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Research Article

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Evidence for the Synthesis and Release of Strongly Immunosuppressive, Noncytotoxic Substances by *Streptococcus intermedius*

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ABSTRACT Products secreted by *Streptococcus intermedius* were studied for their effects on the immune response. Three different preparations of crude extracellular products from *S. intermedius* (CEP-Si) were found to have powerful suppressor activity in vitro as shown by inhibition of human lymphocyte proliferation (uptake of [^{3}H]thymidine) and protein synthesis in response to a wide variety of stimulants, including mitogens and antigens, and suppression of plaque formation by human cells in response to sheep erythrocytes. CEP-Si was noncytotoxic, because cells incubated with high concentrations of CEP-Si and subsequently washed were viable and recovered their ability to respond to mitogens, and because leukocyte migration was not inhibited by CEP-Si, nor was the release of leukocyte migration inhibitory factor from sensitized lymphocytes. The possibility of antigen or mitogen competition was excluded. The effects of CEP-Si in vitro were time dependent and did not require the presence of monocytes. Cells pretreated with CEP-Si and then washed suppressed plaque formation by fresh autologous cells in highly stimulated cultures. CEP-Si injected into C57BL/6 mice also strongly suppressed their immune response to sheep erythrocytes, and the in vivo suppression was correlated with the effects of CEP-Si in vitro.

INTRODUCTION

It has been reported that hosts heavily infected with microorganisms are immunologically unresponsive (1–10). This state of immunosuppression has been attributed to the generation of suppressor monocytes (4, 8–10) or suppressor T lymphocytes (1, 2, 6, 9), and it has been suggested (9) that such suppressor cells are generated as a result of hyperstimulation and deregulation of immunosuppressor control mechanisms in the presence of large amounts of antigen. It is also conceivable, however, as noted by Schwab (11), that the infecting microorganisms could produce substances (not necessarily toxins) directly able to suppress the host's immune response in order to survive in an adverse environment. Previous studies have shown suppressive effects of noncytotoxic cellular extracts from group A streptococci and of streptococcal lipoteichoic acid on immune responses of mice in vivo (12–15); however, in vitro suppression by these substances either was not studied or when studied was not observed (14), although noncytotoxic immunosuppression in vitro and in vivo by enzymes secreted by bacteria, such as ribonuclease (16), L-glutamine (17), and asparaginase (18), has been reported.

The study of substances with immunosuppressive activity that may be released by bacteria is of obvious importance in regard to the control of infection, particularly if the in vitro activity corresponds to a similar phenomenon in vivo and vice versa. Indeed, demonstration of in vitro activity is important for extrapolation of results from experimental animals to human beings. In addition, studies of the mechanism of action of any noncytotoxic immunosuppressor are of interest. Therefore, we have studied the in vitro and in vivo effects and mechanism(s) of action of the suppressor activity present in crude extracellular products secreted by *Streptococcus intermedius* (CEP-Si),[1] which some of us have previously shown

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[1] *Abbreviations used in this paper*: CEP-Si, crude extracellular products secreted by *Streptococcus intermedius*;

(19) to inhibit [^{3}H]thymidine uptake by human mononuclear cells stimulated with phytohemagglutinin (PHA), and we have compared this product(s) with those described previously.

METHODS

Preparation of bacterial products. S. intermedius was cultured, and the products secreted in culture were prepared as described in detail elsewhere (19). Briefly, the microorganisms were grown under anaerobic conditions over a sterile dialysis membrane (cutoff 12,000–14,000 mol wt) layered over a tryptone-glucose agar culture medium. The organisms were grown at 37°C for 24 h. After this growth period, the dialysis membranes were washed with 0.05 M potassium phosphate buffer, pH 7.5, and the wash was clarified by centrifugation at 29,000 *g* for 15 min and concentrated ≅10-fold by positive-pressure ultrafiltration with an Amicon PM-10 filter (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). Three different batches of CEP-Si were used, and the protein content of each batch was determined. As controls, two different preparations were made as above but without microorganisms, i.e., the dialysis membranes were layered over the tryptone-glucose culture medium under the same conditions as above, washed, and the wash was treated in the same manner. These were designated control preparations (CP).

[^{3}H]Thymidine uptake by human mononuclear cells. Peripheral blood mononuclear cells from 10 different donors, 6 of them with strong positive delayed skin reaction to tuberculin, were obtained from defibrinated blood after centrifugation on a Ficoll (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) -sodium metrizoate gradient by the method of Boyum (20). In addition, thymuses obtained from four different children during the course of thoracic surgery were gently teased, and the mononuclear cells were separated in the same manner. The mononuclear cells were cultured according to the method of Du Bois et al. (21) in Nunc plastic tubes (1090, Nunc, Products 8, Algade DK-4000, Roskilde, Denmark). Minimal essential medium (MEM) (MEM-S, Gibco Diagnostics, Chagrin Falls, Ohio) was used as tissue culture medium, supplemented with 20% fresh, heat-inactivated human AB serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). The medium was buffered with Tris-HCl. PHA (Gibco), concanavalin A (ConA, Sigma Chemical Co., St. Louis, Mo.), pokeweed mitogen (PWM), and purified protein derivative of tuberculin (PPD, Serum Statenstinstitut, Copenhagen, Denmark) were used as stimulants at final concentrations of 1/40, 8 μg/ml, 1/100, and 10 μg/ml, respectively. In the case of thymocytes, PHA was the only stimulant used. In some instances, the cells were preincubated in medium 24–96 h before stimulation by PHA.

Cultures of 3×10^5 mononuclear cells/ml in 1 ml of tissue culture medium were performed in triplicate or quadruplicate. In some instances a bidirectional mixed lymphocyte culture (MLC) was performed with 1.5×10^5 mononuclear cells from each of two HLA-incompatible human donors. CEP-Si was added at dilutions ranging from 1/200 to 1/25,600 (i.e., equivalent to 32–0.25 μg of protein detected in CEP-Si) at various times of culture, either before, after, or at the same time as the stimulant. In some experiments, CEP-Si was added daily to some cultures. In some experiments CEP-Si was washed out of the cell suspensions after incubation, and in others CEP-Si-treated cells stimulated with PHA or PPD and subsequently washed were added to fresh cells (not treated with CEP-Si).

CEP-Si was also tested for its effects on suspensions of purified T cells. These cell suspensions were prepared as follows. Mononuclear cell suspensions (2×10^6 cells/ml in MEM with 20% human AB serum) were incubated with carbonyl iron particles for 2 h at 37°C, and phagocytic cells were removed by passage through a strong electromagnetic field. EA-rosette-forming cells were then performed by the method of Bianco et al. (22), and removed by layering the cell suspension after rosette formation over a Ficoll-sodium metrizoate gradient and centrifuging as described above. The remaining cells were incubated with sheep erythrocytes (SRBC) to form E rosettes, according to the method of Bentwich et al. (23). The E-rosette-forming cells were recovered from the bottom of a Ficoll-sodium metrizoate gradient after centrifugation, and the SRBC were lysed with 0.02% NH_4Cl in Tris-HCl buffer, leaving a population of purified T cells containing <0.5% phagocytic cells or nonspecific esterase diffusely staining cells (24). CP were added only at the beginning of PHA- or PPD-stimulated cultures, at dilutions of 1/5–1/200.

All cultures were pulsed with 0.5 μCi [^{3}H]thymidine (6 Ci/mmol) 24 h before harvesting. Cells were routinely harvested at 3 d after the beginning of the culture when PHA was used as the stimulant, at 4 d for ConA stimulation, and at 6 d for PWM, PPD, or MLC. In some experiments, cultures were harvested at different intervals, as indicated below, and some cultures were used for morphological controls.

[^{3}H]Leucine uptake by human mononuclear cells. [^{3}H]-Leucine uptake was measured by the method of Klesius et al. (25), with slight modification. Briefly, some human mononuclear cells were cultured in Falcon microtiter plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) either in MEM without serum, buffered with sodium bicarbonate, as described in the original method, or alternatively in MEM with 20% AB serum as described above for [^{3}H]thymidine uptake. In the former case, 0.2 ml of the cell suspension, which contained 2×10^5 cells, was placed in each well. The cultures were incubated at 37°C in a humid atmosphere with 5% CO_2. PHA and PPD were used as stimulants, at the same concentrations used for [^{3}H]thymidine uptake. The PPD-stimulated cultures were harvested after 24 h and the PHA-stimulated cultures after 24 or 72 h (the latter only in the case of cultures containing AB serum). All cultures were pulsed with 1 μCi [^{3}H]leucine (57.1 Ci/mmol) 5 h before harvesting. CEP-Si was added to test cultures as described above.

Leukocyte migration assay. Leukocyte migration was tested as described by Clausen (26) and Hoffman et al. (27), with slight modification. Leukocytes were obtained from three human donors highly sensitive to PPD. Peripheral blood was collected in sterile disposable syringes with 50 U of heparin (Panheparin, Abbott Laboratories, North Chicago, Ill.) per milliliter of blood. High molecular-weight dextran, 6% in saline (Sigma Chemical Co.), was added at a ratio of 1:5 and allowed to sediment for 40 min at 37°C. Polymorphonuclear cells (PMN) obtained from the supernatant were washed with Dulbecco's phosphate-buffered saline (Gibco Diagnostics) and resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated horse serum. PPD (100 μg/ml) and(or) CEP-Si at various dilutions was added to test cultures. The cultures were adjusted to 1.5×10^8 cells/ml and incubated for 30 min at 37°C.

Migration was assayed in 1% agarose (Indubiose A37, L'Industrie Biologique Francaise S. A., Gennevilliers, France)

ConA, concanavalin A; CP, control preparations; MEM, minimal essential medium; MLC, mixed lymphocyte culture; PHA, phytohemagglutinin; PMN, polymorphonuclear cells; PPD, purified protein derivative of tuberculin; PWN, pokeweed mitogen; SRBC, sheep erythrocytes.

in medium TC 199 supplemented with 10% horse serum (Gibco Diagnostics), penicillin (60 U/ml), and streptomycin (60 μg/ml) and buffered with Tris-HCl. 7-μl samples of the various cultures were placed in wells of 2.3-mm diameter. Each culture was tested in triplicate. The plates were incubated for 20 h at 37°C in a humidified atmosphere containing 5% CO_2. Migration was measured with a calibrated ocular as the mean of two diameters of the migration area.

Immunization of human mononuclear cells against SRBC. To test the generation of human antibody-producing cells against SRBC by specific immunization, we used a slight modification of the method described by Fauci and Pratt (28, 29). Peripheral blood mononuclear cells from human volunteers were separated on Ficoll gradients as described above and adjusted to 2.5×10^6 cells/ml in RPMI 1640 which contained 100 U/ml penicillin and 100 μg/ml streptomycin, buffered with sodium bicarbonate, and supplemented with 10% human heat-inactivated AB serum previously exhaustively absorbed with SRBC in the cold. 1-ml samples (instead of 2-ml samples as in the original method) of the suspension were incubated in Falcon tubes (Cat. 2051, Falcon Labware) on a rocker platform (10 cycles/min) in a humidified atmosphere of 5% CO_2. All the cultures were costimulated with 10^6 mitomycin-treated mononuclear cells from an HLA-incompatible donor (MLC), the SRBC (5×10^6 cells/ml) were added to some cultures.

The effects of CEP-Si were tested in these cultures either by addition of different amounts directly to the cultures containing SRBC or by addition of different concentrations of autologous cells which had been treated with CEP-Si and then washed. In the latter event, the cells were either incubated with CEP-Si under the conditions described above with SRBC or in some instances stimulated with PPD as described above and subsequently washed. In both cases these cells were added to fresh cells which were either unstimulated or stimulated with SRBC. All the cultures were fed with 5 mM L-glutamine daily and incubated for 7 d, after which the cells were washed. All cultures were performed either in duplicate or in triplicate. Direct plaque-forming cells were then assayed by a slight modification of the method of Jerne and Nordin (30), with a mixture of 1/112 final dilution (vol/vol) SRBC and 1.8% fresh guinea pig serum as a source of complement in Bacto-Agar (Difco Laboratories, Detroit, Mich.) at 0.36% final concentration, supplemented with a 0.005% DEAE-dextran (Sigma Chemical Co.) solution. The mixture was poured into Falcon disposable plastic Petri dishes (Falcon Labware), and the assay for hemolytic plaques was performed in a humidified atmosphere for 3 h at 37°C.

In vivo assays. C57BL/6 mice 6-wk-old were used. All mice were immunized by intraperitoneal injection of SRBC diluted 1/20 (vol/vol) in 0.3 ml buffered saline solution. Primary immunization was assayed 5 d later. The mice were killed, their spleens were removed and gently teased, and the separated mononuclear cells were assayed for formation of hemolytic plaques as described above. CEP-Si was injected intraperitoneally either on the day of immunization (day 0), 15, 6, 4, or 2 d before (days −15, −6, −4, −2), or 2 or 4 d afterward (days +2, +4) at a constant dose of 5,000 U of PPD biological activity (as defined below). On day −2, CEP-Si from the three different batches was injected at various doses, measured in units of PHA or PPD biological activity (see below). Groups of three animals each were usually used for each treatment, and experiments were repeated five times. Controls consisted of animals not injected with CEP-Si and animals not receiving any treatment. Some mice were first given 400 or 250 rads total-body irradiation. Those receiving 400 rads were immunized 10 d after irradiation and those receiving 250 rads 3 d after immunization. CEP-Si (5,000 U) was given to these mice only on day −2. In two sets of experiments, mice were injected weekly with 5,000 U of CEP-Si for 5 wk, and 3 mo after discontinuation of the treatment they were immunized with SRBC.

Preliminary assay of purification of CEP-Si. Isoelectric focusing was conducted by the method of Arnaud and Coryssel (31) in thin-layer polyacrylamide gels with a pH gradient of 3.5–8. The samples of CEP-Si were applied by means of 0.5 × 1.0-cm filter paper pads. After electrofocusing, the gels were cut in sections, after determination of the pH. These sections were subsequently removed, incubated in phosphate-buffered saline, and the substances were eluted from the gel by repeated freezing and thawing. The ampholytes were removed by means of gel filtration on Sephadex G-75 (Pharmacia Fine Chemicals, Inc.). As previous experiments indicated that the suppressor substances were eluted on the void volume from Sephadex G-200 gel filtration, only the void volume from Sephadex G-75 of the different fractions was assayed for biological activity.

RESULTS

Effects of CEP-Si on uptake of [^{3}H]thymidine and [^{3}H]leucine in vitro. CEP-Si produced a marked suppression of [^{3}H]thymidine uptake by stimulated human peripheral blood cells when added at the beginning of the cultures. This was observed in more than 20 experiments with different batches of CEP-Si and cells from different donors. Strong suppression was observed in some cases at dilutions as high as 1/12,800 (Table I), corresponding to ≅0.5 μg protein added per culture. The effects depended somewhat upon the kind of stimulant used, being strongest for MLC stimulation, less strong for PPD, and still less for PHA. Strong suppression was also seen in cultures stimulated with PWM or ConA (data not shown). The effects in nonstimulated cultures were less evident than in stimulated cultures. Also, in thymocyte cultures stimulated with PHA the suppression produced by CEP-Si was considerably weaker than in PHA-stimulated peripheral blood mononuclear cell cultures. CP did not produce any significant suppression of [^{3}H]thymidine uptake in PPD- or PHA-stimulated cultures, even when used at dilutions of 1/20. When used at dilutions of 1/5, CP consistently reduced [^{3}H]thymidine uptake in PHA- and PPD-stimulated cultures. The percentage of the control values was never below 60%, and this decrease probably was a result of a relative decrease in medium nutrients, as similar effects can be observed if cultures are performed in medium with equal amount of phosphate-buffered saline.

The effects of CEP-Si varied somewhat from batch to batch, but the results for each batch were consistent and reproducible when expressed as percentages of control values for the different cell suspensions tested (Fig. 1A and B). Therefore, dose-response curves were calculated for the effects of each batch on PHA- and PPD-stimulated cultures. For PHA stimulation, the statistically most probable curve was fitted by a second-

TABLE I

Inhibition of [³H]Thymidine Uptake by CEP-Si in Cultures of Human Peripheral Blood Mononuclear Cells with Different Stimulants and Human Thymocytes Stimulated with PHA

CEP-Si concentration	[³H]Thymidine uptake*				
	Peripheral blood cells				Thymocytes
	PHA	PPD	MLC	Nil‡	PHA
	mean cpm ±SD				
0 (control)	173,294±10,629	150,444±11,018	31,556±3,983	1,623±57	1,842±74
1/200	149±40 (0.08%)(*a*) (0.02–0.15%)(*b*)	68±10 (0.05%) (0.01–0.15%)	79±16 (0.2%) (0.01–0.04%)	53±1 (3%) (2–5%)	64±30 (3.5%) (2.1–9%)
1/400	226±43 (0.13%) (0.1–0.7%)	105±1 (0.07%) (0.01–0.15%)	80±16 (0.2%)	192±101 (12%) (12–20%)	66±29 (3.6%)
1/800	190±39 (0.11%) (0.09–0.15%)	113±23 (0.08%) (0.02–0.23%)	97±40 (0.3%) (0.01–0.5%)	295±30 (18%) (18–23%)	160±67 (8.7%) (4–16%)
1/1,600	188±43 (0.10%) (0.1–5%)	131±20 (0.09%) (0.05–0.45%)	89±31 (0.2%) (0.01–1%)	253±40 (16%) (12–20%)	666±76 (36%) (30–43%)
1/3,200	277±26 (0.16%) (0.1–16%)	218±40 (0.15%) (0.11–0.65%)	95±47 (0.3%)	ND§	1,443±214 (78%)
1/6,400	1,382±387 (0.8%) (0.8–80%)	587±30 (0.39%) (0.39–16%)	86±15 (0.3%) (0.01–0.3%)	318±95 (20%)	1,489±34 (81%) (75–89%)
1/12,800	27,531±131 (16%) (6–90%)	4,290±190 (2.9%) (2–65%)	ND	624±84 (38%) (30–60%)	1,698±26 (92%) (85–98%)

* Numbers in parentheses indicate mean of the results of triplicates of a typical experiment, expressed as percentage of the controls (*a*) and the range for at least four other experiments with different donors but with the same stimulants (*b*).

‡ No stimulation.

§ ND, not done.

degree equation and for PPD stimulation by a third-degree equation (Fig. 1A and B). Because the nature of the active component(s) in CEP-Si is not known, we decided to quantitate the doses used in terms of biological activity. Units of PPD and PHA biological activity were calculated on the basis of the above equations (Fig. 1A and B), where one unit of biological activity was arbitrarily considered to be the amount producing a 50% reduction in [³H]thymidine uptake as compared with controls (PPD- or PHA-stimulated cultures without CEP-Si).

The suppressor effect of CEP-Si on PHA- and PPD-stimulated cultures did not require the presence of monocytes, as [³H]thymidine uptake was strongly suppressed in cultures of pure E-rosette-forming cells (Table II). A small decrease in the effects of CEP-Si was observed for pure T cells as compared with unseparated mononuclear cells when low concentrations of CEP-Si were used; however, this observation probably reflects a reduction in the effects of CEP-Si in poorly stimulated cultures rather than mediation of the effects by monocytes.

The effects of CEP-Si on protein synthesis as assessed by [³H]leucine uptake were highly dependent upon the conditions of culture, being much weaker in serum-free mononuclear cell cultures than in those containing serum, for both PPD and PHA stimulation (Table III). In PHA-stimulated cultures that contained serum, the suppression was greater when the cells were harvested after 3 d than after 24 h. These differences were apparent when the results were expressed either as percentages of control values or as absolute counts.

Dynamics of the effects of CEP-Si on [³H]thymidine

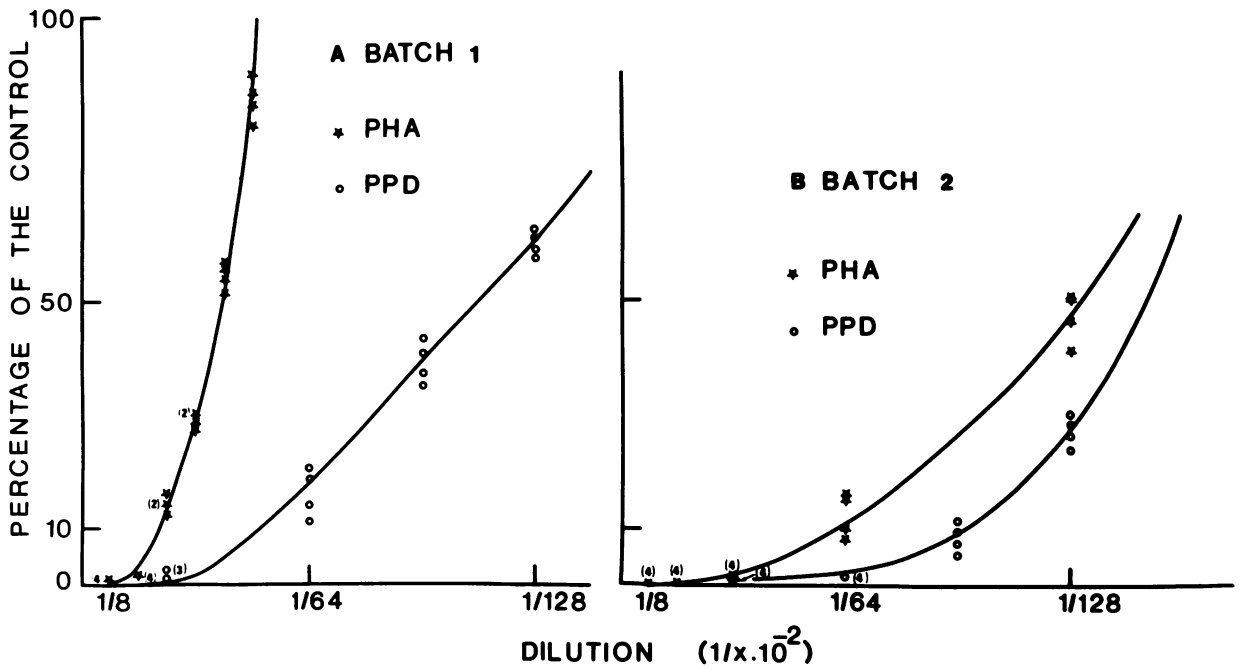

FIGURE 1 Estimated effects of two batches of CEP-Si on PHA- and PPD-stimulated cultures of human peripheral blood mononuclear cells. Curves were calculated by polynomial regression analysis (Dixon, W. J. 1975. *BMDP. Biomedical Computer Programs.* University of California Press, Los Angeles, Calif. 593–620). Results are expressed as the relationship between the final dilution of CEP-Si in the test cultures and the percentage of [^{3}H]thymidine uptake relative to the value for control cultures. The regression equations for the curves drawn for the first batch were $y = 4.99 - 8.51x + 3.64x^2$ and $y = 2.77 - 3.11x + 0.821x^2 - 0.025x^3$ for PHA- and PPD-stimulated cultures, respectively. For the second batch the equations were $y = -0.428 - 0.005x + 0.188x^2$ and $y = -1.30 + 1.39x - 0.256x^2 + 0.018x^3$ for PHA- and PPD-stimulated cultures, respectively. In all equations, $x = 1$ represents a dilution of 1/800; $x = 4$, a dilution of 1/3,200, etc. Theoretical points were calculated on the basis of at least five different dilutions of CEP-Si, corresponding to four different experimental points for both PHA- and PPD-stimulated cultures. In some instances parts of the curves have been omitted for clarity.

uptake in PHA- and PPD-stimulated cultures. When CEP-Si was added to cultures at different times, it was apparent that the suppression was considerably enhanced in PHA-stimulated cultures when the preparation was added before the PHA (Fig. 2A). The effect was progressively reduced when the CEP-Si was added at longer intervals after the initiation of culture, in proportion to the length of its presence in the culture, in both PHA- and PPD-stimulated cultures (Fig. 2A and B). No suppression was seen with small doses of CEP-Si added only 24 h before the cultures were harvested.

[^{3}H]Thymidine uptake values at different times in PHA-stimulated cultures treated with CEP-Si parallelled those in controls for a certain time, after which they increased less rapidly than controls and, after reaching a maximum level, dropped abruptly (Fig. 3). The abrupt drop occurred sooner when higher doses of CEP-Si were used. In general, the minimum values in treated cultures coincided with the time of maximum stimulation in controls. Interestingly, the values in the treated cultures began to increase after reaching the minimum level (i.e., after the controls reached maximum stimulation levels) (Fig. 3 IA and IB). This increase occurred sooner, however, when higher doses of CEP-Si were added. Moreover, as shown in Fig. 3 IIA and B, in two experiments the increase and abrupt drop occurred even sooner when the cultures were supplemented daily with CEP-Si than when they were supplemented only at the beginning of the culture.

Evidence for noncytotoxicity of CEP-Si. To determine whether CEP-Si was cytotoxic to the test cells, doses up to 80 U of biological activity (as defined above) were added for periods up to 48 h to unstimulated cultures and to cultures stimulated with PHA or PPD. When cell viability was checked by trypan blue exclusion after different periods of incubation, more than 95% were viable in every case.

In addition, cells incubated with CEP-Si and then washed twice responded normally to both PHA and PPD. Surprisingly, cells incubated with CEP-Si, washed twice, and cultured without stimulation underwent

TABLE II

Comparison of the Effects of 20 and 2 U of PHA or PPD Biological Activity of CEP-Si on [³H]Thymidine Uptake in PHA- and PPD-Stimulated Cultures, Respectively, of Unseparated Mononuclear Cells (UMc) or Purified T Cells (Tc) from Three Different Donors

	[³H]Thymidine uptake					
	Donor 1		Donor 2		Donor 3	
Culture	PHA	PPD	PHA	PPD	PHA	PPD
	mean cpm±SD					
UMc	75,639±2,456	65,893±5,439	101,899±7,345	80,535±10,345	120,695±11,232	92,345±2,045
UMc + CEP-Si, 20 U	156±11 (0.2%)*	75±25 (0.11%)	180±20 (0.17%)	200±12 (0.24%)	212±10 (0.17%)	150±22 (0.16%)
UMc + CEP-Si, 2 U	5,015±1,967 (6.6%)	8,932±2,503 (13.6%)	9,737±567 (9.6%)	5,647±378 (7.0%)	13,895±1,097 (11.5%)	6,977±432 (7.6%)
Tc	24,001±1,001	16,937±2,021	45,735±3,341	18,945±1,111	40,112±935	30,333±1,935
Tc + CEP-Si, 20 U	106±9 (0.44%)	154±16 (0.90%)	39±20 (0.08%)	200±10 (1.05%)	86±31 (0.21%)	53±32 (0.17%)
Tc + CEP-Si, 2 U	4,375±231 (18.2%)	5,981±437 (36.5%)	4,375±399 (9.6%)	5,987±832 (31.6%)	4,999±301 (12.5%)	3,998±472 (13.2%)

* Numbers in parentheses indicate percentage of control values (UMc or Tc not supplemented with CEP-Si).

spontaneous proliferation, proportional to the dose used. Table IV shows the results of two experiments in which cells were first incubated with a CEP-Si dose of 10 or 40 U of PHA or PPD biological activity for 48 h, then washed twice, and then reincubated with PPD or PHA, respectively. For comparison, some cell preparations were incubated with 10 or 40 U of PPD biological activity, washed twice, and then reincubated without stimulation.

Finally, leukocyte migration experiments revealed that CEP-Si did not inhibit the release of leukocyte migration inhibitory factor from cells of strongly sensitive donors in the presence of PPD and did not inhibit the normal migration of human PMN in the absence of antigen. With cell populations from three different donors, the results (expressed as mean millimeters of migration ±1 SD) were as follows: In control cultures (in the absence of PPD), the areas of migration were 10.5±0.7, 8.9±0.4, and 8.0±0.2 in the absence of CEP-Si and 10.7±0.6, 8.7±0.4, and 8.2±0.3 when CEP-Si was used at 1/40 final dilution. In PPD-stimulated cultures, the values were 5.0±0.1, 6.1±0.1, and 5.1±0.2 in the absence of CEP-Si and 4.9±0.2, 6.2±0.1, and 5.2±0.2 in the presence of a 1/40 final dilution of CEP-Si for donors 1–3, respectively. Lower concentrations of CEP-Si did not produce any significant differences in either control or PPD-stimulated cultures.

Effects of CEP-Si on hemolytic plaque formation by human cells in vitro. When human mononuclear cells stimulated by SRBC plus mitomycin-treated cells were treated with CEP-Si, formation of hemolytic plaques against SRBC in vitro was strongly inhibited, in proportion to the dose of CEP-Si. When these cells were treated with CEP-Si and then washed twice, they induced an increase in hemolytic plaques when added to untreated fresh autologous normal cells stimulated by MLC alone, whereas they induced a slight but consistent decrease when added to the same fresh autologous normal cells stimulated by both MLC and SRBC. This decrease was proportional to the number of CEP-Si-treated cells added (Fig. 4). Cells treated with PPD and subsequently washed either slightly enhanced or had no effect on hemolytic plaque formation by fresh cells stimulated by MLC. Cells stimulated by MLC or by MLC and SRBC but not treated with CEP-Si were not effective on hemolytic plaque formation by fresh cells stimulated by MLC or MLC and SRBC. The viability of cells incubated with CEP-Si did not differ from that of controls. In both cases, the viability was never <90%, and in several instances 95%. These values are considered excellent, taking into account that the cells remained in culture for 7 d, and that the populations of nonresponder cells show a tendency to die in culture.

In vivo experiments. As shown in Fig. 5, CEP-Si at a dose of 5,000 U of PPD biological activity strongly suppressed the primary response of C57BL/6 mice to SRBC when injected before the immunogen. When the CEP-Si was administered 4–6 d before immunization, the number of plaque-forming cells was

TABLE III

Inhibition of [³H]Leucine Uptake by CEP-Si in Cultures of Human Peripheral Blood Mononuclear Cells Stimulated with PHA or PPD

CEP-Si concentration	[³H]Leucine uptake*				
	PHA			PPD	
	A‡	B	C	A	B
			mean cpm±SD		
0 (control)	661±29	8,178±1,927	18,458±2,268	463±83	2,675±113
1/200	411±115 (69%)(*a*) (35–80%)(*b*)	1,189±115 (15%) (10–19%)	342±53 (1.9%) (1–4%)	346±43 (75%) (65–85%)	1,108±86 (41%) (25–52%)
1/400	315±20 (48%) (35–70%)	1,468±32 (18%) (12–20%)	467±13 (2.5%) (1–5%)	332±26 (72%) (63–88%)	1,221±96 (46%) (40–61%)
1/800	413±20 (70%) (60–90%)	1,637±170 (20%) (15–25%)	727±214 (3.9%) (2–7%)	442±103 (95%) (80–102%)	1,198±59 (45%) (43–59%)
1/1,600	549±103 (83%) (75–90%)	2,055±82 (25%) (20–35%)	1,064±274 (5.8%) (3–8%)	440±54 (95%) (90–100%)	1,460±73 (55%) (45–61%)
1/3,200	560±67 (85%) (80–100%)	3,474±420 (43%) (35–47%)	1,798±259 (9.7%) (4–12%)	406±18 (88%) (80–103%)	1,570±65 (59%) (50–77%)
1/6,400	558±2 (85%) (85–95%)	6,245±442 (76%) (65–85%)	ND§	427±56 (92%) (90–100%)	1,926±131 (72%) (65–85%)
1/12,800	601±101 (91%) (90–100%)	ND	6,125±661 (33%) (15–45%)	484±49 (104%) (95–104%)	2,009±162 (95%) (90–107%)

* Numbers in parentheses indicate percentage of control values for a typical experiment and the range for two additional experiments, (*a*) and (*b*), respectively.

‡ A, serum-free culture; B, cultured with human AB serum and harvested after 24 h; C, cultured with human AB serum and harvested after 3 d.

§ ND, not done.

consistently very low, usually <1% of controls, and when it was administered 2 d before immunization the values were always <4% of controls. When injected 15 d before SRBC, CEP-Si produced considerably less inhibition of the primary response than when injected 6, 4, or even 2 d before SRBC (average plaque-forming cell response, 50% of controls). Thus, the activity seems to be partially destroyed after long periods in vivo. CEP-Si injected on the same day as SRBC produced variable results, but in general did not cause a reduction in the number of hemolytic plaques. When injected after SRBC (i.e., on day +2 or +4), CEP-Si caused no reduction in the number of plaques. These results parallel those for [³H]thymidine uptake in vitro.

A highly significant inverse correlation was found between the number of biological units (determined as above for [³H]thymidine uptake in vitro in PPD-stimulated cultures with three different batches) of CEP-Si injected into the mice at day −2 and the number of hemolytic plaques formed ($r = 0.96$, $P < 0.001$). This correlation was defined by a potential equation of the formula $y = 1{,}414.23\ x^{-77}$. A similarly highly significant correlation ($P < 0.001$) was found for the number of biological units determined in PHA-stimulated cultures, although the r value in this case was considerably lower ($r = 0.76$).

Mice irradiated with 250 or 400 rads and then immunized with SRBC 3 or 10 d later, respectively, showed, as expected, much lower plaque-forming cell responses (86±10 and 64±12, respectively) than did

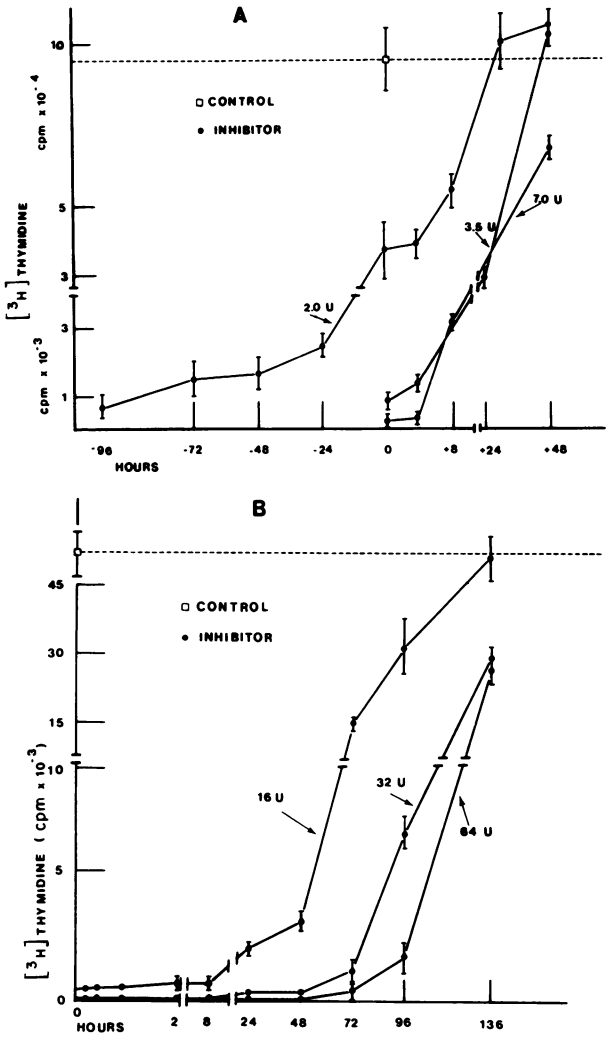

FIGURE 2 Example of [³H]thymidine uptake in PHA-stimulated (A) and PPD-stimulated (B) cultures of human peripheral blood mononuclear cells to which CEP-Si was added at different times and at different doses, compared with values in similar cultures without CEP-Si (controls). Both PHA and PPD were added to the cultures at 0 h. In the case of PHA stimulation, CEP-Si was added to some of the cultures before the mitogen (−96 to −24 h). The doses of CEP-Si are expressed in units of PHA and PPD biological activity (units) in A and B, respectively. Values are means for two different experiments. For clarity, the results of the response to PHA of cells preincubated in media without CEP-Si were not shown. These results indicated that the response to PHA is potentiated if the cells are preincubated with medium. This potentiation is proportional to the time of incubation, being the response to PHA at −96 h, about three-fold the response to PHA observed with cells nonpreincubated.

unirradiated mice (942±177). In these animals CEP-Si also suppressed the primary response to SRBC when injected at day −2, but the decrease was less dramatic than in unirradiated mice; i.e., in three comparable experiments, the number of plaque-forming cells, expressed as a percentage of control values, was 1.1±0.1%, 35±5.5%, and 38±7.2% (mean±1 SD) for mice receiving 0, 250, and 400 rads, respectively.

Mice receiving doses of CEP-Si up to 5,000 U of PPD biological activity showed no weight loss. When test animals were injected weekly with 5,000 U of PPD biological activity for 5 wk, no signs of toxicity were seen, and their primary responses to SRBC 3 mo after the treatment were equivalent to those of controls.

Preliminary fractionation of CEP-Si. The suppressor effect of CEP-Si was detected only in two fractions, out of nine, obtained by isoelectric focusing. One of the fractions was able to suppress PPD- or PHA-stimulated cultures, and the other was able to suppress only PPD-stimulated cultures. The first and the latter of these fractions contained substances with isoelectric point at pH 4.5–5 and 3.5–4, respectively. The suppressor effect on PPD-stimulated cultures was at least five times higher in the latter fraction than in the first. Both fractions were noncytotoxic, and, like for CEP-Si, the suppressor effect in vitro was observed more easily if the fractions were added to the cultures before the stimulant. As the suppressor effects of CEP-Si in vivo correlated excellently with the in vitro suppressor effects on PPD-stimulated cultures, the fraction able to suppress the PPD response was tested in vivo. Like for CEP-Si, this fraction was able to suppress the response to SRBC of C57BL/6 mice only if administered before the immunogen, i.e., strong suppression was observed at days −6, −4, and −2, but no inhibition of the response was observed at days 0, +2, or +4. The effect of this fraction was also proportional to the amount injected into the animals. Preliminary results indicate that the fraction less active on suppressing the PPD response was active in vivo as well but roughly at least four times less active than the other fraction.

DISCUSSION

The possibility that the suppressor effects of CEP-Si were a result of contamination with constituents of the culture medium can be excluded on the basis of the finding that control preparations (Methods) did not cause suppression in PHA- or PPD-stimulated cultures, even at dilutions of 1/20.

As for other substances extracted from or produced by bacteria (11–18) which are not toxins, it was found that CEP-Si was not cytotoxic, as shown by (*a*) the viability and proliferative ability of cells incubated with large doses and then washed, (*b*) its lack of inhibition of normal migration of PMN not incubated with antigen, (*c*) its failure to inhibit the release of leukocyte migration inhibitory factor by sensitized cells, and (*d*) its lack of toxicity when injected into mice.

It has been suggested by others that the suppressor

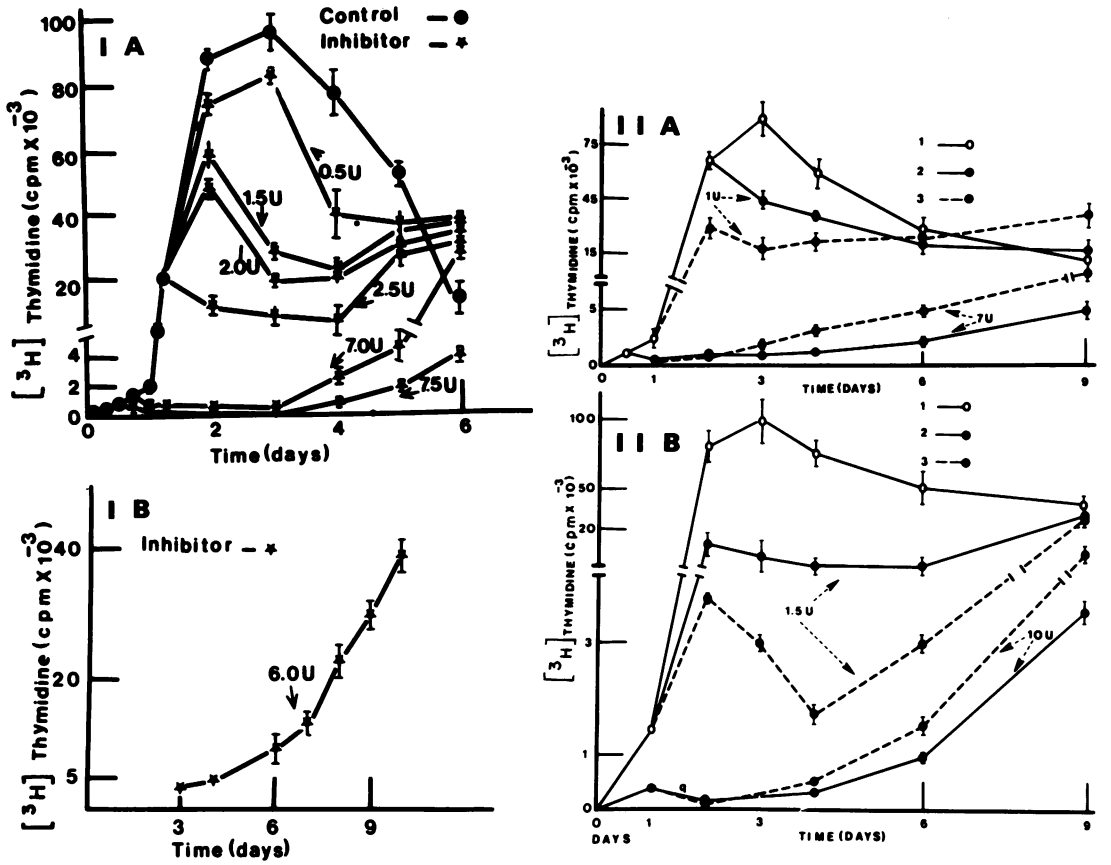

FIGURE 3 Dynamics of [^{3}H]thymidine uptake at different times in PHA-stimulated cultures of human peripheral blood mononuclear cells without CEP-Si (controls) or supplemented with various amounts of CEP-Si (expressed in units of PHA biological activity (U). (I) Values obtained from one of four reproducible experiments in which both PHA and CEP-Si were added at the beginning of the cultures. (II) Comparison of cultures of cells from two different donors (A and B) not supplemented with CEP-Si(1), supplemented with CEP-Si at different concentrations at the beginning of the culture(2), or supplemented daily with CEP-Si(3).

effects of some bacterial substances—e.g., lipoteichoic acid (15)—may represent competition between these substances and antigens as a result of blocking of the relevant receptors (11). These studies clearly exclude this possibility for the products secreted by *S. intermedius*, as they can strongly suppress the immune response to a variety of antigens, as well as cell proliferation in response to several mitogens. It is highly unlikely that one or more substances secreted by a single bacterial species would share antigenic determinants with such a wide variety of stimulants. In addition, although CEP-Si progressively loses its suppressor activity when added to the cultures at later times, its effects are the same whether it is added at the beginning of the culture or 8 h after the addition of PHA. In this respect, it is well known that PHA requires only a short time of contact with lymphocytes to trigger cell proliferation, because the mitogenic effect is equally strong whether the cells are exposed to PHA throughout the culture period or only for a short incubation period after which they are washed and reincubated in medium without PHA.

A major concern in studies of [^{3}H]thymidine uptake is the possibility that a high concentration of unlabeled thymidine in the culture could cause a reduction in uptake that would be falsely interpreted as inhibition (32–34). In this study, the possibility of this artifact was ruled out by several observations. First, morphological controls were carried out, and the results correlated roughly with the results for [^{3}H]thymidine uptake. Second, when several doses of CEP-Si, which caused strong suppressor effects on thymidine uptake by PHA- or PPD-stimulated cells when added at the beginning of the cultures, were added immediately before the cultures were pulsed with labeled thymidine (24 h before harvesting), no inhibition of uptake was seen. Third, in cultures containing CEP-Si, [^{3}H]-thymidine uptake reached a minimum level and then

TABLE IV

Lack of Inhibition of [^{3}H]Thymidine Uptake by Peripheral Blood Mononuclear Cells (from Two Donors) Stimulated with PHA or PPD after being Incubated with CEP-Si for 48 h and then Washed Twice

Donor	Amount of CEP-Si	[^{3}H]Thymidine uptake					
		Nil*		PHA		PPD	
		Unwashed	Washed‡	Unwashed	Washed‡	Unwashed	Washed‡
	U§	mean cpm±SD					
1	0	809±112	672±211	89,765±9,578	99,980±8,556	101,697±8,456	121,332±8,117
	10	422±54	2,846±31	95±29	75,045±1,982	156±15	60,384±2,931
	40	101±15	6,850±275	403±39	90,234±3,462	93±43	140,975±12,567
2	0	578±62	723±101	77,896±5,314	96,777±3,256	121,567±15,345	145,885±22,001
	10	392±82	1,531±35	65±8	46,593±12,031	78±12	133,567±3,468
	40	112±53	4,031±32	53±9	57,857±4,367	51±4	160,698±4,456

* No stimulation.

‡ Cells washed after 48 h of incubation with CEP-Si and, for stimulated cultures, restimulated with PHA or PPD.

§ As defined in Results. One unit is the amount of CEP-Si causing a 50% reduction in [^{3}H]thymidine uptake by cultures stimulated with PHA or PPD.

began to increase again, although the test substance was still present in the cultures. Moreover, an excellent correlation was also observed between the effects of CEP-Si in vitro and in vivo. As in PHA- or PPD-stimulated cultures, the effects were time dependent in test animals, the effect being stronger when the CEP-Si was injected at longer times before SRBC (although at very early injection times, e.g., 15 d before immunization, the effect was negated, probably because the material was degraded in vivo).

Perhaps the most important observation in this respect was the excellent correlation between the suppressor effects of CEP-Si in PPD-stimulated cultures and the effects of various amounts (measured in PPD biological units) injected on day −2. However, it should be emphasized that for PPD stimulation in vitro, we purposely selected donors with exquisite sensitivity to PPD, and thus it is questionable whether such a

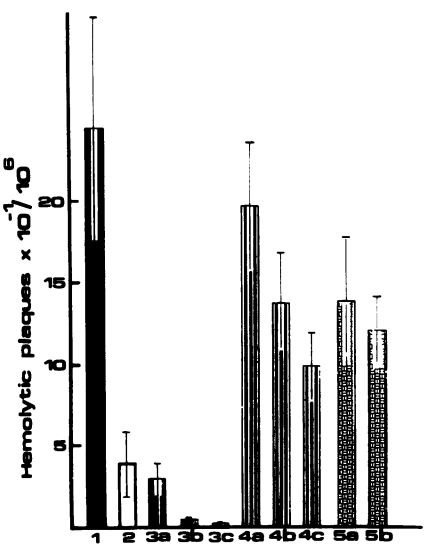

FIGURE 4 Results (mean±1 SD) of four different types of experiments measuring the number of hemolytic plaques per 10^6 human peripheral blood mononuclear cells developed in vitro under different conditions. (1) Cells incubated with SRBC and costimulated by MLC. (2) Cells costimulated by MLC but not incubated with SRBC. (3*a*–*c*) As in 1, with CEP-Si added at doses of 4, 6, and 8 U of PPD biological activity, respectively. (4*a*–*c*) Cells incubated with SRBC and costimulated by MLC, supplemented with 0.5×10^6, 1×10^6, and 2×10^6 autologous cells previously cultured as in 1 and in the presence of 6 U of PPD biological activity of CEP-Si and then washed twice. (5*a*–*b*) Cells treated as in 2 but supplemented with 1×10^6 and 2×10^6 autologous cells treated with CEP-Si and washed as in 4.

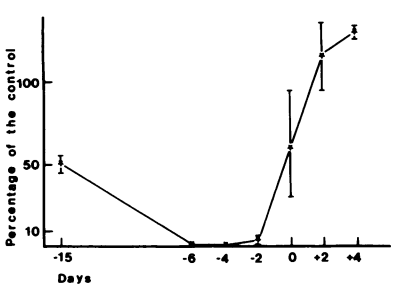

FIGURE 5 Numbers of hemolytic plaque-forming cells in C57BL/6 mice immunized against SRBC and given injections of CEP-Si (5,000 U of PPD biological activity) at various times. The results, which represent five different experiments, are expressed as the percentage of the number of hemolytic plaque-forming cells observed in mice not treated with CEP-Si (controls). Control and treated mice were immunized on day 0, and CEP-Si was administered either before (−15 to −2 days) or after (0 to +4 days) immunization. Control values were 1,255±568 plaque-forming cells per 10^6 spleen mononuclear cells. All animals were killed 5 d after immunization with SRBC.

high correlation would be found for mononuclear cell cultures derived from nonresponsive or slightly sensitive individuals. These correlations between in vitro and in vivo effects of CEP-Si clearly exclude a similarity between this substance(s) and the bacterial extracts of group A streptococci (12–14), which have proven to be totally ineffective in vitro, although both substances are strong noncytotoxic immunosuppressors and are more effective when administered before antigen challenge.

In view of the observations that CEP-Si is noncytotoxic and has suppressor effects which are potentiated with increasing times of contact in vitro and in vivo (with the exception of much longer periods in vivo), it is tempting to speculate, in light of current knowledge of immunosuppression (35), that this substance(s) is able to generate suppressor cells or, alternatively, destroy helper cells. When suppressor or helper cells are involved, one must consider monocytes as being the active cells or inducers thereof (36–39). This study rules out a role of monocytes in the activity of CEP-Si, however, because the activity was equally strong in the presence and absence of monocytes. Our results seem to support the possibility of generation of suppressor cells rather than destruction of helper cells, as suggested by the dynamics of [^{3}H]thymidine uptake in treated cultures. If helper cells were being destroyed or inhibited, the values of [^{3}H]thymidine uptake should be progressively lower than controls rather than following control values and then dropping abruptly. Moreover, generation of suppressor cells is further suggested by the facts that the suppressor effects of CEP-Si are more evident (*a*) in highly stimulated than in poorly stimulated cultures, (*b*) in good culture conditions than in poor conditions (as seen for [^{3}H]leucine uptake in PHA- or PPD-stimulated cultures in AB serum vs. serum-free medium), and (*c*) in healthy animals than in irradiated animals. Indeed, it is more likely that generation of effector cells would be enhanced under favorable conditions in vivo or in vitro and inhibited under unfavorable conditions. The fact that CEP-Si loses its activity in vivo when injected into animals at long intervals before antigen challenge most likely indicates that the active substance(s) is catabolized. Therefore, it is probable that under these conditions only a small amount of suppressor activity is present in the host at the time of antigen challenge, and as a result, only a few suppressor cells are generated by the small amount of activity remaining.

Finally, generation of suppressor cells is also supported by the observations that stimulated cells incubated with CEP-Si began to proliferate after reaching a minimum of [^{3}H]thymidine incorporation, and that unstimulated cells incubated with CEP-Si and then washed proliferated spontaneously. Moreover, in cultures supplemented daily with CEP-Si, the minimum [^{3}H]thymidine uptake and subsequent proliferation both occurred sooner than in cultures supplemented only at the beginning of the culture. These observations fit with the concept of an autologous MLC reaction induced by proliferating suppressor cells, as suggested by Haynes and Fauci (40). Such a reaction could also explain the lack of decrease in [^{3}H]thymidine uptake in cultures of PHA- or PPD-stimulated fresh cells supplemented with CEP-Si-treated cells, because uptake by the proliferating cells would mask any inhibition of the mitogen or antigen response.

It is surprising, however, that human cells treated with CEP-Si, unlike ConA-induced suppressor cells, which suppress formation of hemolytic plaques against SRBC by both mouse (41–45) and human (46) cells, increased rather than reduced the number of human mononuclear cells forming hemolytic plaques when stimulated by MLC but not by SRBC. A clear-cut but small inhibition of plaque formation was observed, however, when fresh normal cells stimulated by both MLC and SRBC were incubated with autologous cells pretreated with CEP-Si and SRBC. One attractive explanation for these latter observations might be the possibility that the observed suppression was specifically induced to the stimulant, inasmuch as cells treated with PPD and CEP-Si, unlike those treated with CEP-Si and SRBC, failed to inhibit hemolytic plaque formation by fresh cells stimulated by MLC and SRBC. This hypothesis would also explain why the suppressor effects of CEP-Si are greater in MLC- than PPD-stimulated cultures and greater in the latter than in PHA-stimulated cultures. These observations, which suggest that CEP-Si can induce the generation of suppressor cells, indicate a lack of similarity between CEP-Si and immunosuppressive enzymes secreted by other bacteria (16–18). Indeed, the enzymatic suppressor effect is assumed to be a result of the destruction of essential cellular nutrients, such as amino acids (11), and therefore their effects are more likely a result of inhibition of helper and effector cells. Consequently, their effects would not be expected to show selectivity for some stimulants or to produce faster recovery of proliferation when added to cultures daily rather than only at the beginning of the cultures, as found for CEP-Si.

In conclusion, we report that *S. intermedius* is able to secrete a noncytotoxic substance(s), unrelated to other bacterial substances described previously, which strongly suppresses the immune responses (most likely by inducing specific suppressor cells) to a variety of antigens and mitogens in vitro, the action of which correlates well with its effects in vivo. We realize that fractionation of CEP-Si is an important step in identifying the active substance(s), and purification of the suppressor activity in CEP-Si is now in progress. Upon preliminary fractionation of CEP-Si it was

observed that the suppressor effects were found in two fractions. No enhancing effects were observed whatsoever, and therefore it is unlikely that the effects observed with crude preparations were a result of the combined effects of opposite (i.e., helper and suppressor) signals.

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