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Induction of suppressor cells by staphylococcal enterotoxin B: identification of a suppressor cell circuit in the generation of suppressor-effector cells

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SUMMARY

We have shown previously that staphylococcal enterotoxin B (SEB) has the capacity to non-specifically inhibit antibody responses *in vitro* through the induction of a suppressor cell population. In the present studies, an analysis of the cellular dynamics has shown that the generation of Lyt-1^-2^+ suppressor-effector cells is dependent on the initial activation by SEB of an Lyt-1^+2^- suppressor-inducer population. Co-culture experiments carried out *in vitro* suggest that the induction of the suppressor-effector population requires at least two signals: one signal is provided by the suppressor-inducer population, and the second signal is provided by SEB. Studies also show that the *in vitro* antibody response is suppressed when the suppressor cells are added as late as Day 4 of a 5-day culture. While the suppressor cell population activated early in the antibody response is Lyt-1^-2^+, depletion studies suggest that the population that acts late in an ongoing response bears the Lyt-1^+2^+ surface phenotype. The results demonstrate that at least three distinct SEB-induced T-cell populations are capable of participating in the suppression of the antibody response. The relationship between the generation of non-specific suppressor cells and the activation of antigen-specific cell circuits is discussed.

INTRODUCTION

A common major pathway for the generation of antigen-induced and antigen-specific suppressor T cells has been proposed recently (Dorf & Benacerraf, 1984). The cellular interactions characteristic of this pathway include the generation of a suppressor-inducer T-cell subset, followed by the subsequent development of one or more suppressor-effector populations. This pathway has been defined for responses to a variety of antigens, and the suppression may be manifested by reduced antibody responses (Eardley *et al.*, 1978; Germain *et al.*, 1978; Sherr & Dorf, 1981), contact sensitivity (Sy *et al.*, 1979; Sunday, Benacerraf & Dorf, 1981; Ptak *et al.*, 1982), delayed-type hypersensitivity (Bach *et al.*, 1978; Weinberger *et al.*, 1979) or T-cytotoxic cell activity (Greene *et al.*, 1982; Sy *et al.*, 1982).

Much less is known about the relationship between non-antigen-specific suppressor cells and the common suppressor cell pathway just described. Alloantigen-induced and antigen-specific Lyt-1^-2^+ suppressor cells have been shown (Beckwith & Rich, 1982) to function by inducing a non-antigen-specific Lyt-1^+2^+ population. In a similar system (Zembala *et al.*, 1982), however, an antigen-specific suppressor population (in this case, Lyt-1^+2^+) has been demonstrated to be required for the subsequent activation of a non-specific suppressor-effector (in this case, Lyt-1^-2^+) of contact sensitivity. Additional information concerning the requirement for antigen stimulation in the activation of each of these (and other) non-specific cell populations is clearly needed.

We have shown previously (Donnelly & Rogers, 1982) that the polyclonal T-cell mitogen staphylococcal enterotoxin B (SEB), a toxin associated with common food poisoning, is capable of inducing non-specific T-suppressor cells. In the present paper we show that the generation *in vitro* of Lyt-1^-2^+ suppressor-effector cells by SEB requires the activation of an Lyt-1^+2^- cell population. The results show that activation of Lyt-1^-2^+ suppressor-effector cells requires interaction with both SEB-primed Lyt-1^+2^- cells and a signal supplied by the SEB. The results also show that the Lyt-1^-2^+ population

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Abbreviations: Con A, concanavalin A; MEM, minimal essential Eagle's medium; NFSC, non-fractionated splenocytes; PFC, plaque-forming cell; SEB, staphylococcal enterotoxin B; SRBC, sheep erythrocytes; TCM, tissue culture medium.

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functions only when present early in an antibody response. A third SEB-induced T-cell population, which appears to express the Lyt-1^+2^+ phenotype, is capable of suppressing the antibody response when added late in an ongoing antibody response.

MATERIALS AND METHODS

Mice

Male BALB/cAnSKH mice were obtained from the breeding facility at the Skin and Cancer Hospital, Temple University School of Medicine. Mice were between the ages of 8 and 12 weeks when used in these experiments. Certain mice received an intraperitoneal injection of 0·2 ml of a 10% suspension of sheep erythrocytes (SRBC) and were used after 2 weeks as a source of immune splenocytes.

Staphylococcal enterotoxin B

Lyophilized SEB (Sigma, St Louis, MO) was reconstituted with minimal essential Eagle's medium (MEM; M.A. Bioproducts, Walkersville, MD) and added to the primary cultures at a final concentration of 10 μg/ml.

Antibodies

Monoclonal anti-Ly-1 and anti-Lyt-2 antibodies, produced by hybridomas 53-7.313 and 53-6.72 (Ledbetter & Herzenberg, 1979; obtained from the American Type Culture Collection, Rockville, MD), respectively, were prepared as culture supernatants and used as the source of first antibody in indirect cytotoxicity depletions at a final dilution of 1 : 10. Goat anti-rat IgG (heavy and light chain specific) was employed as the second antibody in indirect cytotoxicity depletions at a final concentration of 30 μg/ml. The degree of depletion obtained with the anti-Ly antibodies was determined by immunofluorescence. The population of Ly-1-depleted cells was found to contain no greater than 4% Ly-1-positive cells. The population of Lyt-2-depleted cells was found to contain less than 0·5% Lyt-2-positive cells.

Monoclonal anti-L3T4, produced by hybridoma GK1.5 (Dialynas *et al.*, 1983), was obtained from the American Type Culture Collection and was used as a culture supernatant. Depletion of L3T4-positive cells was carried out by treatment with non-diluted supernatant, followed by treatment with complement. This regimen was then repeated for successful generation of less than 1% L3T4-positive cells.

Guinea-pig serum was used as a source of complement for cell depletion by indirect cytotoxicity. It was first absorbed on minced mouse liver for 30 min in the cold. It was then diluted 1 : 2 with medium consisting of MEM supplemented with 1% fetal calf serum and 25 μg/ml gentamicin.

Priming cultures

Cell priming was performed by incubating normal spleen cells (SC) at a concentration of $1{\cdot}5 \times 10^7$ cells/ml in a tissue culture medium (TCM) consisting of MEM supplemented with 1 mM non-essential amino acids, 1 mM sodium pyruvate, 50 μg/ml gentamicin, 2 mM L-glutamine, 10% fetal calf serum and 0·05 mM 2-mercaptoethanol. Cultures with or without SEB were incubated at 37° in a sealed gas chamber in an atmosphere consisting of 10% CO_2, 7% O_2 and 83% N_2.

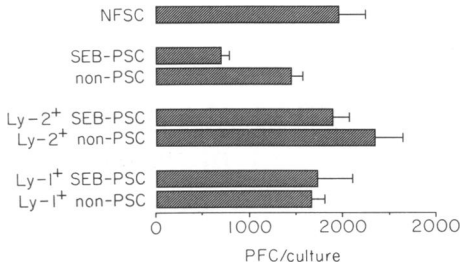

Figure 1. The requirement for Ly-1^+ cells in the induction of suppressor cells by SEB. Naive splenocytes were first either depleted of Ly-1-bearing cells, or left non-fractionated, and then primed for 2 days with 10 μg/ml SEB. The SEB-primed (or non-primed control populations) were then co-cultured with immune splenocytes and SRBC in a Mishell–Dutton culture system. The PFC response was measured 5 days later. The treatment with complement alone, or second antibody and complement alone, has no effect on the responses shown.

Mishell–Dutton cultures

Antibody-forming cells were generated *in vitro* in micro-Mishell–Dutton cultures (Kappler, 1974; Tittle & Rittenberg, 1978). Cells were cultured in flat-bottomed 96-well microculture trays at a density of 2×10^7 cells/ml in the TCM in the atmosphere described above. Each well received 0·05 ml of this cell suspension and 0·05 ml of a 0·1% suspension of SRBC in the TCM. A culture represented a row of eight wells and cells were pooled accordingly at the time of the PFC assay. Co-culture of various primed cells with immune SC was used to evaluate suppressor cell activity. Twenty-four hours after initiation of cultures, the cells were fed 0·05 ml of a nutritional cocktail that consisted of MEM supplemented with a final concentration of 3 mM non-essential amino acids, 3 mM essential amino acids, 6 mg/ml dextrose, 6 mM L-glutamine, 0·67% sodium bicarbonate, 33% fetal calf serum and 41 μg/ml each of adenosine, uridine, cytosine and guanosine.

Detection of the in vitro *immune response*

The plaque-forming cell (PFC) response of Mishell–Dutton cultures was determined by haemolytic plaque assay (Cunningham & Szenberg, 1969). Triplicate determinations of the PFC response were performed for each group, and the mean value per group was determined. Only the direct (IgM) PFC response was measured. Results are expressed as PFC per culture and include the standard error of the mean for each group.

RESULTS

Ly-1^+ cells are required for the induction of Lyt-2^+ suppressor cells

We have demonstrated previously (Donnelly & Rogers, 1982) that SEB induces a Lyt-1^-2^+ T-cell population that is capable of suppressing the anti-SRBC response when added on Day 0 of a 5-day Mishell–Dutton culture. We initially wished to determine whether the induction of suppressor cells by SEB was dependent on the presence of a Ly-1^+ cell population. In this study we first depleted Ly-1-bearing cells with monoclonal antibody, and then cultured these depleted cells with or without SEB under the usual conditions. The results from a representative experiment are shown in Fig. 1. Cultures of Ly-1-depleted cells failed to

Table 1. A Ly-1$^+$ population induces a Lyt-2$^+$ suppressor-effector population

Group	Primed cells*		PFC/culture	
	Day 1	Day 2	Exp. 1	Exp. 2
I	—	—	6040±380	4800±189
II†	Non-Pr NFSC	—	6566±1278	5460±1075
III	SEB-Pr NFSC	—	2300±161	2033±338
IV‡	Non-Pr Ly-1$^+$	Non-Pr Lyt-2$^+$	8820±468	4340±771
V	Non-Pr Ly-1$^+$	SEB-Pr Lyt-2$^+$	6113±167	4500±770
VI	SEB-Pr Ly-1$^+$	Non-Pr Lyt-2$^+$	6633±334	5073±547
VII	SEB-Pr Ly-1$^+$	SEB-Pr Lyt-2$^+$	3446±278	2360±141
VIII§	—	Non-Pr Lyt-2$^+$	10080±20	4960±678
IX	—	SEB-Pr Lyt-2$^+$	7686±790	4260±525
X	—	Non-Pr NFSC	6566±1278	5483±1009
XI	—	SEB-Pr NFSC	5540±1380	5193±336
XII¶	Non-Pr Ly 1$^+$	Wash/add SEB	7260±260	4860±933
XIII	SEB-Pr Ly 1$^+$	Wash/re-culture	6206±607	5040±607

* All primed cultures were harvested and mixed with fresh immune cells (10% primed cells and 90% immune cells) and SRBC in the Mishell–Dutton culture system. All primed cell groups were added on Day 0 of the Mishell–Dutton culture, and the PFC response was assessed on Day 5.

† Certain priming cultures were generated by culturing NFSC for the entire 2-day priming period with or without SEB (groups II and III).

‡ Naive splenocytes were fractionated by depletion with anti-Ly-2 antibody (termed 'Ly-1$^+$' cells) prior to incubation with or without SEB for 1 day. These primed or non-primed Ly-1$^+$ cells were then co-cultured with fresh naive Lyt-2$^+$ cells (splenocytes depleted with anti-Ly-1 antibody) with or without additional SEB for one additional day (groups IV–VII). At the end of this second day of priming, the cells were added on Day 0 of the Mishell–Dutton culture.

§ These priming cultures consisted of NFSC or Lyt-1$^-$2$^+$ cells cultured with or without SEB for only 24 hr prior to harvest and addition to the Mishell–Dutton culture (groups VIII–XI).

¶ Lyt-1$^+$2$^-$ cells were cultured with or without SEB for the first day, harvested and washed, and returned to culture without or with SEB for an additional day (groups XII and VIII, respectively).

develop suppressor cell activity, whereas the SEB-primed non-fractionated splenocyte (NFSC) cultures exhibited significant suppressor cell activity. Cells cultured in the absence of SEB also failed to demonstrate suppressor cell activity. As expected, based on our previous observations, cultures of Lyt-2-depleted cells also failed to develop suppressive activity (data not shown). The results suggest that either the generation or induction of suppressor cells by SEB is dependent on a Ly-1$^+$ cell population.

The induction of Lyt-2$^+$ cells by primed Ly-1$^+$ cells and SEB

The results from the depletion experiments in the induction phase suggest several possible cellular dynamics. We considered that either a Ly-1$^+$ population is required for the induction of the suppressor-effector cell activity, or the Lyt-1$^-$2$^+$-effector population differentiates from a Ly-1$^+$2$^+$ population. In order to approach this question we attempted to determine whether functional suppressor cell activity could develop in co-cultures consisting of both Lyt 1$^+$2$^-$ and Lyt-1$^-$2$^+$ cells. The results of two representative experiments are presented in Table 1. Here again the co-cultures containing SEB-primed NFSC (group III)

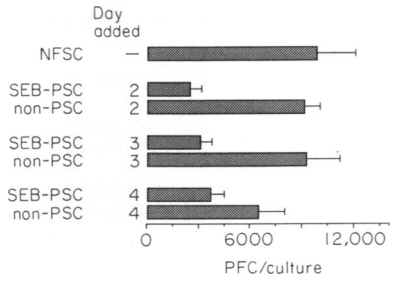

Figure 2. Kinetics of SEB-PSC-mediated suppression of antibody responses to SRBC. The SEB-PSC and non-PSC were generated by incubating splenocytes with or without SEB for 48 hr and then mixed with NFSC in Mishell–Dutton cultures on Days 2, 3 and 4. Each bar represents the mean (±SE) anti-SRBC PFC response determined on Day 5.

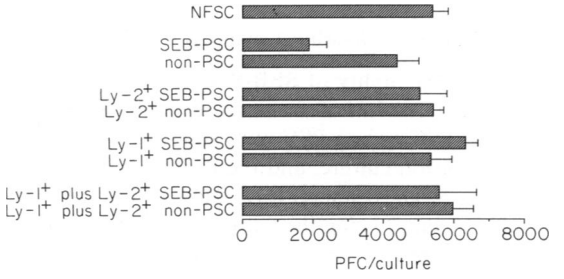

Figure 3. Analysis of Lyt phenotype of SEB-induced suppressor cells which are effective late in an ongoing antibody response. The SEB-PSC and non-PSC were treated with monoclonal anti-Ly-1 or anti-Lyt-2 antibody plus complement or with complement alone. Each selectively depleted cell population or the combination of depleted cell populations were mixed with NFSC in Mishell–Dutton cultures on Day 3. Each bar represents the mean (±SE) anti-SRBC PFC response determined on Day 5.

manifest significant suppression. A Lyt-1$^+$2$^-$ cell population was cultured individually for 24 hr with SEB, harvested, and then co-cultured together with Lyt-1$^-$2$^+$ cells and SEB for an additional 24 hr. These co-cultures exhibited significant suppressor cell activity (43% and 51% suppression) (group VII).

Several additional experimental groups were also analysed as controls. The data show that the Lyt-1$^-$2$^+$ cells cultured alone for 24 hr with SEB (group IX), and the NFSC cultured with SEB for 24 hr (group XI), both failed to manifest maximal suppressive activity. These results suggest that indeed Lyt-1$^+$2$^-$ suppressor-inducer cell population is required for the generation of Lyt-1$^-$2$^+$ suppressor-effector cells. Suppression is apparent only in the case where Lyt-2$^+$ cells are primed with SEB in the presence of previously primed Ly-1$^+$ cells (group VII). There is no apparent requirement for a Lyt-1$^+$2$^+$ cell population, since the cells primed with SEB on both Days 1 and 2 are suppressive even though they are depleted of this population (group VII). The results also indicate that both the Lyt-1$^+$2$^-$ suppressor-inducer and the Lyt-1$^-$2$^+$ suppressor-effector must be triggered by the SEB, since co-cultures of either non-primed Lyt-1$^+$2$^-$ cells (groups IV and V) or non-primed Lyt-1$^-$2$^+$ cells (groups IV and VI) failed to manifest suppressor cell activity.

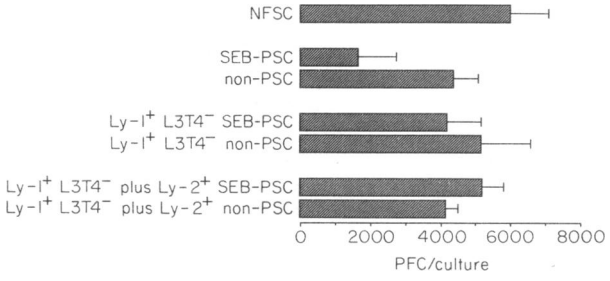

Figure 4. The requirement for L3T4$^+$ cells in the late-acting suppression of the antibody response. Ly-1-bearing and Lyt-2-bearing cells were prepared from SEB-PSC and non-PSC by depletion with monoclonal antibodies and complement, and the Ly-1-bearing cells were further fractionated with anti-L3T4 and complement. Each selectively depleted cell population or the combination of depleted populations was added to NFSC in Mishell-Dutton cultures on Day 3. Each bar represents the mean (± SE) anti-SRBC PFC response determined on Day 5.

Delayed addition of SEB-primed suppressor cells

An analysis of the ability of SEB-PSC to function at various times after the initiation of an antibody response was carried out. The SEB-PSC or non-PSC were added at Day 2, 3 or 4 of the Mishell–Dutton culture, and the PFC response was determined on Day 5. The results (Fig. 2) showed that the SEB-PSC was capable of suppressing the antibody response when added to the ongoing immune response as late as 24 hr before the termination of the Mishell–Dutton culture assay.

Surface phenotype of the late-acting suppressor cell population

Our previous studies have demonstrated convincingly that the SEB-induced Lyt-1$^-$2$^+$ T-cell population inhibits antibody responses when added early in the antibody response. We attempted to assess the surface phenotype of the late-acting suppressor cell population. The results (Fig. 3) show that when SEB-PSC were added to Mishell–Dutton cultures on Day 3, the depletion of either Ly-1$^+$ or Lyt-2$^+$ cells abrogated the suppressive activity. Furthermore, the combination of Ly-1$^+$ and Lyt-2$^+$ cell populations did not appear to manifest the suppressive activity. The results suggest that Lyt-1$^+$2$^+$ cells were required for suppression in the late stages of the antibody response.

We considered an alternative explanation for the inability of the mixture of Ly-1$^+$ and Lyt-2$^+$ cells to suppress the antibody response. Since the Ly-1$^+$ cells would be expected to be made up of both L3T4$^+$ and L3T4$^-$ cells, the Ly-1$^+$ L3T4$^+$ may counteract any Ly-1$^+$ suppressor cell activity either alone or in combination with Lyt-2$^+$ cells. For this reason, we attempted to determine the ability of the Ly-1$^+$ L3T4$^-$ cells to exhibit suppressive activity. The results (Fig. 4) show that Ly-1$^+$ L3T4$^-$ cells fail to inhibit the antibody response. The results also show that Ly-1$^+$ L3T4$^-$ combined with Lyt-2$^+$ cells fail to exhibit suppressive activity. These results offer additional evidence which suggests that the late-acting suppressor cell bears the Lyt-1$^+$2$^+$ surface phenotype.

DISCUSSION

We have shown previously that the immunosuppressive activity of SEB is due, at least in part, to the induction of a suppressor T-cell population. Our interest has been extended recently to the nature of the cellular dynamics involved in the induction of these suppressor cells. Given the vast array of suppressor cell phenomena reported in the literature, we have attempted to put the SEB-induced suppressor cell population in the perspective of the well-established suppressor cell systems.

We have found that the generation of 'suppressor-effector' cells by SEB is dependent on the prior activation of a 'suppressor-inducer' population. Analysis of the surface phenotype of the cells in this experimental system indicates that the suppressor-inducer population is Lyt-1$^+$2$^-$, and the suppressor-effector population is Lyt-1$^-$2$^+$. These 'inducer' and 'effector' phenotypes are consistent with numerous published observations of other regulatory cell circuits. It is particularly significant here that this mitogen-induced, non-antigen-specific suppressor cell population is induced in a manner that is apparently consistent with the antigen-specific suppressor cells (Bach *et al.*, 1978; Dorf & Benacerraf, 1984).

Analysis of other non-antigen-specific suppressor cell circuits have yielded somewhat similar results. The studies of Asano & Hodes (1983) and Malkovsky *et al.* (1983) suggest that carrier-induced non-antigen-specific suppression is mediated by a primed Ly-1$^+$ suppressor-inducer and a non-primed Lyt-1$^-$2$^+$ suppressor-effector. Beckwith & Rich (1982) have also shown that alloantigen-primed suppressor-inducer cells trigger a non-primed suppressor-effector population to mediate non-specific inhibition of the mixed-lymphocyte reaction. Our results differ from these studies in that a requirement clearly exists for priming of the Lyt-1$^-$2$^+$ suppressor-effector together with the participation of the Lyt-1$^+$2$^-$ suppressor-inducer population.

It should be pointed out that the terminology employed in this paper is based strictly on the apparent function. In other words, the Lyt-1$^+$2$^-$ T cell is apparently required for the 'induction' in the activity of a Lyt-1$^-$2$^+$ T-suppressor cell. The latter cell population is termed the suppressor 'effector' because it is capable of inhibiting the antibody response when added to the target cells. Cells with the Lyt-1$^+$2$^-$ phenotype have been reported by Zembala *et al.* (1986) to function as the non-specific suppressor effector of contact sensitivity. The relationship of the SEB-primed Lyt-1$^+$2$^-$ cell population in our studies and the Lyt-1$^+$2$^-$ suppressor effector in the studies of Zembala is not clear.

A complete review of the function and cellular dynamics of suppressor cells is beyond the scope of this discussion. However, it should be recognized that certain antigen-specific suppressor cell circuits do involve the generation of non-specific suppressor cells and factors (Dorf & Benacerraf, 1984; Asherson, 1986). In addition, T-accessory cell populations may function at later stages in the cell circuit in a non-antigen-specific manner. The contribution of additional cell populations, and the involvement of possible suppressor factors, remain undefined in the SEB-induced suppressor cell cascade.

Our results also suggest a possible role for Lyt-1$^+$2$^+$ T-suppressor cells. The cell population added late in an ongoing antibody response is susceptible to treatment with either anti-Ly-1 or anti-Lyt-2 antibody. These results could be explained by a requirement for both Lyt-1$^+$2$^-$ and Lyt-1$^-$2$^+$ suppressor cells in the late stages of the response. The results show, however, that the combination of Lyt-1$^+$2$^-$ and Lyt-1$^-$2$^+$ cells does not exhibit a suppressive effect. These findings suggest that the late-acting suppressor cell is Lyt-1$^+$2$^+$. The results show, moreover,

that the failure of the combined Ly-1$^+$ and Lyt-2$^+$ populations is not due to compensating 'help' provided by a population of Ly-1$^+$ L3T4$^-$ cells. The data suggest that in fact the Ly-1$^+$ L3T4$^-$ cells do not suppress the late antibody response alone or in combination with Lyt-2$^+$ cells.

It is of particular interest that the Lyt-1$^-$2$^+$ population is able to inhibit the antibody response when added early, but not late, in the antibody response. There are at least two explanations for the activity of the SEB-primed Lyt-1$^-$2$^+$ cell population which acts on the antibody response. First, the target cell population may be a cell type that operates only early in the antibody response (e.g. macrophage, T-helper cell) and as such would only manifest a suppressive effect if added in the first days of the response. The second possibility is that the inhibitory activity of SEB-primed Lyt-1$^-$2$^+$ population requires additional time because it operates through an intermediate cell population (which, in this case, is a Lyt-1$^+$2$^+$ cell population). A similar pathway has been described in another system (Beckwith & Rich, 1982).

It should be pointed out that non-specific suppression induced by the T-cell mitogen, Con A, has been thoroughly described by Pierce and his colleagues (Aune & Pierce, 1981a, b; Aune, Webb & Pierce, 1983). The generation (by Con A) of a soluble immune response suppressor factor by a Lyt-1$^-$2$^+$ cell population acts through a macrophage intermediate to directly suppress lymphocyte function. Several characteristics of this experimental system, including the biochemistry of the factor (Aune *et al.*, 1983), the chemical nature of the function of the macrophage intermediate (Aune & Pierce, 1981b) and the biochemical effects on the target cell (Irons *et al.*, 1984) suggest the possibility that these suppressor cells are distinct from the SEB-induced suppressor cell populations. The relationship between the Con A-induced suppressor cell (and the factor) and other antigen-specific or non-specific cell circuits remains to be determined.

The experimental system we have described in the present report offers several advantages in the study of both the induction and expression phases of suppressor cell activity. In this system the entire suppressor cell circuit can be generated *in vitro*. This offers unique opportunities for the analysis of cellular dynamics in this process. In addition, we have observed that the requirement for certain triggering events can be determined in a straightforward fashion. Finally, these techniques for generating suppressor-inducer and suppressor-effector populations should allow for an analysis of possible suppressor factors, and the comparison of the suppressor factors in this system with those of another.

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