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Thomas J. Rogers

The ability of Staphylococcal enterotoxin B (SEB) to stimulate a population of suppressor cells in cultures of murine splenocytes was investigated. A suppressor cell population which was capable of suppressing the ability of normal syngeneic splenocytes to respond to heterologous sheep erythrocytes (SRBC) was induced by purified SEB. Final SEB concentrations ranging from 0.05 to 5.0 ug/ml were capable of activating this suppressor cell population, and significant suppression could be detected with relatively small numbers of SEB-primed spleen cells (SEB-PSC) per culture. Significant inhibition of splenocyte antibody responses occured following intravenous administration of either 0.5, 5.0 or 50 ug of purified SEB per mouse. The plaque-forming cell (PFC) responses of *in vivo* SEB-PSC to both T-dependent (SRBC) and T-independent (DNP-Ficoll and DNP-PVP) antigens were suppressed. Elimination of macrophages before or after priming splenocytes with SEB did not reduce the suppression of PFC responses when SEB-PSC were added to normal cells in Mishell-Dutton cultures. Treatment of cells with anti-thymocyte serum plus complement, before or after priming with SEB, effectively eliminated the suppression. Elimination of B cells and reduction of macrophages resulted in greater suppression of PFC responsiveness when SEB-PSC were added to normal cells. These results suggest

that the suppressor cell population is part of the T-cell series. Indomethacin, a prostaglandin synthetase inhibitor (PGSI), blocked the induction of suppressor cells in primed spleen cell cultures when present during the entire priming period, indicating that prostaglandins participate in the inductive phase of these cells. Once suppressor cells had been activated by SEB, exposure to indomethacin did not reduce the suppression of PFC responsiveness. Inhibition of prostaglandin synthesis with Ro-3-1428 significantly reduced the induction of suppressor cells in T-cell-enriched primed spleen cell cultures, while indomethacin only partially reduced the induction of suppressor cells in T-cell-enriched primed spleen cell cultures. These results establish SEB-induced T suppressor cells as a useful model system for studies of suppressor cell function and regulation of antibody responses.

Immunosuppression Induced by Staphylococcal Enterotoxin B

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Head of Department of Microbiology

Redacted for privacy

Dean of Graduate School

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I. REVIEW OF PERTINENT LITERATURE

Cell types of the immune response

In higher vertebrates, two types of lymphocytes are found in peripheral lymphoid tissues. Both populations originate as pluripotential stem cells in the bone marrow and migrate to specific lymphoid organs for further differentiation. The thymus-derived lymphocytes (T-cells) differentiate under the influence of the thymus and are involved in antigenic recognition in cell-mediated immune reactions including delayed-type hypersensitivity, transplantation rejection and acquired cellular resistance to infection. Other lymphocytes differentiate in peripheral lymphoid tissues, and many become the precurssors of antibody-forming cells. These cells are classically referred to as B lymphocytes (or B-cells) because they were originally discovered to differentiate in the bursa of Fabricius in the chicken. The bursal equivalent tissue in mammals is believed to include the gut-associated lymphoid tissue (GALT) and specific sites in the bone marrow.

The recognition of specific cell surface antigens has allowed the distinction of a number of T-cell subpopulations with characteristic functions. The B-cells typically express surface immunoglobulin (Ig), and T-cells typically express the theta (or Thy 1) alloantigen. The Ly antigens, first described by Cantor and Boyse (1977), have been very useful in characterizing the various subpopulations of thymic-derived lymphocytes. In the mouse, only T-cells which express the Thy 1^+ Ly $1^+2^-3^-$ surface phenotype are capable of helper function.

These cells, referred to as T-helper cells (T_H), are capable of providing a necessary signal which allows B-cells to produce antibody to most antigens (referred to as T-dependent antigens). In contrast, T-cells that express the Thy 1^+ Ly $1^-2^+3^+$ surface phenotype may express suppressor cell function. These cells are characterized by an ability to inhibit the antibody producing capacity of B-cells, as well as the function of other T-cells (Eardley et al., 1978). Thymus-derived cells which kill specific target cells, termed cytotoxic cells (T_C), have also been shown to express the Ly $1^-2^+3^+$ surface phenotype. A third class of T-cells manifest all three Ly components and are the first to appear during ontogeny. This subpopulation of T-cells (Ly $1^+2^+3^+$) comprise approximately 50% of the total peripheral T-cell population and are believed to function as amplifier cells in the immune response. These cells have been shown, for example, to augment both suppressor and helper T-cell activities (Jadinski et al., 1976).

Another class of cells within the immune system, the macrophages, have also been shown to play an important role in the regulation of the immune response. One major function of macrophages is the uptake of antigens (usually by phagocytosis) and the presentation of these molecules in an immunogenic form to the lymphocyte (Rosenstriech & Rosenthal, 1974). Macrophages are necessary accessory cells for the activation of T lymphocytes by antigens, mitogens and allogeneic cells. Macrophages are also required for B lymphocyte activation by soluble protein antigens, cellular antigens, T-independent antigens and most, if not all, mitogens (Rosenstreich & Mizel, 1978).

Suppressor T-cells and suppressor factors

Suppressor T-cells were first demonstrated in mice vaccinated with sheep erythrocytes (SRBC) (Gershon et al., 1972). Numerous soluble and insoluble antigens have now been shown to induce suppressor cell function. Unfortunately, the mechanism of the immunoregulation on a cellular or molecular level is still not clear.

Many models have been proposed to describe the mechanism(s) by which suppressor cells regulate functions of the various cells within the immune system (reviewed in Pierce & Kapp, 1976). It is clear that suppressor T-cells may function in either antigen specific or in antigen non-specific fashions. Numerous reports (Rich & Pierce, 1974; Tada et al., 1973; Namba & Waksman, 1977; Ambrose, 1973; Rogers et al., 1980) have shown that suppressor cells function, at least in part, by releasing peptide mediators which interact with the target cells within the immune system.

Rich and Pierce (1973), while studying the effects of the T-cell mitogen concanavalin A (Con A) on in vitro antibody responses to the T-dependent antigen sheep erythrocytes (SRBC), observed that antibody responses were depressed by the addition of mitogenic doses of Con A to cultures of mouse splenocytes. They showed that the suppression resulted from the induction of suppressor T-cells. Cultures containing Con A-primed cells produced a very low plaque-forming cell response in comparison with cultures containing only normal cells. If the Con A-primed cultures were treated with anti-Thy 1 antisera before or after Con A was added, the suppressive activity was abolished, indicating that the suppression was dependent on functional T-cells. A non-antigen specific cell-free factor has been isolated from cultures of Con A-

primed cells which will produce significant suppression of PFC responses to both T-dependent and T-independent antigens in the absence of Con A-primed cells (Rich & Pierce, 1974). This factor, soluble immune response suppressor (SIRS), appears to be approximately 35,000 to 67,000 daltons in size and will suppress the mitogenic stimulation of B-cells by lipopolysaccharide in additon to its inhibitory effects on the primary in vitro plaque-forming cell response.

Rogers et al. (1980) have reported the induction and expression of a prostaglandin-induced suppressor factor in cultures of glass wool adherent splenic T-cells which was capable of non-specifically suppressing PHA- and LPS-induced blastogenesis. This suppressor factor, referred to as prostaglandin-induced T-cell-derived suppressor factor (PITS), was shown to be resistant to boiling, RNase and DNase, but was sensitive to the proteolytic enzymes, proteinase K, trypsin and pronase. Fractionation of culture supernatants by Sephadex G-100 chromatography produced two distinct factors: PITSα, a 35,000 dalton factor, and PITSβ, a 2000 to 5000 dalton factor.

The stimulation of T-suppressor cells may result in the release of either antigen specific or non-specific factors. These factors may be directly suppressive, or they may induce other cells to become suppressive. The nature of these very complex cellular interactions remains to be resolved.

Prostaglandins

Prostaglandins (PG) are a class of twenty-carbon, aliphatic unsaturated fatty acids which are produced by virtually every mammalian cell type with the notable exception of the mature erythrocyte. The primary

prostaglandins (PGE_2, $PGF_{2\alpha}$ and PGD_2) have been shown to be synthesized through the oxidation of arachidonic acid by a cyclooxygenase to yield the endoperoxide intermediates, PGG_2 and PGH_2. These intermediates are then isomerized to PGE_2 and/or PGD_2 or reduced to $PGF_{2\alpha}$. Prostaglandins of the E and F series are rapidly metabolized at the local site(s) of their synthesis and do not accumulate intracellularly. Release of arachidonic acid from membrane phospholipids appears to be the rate limiting step in PG biosynthesis (Pelus & Strausser, 1977).

A large volume of experimental evidence, accrued largely over the last ten years, has supported the proposal that prostaglandins actively participate in the regulation of immunological processes. Prostaglandins, primarily of the E series, have been shown to inhibit the blastogenic transformation of human peripheral lymphocytes in response to phytohemagglutinin (PHA) (Stockman & Mumford, 1974; Smith et al., 1971). Melmon et al. (1974) showed that when sensitized murine splenic lymphocytes were exposed to concentrations of PGE_1 and PGE_2 ranging from 350 ng to 35 ug/ml, a dose-dependent reduction of the plaque-forming cell response was observed. In addition to the suppressive effects on mitogen-induced DNA synthesis and antibody formation, PGE_1 and PGE_2 have been shown to inhibit the release of certain lymphokines (Bray et al., 1976), expression of cell-mediated cytotoxicity (Henney et al., 1972) and the IgE-mediated release of histamine from human basophils (Henney & Bubbers, 1973).

A role for prostaglandins in mediating immunological unresponsiveness in certain tumor cell lines has been inferred from evidence which has shown that large quantities of PGE_2 are present in many naturally occurring and experimentally induced tumors (Goodwin et al., 1980).

Syngeneic murine tumor cells which synthesize high concentrations of PGE_2 were shown to significantly suppress the PFC responses of normal mouse splenocytes when added to *in vitro* cultures of these cells along with an immunogenic dose of SRBC. The tumor-induced immunosuppression could be mimicked by the addition of PGE_2 in submicromolar concentrations. In addition, the tumor-induced immunosuppression could be blocked by indomethacin and other prostaglandin synthetase inhibitors (Plescia *et al.*, 1975; Grinwich & Plescia, 1977).

Other workers have shown that the treatment with PG synthetase inhibitors (PGSI) *in vitro* or *in vivo* partially restores the suppressed mitogen responses of splenocytes to PHA and bacterial lipopolysaccharide (LPS) in tumor-bearing mice (Pelus & Strausser, 1976). Droller *et al.* (1978) have reported that PG synthetase inhibitors are capable of enhancing natural and antibody-dependent cytotoxicity by lymphocytes against tumor cell lines that produce PGE_2.

Staphylococcal enterotoxins: Chemical and physiological characteristics

Staphylococcus aureus has been shown to produce a variety of serologically distinct exotoxins and exoenzymes. Many of these compounds are capable of significantly altering the immunological status of the host (Schwab, 1975). The mechanisms by which these compounds affect the immune system is only beginning to be understood.

Staphylococcal enterotoxin (SE) is currently the single most common cause of food-borne disease in the United States (Bergdoll, 1972). Staphylococcal food poisoning results from the ingestion of food containing preformed toxin or from the transient colonization of any portion of the gastrointestinal tract and subsequent synthesis and release of

enterotoxin. The illness which develops after an incubation period of from one to six hours is characterized by an explosive onset of vomiting, abdominal cramps and diarrhea (Effersoe and Kjerulf, 1975).

Five serologically distinct Staphylococcal enterotoxins are now recognized: SEA (Casman, 1960), SEB (Bergdoll et al, 1959), SEC (Bergdoll et al., 1965), SED (Casman et al., 1967) and SEE (Bergdoll et al., 1971). Two enterotoxin C's from different strains of Staphylococcus aureus have been purified (Bergdoll & Borja, 1967; Avena & Bergdoll, 1967) and are classified as C_1 and C_2 on the basis of their different isoelectric points (8.6 and 7.0, respectively). The target of their activity appears to be peripheral gastrointestinal sensory receptors, which, following exposure to enterotoxin, transmit a neural impulse by the vagus nerve to the center for intestinal hypermotility in the hypothalmic region of the brain. This gives rise to the characteristic vomitting and intestinal hypermotility (Elwell et al., 1975). The quantity of SE at the activation site is critical. The threshold levels of SE required for development of physiological symptoms are approximately 1 ug (SEA) and 20-35 ug (SEB or SEC) in adult humans (Sourek, 1980). These quantities are arbitrary as many factors govern an individual's susceptiblity to Staphylococcal enterotoxicosis.

Five enterotoxins have been purified: SEA (Chu et al., 1966; Schantz et al., 1972), SEB (Schantz et al., 1965), SEC_1 (Borja & Bergdoll, 1969), SEC_2 (Avena & Bergdoll, 1967) and SEE (Borja et al., 1972). The purified enterotoxins are resistant to the proteolytic enzymes, trypsin, chymotrypsin, renin and papain, and are generally considered to be quite heat resistant. Hilker et al. (1968) reported that crude enterotoxin A required considerable heating at 100°C in order

to reduce the active toxin content significantly.

The enterotoxins are single polypeptide chains containing relatively large amounts of lysine, aspartate, glutamate and tyrosine. They contain only one or two residues of tryptophan and only one cystine residue. There are no free sulfhydryl groups. The disulfide bridge can be reduced and the sulfhydryl groups alkylated with either iodoacetamide or iodoacetate without affecting the toxic or antigenic properties of the molecule (Dalidowicz et al., 1966). The complete amino acid sequence of SEB was determined by Huang and Bergdoll (1970) and revealed the presence of 239 amino acid residues. The molecular weights of the enterotoxins range from about 27,000 to 32,000 daltons.

It is generally accepted that SEC_1 and SEC_2 share common major antigenic determinants (Lee et al., 1980) as do SEB and SEC_1 (Johnson et al., 1972) and SEA and SEE (Bergdoll et al., 1971). Spero et al. (1978) performed both antigen binding and competitive inhibition studies with homologous antiserum for SEA, SEB and SEC_1. Their results clearly indicated that SEA shares no major antigenic determinants with either SEB or SEC_1, although some degree of cross reactivity does exist between SEB and SEC_1.

Staphylococcal enterotoxins: Interaction with the immune system

Results reported by Peavy et al. (1970) show that Staphylococcal enterotoxins mitogenically activate human and mouse lymphocytes. Blast transformation was stimulated by SEB in 50 to 60% of the cell population by the end of the third day of culture. Lymphocyte blast transformation in response to mitogenic stimulation is generally considered to be non-specific in nature, although the non-specific activation of lymphoreti-

cular cells *in vivo* by bacterial or viral mitogens might provide the necessary signal for the elaboration of one or more of a number of soluble lymphokines, stimulation of helper, suppressor or cytotoxic cells or some combination of these events. Taranta (1974), studying lymphocyte activation by Staphylococcal and Streptococcal mitogens, proposed the definition "indirect toxins" to explain how some bacterial products could stimulate lymphocytes *in vivo* to damage other cells. It has been shown that SEA and SEC_1 are capable of lymphocytic stimulation equal to that generated with SEB (Warren *et al.*, 1975). Spero *et al.* (1975) reported that a significant portion of the mitogenic activity of SEB was retained after detoxification of the enterotoxin by formaldehyde at pH 5.0, 7.5 or 9.5. This suggested that the mitogenic and toxic activities are not shared on the same sites on the molecule. Formalinization of SEB was shown to bring about extensive polymerization of the normally loose globular structure (Warren *et al.*, 1973). Greaves *et al.* (1974) showed that human T-cells from tonsil tissue responded well to SEB; whereas, B-cells did not. It was concluded that SEB is primarily a T-cell mitogen. Archer *et al.* (1980) have verified that the enterotoxins are potent T lymphocyte mitogens *in vivo*. They observed that mouse (C57B1/6) spleen cells were activated significantly at both 24 and 48 hours post injection of 10 ug SEA and returned to normal levels at about 72 hours post innoculation. Spleens from athymic nude mice (nu/nu), which lack functional T-cells, were not activated by SEA; in fact, a suppression of DNA synthesis by 50% was observed in this case.

Warren *et al.* (1975), studying the inhibitory effects of specific antitoxin and a variety of monosaccharides on the mitogenicity of SEA, SEB and SEC_1, concluded that lymphocyte activation by SE requires direct

interaction of the toxin with lymphocyte receptors of low avidity for the protein. Monosaccharide inhibition studies indicated that lymphocyte receptors for SE lack determinants for mannoside, galactose, acetylgalactosamine or fucose, and, therefore, differ significantly from plant lectins such as concanavalin A (Con A) or phytohemagglutinin (PHA). Incubation of murine spleen cells with an optimal dose of SEB plus an optimal dose of Con A stimulated a nearly two-fold increase in the level of ^{3}H-thymidine uptake over cells which received SEB alone, indicating that the two mitogens bind to different lymphocyte receptors. Further evidence for non-identity of Con A and SEB spleen cell receptors was shown by an experiment in which αmethyl mannoside and αmethyl glucoside, monosaccharides which specifically block Con A-induced lymphocyte activation, were unable to block stimulation of DNA synthesis by SEB.

Kaplan (1972) reported that SEB was capable of inducing normal lymphocytes to release macrophage migration inhibition factor (MIF). Antibody to SEB specifically blocked the inhibitory effect of SEB on migration of normal guinea pig peritoneal exudate cells. Smith and Johnson (1975) showed that the plaque-forming cell (PFC) response to sheep red blood cells (SRBC), a T-dependent antigen, in cultures of murine splenocytes, was reduced more than 90% by the addition of either 0.1 ug of SEA, 3 to 6 ug of SEB or 0.78 ug of Con A . The presence of SEA during the first 24 hours of culture and subsequent removal was still as effective in inhibiting the PFC response as when it was present throughout the entire culture period. These investigators concluded that the PFC inhibitory properties of SE appear to affect some early event(s) in the <u>in vitro</u> immune response.

Experiments by a number of investigators (Johnson et al., 1975a; Johnson et al., 1975b) have shown that crude and partially purified viral-induced interferon inhibits the primary in vitro PFC response of murine splenocytes to both T-dependent (SRBC) and T-independent (Escherichia coli 0127 lipopolysaccharide) antigens. Based on these results, Johnson and Baron (1976) have proposed that the suppressive effect of SE is mediated by the induction and expression of what they refer to as "mitogen-induced interferon." If in fact the suppression is resulting from the induction of interferon by SE, no proof has been given that this was separable from direct suppression mediated by SE. It is interesting to note that antibody to classical viral-induced interferon does not neutralize the ability of SEA to inhibit the PFC response, while the same antibody is capable of eliminating inhibition of the PFC response generated with viral-induced interferon.

It is evident that SE is capable of modulating both antigen specific and non-specific functions of the immune response. Staphylococcal enterotoxins share many of the same biological functions of the plant lectins, PHA and Con A. These functions include the ability to non-specifically activate T lymphocyte DNA synthesis and to suppress the maturation of normal plaque-forming cell responses. Because these plant lectins have been shown to stimulate the induction of suppressor cells, it is important to determine whether SE is also capable of modulating immunological responsiveness by the activation of suppressor cells. In addition, prostaglandin synthesis has been implicated as an essential stimulus in the activation and expression of a number of suppressor cell populations. It is, therefore, essential to determine whether PG synthesis is necessary for the activation of suppressor

cells by Staphylococcal enterotoxin.

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II. IMMUNOSUPPRESSION INDUCED BY STAPHYLOCOCCAL ENTEROTOXIN B

INTRODUCTION

A number of investigators have demonstrated the ability of certain T-cell mitogens to induce a population of suppressor cells, which, in turn, possess the capacity to non-specifically inhibit primary and secondary antibody responses. Rich and Pierce (1973) showed that a population of T lymphocytes activated by concanavalin A (Con A) *in vitro* or *in vivo*, could suppress the plaque-forming cell (PFC) response of B-cells. Generation of suppressor cells with Con A was also demonstrated by Dutton (1972). Incubation of splenocytes in the presence of Con A for 48 hours and subsequent combination of these Con A-activated cells with normal cells resulted in significant suppression of antigen-specific PFC responses. More recent experiments showed that a factor is elaborated by Con A-activated cells (Rich & Pierce, 1974; Rich & Rich, 1975). This factor, soluble immune response suppressor (SIRS), is capable of producing inhibitory effects similar to those of the Con A-activated suppressor cells.

Suppressor T-cells are believed to express the Thy 1^+ Ly $1^-2^+3^+$ surface phenotype (Jadinski *et al.*, 1976). Tse and Dutton (1977) have demonstrated that Con A-activated suppressor T-cells are large blast cells, and their induction is not inhibited by treatment with mitomycin C or irradiation, both of which inhibit DNA synthesis. This indicates that replication is not required for expression of suppressor activity by Con A-activated cells.

Fisher *et al.* (1981) have recently proposed that a double signal is required for the activation of suppressor T-cells by Con A. One

signal is provided by sufficient binding of Con A to cell surface receptors, and a second signal is provided by the release of prostaglandin E_2 (PGE_2) from these same cells. It has been known for some time that PGE_2 is capable of mimicking the suppression of specific immune responses in a number of model systems (Goodwin et al., 1977; Goodwin, 1980; Fulton & Levy, 1980). Webb et al. (1976) have shown that suppression of mitogen-induced blast transformation, stimulated by both high and low doses of phytohemagglutinin (PHA) and high doses of Con A, could be reversed by the addition of certain inhibitors of prostaglandin synthesis. Others have reported similar abrogation of suppressive effects by prostaglandin synthetase inhibitors (PGSI) in model systems designed to study the effects of PG synthesis on the suppression of PFC responses (Webb & Osheroff, 1976; Webb & Nowowiejski, 1977; Fulton & Levy, 1980).

Enterotoxins produced by certain strains of Staphylococcus aureus have been shown to be potent mitogens for both human and mouse T lymphocytes in vitro (Peavy et al., 1970; Langford et al., 1978) and in vivo (Archer et al., 1980). Staphylococcal enterotoxins (SE) have also been shown to directly inhibit the primary in vitro immune response of murine splenocytes to T-dependent antigens (Smith & Johnson, 1975) at concentrations similar to those observed for PHA and Con A (Rich & Pierce, 1973).

The experiments reported here were undertaken to determine if SE stimulates suppressor cells which inhibit the antibody-forming capacity of normal cells. Results show that T-suppressor cells are induced by purified SEB, and that the function of these T-suppressor cells may be reversed by inhibitors of prostaglandin synthesis. Although suppressor

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MATERIALS AND METHODS

Animals. Male BALB/c mice were obtained from Simonsen Laboratories, Gilroy, Ca. All experiments were performed using mice between the ages of eight and sixteen weeks.

Antigens and complement. Sheep erythrocytes (SRBC) were obtained from one experimental animal and aged five to fourteen days before use. A final 0.1% suspension of SRBC for use in immunizing cells in the micro Mishell-Dutton cultures, was prepared in tissue culture medium (TCM) which consisted of suspension minimal essential medium (MEM; Microbiological Associates (MBA), Walkerville, Md.) supplemented with a final concentration of 1.0 mM essential amino acids (MBA), 1.0 mM sodium pyruvate (MBA), 50 ug/ml gentamycin (Schering Corp., Kenilworth, N.J.), 2.0 mM L-glutamine (Grand Island Biological Co. (GIBCO), Grand Island, N.Y.), 10% fetal calf serum (FCS; GIBCO), 0.05 mM 2-mercapto-ethanol (Sigma, St. Louis, Mo.) and 10 ug/ml each of adenosine, uridine, cytosine and guanosine (Sigma). A 20% suspension, prepared in modified barbital buffer (MBB), pH 7.4, was used for the generation of a continuous monolayer in the hemolytic plaque assays.

DNP-Ficoll was obtained from Biosearch Inc., San Rafael, Ca. (DNP_{42}-AECM-$Ficoll_{400}$). A final concentration of 2.0×10^{-3} ug/ml was prepared in TCM for use in immunizing cell cultures. Sheep red blood cells were conjugated with trinitrophenyl (TNP) according to the method of Rittenberg and Pratt (1969). Briefly, SRBC were washed three times in physiological saline and 1 ml of undiluted SRBC was combined with 20 mg of 2,4,6-trinitrobenzene sulfonic acid (TNBS; Sigma) and 7 ml of 0.28 M cacodylate buffer, pH 6.9, in a foil-covered centrifuge tube and gently rotated for 10 min at room temperature. At the end of this

reaction period, the TNP conjugated SRBC were washed three times in MBB and a 20% suspension was prepared for use in the hemolytic plaque assay.

Fresh guinea pig serum (Rockland Serum Co., Rockland, Md.), adsorbed twice against SRBC, was used as a source of complement in the plaque-forming cell assays at a final dilution of 1:20 in MEM.

Purification and analysis of SEB. Purified Staphylococcal enterotoxin B was prepared according to the method of Schantz et al. (1965). Briefly, one liter of 4% N-Z Amine A (Humko Sheffield Chemical Co., Memphis, Tn.), a pancreatic digest of casein, was supplemented with 1.0% yeast extract (Difco, Detroit, Mi.) and 10 mg of magnesium chloride (J.T. Baker Chemical Co., Phillipsburg, N.J.) and inoculated with Staphylococcus aureus strain S-6. This culture was incubated at 37°C for 24 hr on a gyratory shaker (New Brunswick Scientific, N.J.) at 250 RPM. The Staphylococcal enterotoxin B (SEB)-containing supernatant was centrifuged at 10,000 RPM for 20 min to remove the bulk of the cells. The supernatant was then diluted with two volumes of water and adjusted to pH 6.4. The toxin was removed from the diluted culture by adsorption to Amberlite CG-50 (Sigma) equilibrated with 0.05 M phosphate buffer (PB), pH 6.4. The toxin-bound resin was loaded into a Pharmacia glass column (1.6 X 20 cm), washed with one column volume of water and fractionally eluted with 0.5 M PB, pH 6.8, in 0.25 M NaCl. After uV spectrophotometric analysis (Bekman model #35) at 280 nm, peak protein fractions were pooled and dialyzed for 48 hr against 0.008 M PB, pH 6.8, and the toxin was readsorbed onto a second Amberlite CG-50 column equilibrated with 0.05 M PB, pH 6.8. The toxin was fractionally eluted with 0.15 M disodium phosphate. Fractions were again assayed for peak protein, pooled and dialyzed against 0.008 M PB, pH 6.2. The combined

fractions from the second Amberlite CG-50 column were adsorbed and chromatographed on a carboxymethyl cellulose column (CM-52; Whatman Ltd., Clifton, N.J.) equilibrated with 0.01 M PB, pH 6.2. The toxin was fractionally eluted with a linear gradient of PB from 0.02 M at pH 6.2 to 0.07 M at pH 6.8.

Purified SEB was also obtained from Sigma. Purified SEB produced one precipitin band with rabbit anti-SEB serum (Sigma) on a microslide double diffusion gel and produced no precipitin line in the presence of anti-SEA or control normal rabbit serum at the same concentrations.

Preparation of spleen cell suspensions. Mice were sacrificed by cervical dislocation, spleens removed aseptically and single-cell suspensions prepared as described by Rogers and Balish (1978) with the following modifications: cells were harvested, washed two times in holding medium (HM) consisting of MEM (GIBCO) supplemented with 10% FCS (GIBCO) and adjusted to the final cell concentration in TCM.

Cell cultures. Splenocytes were cultured in micro Mishell-Dutton cultures as described by Kappler (1974) and modified by Tittle and Rittenberg (1978). Cells were cultured in sterile flat-bottom 96-well microculture trays (Falcon Plastics, Oxnard, Ca.). After dilution to the appropriate cell concentration (2.0×10^7 cells/ml) in TCM, each well received 0.05 ml of this cell suspension and 0.05 ml of the appropriate antigen. A culture represented a row of eight wells, and cells were pooled accordingly at the time of the plaque-forming cell assays five days after culture initiation. Plates were maintained in a sealed gas chamber at 37°C in an atmosphere consisting of 10% CO_2, 7% O_2 and 83% N_2. Twenty-four hours after the initiation of cultures, the cells were fed 0.05 ml per well with a nutritional cocktail mixture consist-

ing of MEM (GIBCO) supplemented with a final concentration of 2.8 mM non-essential amino acids, 2.8 mM essential amino acids, 5.5 mg/ml glucose (GIBCO), 5.5 mM L-glutamine, 0.63% sodium bicarbonate, 33% FCS and 41 ug/ml each of adenosine, uridine, cytosine and guanosine. Cultures were gassed once daily during the five-day incubation period with the gas mixture previously described.

Spleen cell priming with SEB. Splenocyte cultures were primed with SEB in a manner similar to that described by Archer et al. (1978) with the following modifications: after adjusting the cell concentration to 1.5 X 10^7 cells/ml in TCM, 1.9 ml of this cell suspension was dispensed into individual tissue culture dishes (35 X 100 mm; Falcon) along with 0.1 ml SEB (primed spleen cells; SEB-PSC) or 0.1 ml TCM (non-primed spleen cells; NON-PSC). Three or more cultures were established and pooled accordingly at the time of cell harvesting. Cultures were incubated for 48 or 96 hr, harvested, pooled appropriately, washed three times and cell concentrations adjusted accordingly before combination with fresh normal nonfractionated spleen cells (NFSC or NSC).

Trypan blue exclusion cell viablity determinations were performed on primed spleen cell suspensions after incubation to analyze the potential cytotoxicity of SEB (Appendix II). No significant difference was found between the NON-PSC and SEB-PSC populations at the concentration levels of SEB used in the in vitro cultures for these experiments (5 ug/ml).

Elimination of macrophages. Spleen cell suspensions were depleted of macrophages by two seperate procedures in different experiments. The first procedure involved the carbonyl iron technique first described by Sjoberg et al. (1972). Briefly, the spleen cell suspension (either

primed spleen cells or fresh normal nonfractionated spleen cells to be used in generating PSC cultures) was washed two times in HM and the final cell concentration adjusted to 10^7 cells/ml in macrophage separation media (MSM) which consisted of RPMI-1640 (GIBCO) supplemented with a final concentration of 4.0 mM L-glutamine, 50 ug/ml gentamycin and 20% FCS. One hundred million cells (at a concentration of 1.0 X 10^7 cells/ml) were combined with 0.6 gm of carbonyl iron powder (Atomergic Chemical Corp., Plainview, N.Y.) and the mixture swirled on a gyratory shaker-incubator (250 RPM) for 1 hr at 37°C. At the end of the incubation period, the non-adherent cells were recovered by decanting the supernatant into a second sterile centrifuge tube while retaining the adherent cells (primarily macrophages and other accessory cells) under the force of a large hand-held magnet. The macrophage-depleted populations were washed three times in HM and cell concentrations redetermined and readjusted before combination with fresh normal NSC in the micro-Mishell-Dutton cultures.

Macrophages were also removed from NSC suspensions by passage through a Sephadex G-10 column as described by Ly and Mishell (1974). Briefly, a Sephadex G-10-120 (Sigma) slurry, suspended in 0.01 M phosphate buffered saline (PBS), pH 7.2, and sterilized, was loaded into a glass wool-plugged, 10 ml syringe (Becton Dickinson, Rutherford, N.J.) and allowed to settle up to about the 7 ml mark. The column was rinsed with three bed volumes of warm (37°C) MSM. A total of 5 X 10^7 cells in 1.5 ml were loaded onto each column and allowed to enter the resin bed before closing the elution valve and layering 1 ml of warm MSM on top of the column bed. The columns were incubated at 37 C for 30 min and the non-adherent cell population was eluted with MSM (15 ml

of MSM per column).

Elimination of T-cells. Spleen cell suspensions were depleted of T-cells by treatment with rabbit anti-mouse T-cell serum plus rabbit complement (Cedarlane Laboratories Ltd., Ontario, Canada). The concentrations of all cell suspensions were adjusted to 10^7 cells/ml in cytotoxicity medium which consisted of RPMI-1640 plus 0.3% bovine serum albumin (BSA; Miles laboratories, Inc., Elkhart, In.). The anti-mouse T-cell serum was added to the cell suspension (1:20 dilution of serum) and incubated at 4°C for 1 hr. At the end of this incubation period, the cells were centrifuged, the supernatant decanted and the cell pellet resuspended to its original volume and concentration in cytotoxicity medium. Rabbit complement was added to the cell suspension at a final dilution of 1:10, and this mixture was incubated at 37°C for 1 hr. After this incubation period, the cells were washed three times in cytotoxicity media and resuspended in TCM before determining the final cell concentration.

Elimination of B-cells. Spleen cell suspensions were depleted of B-cells by passage of cells over a glass wool column according to the method of Julius et al. (1973) as modified by Folch et al. (1973), followed by selective binding of residual B cells to rabbit anti-mouse kappa and lambda light chain-coated plates according to the "panning" method of Wysocki and Sato (1978).

Glass wool columns for the separation of the glass wool-adherent cell population (primarily B-cells and macrophages) and the glass wool-non-adherent cell population (primarily T-cells) were prepared by loosely packing 0.6 gm of glass wool (Corning Glass Works, Corning, N.Y.) into individual 10 ml syringes. Each column received 5×10^8 cells in

5 ml of separation media consisting of RPMI-1640 supplemented with 5% BSA. The loaded column was then incubated for 1 hr at 37°C in a humidified CO_2 incubator. The non-adherent cells (primarily T-cells) were eluted by passage of approximately 40 ml of warm (37°C) separation media per column.

Any contaminating B-cells in this glass wool-fractionated cell suspension were eliminated by selective binding to anti-mouse kappa and lambda light chain antibody which had been previously bound to plastic petri dishes. Polystyrene petri dishes (100 X 15 mm, bacteriological grade; Falcon) were coated with rabbit anti-mouse kappa and lambda light chain antiserum (Bionetics, Kensington, Md.) diluted to a final concentration of 5 ug/ml in 0.05 M Tris buffer, pH 9.5. After 1 hr of incubation at room temperature, the buffer was decanted and the dishes were repeatedly washed with 0.01 M PBS, pH 7.2. Each antibody-coated plate then received 2.0×10^7 cells resuspended in 3 ml of 0.01 M PBS plus 5% FCS, pH 7.2. The plates were incubated at 4°C for 1 hr, and the non-binding cell population (B-cell depleted) was recovered by decanting the supernatant into a sterile 50 ml centrifuge tube. The plates were gently washed with three additional 5 ml aliquots of 0.01 M PBS plus 1.0% FCS, pH 7.2, and the supernatant from these washes pooled appropriately.

Esterase stain for the detection of macrophages. To verify that macrophages had been depleted from the spleen cell suspensions by passage of cells through a Sephadex G-10 column or by carbonyl iron adherence, representative samples of the macrophage-depleted cell suspensions were prepared and fixed for selective esterase staining as described by Koski et al. (1976). The esterase stain is specific for

macrophages and monocytes as these cell types contain high levels of non-specific esterase; whereas, lymphocytes, neutrophils, eosinophils, basophils and plasma cells do not contain detectable esterase activity. Results showed that the unfractionated spleen cell suspensions from BALB/c mice contained approximately 6.8% esterase positive cells. Carbonyl iron treatment significantly reduced the macrophage population to about 1.0%, but did not successfully eliminate macrophages entirely. Sephadex G-10 treatment, on the other hand, was very effective at eliminating macrophages from a mixed spleen cell suspension as no esterase-positive cells could be found on representative slide preparations after viewing well over 500 cells.

Blastogenesis assay. To verify the functional depletion of T-cells from a mixed spleen cell suspension by treatment with rabbit anti-mouse T-cell serum plus complement, a portion of the treated cell suspension was analyzed for its mitogenic response to phytohemagglutinin (PHA; Wellcome Reagents, Ltd., Beckenham, England), a potent T-cell mitogen. The blastogenesis assays were performed according to the method of Fulton et al. (1975) as modified by Rogers and Balish (1978). Briefly, representative samples of the cell suspensions (either normal NSC or T-cell-depleted populations) were dispensed into Falcon microtiter plates after adjusting the cell concentrations to 10^7 cells/ml in complete RPMI which consisted of RPMI-1640 supplemented with a final concentration of 4.0 mM L-glutamine and 50 ug/ml gentamycin. Each well received 0.1 ml of the appropriate cell suspension plus 0.05 ml of PHA at one of three final concentrations (0.5, 1.0 or 2.0 ug/ml). Control cultures were established and consisted of wells supplemented with media alone (complete RPMI). Six culture wells of each experimental

and control group were established. Plates were incubated for 48 hr at 37°C in a humidified 5% CO_2 incubator before adding 1 uCi of (^{3}H) thymidine (specific activity, 119 Ci/mM; New England Nuclear, Boston, Ma.) in a volume of 0.05 ml of RPMI. Cultures were incubated for an additional 24 hr and then harvested with a MASH II automatic cell harvestor (MBA) onto glass microfiber filter papers (Whatman). After the filter papers were air dried, they were placed into glass scintillation vials with 6 ml of scintillation fluid (Econofluor; New England Nuclear) and counted in a model LS 8000 liquid scintillation spectrometer (Beckman Instruments, Fullerton, Ca.).

The PHA-induced blastogenic response of T-cell-depleted cultures was compared with normal NSC cultures by first determining the average number of counts per minute for each group and the corresponding standard deviation and then calculating the percent PHA response relative to control NSC (Appendix I).

Identification of B-cells by cell surface labelling. To confirm that B-cells had been effectively depleted from glass wool-fractionated and specifically "panned" cell suspensions, the number of immunoglobulin positive (Ig^+) cells was determined by selective binding of anti-mouse Ig-coated latex beads (Immunobeads; Bio-Rad Laboratories, Richmond, Ca.). An Ig^+ cell is defined as any cell which had bound three or more latex beads after incubation for 24 hr. The percent Ig^+ cells was determined based on cell counts of 500 or more individual cells. Results showed a reduction to less than 1% contaminating B-cells by the aforementioned glass wool and panning treatments while non-fractionated splenocyte suspensions from BALB/c mice contained an average of 45.5% B-cells.

Prostaglandin synthetase inhibitors (PGSI). Indomethacin, a non-reversible inhibitor, was obtained from Sigma. Indomethacin (1(p-chlorobenzoyl)-5-methoxy-2-methyl indole-3-acetic acid) inhibits the cyclooxygenase pathway. Ro-3-1428 (5,8,11,14-eicosatetraynoic acid), an acetylenic analogue of arachidonic acid, was a gift from Hoffman La Roche Inc., Nutley, N.J. Ro-3-1428 is a reversible inhibitor of both the lipoxygenase and cyclooxygenase pathways. Both of these compounds were added to cell cultures at a final concentration of 10^{-7} M. Preliminary experiments showed that, at this concentration, these compounds have no effect on cell viablity. Trypan blue cell viablity determinations were performed on splenocyte cultures which had been exposed to indomethacin or Ro-3-1428 and are reported in Appendix II.

Detection of the primary in vitro anti-SRBC response. The plaque-forming cell (PFC) response of Mishell-Dutton cultures was determined by the Cunningham slide modification of the Jerne hemolytic plaque assay (Cunningham & Szenberg, 1968). Micro cultures were harvested using a multi-channel microtiter pipette (Titertek, Division of Flow Labs, Finland), pooled appropriately, washed two times in MEM and resuspended in a final volume of 1 ml of MEM. Quadruplicate determinations of the PFC response were performed for each group, and the average value per group was determined. Slide chambers were prepared by combining 100 ul of the cell suspension, 20 ul of a 20% SRBC suspension prepared in MBB (pH 7.2), 20 ul of complement (diluted 1:2 in MEM) and 15 ul of MEM. These components were gently mixed, loaded into Cunningham chambers (3 X 1 in. pre-cleaned microslides; VWR Scientific Inc., San Francisco, Ca.), sealed with a paraffin-vaseline mixture and incubated for 1 hr at 37°C. Only the direct (IgM) PFC response was measured. Results are expressed

as PFC per culture or PFC per 10^6 cells (assuming 35% recovery) and included the standard deviation (SD) for each group.

Experimental design and statistical analysis. Many of the experiments were designed to analyze the effect of combining relatively small proportions of primed spleen cells with normal non-fractionated spleen cells (NSC or NFSC) and assaying the PFC response 5 days after primary antigenic stimulation *in vitro*. The final concentration of all cultures remained constant. The percent suppression of PFC responses was determined relative to cultures containing only fresh normal (non-primed, non-fractionated) control cells. Statistical significance was determined by using Student's t-test. A p-value of less than 0.01 was considered statistically significant.

The initial experiments were designed to characterize the optimal *in vitro* conditions for maximal induction and expression of suppressor cells by SEB. The second set of experiments were directed at determining the effector cell type(s) induced by SEB. By employing a number of different cell separation techniques, it was possible to characterize the suppressor cell population. The final experiments were designed to investigate the role of prostaglandin synthesis in the induction and expression of SEB-activated suppressor cells.

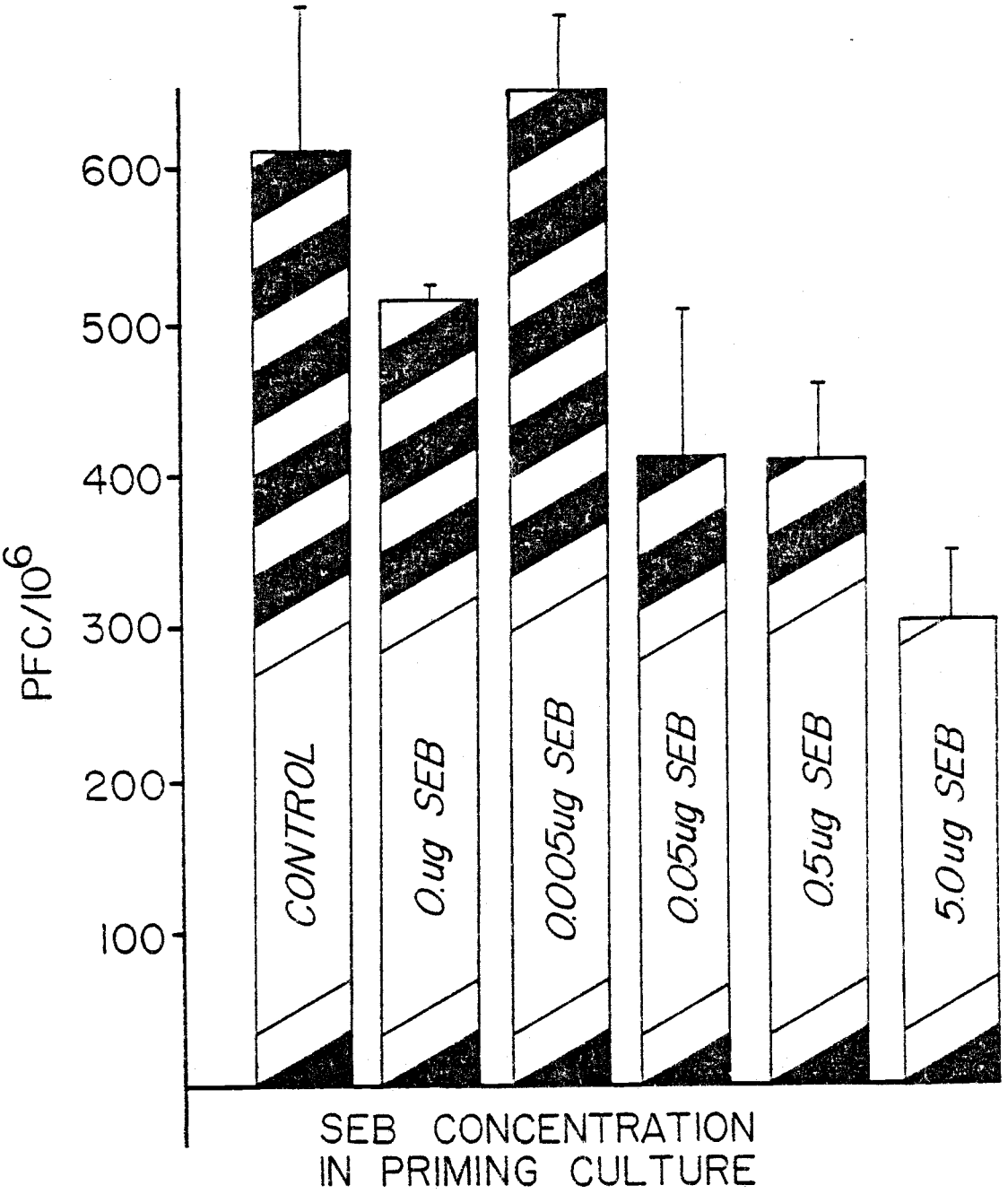
RESULTS

Determination of the optimal priming concentration of SEB for the induction of suppressor cells in vitro. The addition of Staphylococcal enterotoxin B (SEB) directly to cultured cells in a Mishell-Dutton culture has been shown to dramatically inhibit the ability of splenocytes to generate a primary in vitro antibody response to a T-dependent antigen, sheep red blood cells (Smith & Johnson, 1975). The results of similar experiments performed in our laboratory verified that sub-microgram quantities of SEB could essentially abrogate the anti-SRBC response when SEB was added simultaneous with antigen at the initiation of culture (data not shown). The PFC response of five-day cultures was inhibited approximately 80% by the addition of SEB at concentrations as low as 0.1 ug/ml.

To determine the optimal concentration of SEB necessary for maximal suppressor cell induction, various concentrations of SEB were added to prime spleen cell cultures. Cells were harvested after 48 hr, washed three times and primed spleen cells (PSC) were combined with fresh normal NSC. Replicate cultures of each dose and the appropriate controls were pooled accordingly at the time of cell harvesting. Final concentrations of 0.005 to 50.0 ug/ml were tested for their ability to induce suppressor cells in the PSC cultures. The results shown in Figure 1 illustrate that SEB concentrations ranging from 5 ug/ml to 0.05 ug/ml are capable of inducing a suppressor cell population which will significantly inhibit the capacity of normal cells to respond to heterologous SRBC antigens. Subsequent experiments were performed employing a final SEB concentration of 5 ug/ml for the generation of suppressor cells in vitro.

Figure 1. Effect of different priming doses of SEB on the subsequent transfer of suppression to normal cells *in vitro*. All cultures contained 10% primed spleen cells. All priming cultures were incubated for 2 days before harvesting. Each bar represents the mean of quadruplicate plaque counts.

Figure 1.


600
500
400
300
200
100
PFC/10^6
CONTROL
0.ug SEB
0.005ug SEB
0.05ug SEB
0.5ug SEB
5.0ug SEB
SEB CONCENTRATION
IN PRIMING CULTURE

Trypan blue exclusion cell viablity determinations of SEB-PSC showed that this concentration of SEB did not significantly reduce cell viablity relative to cells which were not exposed to SEB (ie., NON-PSC) (Appendix II). These results are consistent with data reported by Buxser and Bonventre (1981).

Optimal priming incubation period and concentration of SEB-PSC for maximal suppression of the anti-SRBC PFC response. To determine the optimal duration of exposure of cells to SEB and subsequent maximal induction of suppressor cells and resulting suppression of the PFC response, a comparison of cells primed for 48 hr and 96 hr with SEB (5 ug/ml) was performed. Results shown in Figure 2 illustrate that better induction of suppressor cells occurred by culturing cells in the presence of SEB for 48 hr than by culturing cells for 96 hr, indicating that the induction of suppressor cells by SEB is a rapidly occurring event and subsequently, relatively short-lived. If cells are cultured for 96 hr in the presence of SEB (5 ug/ml), harvested and subsequently combined with normal NSC, the PFC response to SRBC was not as significantly suppressed.

A dilution analysis of the effect of PSC on the anti-SRBC antibody responses of NSC shows that cultures containing as few as 2.5% SEB-PSC are significantly suppressed (Figure 3). The results show a dose-dependent suppression of the anti-SRBC response occurred over the range of 1, 2.5, 5, 10 and 20% SEB-PSC. The control NON-PSC combinations at the same proportions did not produce this suppression, but generally exhibited a significantly enhanced PFC response to SRBC relative to cultures comprised of 100% NSC.

Figure 2. Effect of *in vitro* SEB-primed splenocyte populations on the anti-SRBC responses of normal cells. The first bar of each pair represents cultures primed for 2 days with SEB. The second bar of each pair represents cultures primed for 4 days with SEB. A final SEB concentration of 5 ug/ml was employed as the priming dose for both 2 and 4 day cultures. Each bar represents the mean of quadruplicate plaque counts.

Figure 2.

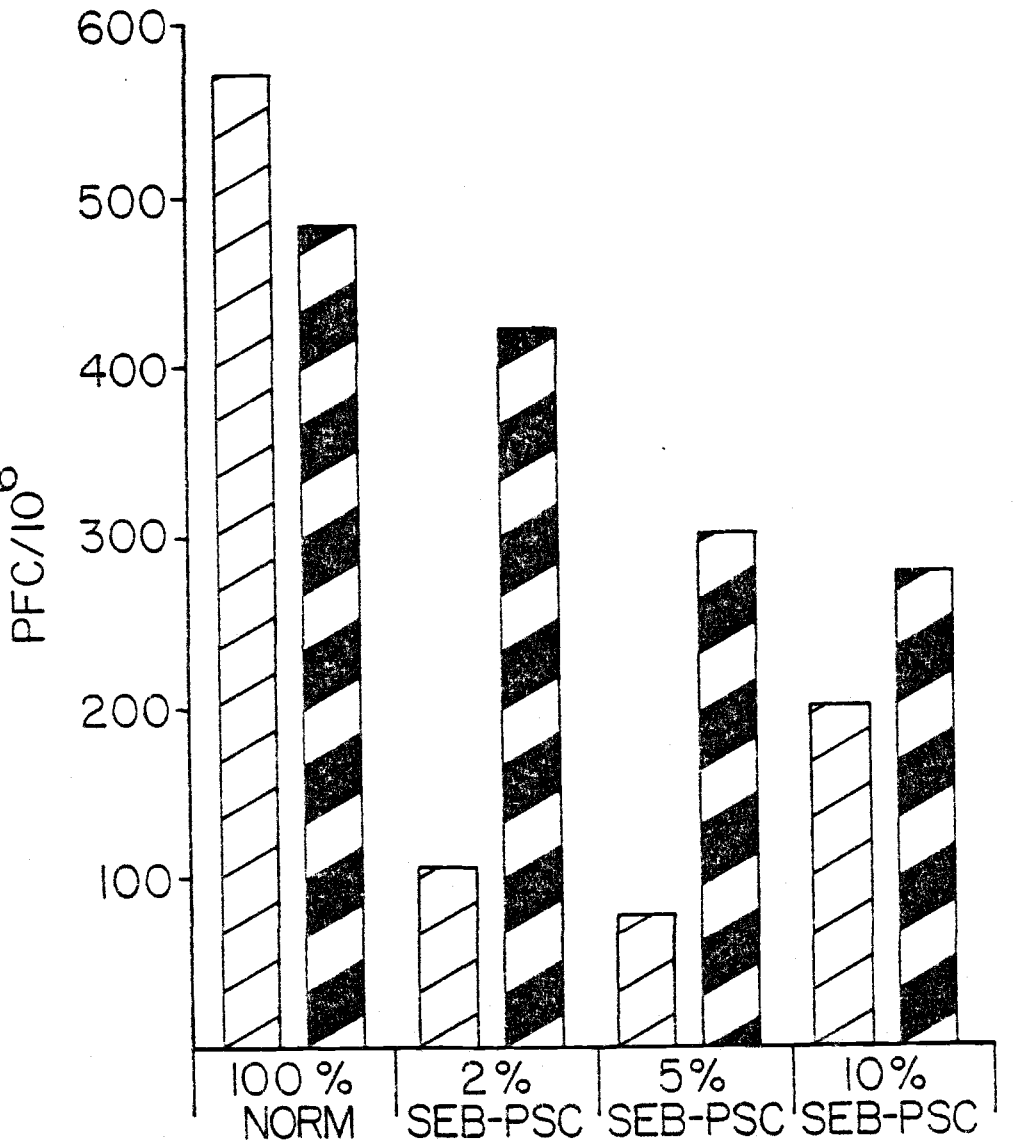
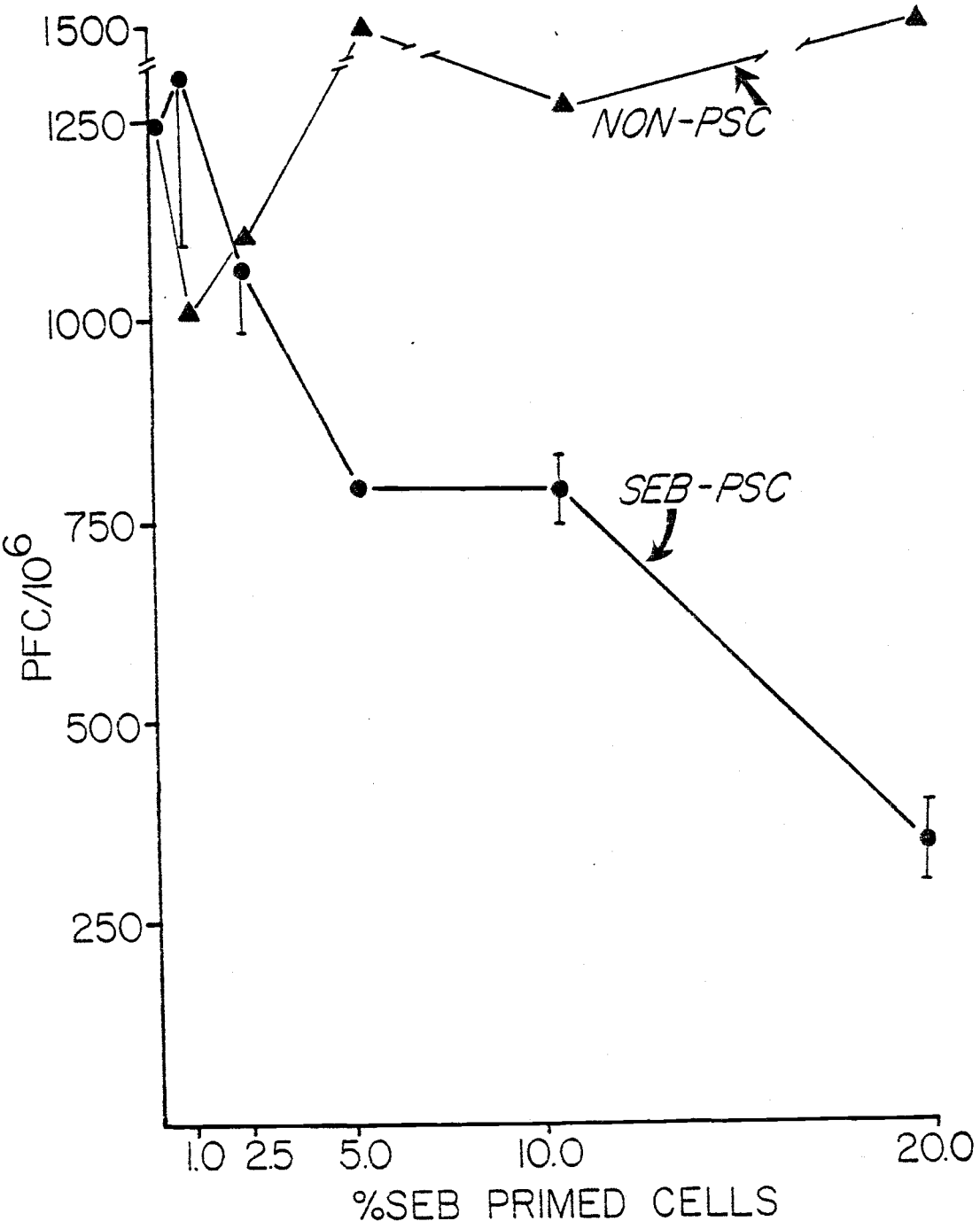

600
500
400
300
200
100
PFC/10^6
100 %
NORM
2%
SEB-PSC
5%
SEB-PSC
10%
SEB-PSC

Figure 3. Effect of SEB-primed spleen cell (SEB-PSC) concentration on the ability to suppress normal spleen cell responses to SRBC antigens. SEB-PSC cultures were generated by incubating splenocytes in the presence of SEB at a final SEB concentration of 5 ug/ml for 48 hr. NON-PSC cultures were generated by culturing splenocytes for 48 hr without the addition of SEB. Data points represented here are the mean of quadruplicate plaque counts.

Figure 3.

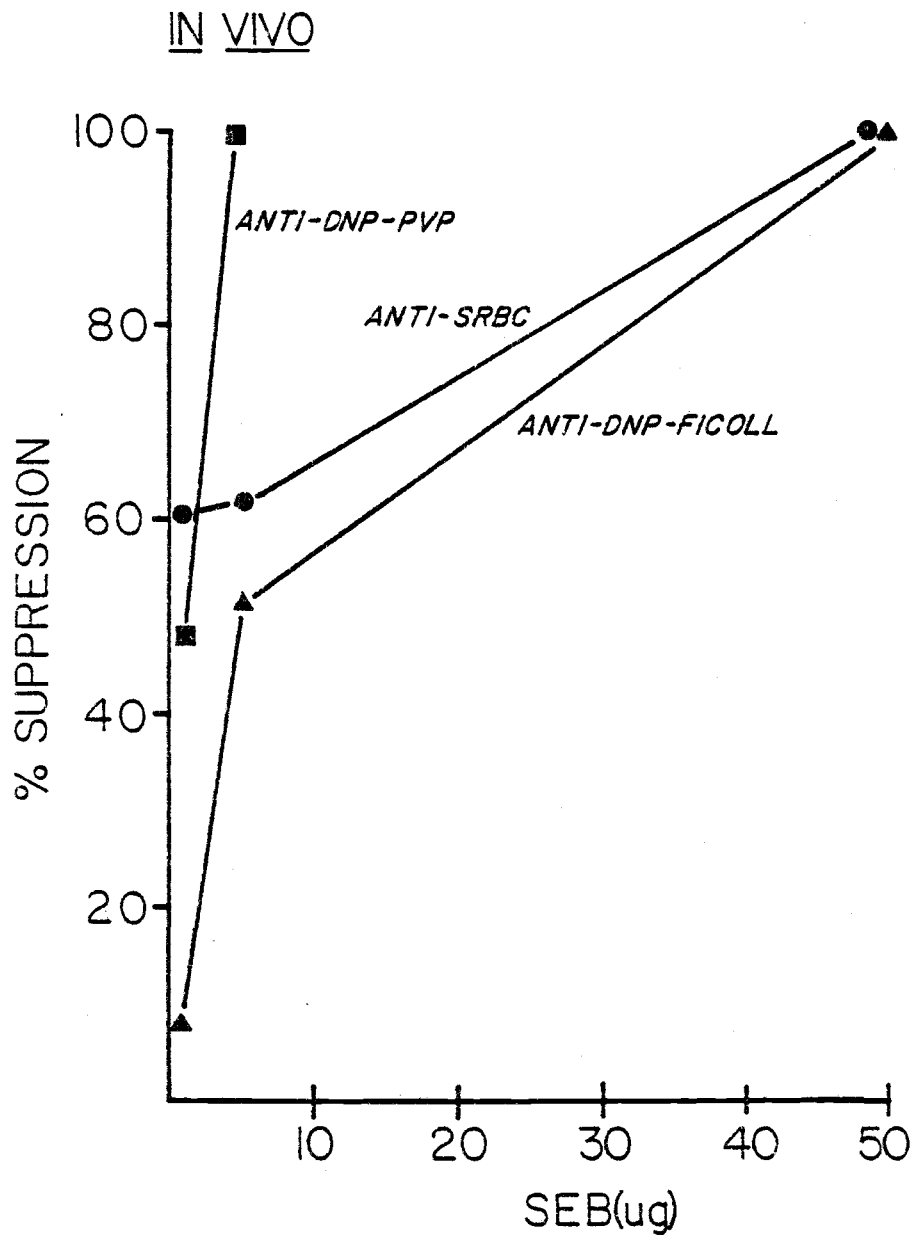

1500
1250
1000
750
500
250
PFC/10^6
NON-PSC
SEB-PSC
1.0
2.5
5.0
10.0
20.0
%SEB PRIMED CELLS

Generation of SEB-primed splenocytes in vivo. It is clear from the results presented thus far, that SEB-PSC generated in vitro are capable of suppressing the antibody response of normal USC to a T-dependent antigen. We next examined the ability of SEB to induce suppressor cells in vivo. SEB-PSC were generated in vivo by intravenous inoculation of groups of 3 mice (BALB/c) with either 0.5, 5.0 or 50 ug/mouse of purified SEB in 0.2 ml of physiological saline. Three days after inoculation, animals were sacrificed, spleens removed and single-cell suspensions prepared for the micro Mishell-Dutton cultures. Cultures of SEB-PSC were immunized with either DNP-Ficoll at a final concentration of 2.0×10^{-3} ug/ml, DNP-PVP at a final concentration of 2.5×10^{-4} ug/ml or 0.1% SRBC diluted to the appropriate concentration in TCM. At the end of the five-day incubation period, the PFC response was determined.

The results (Figure 4) illustrate that the PFC response to DNP-Ficoll of splenocytes from mice primed with 5.0 ug of SEB was suppressed approximately 50%. The response to DNP-PVP was completely suppressed at this priming concentration, and the response to SRBC was suppress- greater than 60%. When 50 ug of SEB per mouse was used as the priming dose in vivo, the PFC response to all three antigens was reduced to background levels. With 0.5 ug, the same concentration used to generate SEB-PSC in vitro, the PFC response to DNP-Ficoll was not significantly reduced (less than 10% suppression), but the anti-DNP-PVP PFC response was reduced by about 50%, and the anti-SRBC response by about 60%.

Figure 4. In vivo SEB-primed spleen cell responses to T-dependent (SRBC) and T-independent (DNP-PVP, DNP-Ficoll) antigens. Groups of three mice (BALB/c) were primed by intravenous inoculation of either 0.5, 5.0 or 50 ug of purified SEB three days prior to sacrifice.

Figure 4.


IN VIVO
100
80
60
40
20
% SUPPRESSION
ANTI-DNP-PVP
ANTI-SRBC
ANTI-DNP-FICOLL
10
20
30
40
50
SEB(ug)

The role of macrophages in the induction and expression of suppressor cells by SEB. To determine whether or not macrophages were required for the expression of SEB-activated suppressor cells, splenocytes (NSC) were primed *in vitro* with SEB (5 ug/ml) for 48 hr and then harvested, washed and depleted of macrophages by passage through a Sephadex G-10 column as outlined in the Materials and Methods section. These SEB-primed, macrophage-depleted spleen cells were combined with normal NSC at various proportions, and the PFC response determined five days after primary antigenic stimulation with SRBC. Control non-Sephadex-passaged NON-PSC and SEB-PSC cultures were included for comparison. The results (Figure 5) clearly indicate that elimination of macrophages from SEB-PSC populations after priming for 48 hr does not reduce the degree of suppression of the PFC response when these cells are combined with normal NSC. The combination of NON-PSC with normal NSC resulted in an anti-SRBC PFC response which was equal to and even significantly enhanced over control 100% NSC responses. The PFC responses of SEB-PSC mixed with NSC cultures was significantly suppressed (an average of 70% suppression). The response of macrophage-depleted SEB-PSC was also significantly suppressed (73% suppression with 10% macrophage-depleted SEB-PSC and 98% suppression with 5% macrophage-depleted SEB-PSC). These results indicate that macrophages do not play a contributory role in the transfer of suppression to normal cells by SEB-PSC.

To determine whether or not macrophages were required for the induction of a suppressor cell population in PSC cultures, NSC suspensions from normal BALB/c mice were passaged through a Sephadex G-10 column before establishing PSC cultures and exposure to SEB. Results shown in Figure 6 illustrate that the elimination of macrophages before

Figure 5. Effect of macrophage-depletion of SEB-primed splenocytes on the subsequent expression of suppressor cells. SEB-PSC were generated *in vitro* by culturing splenocytes in the presence of SEB at a final concentration of 5 ug/ml for 48 hr before being harvested, washed and mixed with normal unfractionated splenocytes in micro-Mishell-Dutton cultures at the proportions shown. SEB-PSC and NON-PSC were depleted of macrophages by passage through a Sephadex G-10 column. Control cultures including unfractionated NON-PSC and SEB-PSC at equivalent proportions are illuatrated.

Figure 5.

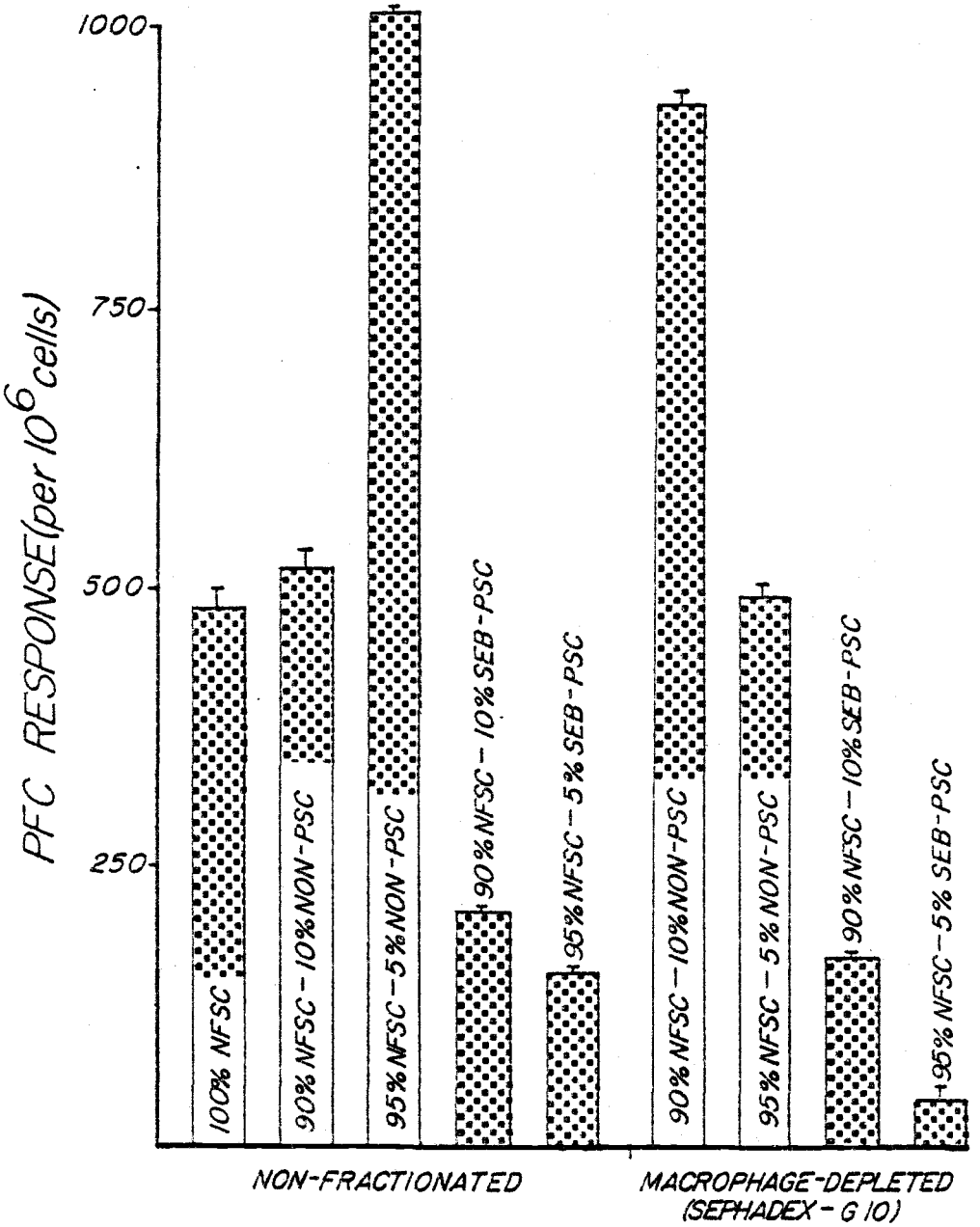
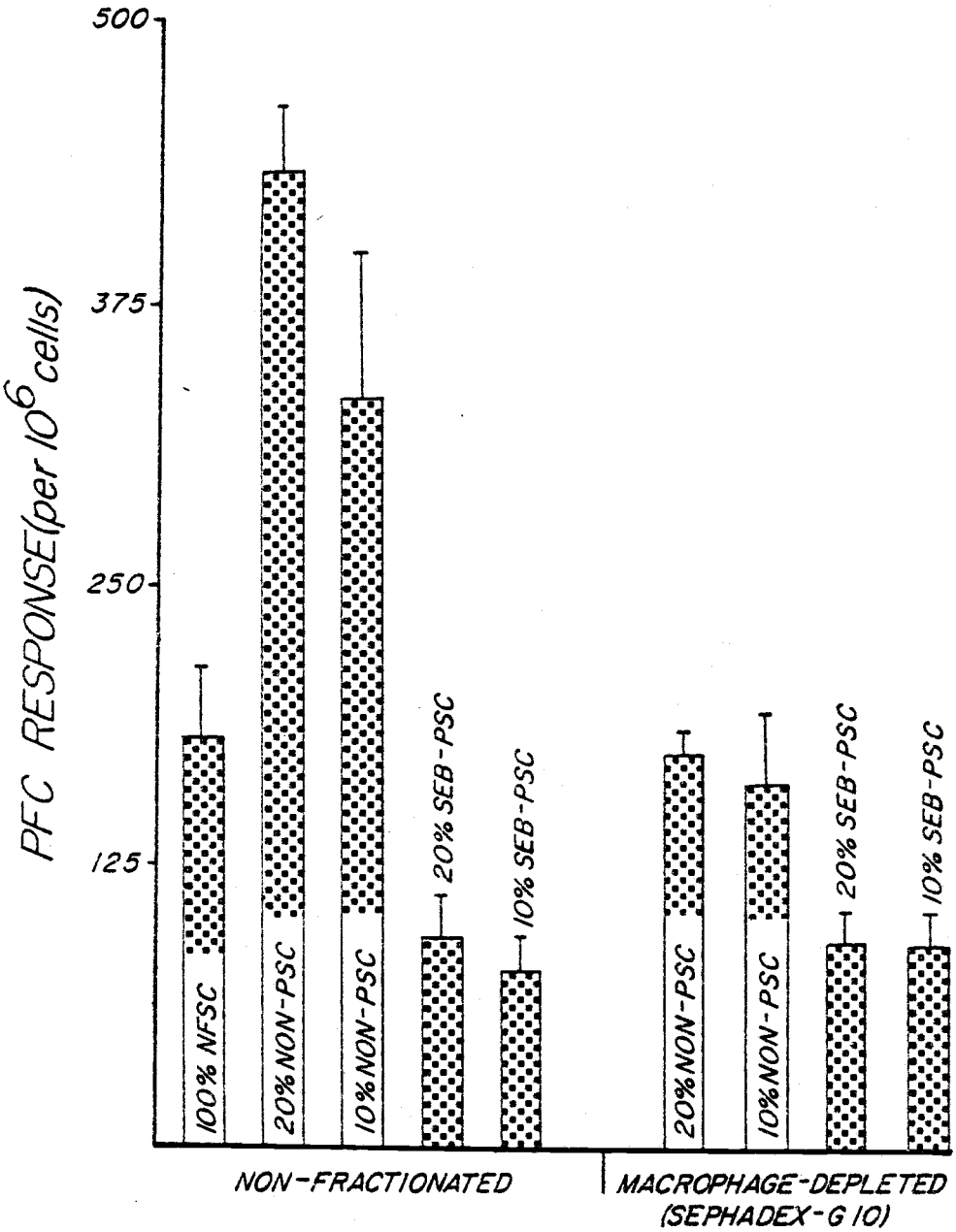

PFC RESPONSE(per 10^6 cells)
1000
750
500
250
100% NFSC
90% NFSC – 10% NON-PSC
95% NFSC – 5% NON-PSC
90% NFSC – 10% SEB-PSC
95% NFSC – 5% SEB-PSC
90% NFSC – 10% NON-PSC
95% NFSC – 5% NON-PSC
90% NFSC – 10% SEB-PSC
95% NFSC – 5% SEB-PSC
NON-FRACTIONATED
MACROPHAGE-DEPLETED (SEPHADEX – G 10)

Figure 6. Effect of macrophage-depletion on the induction of suppressor cells by SEB *in vitro*. Normal splenocyte suspensions were depleted of macrophages by passage through a Sephadex G-10 column prior to the establishment of SEB-PSC or NON-PSC cultures. Control cultures including NON-PSC or SEB-PSC generated in priming cultures which consisted of unfractionated splenocytes are shown.

Figure 6.


500
375
250
125
PFC RESPONSE(per 10^6 cells)
100% NFSC
20% NON-PSC
10% NON-PSC
20% SEB-PSC
10% SEB-PSC
20% NON-PSC
10% NON-PSC
20% SEB-PSC
10% SEB-PSC
NON-FRACTIONATED
MACROPHAGE-DEPLETED
(SEPHADEX-G 10)

priming cells with SEB did not alleviate the levels of suppression in cultures of SEB-PSC mixed with NSC. The antibody responses of cultures containing non-fractionated SEB-PSC was equivalent to that of cultures containing Sephadex G-10-passaged (macrophage-depleted) SEB-PSC (in both cases, approximately 55%). The responses of cultures containing non-fractionated NON-PSC were significantly enhanced over control 100% NSC cultures. The responses of cultures containing Sephadex G-10-passaged NON-PSC were slightly below the baseline PFC response levels, but this result is probably due to the reduction in numbers of both macrophages and antibody-producing cells as noted by Mishell and Shiigi (1980). The enhancement of antibody responses occurring as a result of combining non-fractionated NON-PSC with normal NSC was observed repeatedly with similar cell transfer experiments and can only be explained at this time as resulting from the enrichment for a specific cell population in PSC cultures which augments the PFC response to T-dependent antigens. Based on these results, macrophages are not required for the induction of suppressor cells by SEB in PSC cultures since the removal of macrophages from splenocyte suspensions before priming with SEB did not alter the activity of the suppressor cell population.

The role of T-cells in the induction and expression of suppressor cells by SEB. To determine if the suppression of splenocyte PFC responses by SEB was due to the expression of a T-suppressor cell population, cell suspensions of SEB-PSC and control NON-PSC were treated with rabbit anti-mouse T-cell serum plus rabbit complement after priming cells for 48 hr. (An analysis of specificity of T-cell depletion by Cedarlane rabbit anti-mouse T-cell serum plus rabbit complement is shown in Appendix I). If the suppression of PFC responses was result-

ing from a T-cell subpopulation (ie., T-suppressor cells), treatment of the cell suspension with anti-thymocyte serum plus complement would effectively eliminate the ensuing suppression. Results (Figure 7) illustrate that the expression of suppressor cells can indeed be eliminated by treatment of SEB-PSC with anti-T cell serum plus complement. Although the suppression of normal NSC PFC responses by SEB-PSC was not as strong as in previous PSC transfer experiments, it is evident that the PFC responses of cultures containing SEB-PSC (non-treated) were suppressed about 20%, and cultures containing NON-PSC at equivalent proportions produced PFC responses significantly greater than the control cultures containing NSC alone. Cultures containing T cell-depleted-SEB-PSC produced PFC responses at or above the response of control cultures containing NSC only. The PFC response of control cultures containing T cell-depleted-NON-PSC were still significantly enhanced. Expression of suppressor cells in SEB-PSC populations clearly resides in the T-cell population.

To determine if the induction of suppressor cells by SEB in splenocyte cultures occurs through the stimulation of T-cells directly, a T-cell purified population was prepared before establishing PSC cultures. Glass wool-non-adherent cells (primarily T cells) were depleted of any residual B-cells by selective binding of contaminating B-cells to rabbit anti-mouse kappa and lambda light chain-coated petri dishes according to the "panning" method. Control non-T-cell-purified NON-PSC and SEB-PSC cultures were established for comparison. Results (Figure 8) show that suppression was still transferable to normal cells in a T-cell purified SEB-PSC population. Control cultures containing NON-PSC were slightly enhanced as previously shown. The PFC responses of cultures

Figure 7. Effect of T-cell depletion of SEB-primed splenocytes on the subsequent expression of suppressor cells. SEB-PSC and NON-PSC were depleted of T-cells by treatment with rabbit anti-mouse T-cell serum plus complement after priming splenocytes for 48 hr *in vitro*. Control cultures including unfractionated NON-PSC and SEB-PSC at equivalent proportions are illuatrated.

Figure 7.

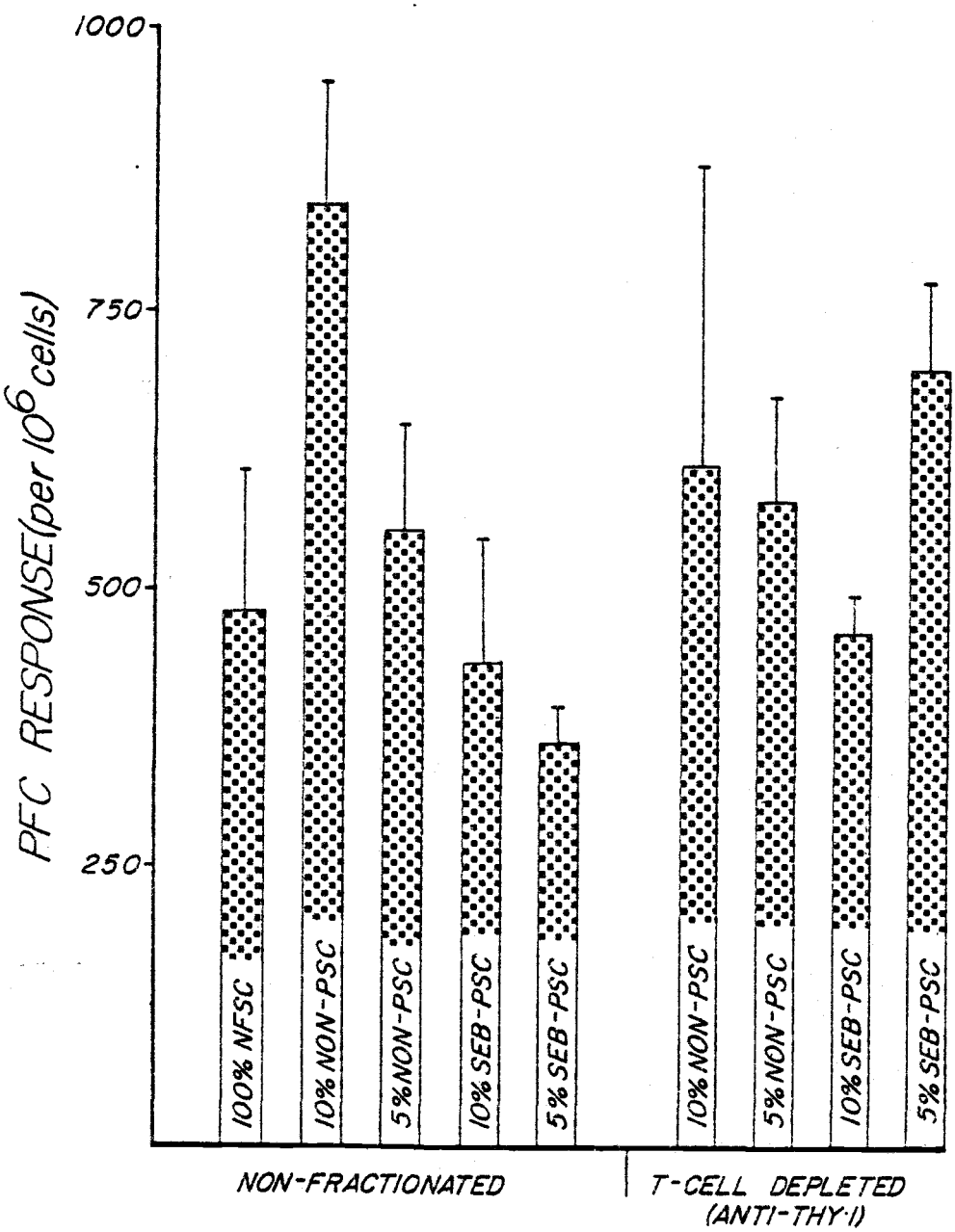
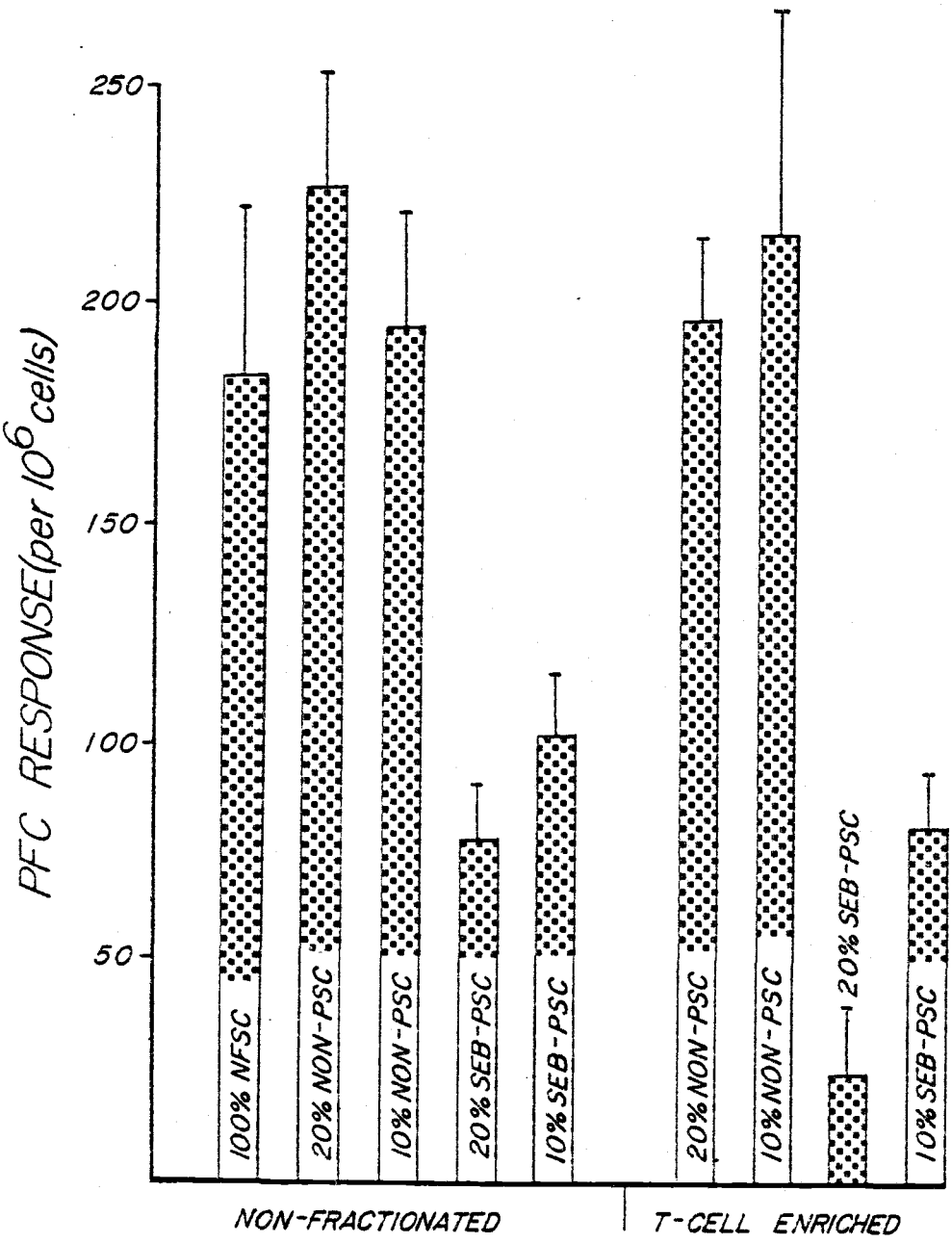

1000
750
500
250
PFC RESPONSE(per 10^6 cells)
100% NFSC
10% NON-PSC
5% NON-PSC
10% SEB-PSC
5% SEB-PSC
10% NON-PSC
5% NON-PSC
10% SEB-PSC
5% SEB-PSC
NON-FRACTIONATED
T-CELL DEPLETED
(ANTI-THY·1)

Figure 8. Induction of suppressor cells in a T-cell-enriched population. Enriched T-cell populations were prepared by glass wool fractionation to remove the glass adherent fraction (primarily B-cells and macrophages) followed by selective binding of residual B-cells to anti-kappa and lambda chain-coated petri dishes. Control cultures including SEB-PSC and NON-PSC generated in priming cultures which consisted of nonfractionated splenocytes are shown.

Figure 8.

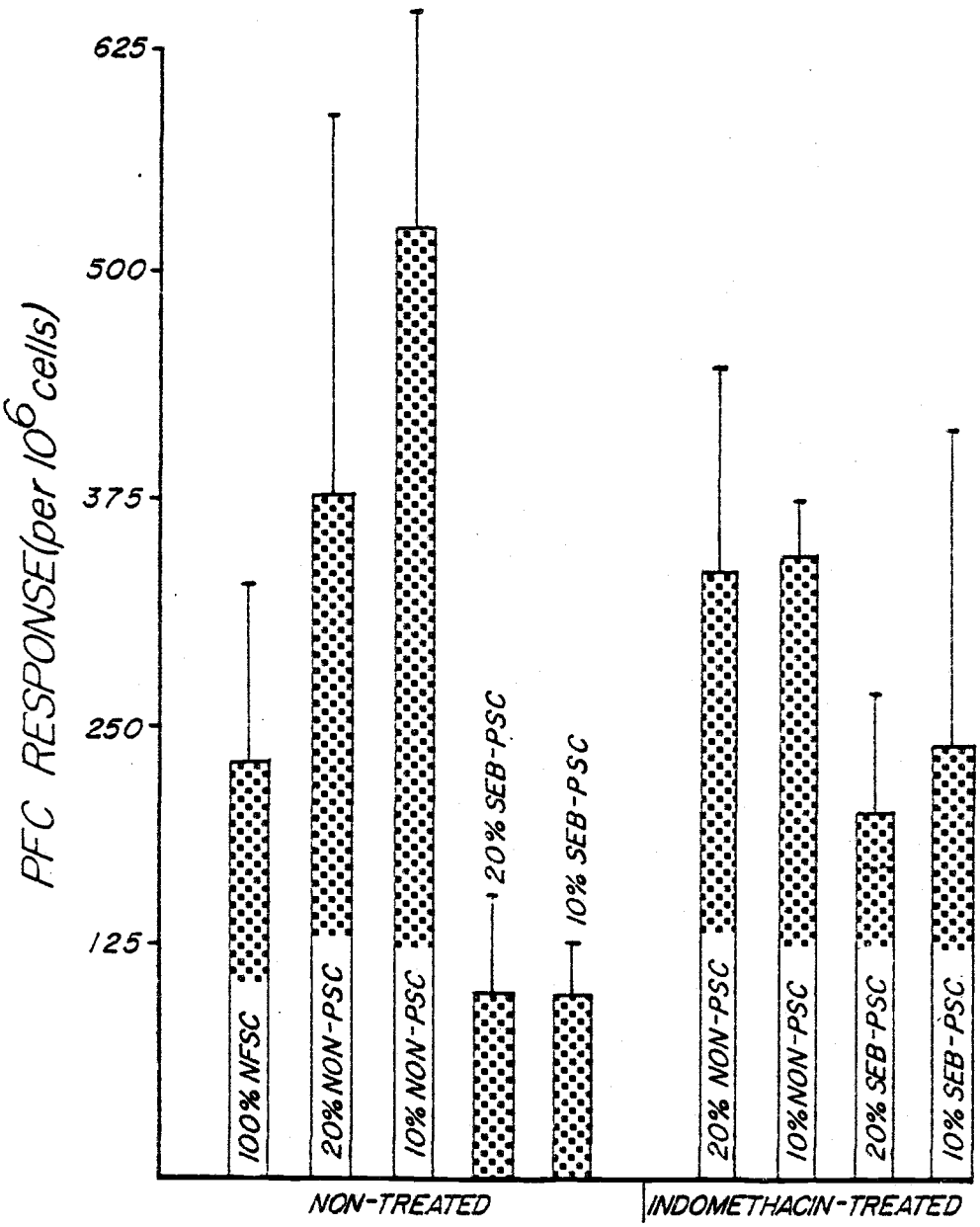

PFC RESPONSE(per 10^6 cells)
250
200
150
100
50
100% NFSC
20% NON-PSC
10% NON-PSC
20% SEB-PSC
10% SEB-PSC
20% NON-PSC
10% NON-PSC
20% SEB-PSC
10% SEB-PSC
NON-FRACTIONATED
T-CELL ENRICHED

containing T-cell-purified SEB-PSC were suppressed to an even greater extent than cultures containing non-fractionated SEB-PSC at the same proportions. Cultures containing 20% SEB-PSC were now suppressed 87.9%, and cultures containing 10% SEB-PSC were suppressed 59.2%. These data indicate that T-cells alone were capable of suppressing the anti-SRBC PFC response of normal cells.

The effect of indomethacin on the induction and expression of suppressor cells by SEB in vitro. To determine whether or not the induction of suppressor cells by SEB was dependent on prostaglandin synthesis, cultures of NON-PSC and SEB-PSC were treated with indomethacin (10^{-7} M). After 48 hr, cultures were harvested, washed three times and resuspended in fresh TCM. NON-PSC (non-treated and indomethacin-treated) as well as SEB-PSC (non-treated and indomethacin-treated) were combined with normal NSC and their subsequent response to primary antigenic stimulation with SRBC was determined five days later. Results (Figure 9) demonstrate that indomethacin was capable of blocking the induction of suppressor cells by SEB. Cultures containing non-indomethacin-treated NON-PSC were at or above normal NSC response levels, and cultures containing non-indomethacin-treated SEB-PSC were significantly suppressed at all cell proportions (ie., 20, 10 and 5% SEB-PSC). The PFC responses of cultures containing indomethacin-treated NON-PSC remained essentially the same; however, the PFC responses of cultures containing cells primed with SEB in the presence of indomethacin were no longer suppressed. Cultures containing 20% indomethacin-treated SEB-PSC were suppressed only 4.8% versus cultures containing 20% non-indomethacin-treated SEB-PSC which were suppressed 59.5%. The PFC response of cultures containing 10% indomethacin-treated SEB-PSC was

Figure 9. Effect of indomethacin on the induction of suppressor cells in cultures of nonfractionated splenocytes. Indomethacin was added to cultures of SEB-PSC or NON-PSC at a final concentration of 10^{-7} M. Primed spleen cell cultures (with or without indomethacin) were incubated for 48 hr, harvested, washed and mixed with normal NSC in the proportions shown. Control cultures including non-indomethacin-treated NON-PSC and SEB-PSC are illustrated.

Figure 9.


625
500
375
250
125
PFC RESPONSE(per 10^6 cells)
100% NFSC
20% NON-PSC
10% NON-PSC
20% SEB-PSC
10% SEB-PSC
20% NON-PSC
10% NON-PSC
20% SEB-PSC
10% SEB-PSC
NON-TREATED
INDOMETHACIN-TREATED

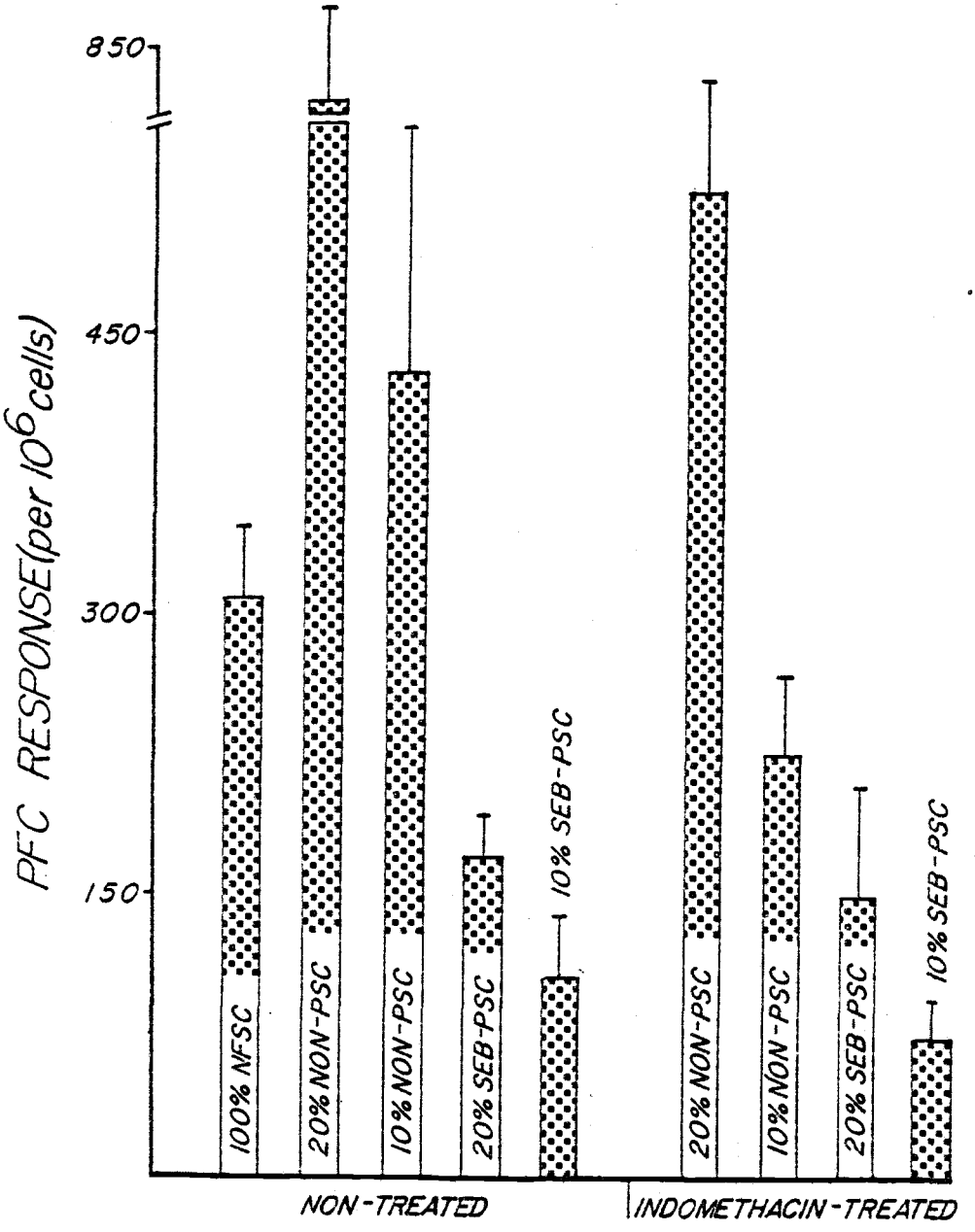
even somewhat enhanced over the PFC response of control cultures containing 100% NSC. It is apparent that prostaglandin (PG) synthesis is required for the induction of suppressor cells by SEB.

To determine if the expression of SEB-primed cells is dependent on PG synthesis, PSC were exposed to indomethacin (10^{-7} M) for four hours after priming in vitro for 48 hr. After harvesting PSC cultures, the cells were washed and adjusted to 10^{7} cells/ml in TCM. The cells were incubated in the presence of indomethacin (10^{-7} M) for 4 hr, washed three times and combined with normal NSC in the Mishell-Dutton cultures. The results (Figure 10) show that the PFC responses of cultures containing PSC which had been exposed to indomethacin were still significantly suppressed. The PFC response of cultures containing indomethacin-treated NON-PSC were reduced relative to the PFC response of cultures containing non-indomethacin-treated NON-PSC, but the PFC response of cultures containing indomethacin-treated SEB-PSC was still significantly reduced as were cultures containing non-treated SEB-PSC. Prostaglandin synthesis is not required for the expression of suppressor cells induced by SEB once these cells have been activated.

The effect of two PGSIs on the induction and expression of suppressor cells by SEB in a T-cell-purified population. By eliminating B-cells and the majority of macrophages from NSC suspensions before establishing PSC cultures, it was shown that the level of suppression could be significantly increased. This resulted from an apparent enrichment for T-cells, the effector cell population stimulated by SEB. With this SEB-induced suppressor cell system now modified by B-cell-depletion and macrophage reduction prior to the establishment of PSC cultures, experiments were next designed to determine the effect of

Figure 10. Effect of indomethacin on the expression of suppressor cells in cultures of unfractionated splenocytes. SEB-PSC and NON-PSC were treated with indomethacin (10^{-7}M) for 4 hr after priming splenocytes in vitro for 48 hr. After indomethacin-treatment, primed spleen cells were washed and combined with normal NSC in the proportions shown. Control cultures including non-indomethacin-treated NON-PSC and SEB-PSC are shown.

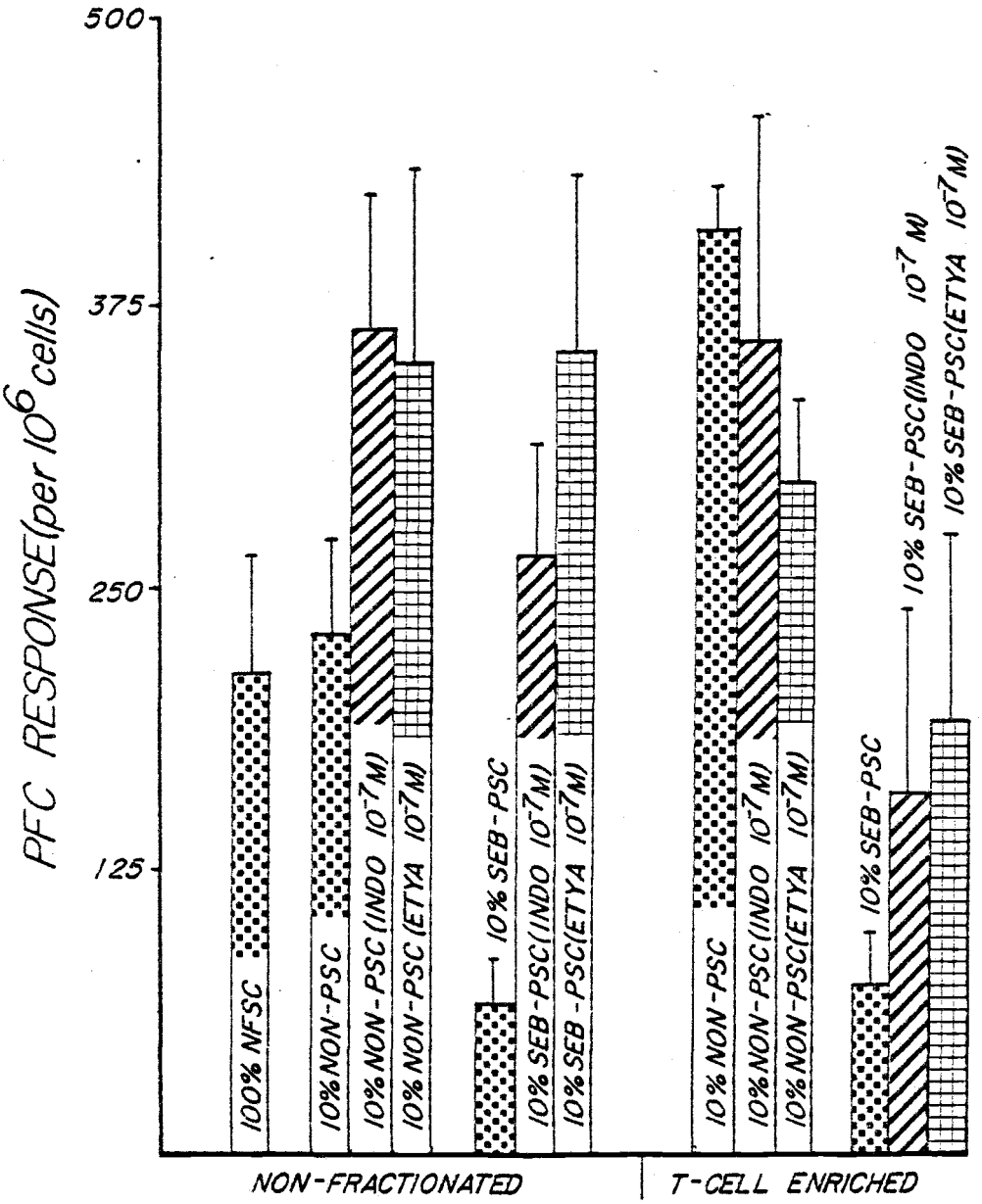
Figure 10.


850
450
300
150
PFC RESPONSE(per 10^6 cells)
100% NFSC
20% NON-PSC
10% NON-PSC
20% SEB-PSC
10% SEB-PSC
20% NON-PSC
10% NON-PSC
20% SEB-PSC
10% SEB-PSC
NON-TREATED
INDOMETHACIN-TREATED

PGSI on the induction of purified suppressor T-cells by SEB. Purified T-cell cultures were prepared and treated with SEB and either indomethacin of Ro-3-1428 (ETYA) at a final concentration of 10^{-7} M. Control priming cultures included SEB and no PGSI or media alone and no PGSI. All Mishell-Dutton cultures consisted of 90% NSC plus 10% PSC. Results of the PFC responses of cultures were compared to cultures containing only normal NSC (Figure 11). SEB-PSC (NSC) and SEB-primed T-cells were equally suppressive when added to normal cells. The presence of either indomethacin or Ro-3-1428 in cultures of SEB-PSC (NSC) during the entire priming period resulted in complete elimination of suppressor cell induction by SEB. The PFC responses of these cultures was significantly increased over the PFC response of cultures containing 100% NSC and appeared to respond similar to cultures containing NON-PSC. Treatment of SEB-primed T-cells with Ro-3-1428 allowed for nearly complete inhibition of suppressor cell induction by SEB. The response of cultures containing 10% SEB-primed T-cells plus Ro-3-1428 was 195 PFC/10^6 cells versus 205 PFC/10^6 cells for cultures of 100% NSC. Similar, although less significant results, were obtained with indomethacin-treated cultures.

Figure 11. Effect of indomethacin and Ro-3-1428 on the induction and expression of suppressor cells in a T-cell-enriched population. T-cell-enriched populations were prepared by glass wool fractionation to remove the glass-adherent population (primarily B-cells and macrophages) followed by selective binding of residual B-cells to anti-mouse kappa and lambda light chain-coated petri dishes. Indomethacin or Ro-3-1428 was added to cultures of SEB-primed T-cells or NON-primed T-cells at a final concentration of 10^{-7} M. Control cultures including non-indomethacin-treated SEB-primed T-cells and NON-primed T-cells are included as well as the corresponding indomethacin-treated and non-indomethacin-treated nonfractionated PSC.

Figure 11.


500
375
250
125
PFC RESPONSE(per 10^6 cells)
100% NFSC
10% NON-PSC
10% NON-PSC(INDO 10^{-7}M)
10% NON-PSC(ETYA 10^{-7}M)
10% SEB-PSC
10% SEB-PSC(INDO 10^{-7}M)
10% SEB-PSC(ETYA 10^{-7}M)
10% NON-PSC
10% NON-PSC(INDO 10^{-7}M)
10% NON-PSC(ETYA 10^{-7}M)
10% SEB-PSC
10% SEB-PSC(INDO 10^{-7}M)
10% SEB-PSC(ETYA 10^{-7}M)
NON-FRACTIONATED
T-CELL ENRICHED

DISCUSSION

Experimental results reported here demonstrate that purified SEB is capable of activating a population of suppressor lymphocytes in cultures of mouse spleen cells. Induction of suppressor cells by SEB does not appear to require prior processing or simultaneous interaction with either macrophages or B-cells or both. SEB-activated suppressor cells are able to significantly inhibit the plaque-forming cell responses of normal cells to the T-dependent antigen, sheep red blood cells. Elimination of T-cells, either before or after induction of the suppressor cell population by SEB, significantly reduces the subsequent suppression of PFC responses. Enrichment for T-cells by selective depletion of B-cells and macrophages increases the suppressive capacity of SEB-primed spleen cells. This evidence strongly supports the conclusion that the activated suppressor cell is a T-cell.

The induction of suppressor cells by SEB can be significantly inhibited by certain prostaglandin synthetase inhibitors, when present in cultures during the entire priming period. Once suppressor cells have been activated by SEB, exposure to PGSI does not reduce the levels of suppression which occur as a result of adding small numbers of SEB-induced suppressor cells to cultures of normal cells.

The results show that SEB is capable of activating suppressor cells at final concentrations ranging from 0.05 to 5.0 ug/ml. Higher concentrations of SEB were also capable of actively inducing suppressor cells, but the net suppression of PFC responses was not increased. To reduce the possiblity of carry-over when combining SEB-PSC with normal cells, most of the experiments were performed with a final <u>in vitro</u> priming concentration of 5.0 ug/ml. To assess the relative toxicity of SEB on

the cell viablity of in vitro primed murine splenocytes, representative samples of normal and SEB-primed spleen cells were analyzed by the standard trypan blue dye exclusion technique. The results (Appendix II) demonstrate that SEB at the maximal concentrations used in these experiments, does not significantly reduce cell viablity. A recent report by Buxser and Bonventre (1981) has shown that SEA and SEB, at final concentrations twenty times that which was used here (100 ug/ml), do not exhibit any significant toxicity for either human intestinal epithelial cells (Henle 407) or murine fibroblasts (10T1/2) after 24 hours of exposure in media containing 10% fetal calf serum. Staphylococcal enterotoxin B concentrations in the range of 0.05 to 5.0 ug/ml are known to be mitogenic for T lymphocytes (Peavy et al., 1970).

Optimal activation of suppressor cells by SEB in vitro in this system occurs after exposure of splenocytes to SEB for 48 hours. Exposure of cells to SEB for 96 hours will still allow for the induction of a suppressor cell population, but the resulting suppression of normal PFC responses is not as significant. Priming periods less than 48 hours did not allow sufficient exposure of splenocytes to SEB for activation of suppressor cells. Generation of suppressor cells with Con A also required a 48 hour priming period in vitro. The fact that the PFC responses of cultures containing SEB-PSC (96 hr) appeared to be much less suppressed than cultures containing equivalent proportions of SEB-PSC (48 hr) may indicate that an active anti-SEB antibody response is beginning to be genrated by the splenocytes.

A number of investigators, studying the effects of SE on in vitro antibody responses, have proposed that splenocytes in culture are stimulated to synthesize and release a soluble factor, referred to as

"mitogen-induced interferon." These authors maintain that the suppressive effects of SE on normal antibody responses is mediated solely by the induction and expression of this single substance (Smith & Johnson, 1975; Johnson & Baron, 1976). It may be that suppression of PFC responses is mediated, in part, by a suppressor factor produced by the suppressor cell population we report here, but these investigators have not provided acceptable proof of the separation of suppressive effects mediated directly by SE from those mediated by their proposed mitogen-induced interferon.

In vivo activation of SEB-PSC by intravenous administration of purified SEB to mice three days prior to sacrifice resulted in significant suppression of both anti-T-dependent and anti-T-independent antigen antibody responses by these cells. Chen et al. (1980) have recently shown that oral administration of 10 ug of purified SEA to BDF mice 48 hours before immunization with 100 ug of ovalbumin dramatically suppresses the homocytotropic antibody response to this antigen. It remains to be determined whether or not in vivo-activated suppressor cells are capable of suppressing normal antibody-forming responses in vitro. Experiments should be performed to determine if in vivo-activated suppressor cells capable of suppressing the antibody responses of normal cells, can be induced by oral administration of SE.

The ability of SEB to activate suppressor cells in cultures of murine splenocytes depleted of macrophages indicates that macrophages are not required for the induction of suppressor cells in this system. The elimination of macrophages from cell suspensions already primed with SEB had no effect on the subsequent ability of SEB-induced suppressor cells to suppress the antibody responses of normal cells. Based on

this evidence, we have concluded that macrophages are not required for either the induction or expression of SEB-activated suppressor cells.

A report by Tadakuma and Pierce (1976) demonstrated that macrophages are an absolute requirement for the expression of SIRS generated in cultures of murine splenocytes by exposure to Con A for 48 hours. Treatment of isolated macrophage populations with SIRS for 2 hours and subsequent combination with splenic lymphoid cells resulted in significant suppression of PFC responses. Exposure of macrophage-depleted splenocyte populations to SIRS under the same conditions did not suppress normal antibody responses to SRBC. Further evidence which supported macrophages as the cell population acted upon by SIRS, was provided by the fact that the addition of normal peritoneal exudate cells (approximately 85% macrophages), but not T or B-cells, to cultures containing SIRS-treated splenocytes restored normal PFC responsiveness. It is not yet clear whether the suppression mediated by SIRS-activated macrophages is due to direct cell contact involving T-cells, B-cells or both or to soluble factors. Unlike the macrophage-dependent suppression mediated by SIRS in the Con A-activated suppressor cell system, SEB clearly does not require interaction with macrophages during or after induction to generate a potent suppressor cell population. Furthermore, SEB appears to act directly upon T-cells, and a subpopulation of T-cells alone is capable of suppressing normal splenocyte PFC responses.

The ability of certain inhibitors of prostaglandin synthesis to reverse the suppression of antibody responses by suppressor cells has been reported by a number of investigators (Webb & Osheroff, 1976; Webb & Nowowiejski, 1977; Fulton & Levy, 1980). Our results indicate that the induction of suppressor cells by SEB <u>in vitro</u> can be effectively

blocked by the addition of certain prostaglandin synthetase inhibitors, notably indomethacin and Ro-3-1428. It is evident from these results that PG synthesis is essential to the induction of suppressor cells by SEB. Experiments designed to assess the role of PG on the expression of SEB-activated suppressor cells demonstrated that exposure of SEB-PSC to prostaglandin synthetase inhibitors (PGSI) had no significant effect on the subsequent suppression of normal antibody responses. It is concluded from this evidence that the induction of suppressor cells by SEB is prostaglandin-dependent, and that once activated, the expression of SEB-induced suppressor cells occurs independent of prostaglandins.

Other investigators have shown that the induction and expression of suppressor cells is dependent on PG synthesis. Webb et al. (1976) reported that mouse spleen cells contain a population of glass wool adherent T lymphocytes which possess the ability to suppress non-glass wool adherent (NAL) responses to PHA and Con A. Pretreatment of the glass adherent spleen cell population (GAL) with anti-thymocyte serum plus complement, but not anti-IgG, eliminated suppression, indicating that the suppressor cell is a T-cell. Most importantly, these investigators showed that pre-incubation of suppressor GAL with either indomethacin or Ro-20-5720 (another PGSI) blocked the normal suppression of mitogen-induced DNA synthesis when GAL were mixed with NAL. Goodwin et al.(1977) demonstrated the presence of a similar glass adherent cell population in cultures of human peripheral blood mononuclear cells which was capable of suppressing T-cell mitogenic responses through production of prostaglandins (PGE_1 and PGE_2). Addition of indomethacin or Ro-20-5720 to cultures of peripheral blood mononuclear cells enhanced the stimulation of T lymphocytes by PHA. These investigators also

showed that the human Con A-activated suppressor cell is not affected by indomethacin; therefore, the Con A-activated suppressor cell does not inhibit mitogen-induced DNA synthesis via PG production as does the glass adherent PG-producing suppressor cell. Rogers et al. (1980) reported that murine glass adherent splenic T-cells cultured in the presence of PGE_2 (10^{-5} M) are stimulated to elicit a suppressor factor with the ability to nonspecifically inhibit PHA- and LPS-induced DNA synthesis. This factor, prostaglandin-induced T-cell-derived suppressor factor (PITS), is released by a certain population of glass adherent T lymphocytes in response to PGE_2 production by glass wool non-adherent lymphocytes in culture.

Staphylococcal enterotoxins are responsible for a very common form of human food poisoning. The development of Staphylococcal enterotoxicosis is usually mediated by the ingestion of foods contaminated with microgram quantities of SE. The results reported here suggest that SE might mediate a transient state of immunosuppression via localization in the gut-associated lymphoid tissues (GALT) and/or spleen and subsequent activation of T-suppressor cells. More work is clearly needed to determine the clinical significance of such an enterotoxin-mediated effect.

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IV. APPENDICES

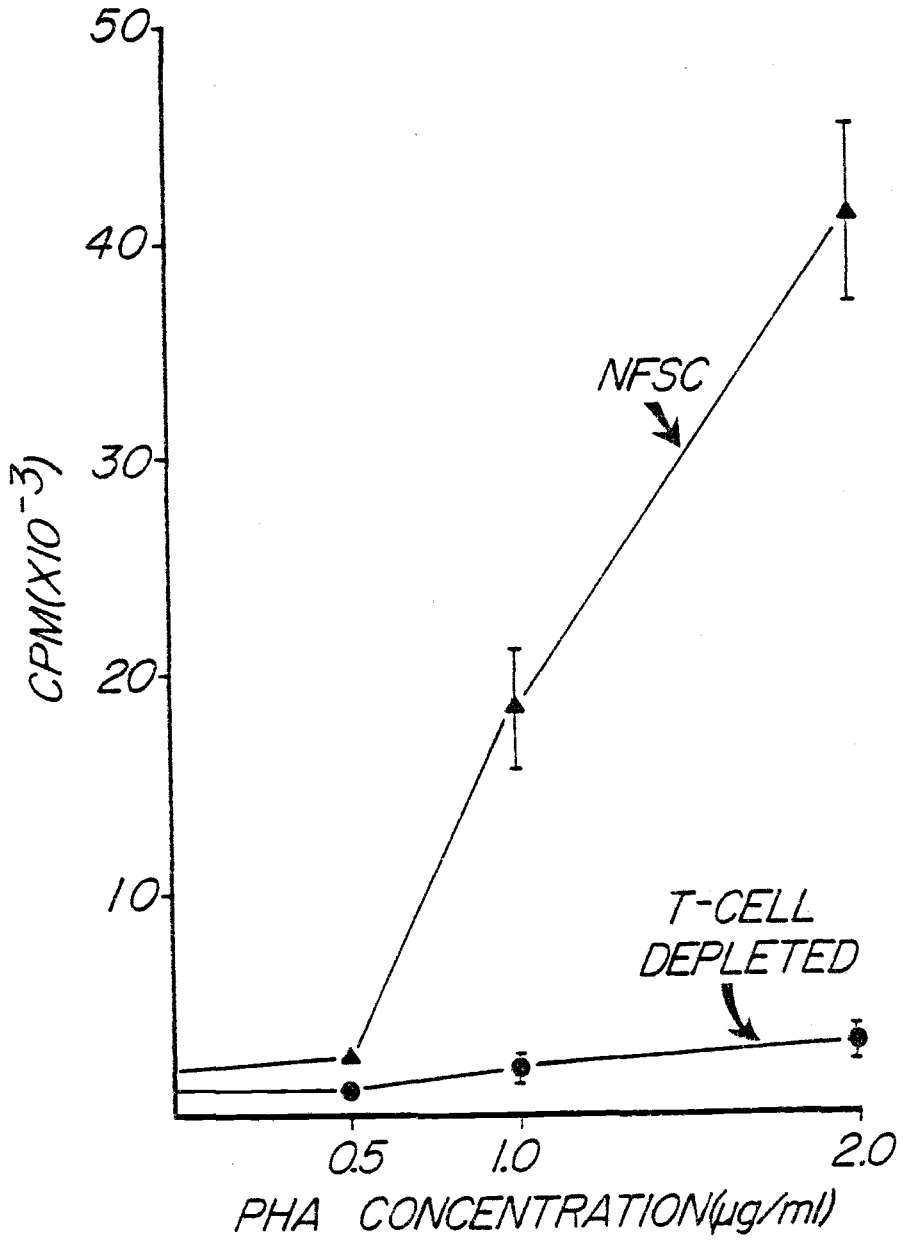
APPENDIX I

To verify that the functional depletion of T cells from a heterologous spleen cell suspension by treatment with rabbit anti-mouse T-cell serum plus complement had occurred, representative samples of treated and control non-treated cell suspensions were analyzed for their blastogenic response to purified phytohemagglutinin (PHA; Wellcome Reagents Ltd., England). Results shown in Appendix Figure I illustrate that the mitogenic response to PHA in T cell-depleted splenocyte cultures was significantly reduced relative to normal NSC cultures at the same cell concentration (10^7 cells/ml) and PHA concentrations (0.5, 1.0 and 2.0 ug/ml, respectively). After correcting for background responses (no PHA), the percent PHA response of T cell-depleted cultures was determined for the three PHA concentrations employed.

		NSC	T-cell-depleted	% PHA Response of Control
PHA_1	0.5 ug/ml	2387+/-483	423+/-16	17.7%+/-3.3%
PHA_2	1.0 "	17134+/-2501	1109+/-136	6.5%+/-5.4%
PHA_3	2.0 "	41092+/-3558	1592+/-115	3.9%+/-3.2%

Average percent PHA response of T cell-depleted cultures: 9.4%+/-3.97%.

The depletion of T cells by Cedarlane rabbit anti-mouse thymocyte serum plus rabbit complement resulted in reducing the PHA-induced blastogenic response to an average of less than 10% that of the control (NSC) response.


50
40
30
20
10
CPM(X10⁻³)
NFSC
T-CELL
DEPLETED
0.5
1.0
2.0
PHA CONCENTRATION(µg/ml)

APPENDIX II

The relative cell viablity of *in vitro* primed spleen cells was determined after incubation for 48 hours at 37°C in TCM in the presence of SEB at a final concentration of 5 ug/ml or SEB plus indomethacin or Ro-3-1428 at a final concentration of 10^{-7}M. Representative samples of these PSC cultures were analyzed by the standard trypan blue dye exclusion technique. The percent viable cells was determined by the following formula:

$$\% \text{ viable cells} = \frac{\text{number of viable cells (non-stained)}}{\text{total number of cells}}$$

Culture		% Viablity
NON-PSC	160/200	80.0%
NON-PSC + Indo (10^{-7}M)	165/200	82.5%
NON-PSC + Ro-3-1428 (10^{-7}M)	145/200	72.5%
SEB-PSC	149/200	74.5%
SEB-PSC + Indo (10^{-7}M)	160/200	80.0%
SEB-PSC + Ro-3-1428 (10^{-7}M)	148/200	74.0%

The average percent viablity was 77.25%, and no significant deviation from this value was noted as a result of exposure to either SEB or SEB plus indomethacin or Ro-3-1428.