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Con A Induced Suppressor Cells: Suppression with *I* Region Incompatibility[1]

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Allogeneic Con A induced suppressor cells differing in the *I* and *S* region but not *H-2K* or *H-2D* regions were as efficient as syngeneic cells in suppressing the secondary IgM and IgG response to burro erythrocytes. Con A activated suppressor cells were not sensitive to anti-Ia serum and complement. However, if the spleen cell population was treated with anti-Ia serum and complement before stimulation with Con A, suppressor cells were not generated.

INTRODUCTION

Cell cooperation between thymus-derived T lymphocytes and bone marrow-derived B lymphocytes is necessary for generation of an antibody response to most antigens (1). The precise nature of this cooperation is not known; however, it has recently been shown that identity within the *I* region of the major histocompatibility locus (H-2) is necessary for cooperation between carrier specific T cells and hapten specific B cells (2–8). Spleen cells identical at the *I* region but differing at the *K, S,* or *D* regions cooperate effectively, whereas cells differing only in the *I* region, more specifically the *I-A* and *I-B* subregions, do not (6–8). Katz and Benacerraf suggest that products of genes mapping in the *I* region, are cell cooperation or interaction proteins (9). Consistent with their observations are experiments from this laboratory in which alloantisera specific for Ia antigens inhibited the primary and secondary *in vitro* response to heterologous erythrocytes (10). No inhibition was observed with antisera specific for *H-2K* or *H-2D* determinants.

In addition to the cooperative or helper function, T cells can be suppressive (1, 11) and there is evidence to suggest that separate T cell subpopulations are responsible for these different functions (11–14). In our studies on the correlation of the functional properties of T cell subsets with the presence or absence of Ia determinants we have demonstrated that this cell surface antigen is present on a

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subpopulation of T cells and that this subpopulation is Con A sensitive but not PHA or Leukoagglutinin sensitive (15, 16).

The following experiments were designed to determine if non-specific suppressor T cells are present in the Ia sensitive T cell subpopulation and whether for suppressor function it would be necessary to have *I* region identity. We have used a system described by several investigators 12, 17, 18, in which Con A induced suppressor cells are added to fresh spleen cell cultures and the PFC response examined after 5 days. The suppressor cell population is tested for suppressor function after treatment with anti-Ia sera and complement and for the ability to suppress PFC responses of allogeneic as well as syngeneic spleen cells. In these experiments the allogeneic cells differ only in the *I* region of the H-2 complex.

MATERIALS AND METHODS

Mice. All mice were raised in the mouse colony at the University of Michigan.

Antisera. Antisera specific for Ia antigens were produced by reciprocal immunizations of A.TH and A.TL as previously described (19). Anti-Thy 1.2 serum was prepared by immunizing A.AKR mice (H-2^{al}, Thy-1^{a}) with A.AL (H-2^{al}, Thy-1^{b}) thymocytes. Rabbit sera, used as a source of complement, was EDTA treated and agarose absorbed.

Cell culture. Spleen cell cultures were established according to the method of Mishell and Dutton modified as described (10). Briefly, spleen cell suspensions were cultured in Hank's MEM supplemented with L-glutamine, essential and non-essential amino acids, sodium pyruvate, vitamins, hepes buffer, 2-mercaptoethanol, 10% fetal calf serum (Reheis Chemical Co., Kewanee, Illinois), and gentamycin. Antigen stimulated cultures contained 2×10^7 BRBC per 10^7 spleen cells in a volume of 1 ml. For secondary responses spleen cell donors were immunized at least 3 weeks before use with 8×10^8 BRBC (Colorado Serum Company).

Suppressor cells were generated with Con A (0.75 μg per 10^7 spleen cells) according to Rich and Pierce (17). Cells from Con A activated cultures were harvested at 48 hr, washed by centrifugation in media 3 times, resuspended to 10^7 cells per ml and 0.1 ml of this suspension added directly to fresh spleen cell cultures or irradiated before addition to reconstituted cultures containing anti-Thy-1.2 resistant cells and irradiated helper cells. Rich and Pierce have reported the radiation-resistance of Con A induced suppressor cells (17).

To obviate the problem of the allogeneic Con A activated cells, we used a culture system containing a mixture of anti-Thy 1.2 treated cells (8×10^6/culture) and irradiated helper cells (6×10^6/culture) prepared from the same population of spleen cells (20). Suppressor cells were irradiated before addition to these cultures.

Cell irradiation. Cells were irradiated with a Westinghouse Coronado model X-ray Therapy unit (250 kV, 15 mA) with a dose of 1467 R at 63 R/min through 0.5 mm Cu: 1 mm AL filters. After irradiation, cells were centrifuged once in media and resuspended to the desired cell density.

Antisera treatment. Cells used as a source of suppressor cells were subjected to lysis by anti-Ia serum prior to culturing with Con A for 48 hr or after 48-hr culture period. Cells were suspended in a 1:10 dilution of anti-Ia serum or normal mouse serum for 30 min at 37°C, centrifuged and resuspended in an appropriate dilution of agarose absorbed rabbit complement for 30 min at 37°C. The cells were centrifuged twice in media before being added to cultures.

Hemolytic plaque assay. Direct and indirect PFC responses to BRBC were determined on Day 5 by the method of Jerne and Nordin (21) with modifications as described (*22, 23*).

RESULTS

Ia positive suppressor T cells. To test whether suppressor T cells are Ia positive T cells or whether for their generation with Con A they are dependent on Ia positive cells, normal spleen cells were subjected to treatment by anti-Ia serum and complement. The resistant spleen cells were either incubated with Con A or without Con A for 48 hr (17). In Table 1, spleen cells not stimulated with Con A did not demonstrate suppressor activity when cultured with syngeneic primed spleen cells. Con A suppressor cells, however, markedly reduced both IgM and IgG *in vitro* responses unless they had been treated with anti-Ia serum and complement before Con A stimulation (line 6 and 7, Table 1).

The significantly decreased number of Con A induced suppressor cells in populations depleted of Ia positive cells indicated that the suppressor T cell was either generated from the small number of Con A reactive Ia positive T cells or was dependent on this population for differentiation. In recent experiments, when small numbers of normal T cells were added to Ia depleted T cells, as few as 20% normal T cells could completely restore the response of Ia depleted T cells. The T6 chromosomal marker was used to further demonstrate that Ia negative T cells were being recruited by Ia positive Con A reactive cells (24).

In two experiments shown in Table 2, spleen cells from non-immunized A.SW ($H\text{-}2^s$) mice were treated with A.TL anti-A.TH serum (anti-Ia^s) or normal A.TL serum and complement before incubation with Con A or after the 48 hr incubation. Antisera plus complement treatment resulted in a 30–45% cell loss. Suppressor cells were added to fresh cultures of primed A.SW spleen cells and IgM and IgG PFC assayed on Day 5. Suppression was not altered by anti-Ia serum treatment of the suppressor population once it was generated (line 6). However,

TABLE 1

Treatment of Spleen Cells with Anti-Ia Antisera before Generation of Con A Suppressors[a]

	PFC/Culture[b]	
	IgM	IgG
1. B10.BR	8	5
2. B10.BR + BRBC	1226	152
3. B10.BR + BRBC + Non-Con A cells	1064	94
4. B10.BR + BRBC + Non-Con A cells (NMS)	1252	152
5. B10.BR + BRBC + Non-Con A cells (α Ia^k)	2473	149
6. B10.BR + BRBC + Con A suppressor cells	145	19
7. B10.BR + BRBC + Con A suppressor cells (αIa^K)	512	84

[a] B10.BR ($H - 2^k$) spleen cells were treated with normal A.TH serum (NMS) or A.TH anti-A.TL (α Ia^k) serum and complement. The cells resistant to this treatment were cultured with Con A or for control without Con A stimulation. After 48 hr the cells were removed from culture and co-cultured with primed syngeneic spleen cells.

[b] Direct and indirect PFC were assayed on Day 5. The PFC/culture represents a pool of four dishes sampled twice.

TABLE 2

Treatment of Suppressor Cells with Anti-Ia Antisera[a]

	PFC/Culture			
	Experiment 1		Experiment 2	
	IgM	*IgG*	*IgM*	*IgG*
1. ASW	40	40	10	10
2. ASW + BRBC	1076	648	2052	2255
3. ASW + BRBC + Suppressor	153	135	277	335
4. ASW + BRBC + Suppressor (α Ia^s, 0 hr)	675	285	975	1075
5. ASW + BRBC + Suppressor (NMS, 0 hr)	110	37	315	315
6. ASW + BRBC + Suppressor (α Ia^s, 48 hr)	135	60	20	55
7. ASW + BRBC + Suppressor (NMS, 48 hr)	150	115	205	300

[a] ASW (H − 2^s) suppressor cells were treated with A.TL anti-A.TH (αIa^s) or normal A.TL serum (NMS) and complement before (0 hr) or after 48 hr incubation with Con A (48 hr). PFC responses to BRBC were measured on Day 5.

elimination of the Ia, positive cells before incubation with Con A decreased the ability of the remaining cells to suppress (line 4). The persistent difference between lines 4 and 2 indicates that anti-Ia serum and complement pretreatment of the spleen cell population significantly reduces the number of Con A suppressors but does not completely eliminate their generation. These results indicate that Ia positive cells are necessary for the generation of suppressor cells but once generated either they do not express Ia determinants or their function is not dependent on the presence of Ia bearing cells.

I-region compatibility. If Ia bearing cells can be removed from a suppressor

TABLE 3

Suppression by Syngeneic and Allogeneic Cells[a]

	PFC/Culture[b]					
	1		2		3	
	IgM	IgG	IgM	IgG	IgM	IgG
Spleen cells	15	0	30	75	—	—
Spleen cells + BRBC	315	150	450	555	2307	893
Spleen cells + BRBC + syngeneic suppressors	90	0	255	135	—	—
Irradiated helper cells + BRBC	0	0	0	0	0	0
Anti-Thy 1.2 treated cells + BRBC	145	45	210	360	1381	162
Anti-Thy 1.2 treated cells + irradiated helper cells + BRBC	495	400	695	845	2170	2020
Anti-Thy 1.2 treated cells + irradiated helper cells + BRBC + syngeneic suppressors	315	135	382	652	1418	343
Anti-Thy 1.2 treated cells + irradiated helper cells + BRBC + allogeneic suppressors	315	115	412	390	1487	662

[a] Secondary PFC response to BRBC was measured 5 days after the addition of 10^6 irradiated suppressor cells to cultures of 10^7 primed spleen cells or 8×10^6 anti-Thy 1.2 treated cells and 6×10^6 irradiated helper cells. Experiments 1 and 2 were A.TH (H-2: $K^sI^sS^sD^d$) and A.TL (H-2: $K^sI^kS^kD^d$) combinations and experiment 3 was A.TL and B10.S(7R) (H-2: $K^sI^sS^sD^d$).

[b] PFC/culture represents a pool of four dishes sampled twice.

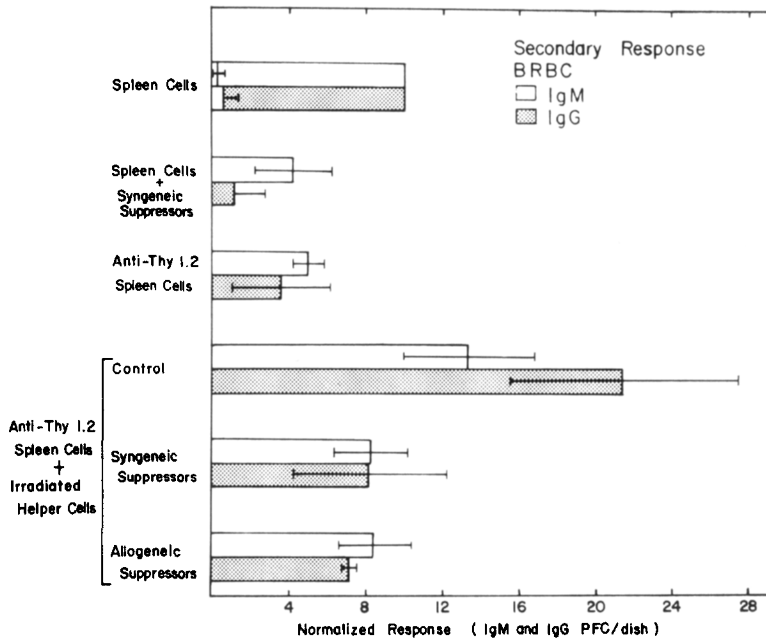

FIG. 1. The addition of *I* region compatible or incompatible suppressor cells to BRBC primed reconstituted cultures. Responses are plotted on a scale in which the response of primed spleen cells is normalized to 10 PFC/culture and expressed as the mean ± SD of three normalized experiments. The suppression of the IgM response was significant at the $P < 0.025$ level with both *I* region compatible and incompatible suppressor cells and the suppression of the IgG response was significant at the $P < 0.005$ level.

population without effect on its function, then one might suppose that *I* region histocompatibility, required for helper function, is not necessary for any cell-cell interaction involved in suppressor cell activity. Therefore, we tested the ability of Con A induced suppressor cells to decrease the PFC response of allogeneic as well as syngeneic spleen cell cultures.

It was necessary to use a reconstituted cell culture system in order to eliminate possible allogeneic effects which could enhance the PFC response. Spleen cells were divided into two groups; one was treated with anti-Thy 1.2 serum and complement to remove T cells and the other was irradiated. The irradiated cells served as a source of helper T cells for the anti-Thy 1.2 treated B cell population. Suppressor cells which were either syngeneic or *I* region incompatible were irradiated before addition to the cultures. The secondary PFC response to BRBC was measured 5 days later. Control cultures contained untreated spleen cells from the same spleen cell pool and were tested for their response and for the suppression of that response with syngeneic suppressor cells. Control cultures, in which cells preincubated for 48 hr without Con A and then added to spleen cell cultures, were not significantly different from cultures to which media alone was added.

Table 3 summarizes the results of three experiments. Allogeneic suppressor cells are able to decrease the response to the same extent as syngeneic cells. The cells differed in the *I* and *S* region but not in the *K* or *D* regions (H-2: $K^sI^kS^kD^d$ and $K^sI^sS^sD^d$). In one experiment involving genetic background differences in addition to these H-2 differences [A.TL$^{H-2^{tl}}$ and B10.S(7R)$^{H-2^{tl}}$] the results are the same.

The normalized results of these three experiments are graphically presented as Mean ± SD PFC per culture dish in Fig. 1.

DISCUSSION

In order to study the relationship of serologically distinct Ia antigens to functional subpopulations of T cells, we have utilized both the *in vitro* response to mitogens and the *in vitro* humoral response to thymus dependent and independent antigens. Although we have been able to significantly block primary and secondary PFC responses and the proliferative response to several B cell mitogens by pretreating spleen cells with anti-Ia serum, it has only been recently that we have been able to associate the presence of the Ia antigen with specific cell functions. For example, treatment of spleen cells or nylon wool purified T cells with anti-Ia serum and complement eliminates their response to the T cell mitogen Con A but not to another T cell mitogen PHA (15). Furthermore, experiments with antisera of restricted specificity indicate that not all *I* subregions code for antigens on Con A reactive cells (16). Recently, another Ia antigen related specific cell function was demonstrated by experiments in which anti-Ia sera specific for the *I-J* subregion inhibited the ability of macrophages to support an *in vitro* humoral response (25).

In the experiments reported here Con A activated suppressor cells were not sensitive to Ia serum and complement and *I* region compatibility between suppressor cells and antigen stimulated spleen cells was not necessary for suppressor function. However, Ia positive cells were necessary for the generation of suppressor cells by Con A. It is not clear whether cells which have the Ia antigen on their surface lose this antigen during their differentiation into suppressor cells, express a new set of different Ia determinants not detectable by the anti-Ia sera used, or whether Ia reactive cells serve as accessory cells to aid the activation of other non-Ia bearing cells into terminally differentiated suppressor cells.

This latter explanation could account for the observation that some degree of suppressor activity can be generated by stimulating spleen cells depleted of Ia positive T cells as judged by their loss of the Con A stimulated proliferative response. We have found that as few as 20% Ia positive Con A reactive T cells can completely restore the proliferative response of Ia negative, non-Con A reactive T cells (24). It is therefore possible that a very small number of Con A reactive cells escape lysis by anti-Ia serum and complement and are sufficient to recruit a limited number of non-Ia bearing suppressor T cells. It is, of course, possible that these Con A induced suppressor cells express Ia antigen(s) detectable only by non-complement binding anti-Ia antibodies. Despite the lack of sensitivity of Con A induced suppressor cells to anti-Ia serum and complement, at least a portion of the splenic blast cell population generated by Con A stimulation can be lysed by anti-Ia serum and complement (26). These blast cells can absorb Ia antibodies which when eluted are cytotoxic for T and B lymphocytes (26).

In experiments shown in Table 3 the response in the reconstituted cultures was not suppressed to the same degree as the untreated cultures. This was most likely the insufficient removal of T cells by anti-Thy 1.2 serum with the remaining T cells providing excess "helper" cells to compete with the added suppressor cells. The finding that *I* region compatibility was not necessary for suppressor cell function is in contrast to the results of Katz *et al.*, demonstrating the necessity for *I* region compatibility between carrier primed "helper" T cells and hapten primed B cells

(6). These investigators propose that the Ia antigens on the surface of T and B cells need to be homologous for the cooperative interaction to cccur. Further support for this has been the demonstration of *I* region determinants on helper T cells in the adoptive secondary IgG response to DNP-KLH (27). It has also been found that the enhancing properties of soluble factors, either from supernatants of cultured allogeneic cells (28) or from antigen stimulated thymocytes (29) can be specifically removed with anti-Ia immunoabsorbants.

Rich and Pierce (17) suggest that Con A activated suppressor cells act by inhibiting the clonal expansion or terminal differentiation of already "activated" B cells. In contrast, the T cell dependent "helper" phase of a response is thought to occur early, during the antigen recognition phase (30, 31). Dutton's experiments on the inhibitory and stimulatory effects of Con A on the PFC response of mouse spleen cells indicate that there are two cell targets for Con A stimulation, one activated to inhibit the response and one activated to enhance (12). His data further suggest that the helper cell responds to Con A but not PHA and the suppressor cell respond to both.

Pierce and coworkers have also isolated a supernatant factor from the Con A activated T cells which can mediate suppression of the PFC response. It is not known if this fatcor, designated SIRS, has Ia determinants. In contrast to Con A activated suppressor cells which are still inhibitory when added to 48 hr cultures, SIRS is not effective if added after 24 hr of culture. SIRS, therefore, acts early during activation even though kinetic data show that the effect of this action occurs during the later stages of differentiation and proliferation (14). These findings suggest that the Con A induced suppressors may function via two distinct pathways. It is also possible that the cells responsible for SIRS secretion and the suppressor cells are distinct cell populations. For example, both populations would be required for the production of SIRS, one an Ia positive active suppressor cell, the other an Ia negative SIRS secreting cell. This alternative, would be in agreement with the observations reported here that depletion of the Ia positive subpopulation will prevent the development of significant suppression, but that not all suppressor activity can be eliminated by anti-Ia antibodies and complement. This residual suppression might be due to SIRS production by the remaining Ia negative cells. The fact that SIRS functions without strain specificity could also explain why no *H-2* restriction of the suppressor activity was found.

The lack of strain specificity for SIRS activity is different from the antigen-specific suppressor factor characterized by Tada and co-workers (32, 33). There soluble suppressor factor can be absorbed by antibodies to *I* region determinants controlled by loci of the *I-J* subregion (34). It has also been demonstrated that anti-Ia sera and complement lyse allotype suppressor T cells (27). They found that these suppressor T cells carried the Ia-4 determinants which has defined the new *I-J* subregion (35). Thus, the relationship of Con A induced suppressor T cells and their soluble factors (SIRS) to other mechanisms is not clear. It does, however, appear that several distinct pathways for suppression exist and that different *in vitro* or *in vivo* systems measure different mixes of each mechanism.

It is interesting and perhaps very significant that the new *I-J* sub-region should control determinants which have been associated with a number of cellular immune functions. For example, these determinants have been shown to be present on the T cell subpopulation responding to Con A (15, 16), the allotype suppressor T

cells (35), as part of soluble factors which suppress antigen specific responses (35), and on macrophages involved in the initiation of the *in vitro* humoral response (25). The ability to assign different functional properties to Ia positive and negative cell populations and even more significantly to different *I* region loci is of tremendous importance to our understanding of the complex processes involved in the immune response.

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