/10 × CBA) were used at an age of 3–6 months.

Antigen and immunization

SRC, always obtained from the same sheep donor and stored in Alsever's solution, were washed in phosphate buffered balanced salt solution (BSS) (Mishell & Dutton, 1967) before use. Approximately 10^7 SRC were added to each culture dish.

Mitogen

Con A was purified and used in the cultures as described before (Andersson *et al.*, 1972a).

Cell cultures for antibody productions

The technique for primary immunization of mouse spleen cells *in vitro* described by Mishell & Dutton (1967) was followed with minor modifications as described earlier (Sjöberg *et al.*, 1972b).

Preparation of non-adherent cells

Adherent cells were removed by treatment with iron carbonyl powder as described before (Sjöberg *et al.*, 1972b). Spleen cells were incubated at a concentration of 20×10^6 spleen cells/ml with iron powder for 30 min at 37°C. The iron was thereafter removed by a magnet.

Preparation of adherent cells

Adherent cells were prepared from spleen cells. One millilitre of a suspension of spleen cells in culture medium was incubated in each culture dish for 30 min at 37°C at a concentration of 10×10^6 cells/ml. Thereafter the dishes were washed with cold BSS three times (Mosier, 1967).

Treatment with anti-θ serum

Anti-θ serum was raised in adult AKR mice by weekly intraperitoneal injections of C3H thymocytes (Reif & Allen, 1964). Serum was collected 6 days after the last immunization. The cytotoxic titre of the serum against C3H thymus cells as assayed with the trypan blue exclusion technique was 1:512–1:1024. To deplete a spleen cell suspension of T cells, the cell suspension was incubated with a 1/4 dilution of anti-θ serum in medium at a cell density of 100×10^6 cells/ml for 30 min at +4°C. Thereafter, the cells were washed once and resuspended in guinea-pig complement diluted 1/10 in medium and incubated for 30 min at +37°C. After washing the cells were suspended in culture medium and used. The complement was absorbed with agarose (Cohen & Schlesinger, 1970) and spleen cells.

Local haemolysis in gel assay

The number of PFC was assayed according to the method of Jerne & Nordin (1963) as modified by Mishell & Dutton (1967). The cultures were harvested on day 4, if not otherwise stated, and cells from three dishes were pooled. After washing the cells were suspended in BSS; duplicate slides were made for each cell suspension and direct PFC were developed by incubation for 3 hr at 37°C with guinea-pig complement diluted 1/20 in BSS.

Determination of DNA synthesis

Acid precipitable radioactivity was determined after labelling with 1 μCi/ml of ^{3}H-thymidine (spec. act. 5 Ci/mmol) for the last 24 hr of a 72-hr culture period. The procedure has been described in detail elsewhere (Andersson *et al.*, 1972a).

RESULTS

Effect of Con A on the primary PFC response against SRC by normal spleen cells

6×10^6 normal spleen cells were cultured together with various concentrations of Con A and the antibody and cell proliferation responses were determined. For assay of DNA synthesis the cultures were harvested on day 3, whereas the PFC responses were determined in parallel cultures after 4 days culture. The results are shown in Figs 1 and 2. Con A caused a marked stimulation of DNA synthesis in cultures without SRC as well as in cultures with SRC added. DNA synthesis started to increase when the concentration of Con A was 0·25 μg/ml and reached a maximum at 2–4 μg/ml in agreement with earlier

results. The effect of Con A on the antibody response varied with the presence or absence of SRC. In cultures without SRC, 0·25 and 0·5 μg of Con A caused a moderate increase of the number of PFC, the peak values rarely exceeding three times those in cultures without Con A (Fig. 1). No increase was obtained with Con A concentrations of 1 μg/ml or higher. Con A did not in general stimulate the PFC response in SRC-treated cultures (Fig. 2), and when stimulation occurred the anti-SRC PFC response was generally low without Con A. However, with a concentration of Con A higher than 0·5 μg/ml there was invariably an

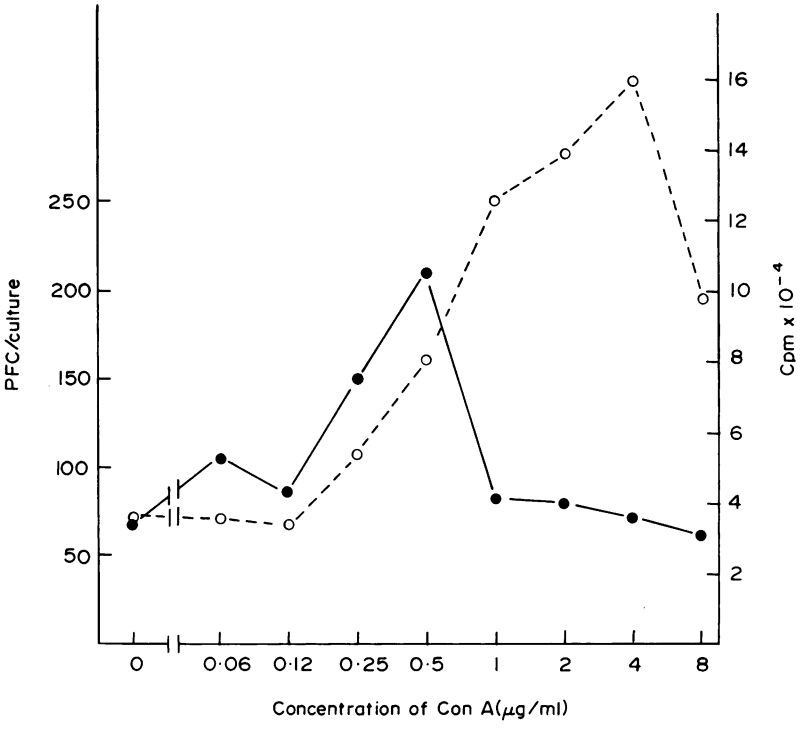

FIG. 1. 6×10^6 spleen cells were cultured with various concentrations of Con A. (○), DNA synthesis determined by ^{3}H-TdR uptake on day 3. (●) PFC numbers/culture determined on day 4.

inhibition of the immune response. Thus, Con A could stimulate the antibody response to a limited degree when the PFC response was low, but high doses of Con A were inhibitory and the stimulation of the PFC responses did not follow the stimulation of cell proliferation.

The antibody response was determined on day 4 above, whereas the cell proliferation was assayed on day 3. To exclude that the discrepancy between DNA and antibody synthesis was caused by an early stimulation and late inhibition of the antibody response in cultures with high concentrations of Con A, the kinetics of the PFC response against SRC were tested in cultures with various amounts of Con A. Table 1 shows that 4 μg of Con A added to the cultures caused a depression of the antibody response already early during the culture period. Therefore, the discrepancy between cell proliferation and antibody formation could

not be explained by an inhibition of the antibody response occurring during the last day in culture.

Reconstitution of the PFC response of T cell-deficient spleen cells by Con A-activated T cells

As shown in Tables 2 and 3, cultures of spleen cells from thymectomized, X-irradiated and bone marrow-protected mice (T × B mice) or of spleen cells treated with anti-θ serum gave no or only a small antibody response to SRC. This has also been demonstrated by others (Mosier *et al.*, 1970; Munro & Hunter, 1970; Chan *et al.*, 1970; Schimpl & Wecker,

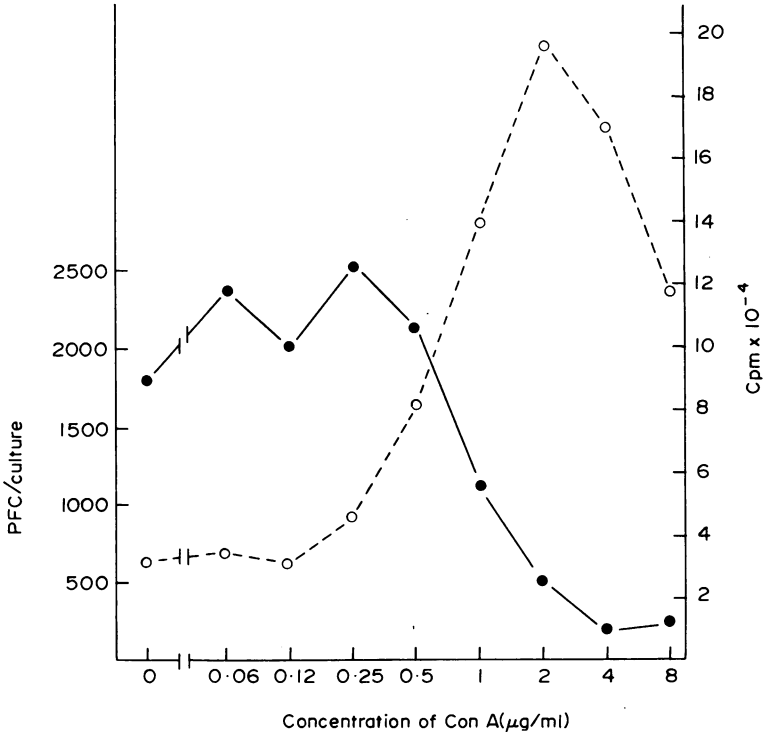

FIG. 2. 6×10^6 spleen cells were cultured with SRC and various concentrations of Con A. DNA synthesis and PFC numbers were determined as in Fig. 1.

1971). Addition of certain concentrations of Con A could reconstitute the response of the T cell deficient spleen cells (Table 2). Thus, addition of Con A at a concentration of 0·5 μg/ml or more to anti-θ-treated spleen cells had a marked stimulatory effect on the PFC response. On the other hand, addition of Con A at concentrations higher than 0·5 μg/ml to cultures of the normal cells depressed the immune response. Anti-θ serum-treated spleen cells always contain a few T cells (Miller & Sprent, 1971). It seems likely that Con A restores the immune response of spleen cells treated with anti-θ-serum by activating these remaining cells.

It was also possible to get a stimulation of the immune response of B cells by adding certain numbers of thymocytes which had been activated by cultivating them for 24 hr with Con A (Table 3). The Con A was removed by incubation with 20 mg/ml of methyl-α-D-manno-pyranoside for 60 min at 37°C and the activated T cells were added to the B cells.

The response to SRC increased with increasing numbers of added T cells until an optimal number was reached. When the number of T cells was increased further there was a sharp reduction in the number of PFC. Untreated thymocytes gave only a marginal and inconsistent restoration of the immune response. Thymocytes which had been treated with Con A for 1 hr to allow binding of Con A to the cells were also used. This control was performed, because Con A by itself could stimulate the response of T cell depleted spleen cells. It was

TABLE 1. Kinetics of the PFC response against SRC after addition to the cultures of various doses of Con A

Exp.	Concentration of Con A	PFC/culture on day				
		1	2	3	4	5
1	0	0	263	5320	14880	—
	0·25	0	233	4040	5640	—
	4	0	40	80	470	—
2	0	3	0	185	1540	2640
	0·25	3	35	275	2400	4640
	4	5	55	160	680	1360

7×10^6 spleen cells from untreated mice were cultured with SRC and various concentrations of Con A. PFC responses were determined on various days.

TABLE 2. Effect of Con A on the response of normal or T cell-depleted spleen cultures to SRC

Treatment of spleen cells	PFC response/culture to SRC at the following Con A concentrations (μg/ml)					
	0	0·25	0·5	1	2	4
Normal mouse serum + C′	1810	1840	2140	780	700	170
Anti-theta serum + C′	30	30	540	2410	3530	2590

8×10^6 spleen cells treated as indicated were cultured together with SRC.

found that thymocytes, which had been incubated with Con A for 1 hr, were not more effective than untreated thymocytes in reconstituting the PFC response, thus making carry-over of Con A to the B cell cultures unlikely.

Inability of Con A-activated T cells to substitute for adherent cells in the primary antibody response

Normal spleen cells or cells depleted of adherent cells by treatment with iron powder were cultured with SRC and various concentrations of Con A (Table 4). It can be seen that addition of Con A sometimes increased the antibody response of the non-adherent

TABLE 3. Reconstitution of the immune response of B cells by Con A stimulated thymocytes

Exp.	Pretreatment of thymocytes	PFC/culture to SRC after addition of indicated No. of thymocytes ($\times 10^6$)						
		0	0·5	1	2	4	6	8
1	Con A for 24 hr	510	310	680	2950	170	—	—
2	Con A for 24 hr	10	—	130	285	315	90	5
3	Con A for 24 hr	0	140	140	325	315	—	30
3	Con A for 1 hr	0	0	25	25	0	—	0
3	None	0	0	45	40	0	—	10

Thymocytes were cultured for 24 hr. Con A at an optimally stimulating dose of 5 μg/ml was added at the beginning of the culture or in one experiment, 1 hr before harvest of the cells or not at all. The thymocytes were then treated with methyl-α-D-mannopyranoside to remove Con A and added at various numbers to 4×10^6 B cells. The B cells were obtained from T × B mice in experiments 1 and 2 and from normal spleen cells treated with anti-θ serum in experiment 3. The bone marrow cells were treated with anti-theta serum before transfer into the mice in experiment 2.

cells. However, in these cases also the PFC response of the normal spleen cells increased with addition of Con A, and the response in the iron-treated cultures was still markedly lower than that in the normal cell cultures. Furthermore, addition of Con A-activated thymocytes could not reconstitute the antibody response of non-adherent cells, although some stimulation of the PFC response could occur (Table 5). Adherent cells added to the cultures were more efficient reconstitutors of the antibody response. Thus, T cells activated by Con A cannot substitute for adherent cells in the antibody response.

TABLE 4. Effect of Con A on the PFC response in normal or adherent cell-depleted cultures

Concentration of Con A	Exp. 1		Exp. 2			
	Day 4		Day 3		Day 4	
	Normal	Depleted	Normal	Depleted	Normal	Depleted
0	3940	385	570	5	3040	50
0·12	4440	600	—	—	—	—
0·25	4120	570	5780	485	6620	1125
0·5	2550	790	1240	355	3160	535
1	1000	530	920	70	1540	395
2	760	310	320	70	970	470
4	230	220	70	65	1180	555

Seven or 10×10^6 normal or adherent cell-depleted spleen cells were cultured with various doses of Con A and the PFC responses/culture were determined on day 4.

TABLE 5. Inability on Con A-activated thymocytes to reconstitute the antibody response to SRC in adherent cell-depleted cultures

Cells	No. of Con A-activated thymocytes ($\times 10^{-6}$)	PFC/culture		
		Exp. 1	Exp. 2	Exp. 3
Untreated	0	11580	7200	3300
Iron-treated	0	33	220	10
Iron-treated	0·5	30	210	30
Iron-treated	1	80	350	30
Iron-treated	2	1110	630	40
Iron-treated	4	870	1310	110
Iron-treated + adherent cells	0	—	3170	800

Thymocytes were activated by cultivation with Con A for 24 hr and thereafter washed with methyl-α-D-mannopyranoside to remove Con A. Indicated numbers of the activated thymocytes or alternatively adherent spleen cells were then added to non-adherent cells.

DISCUSSION

The effect of the T cell mitogen Con A on the antibody response *in vitro* against SRC was studied in an attempt to get some information on the function of T cells. The stimulation of DNA synthesis and of the production of PFC did not correlate. Con A doses higher than 0·5 μg/ml were inhibitory to the antibody response, whereas maximal cell proliferation occurred at Con A concentrations of 2–4 μg/ml, in agreement with earlier results (Andersson *et al.*, 1972a). These findings contrast to earlier results with the B cell mitogen LPS. Thus, LPS causes a stimulation of antibody production, which closely parallels activation of cell proliferation (Andersson *et al.*, 1972b). In addition, LPS induces an early appearance of PFC, whereas no such stimulation was recorded after stimulation with Con A (Sjöberg *et al.*, 1972a). Thus, the differences between Con A and LPS with regard to their selective effects on T and B cells are reflected in their effects on the antibody response.

Different alternative explanations of the T and B cell cooperation have been suggested. According to one group of explanations, close contact between T and B cells is necessary and the T cells act either by binding the antigen onto their receptors, thereby offering a higher local concentration (Mitchison *et al.*, 1970; Möller, 1970; Taylor & Iverson, 1971) or by cross-linking the epitopes to the B cells in this way covering the formation of a stable lattice (Bretscher & Cohn, 1970; Feldman & Basten, 1971). According to another group of explanations, the T cells may stimulate the B cells by releasing soluble factors which are either specific receptors, which help in triggering the B cells after binding to macrophages (Lachman, 1971; Feldman & Basten, 1972), or non-specific stimulators of antibody synthesis affecting the B cells directly (Dutton *et al.*, 1971; Schimpl & Wecker, 1972; Sjöberg *et al.*, 1972c).

The present results show that thymocytes and peripheral T cells, which have been non-specifically activated by the T cell mitogen Con A acquire the capacity to stimulate the

response of B cells to the thymus-dependent antigen SRC. Soluble Con A added to cultures of anti-θ serum-treated spleen cells also restored the antibody response. This reconstitution is probably due to activation of residual T cells, always present after anti-θ serum treatment (Miller & Sprent, 1971).

Since Con A is likely to activate T cells irrespective of their immunological specificity, the results in this study suggest that once T cells are activated they can non-specifically support the antibody response of B cells, regardless of the immunological specificity of the latter. There are other recent results, which support a non-specific mechanism for the stimulatory effect of T cells on B cells. Thus, it has been reported that a graft-versus-host reaction may stimulate the response under certain conditions *in vivo* (Katz *et al.*, 1971; Kreth & Williamson, 1971). A mixed lymphocyte culture may also result in an augmented antibody response *in vitro* (Dutton *et al.*, 1971; Schimpl & Wecker, 1971; Sjöberg, 1972). Activation of a large number of T cells could be expected in all these experimental conditions, because the frequency of cells reacting against histocompatibility antigens is very high (Nisbet *et al.*, 1969; Wilson *et al.*, 1968). In fact, there are findings demonstrating that soluble factors, which are stimulatory to the antibody response of T cell depleted spleen cell cultures, are present in the medium from mixed lymphocyte cultures (Britton, 1972; Dutton *et al.*, 1971; Schimpl & Wecker, 1972; Sjöberg *et al.*, 1972c).

The apparent discrepancy between these results and the documented necessity for the hapten and the carrier to be present on the same molecule in order to induce a pronounced secondary response in animals primed with the hapten-carrier complex (Mitchison *et al.*, 1970) can be overcome by assumption that although the T cells are triggered specifically by the antigen, the T cells stimulate the B cells unspecifically, probably via a humoral factor. The hapten carrier complex may act as a bridge between the T and B cells thereby increasing the concentration of the presumptive factor in the vicinity of the relevant B cells.

It is not yet clear whether macrophages act only on T cells, B cells or both. In an attempt to study this, we investigated whether non-specifically activated T cells could substitute for macrophages. Although the activated T cells increased the antibody response of the non-adherent cells to some extent, no marked reconstitution was obtained. In contrast, adherent cells efficiently restored the PFC response in depleted cultures. Therefore, macrophages seem to have a direct effect on B cells. The results obviously do not exclude that the macrophages are important for the functions of T cells as well. Actually some recent results suggest that macrophages are important for the production of a soluble factor, which can substitute for T cells in the antibody response (Sjöberg *et al.*, 1972c) and it has also been shown earlier that antigen-induced proliferation *in vitro*, which is thought to be a T cell mediated phenomenon, is dependent on macrophages (Hersch & Harris, 1968).

A large number of activated T cells, obtained either by adding high doses of Con A to cultures of normal spleen cells or by adding large numbers of activated T cells to cultures with B cells, caused a marked reduction of the antibody response. The suppressive effect of large numbers of activated T cells is analogous to what has been observed *in vivo* (Möller, 1971a) and *in vitro* (Sjöberg 1971; 1972) during GVH reactions leading to antigenic competition. This phenomenon has been shown to be mediated by T cells (Möller, 1971b; Sjöberg & Britton, 1972; Gerschon & Kondo, 1971) and may represent an intense stimulation of T cells resulting in a suppressive effect on B cells. Thus, certain numbers of Con A activated T cells could reconstitute the immune response to red cells in T cell depleted spleen cultures, whereas larger numbers suppressed the response. It cannot presently be decided,

whether the same or different mechanisms are responsible for both reconstitution and suppression of the immune response.

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