Fractionation and Characterization of the Immunosuppressive Substance in Crude Extracellular Products Released by *Streptococcus intermedius*

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ABSTRACT The noncytotoxic immunosuppressive substance detected in crude extracellular products of *Streptococcus intermedius* (CEP-Si) was fractionated by two steps of preparative isoelectric focusing in sucrose gradients using ampholytes of pH range from 3.5 to 6 and 4 to 5, respectively. The in vitro and in vivo suppressor effects of the most highly purified fraction of CEP-Si, designated fraction 3′ (F3′EP-Si), corresponded well with those of the original CEP-Si. F3′EP-Si was sensitive to the effects of alpha, gamma, and delta chymotrypsin, trypsin, and heating. It contained ~1% of the total amount of protein found in the original CEP-Si, corresponding to a single band on analytical isoelectric focusing, stainable by Coomassie Blue and of isoelectric point of 4.25. The absorption spectrum of F3′EP-Si had a maximum at 260 nm but its biological activity was resistant to deoxyribonuclease and ribonuclease A and it did not contain material stainable by methylene blue. It was also resistant to neuraminidase and did not contain material stainable by periodic acid Schiff. We conclude that the substance responsible for the suppressor activity of CEP-Si is a protein of molecular weight ~90,000, which adheres to Sephadex or cellulose acetate and forms complexes with other, nonactive constituents of CEP-Si.

Publication No. 5 from the Department of Immunology, Instituto de Ciencias Biomedicas "Abel Salazar" and publication no. 419 from the Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina.

Received for publication 12 June 1980 and in revised form 4 March 1981.

INTRODUCTION

We recently reported that crude extracellular products of *Streptococcus intermedius* (CEP-Si)[1] contain an extremely powerful noncytotoxic immunosuppressor substance(s) active both in vivo and in vitro (1). We compared the behavior of CEP-Si with other previously described noncytotoxic immunosuppressor substances obtained from microorganisms such as streptococcal extracts (2), streptococcal lipoteichoic acid (3), and bacterial enzymes like L-asparaginase (4), L-glutaminase (5), and ribonuclease (6); we concluded that CEP-Si was different from these substances mainly in the time-course of its effects and in the possible specificity of the resulting immunosuppression in relation to the stimulant. Other differences between the in vivo and in vitro effects of CEP-Si and both lipoteiochoic acid and streptococcal extracts were also demonstrated, and preliminary results on the purification of CEP-Si were reported (1). We have now confirmed our preliminary results, and in this report we describe the biochemical characteristics of the highly purified immunosuppressive material obtained from CEP-Si.

METHODS

Extracellular products of Streptococcus intermedius. *S. intermedius* was cultured and CEP-Si was obtained by the procedures described previously in detail (1, 7).

[1] *Abbreviations used in this paper*: CEP-Si, *Streptococcus intermedius*; MLC, mixed lymphocyte cultures; PHA, phytohemagglutinin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SRBC, sheep erythrocytes; TEMED, *N,N,N′,N′*-tetramethylenediamine.

J. Clin. Invest. © *The American Society for Clinical Investigation, Inc.* · 0021-9738/81/07/0294/09 $1.00
Volume 68 July 1981 294–302

[^3H]Thymidine uptake by human mononuclear cells. Peripheral blood mononuclear cells from six different donors were obtained from defibrinated blood after centrifugation on a gradient of Ficoll (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) and metrizoate by the method of Boyum (8) and cultured, as described previously (1), according to the method of Du Bois et al. (9) in minimal essential medium (Gibco Diagnostics Laboratories, Lawrence, Mass.) supplemented with 20% fresh heat-inactivated human AB serum and antibiotics. Cultures were pulsed with 0.5 μCi[^{3}H]-thymidine 24 h before harvesting, which occurred usually after 3 d in cultures stimulated with phytohemagglutinin (PHA, Gibco Diagnostics Laboratories) and after 6 d in unstimulated cultures or in cultures of bidirectional histoincompatible mixed lymphocyte cultures (MLC). In some experiments, however, cultures stimulated with PHA were harvested at different intervals. CEP-Si or its subfractions were added at the beginning of the MLC or of unstimulated cultures and at different intervals in the case of PHA-stimulated cultures. The suppressor effects of the most purified subfraction on both PHA-and MLC-stimulated cultures were estimated by means of the mathematical formulas described previously (1). On the basis of these questions, 1 U of PHA or MLC biological activity was considered the amount of suppressor substance producing a 50% reduction in [^{3}H]-thymidine uptake as compared with controls (PHA- or MLC-stimulated cultures without CEP-Si or its subfractions) (see Fig. 3).

Immunization of human mononuclear cells against sheep erythrocytes. Immunization of human mononuclear cells was assayed using the in vitro assay of human antibody-producing cells against sheep erythrocytes (SRBC) with costimulation by mitomycin-treated histoincompatible leukocytes as originally described by Fauci and Pratt (10, 11) and as exhaustively reported before (1). The effects of CEP-Si and of its most purified subfraction were tested by addition of different amounts at the beginning of the cultures. Direct plaque-forming cells were assessed using a slight modification of the method of Jerne and Nordin (12), as also described previously in detail (1).

In vivo assays of semipurified and purified fractions of CEP-Si. The methodology for in vivo assays was as described earlier (1). Briefly, C57BL/6 mice 6 wk old were injected with 5,000 U of MLC biological activity of each CEP-Si subfraction on days −2 and +2, i.e., 2 d before and after the administration of SRBC to the mice. The primary immune response to SRBC was assessed 5 d after immunization by means of the direct hemolytic plaque assay performed as above.

Preparative isoelectric focusing. Preparative isoelectric focusing of CEP-Si or its semipurified fractions was performed on an LKB 8100-1 column (LKB Instruments, Bromma, Sweden) of 110-ml capacity. In the case of CEP-Si, a pH gradient of 3.5–6 was prepared by mixing equal proportions of ampholytes (LKB 1809-111, pH 3.5–5; and LKB 1809-116, pH 4–6). A second preparative isoelectric focusing was subsequently performed to further separate the active constituents of semipurified CEP-Si fractions, using a pH gradient of 4–5 by means of adequate ampholytes (pH 4–5, Servalyt, Feinbiochemica, Heidelberg, West Germany). In all cases the final concentration of carrier ampholytes was 2% (wt/vol). Convection was avoided and the pH gradient stabilized by means of a vertical density gradient of sucrose (RNA-free, Polysciences, Inc., Warrington, Pa.) from 50 to 5% (wt/vol). The anode and the cathode solutions were 1.0 M phosphoric acid and 1.0 M sodium hydroxide, respectively. The samples were dialyzed overnight at 4°C against 1% glycine (wt/vol) and consisted of 3–6 ml of three different batches of CEP-Si and two batches of the semipurified fractions of CEP-Si, containing 1.3–5 and 0.06–0.2 mg/ml protein, respectively, as measured by the method of Lowry et al. (13). The run was performed at 4°C with an initial constant voltage of 1,600 V for 16 h, subsequently increased up to 1,800 V for another 4 h. Elution of the column was carried out at a flow rate of ~25 ml/h, and the eluate was collected in fractions of 1.5 ml. The pH values for each fraction were determined, and the fractions were pooled according to their optical density at 280 nm.

The ampholytes and sucrose were removed from the fractions by dialysis against a large volume of 1% glycine solution, followed by two steps of vacuum dialysis of the samples, which were diluted with 50 vol of minimal essential medium before each step. After vacuum dialysis, the CEP-Si fractions were reconstituted up to the same volume as the original samples of the unfractionated crude material.

Alternatively, preparative isoelectric focusing of CEP-Si was performed in layers of granulated gels according to Radola (14), using Sephadex G-75 (Ultrodex, LKB Instruments) also with ampholyte concentration of 2% (wt/vol) and a pH gradient of 3.5–6, prepared as described above. After the run the gel was cut, using a fractionating grid, and the fractions were eluted with phosphate buffer (2 gel vol per fraction), then dialyzed, and concentrated as above.

Treatment of F3'EP-Si with enzymes. Trypsin (Worthington Biochemical Corp., Freehold, N. J.), alpha, delta, and gamma chymotrypsin (Sigma Chemical Co., St. Louis, Mo.; type II, regular, and type II, respectively), neuraminidase (Perfringens, Cleveland, Ohio), deoxyribonuclease (DNase), and ribonuclease A (RNase, both from Sigma Chemical Co.) were used for enzymatic treatment of F3'EP-Si. All enzymes were used at concentrations equivalent to 1/50 of the amount of protein in the substrate. Enzymatic treatment was performed overnight at 37°C at pH 7.2, or at pH 5, in the case of neuraminidase.

Analysis of CEP-Si and its most purified active fraction (F3'EP-Si). Analytical isoelectric focusing was performed with a pH gradient of 4–6 on samples of unpurified CEP-Si and its most purified fraction, F3'EP-Si. For this purpose, samples of 10 μl of CEP-Si and F3'EP-Si (the latter concentrated four- or tenfold were applied to polyacrylamide gels, which were made of acrylamide, *N,N'*-methylenebisacrylamide (BDH Chemicals Ltd., Poole, England), and sucrose (Polysciences, Inc.) mixed so that the final parameters of the gel were T = 5% and C = 3% (15). Carrier ampholyte solution (LKB Instruments, 1809-116) was used at a final concentration of 2% (wt/vol) in a 0.8-mm thick gel, and the gel was polymerized with a final concentration of 1% ammonium persulfate (BDH Chemicals) (16, 17). The anode and cathode solutions were the same as those used for preparative purposes (see above). The run took 3 h, starting with a voltage of 300, which was gradually increased up to 800 V by means of a Camag power supply (Camag, Inc., New Berlin, Wis.) and LKB 2117 Multiphor equipment. Alternatively, a sample of the most purified fraction of CEP-Si, containing 50 μg protein, was applied on cellular acetate strips and cellulose acetate electrophoresis was performed according to the method of Kohn (18).

Both polyacrylamide gels and cellulose acetate strips were stained with Coomassie Brilliant Blue R (19), and the cellulose acetate strips were also stained with methylene blue (20). In some instances pH measurements were made on unstained acrylamide gels; in this event, gel slices of 10 × 5 mm were cut, the constituents were eluted with 1 ml distilled water, and the pH of the eluted solution was measured. Also in some cases the CEP-Si fraction containing the suppressor activity was electrophoretically eluted from the slices by the method of Thang et al. (21).

For additional analysis of F3'EP-Si, an aliquot of this

fraction containing 100 μg protein was applied on a small spot of a strip of Whatman 1 filter paper (Whatman, Inc., Clifton, N. J.), and the possible presence of glycoproteins was checked by periodic-acid Schiff stain. As positive and negative controls for the staining procedures, 25 μg dextran (Sigma Chemical Co.) or glycogen (Merck & Co., Rahway, N. J.) and 100 μg purified human albumin were used.

Molecular weight determination of the Coomassie-stainable material in F3'EP-Si. The molecular weight of the Coomassie-stainable material detected in F3'EP-Si was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Weber and Osborn (22), using SDS from Sigma Chemical Co. and a 5% polyacrylamide gel. The samples consisted of CEP-Si, F3'EP-Si 10 times concentrated, and 50 μg of protein and controls which consisted of purified human albumin, ovalbumin, and chymotrypsinogen (Sigma Chemical Co.; 66,000, 43,000, and 27,000 mol wt, respectively), treated with 1% (vol/wt) SDS and mixed with Bromphenol blue, and layered over the gels.

To determine whether the biological activity of CEP-Si was related to the Coomassie-stainable material detected in F3'EP-Si, the mobilities of both were compared with the mobilities of the same proteins used as controls on SDS-PAGE, in Davis and Ornstein's classical PAGE (23, 24) performed with 5% and 7% polyacrylamide gels with and without a pre-run of 2 h (to remove excess *N,N,N',N,*-tetramethylenediamine [TEMED]). After electrophoresis of either F3'EP-Si or CEP-Si, the gels were cut longitudinally in two halves, one of which was stained with Coomassie Blue and the other sliced. The constituents of the slices were eluted by electrophoresis as above, by the method of Thang et al. (21), and then tested for their effects on [^{3}H]thymidine uptake by PHA-stimulated cultures of human peripheral blood mononuclear cells. The relative mobility of both the Coomassie-stainable material and the biological activity of F3'EP-Si in the 5 and 7.5% gels, as compared with controls, allowed the determination of its molecular weight by the method of Thorum and Maurer (25).

RESULTS

Fractionation of CEP-Si by preparative isoelectric focusing. Seven main fractions of CEP-Si (FI-FVII) were obtained by preparative isoelectric focusing (Fig. 1). Suppressor activity for PHA- and MLC-stimulated cultures was found in FV and primarily in FVI, which had isoelectric points of 4.52-4.68 and 4.70-5.25, respectively. FV significantly inhibited [^{3}H]thymidine uptake by PHA- and MLC-stimulated cultures of human peripheral blood mononuclear cells up to a final concentration of 1/400 for PHA (43% of the control response) and 1/800 for MLC-stimulated cultures (35%). FVI suppressed PHA-stimulated cultures up to a final concentration of 1/6,400 (30%) and MLC up to 1/9,600 (15%). Additionally FIII, with isoelectric points between 4.11 and 4.41, suppressed MLC-stimulated cultures but only up to a final concentration of 1/40 (15% of the control response) and was unable to suppress PHA-stimulated cultures. The coefficients of variation for all these cultures ranged between 5 and 15%.

The combined FV and FVI [F(V + VI)EP-Si] represented roughly 6% of the total amount of CEP-Si in terms of protein content, i.e., F(V + VI), reconstituted to the volume of the original material contained 90 μg/ml protein, compared with 1,490 μg/ml in the original batch of CEP-Si. F(V + VI)EP-Si was further fractionated into five subfractions, F1'-F5', by preparative isoelectric focusing with ampholytes between pH 4 and 5 (Fig. 2). Interestingly, with the exception of F4' which had no detectable protein, all

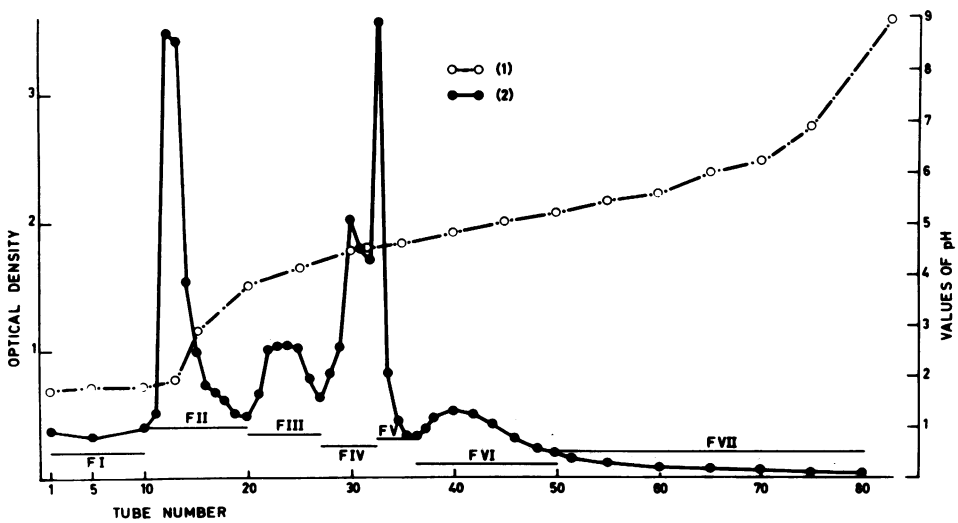

FIGURE 1 Diagram of the fractionation of CEP-Si by preparative isoelectric focusing in a sucrose gradient with an ampholyte range of pH 3.5–6. The fractions of CEP-Si (FI/FVII) were pooled as shown. The optical density measured at 280 nm (●) and the pH values of the eluted fractions (○) are indicated.

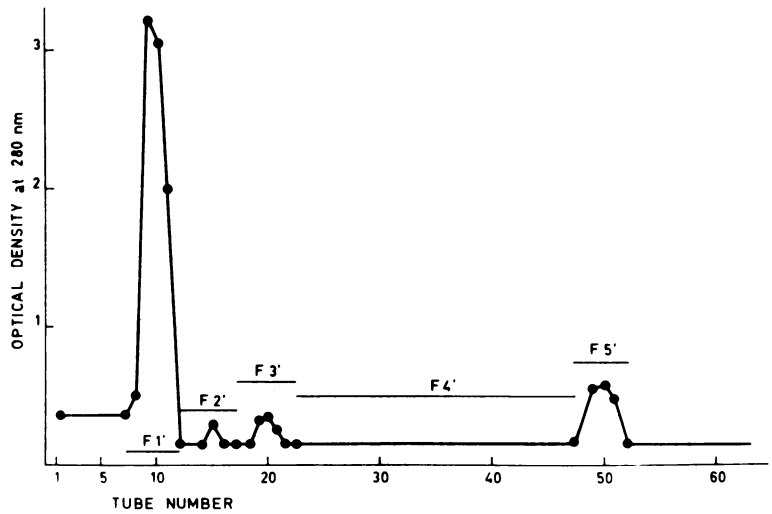

FIGURE 2 Diagram of the fractionation of F(V + VI)EP-Si by preparative isoelectric focusing in a sucrose gradient with an ampholyte range of pH 4–5. The fractions (F1'–F5') were pooled as shown. The optical density at 280 nm (●) is indicated.

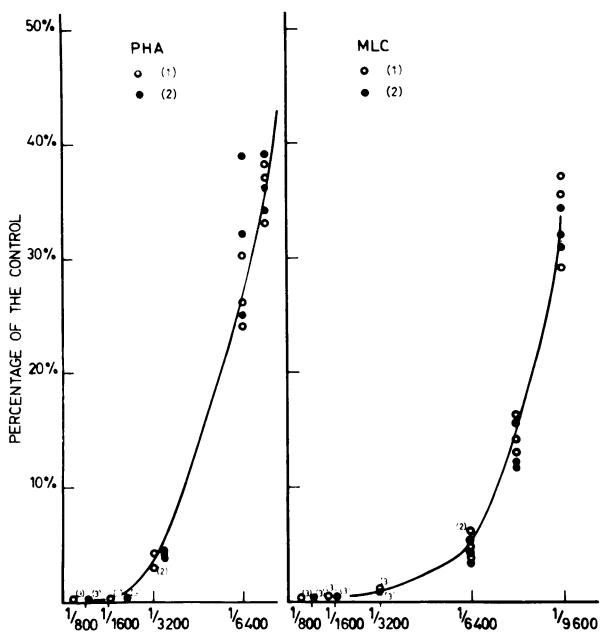

FIGURE 3 Estimated effects of F3'EP-Si on PHA- and MLC-stimulated cultures of human peripheral blood mononuclear cells, and comparison with the results obtained for the original CEP-Si. Results are expressed as the relationship between the final dilutions of F3'EP-Si or CEP-Si in the tissue cultures and the percentage of [^{3}H]thymidine uptake vs. control cultures. The curves for the effects of F3'EP-Si were calculated on the basis of four different dilutions, corresponding to three experimental points (○). The regression equations for the curves drawn for the effects of F3'EP-Si on PHA- and MLC-stimulated cultures were, respectively, $y = 0.02059 - 0.02317x + 0.00681x^2$ and $y = -0.00081 + 0.00665x - 0.00248x^2 + 0.00034x^3$, with $P < 0.001$ in both cases. In both equations, x = 1 represents a dilution of 1/800, x = 4 a dilution of 1/3,200, etc. Also shown are the results obtained with the same dilutions of CEP-Si (●). Part of the curve for MLC responses has been omitted for clarity.

the remaining fractions had isoelectric points below or above that of F(V + VI)EP-Si, which was 4.5-5.25. The immune suppressor activity was found in F3'. This fraction contained 15 μg/ml protein, or ~1% of the protein concentration in the original CEP-Si preparation. It was designated F3' of extracellular products of *S. intermedius*, (F3'EP-Si) and contained roughly 8,400 and 11,200 U/ml of PHA and MLC biological activity, respectively (Fig. 3), which means that 1 ng of protein of F3'EP-Si was equivalent to 0.56 and to 0.75 U of PHA and MLC biological activity, respectively.

The equations representing the mathematical estimations of the suppressive effects of F3'EP-Si and CEP-Si on both PHA- and MLC-stimulated cultures fit perfectly (Fig. 3). Moreover, in both cases the values of [^{3}H]thymidine uptake by mononuclear cells stimulated with PHA and supplemented with inhibitor (CEP-Si or F3'EP-Si) initially followed control values, reached a maximum, dropped abruptly, and started to increase again later on. Also in both cases, the abrupt inhibition and the later proliferation of the mononuclear cells occurred sooner when more biological units of the preparation were added to the cultures. The suppressive effects of both preparations increased in proportion to the time of contact with the target cells, and their effects on the suppression of human anti-SRBC plaque formation in vitro were similar (Fig. 4). Moreover, the suppressor effects of F3'EP-Si,

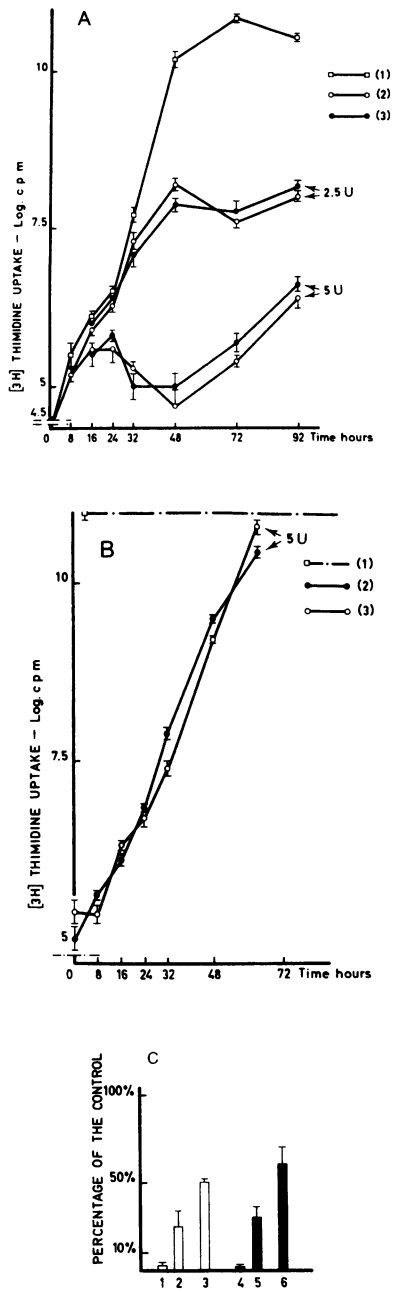

FIGURE 4 Comparison between the in vitro effects of F3'EP-Si and CEP-Si. (A) [^{3}H]Thymidine uptake at different times in PHA-stimulated cultures of human peripheral blood mononuclear cells, either without F3'EP-Si or CEP-Si or supplemented at the beginning of the culture with 2.5 or 5 U of PHA biological activity of either F3'EP-Si or CEP-Si (B) [^{3}H]Thymidine uptake after 3 d in PHA-stimulated cultures of human peripheral blood mononuclear cells, either without F3'EP-Si or CEP-Si or supplemented with 5 U of either F3'EP-Si or CEP-Si added at different times of culture (C) Comparison of the number of human hemolytic plaque-forming cells generated in vitro against SRBC in cultures treated with 8, 4, or 2 U of MLC biological activity of either F3'EP-Si (1, 2, and 3, respectively) or CEP-Si (4, 5, and 6, respectively).

as in the case of CEP-Si, were more evident on MLC- than on PHA-stimulated cells and more on highly stimulated cells than on poorly stimulated ones (Fig. 3, Table I). Finally, the in vivo suppressor effects of F3'EP-Si were also demonstrated. C57BL/6 mice injected with 5,000 U of MLC biological activity of two different batches of F3'EP-Si 2 d before immunization with SRBC produced an average of 25±15 direct plaque-forming cells per 10^6 cells, compared with 980±310 for control mice immunized, but not treated with F3'EP-Si.

Preparative isoelectric focusing with ampholyte range as above (pH 3.5-6) was performed on four different batches of CEP-Si on different occasions. Two active fractions with suppressor activity were always obtained provided the separations were performed using either a sucrose gradient or polyacrylamide gel as support. On two occasions Sephadex G-75 was used as support, and the CEP-Si suppressor activity was not recovered in any of the eluted fractions. As reported previously (1), the CEP-Si fraction able to suppress the response to PHA and to specific antigens (either MLC or purified protein derivative of tuber-

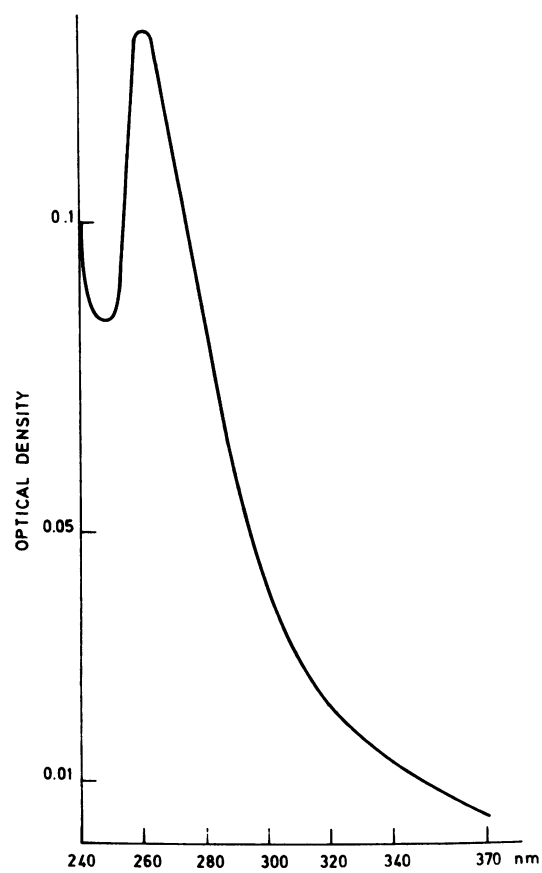

FIGURE 5 Absorption spectrum of F3'EP-Si measured between 240 and 370 nm.

TABLE I

Comparison of the Effects of CEP-Si and of its Subfraction F3'EP-Si on the Inhibition of [³H]Thymidine Uptake of Nonstimulated Cultures (Nil) and PHA- or MLC-stimulated cultures

CEP-Si or F3'EP-Si concentration	[³H]Thymidine uptake					
	CEP/Si			F3'EP-Si		
	PHA	MLC	Nil	PHA	MLC	Nil
	mean cpm ± SD					
0 (control)	35,821±1,889	33,571±1,169	831±116	35,821±1,889	33,571±1,169	831±116
1/200	46±5 (0.12%)*	53±6 (0.15%)	37±12 (5%)	ND	ND	50±9 (6%)
1/400	42±6 (0.11%)	54±23 (0.15%)	125±25 (15%)	56±11 (0.15%)	64±19 (0.19%)	91±6 (11%)
1/800	52±8 (0.14%)	64±7 (0.19%)	166±17 (20%)	75±7 (0.21%)	134±11 (0.4%)	149±15 (18%)
1/1,600	68±6 (0.2%)	90±13 (0.27%)	165±53 (20%)	107±15 (0.3%)	171±15 (0.5%)	191±31 (23%)
1/3,200	1,148±292 (3%)	200±25 (0.6%)	ND	1,074±111 (3%)	235±11 (0.7%)	232±9 (28%)
1/6,400	10,683±334 (30%)	1,982±12 (6%)	218±15 (28%)	9,393±396 (26%)	2,014±181 (6%)	266±35 (32%)

* Numbers in parentheses indicate mean of the results of triplicates of a typical experiment, expressed as percentage of the controls. In at least two additional experiments with different donors, but with the same stimulant, the percentages of controls showed a similar degree of similarity of effects between CEP-Si and F3'EP-Si.

culin was found to have an isoelectric point of 4.5-5, whereas the fraction able to suppress only antigen-stimulated cultures (MLC or purified protein derivative of tuberculin) had a 3.5-4 PI. Only in vivo suppressor activity was found in the first fraction (4.5-5), and the amount of this activity varied considerably from batch to batch of CEP-Si. The antigen-specific suppressor effects were also always greater in this fraction than in the second fraction (3.5-4).

Biochemical analysis of F3'EP-Si. The absorption spectrum of F3'EP-Si, read between 240 and 370 nm, indicated maximum optical density at 260 nm (Fig. 5). As shown in Fig. 6A, F3'EP-Si was quite sensitive to the proteolytic activity of alpha, delta, and gamma chymotrypsin (in the order alpha > gamma > delta) and somewhat less sensitive to the action of trypsin. It was resistant to neuroaminidase, DNase, and RNase but extremely sensitive to heating. Its activity was reduced after incubation at 37°C for 24 h, almost destroyed by incubation for 1 h at 56°C, and totally destroyed by incubation at 70°C for 1 h (Fig. 6B). F3'EP-Si contained only one Coomassie-stainable protein band detectable by isoelectric focusing, with a 4.25 PI (Fig. 7A). This band remained at the starting point after electrophoresis on cellulose acetate strips. No material stainable by methylene blue or periodic-acid Schiff was detected in F3'EP-Si.

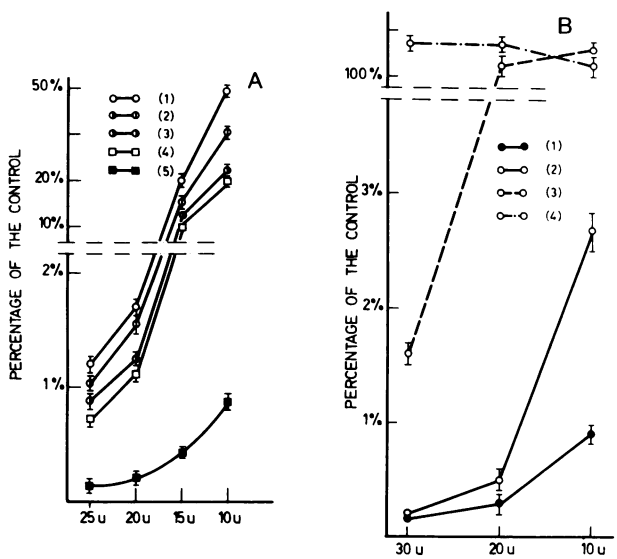

FIGURE 6 Effects of proteolytic enzymes and temperature on the biological activity (expressed as percentage of control values) of F3'EP-Si on PHA-stimulated cultures. (A) Suppressor effects of 25, 20, 15, and 10 U of PHA biological activity of F3'EP-Si, either untreated (1) or treated with alpha chymotrypsin (2), delta chymotrypsin (3), gamma chymotrypsin (4), or trypsin (5). (B) Effects of 30, 20, and 10 U of PHA biological activity of F3'EP-Si, either untreated (1) or previously incubated at 37°C for 24 h (2), 57°C for 1 h (3), or 70°C for 1 h (4).

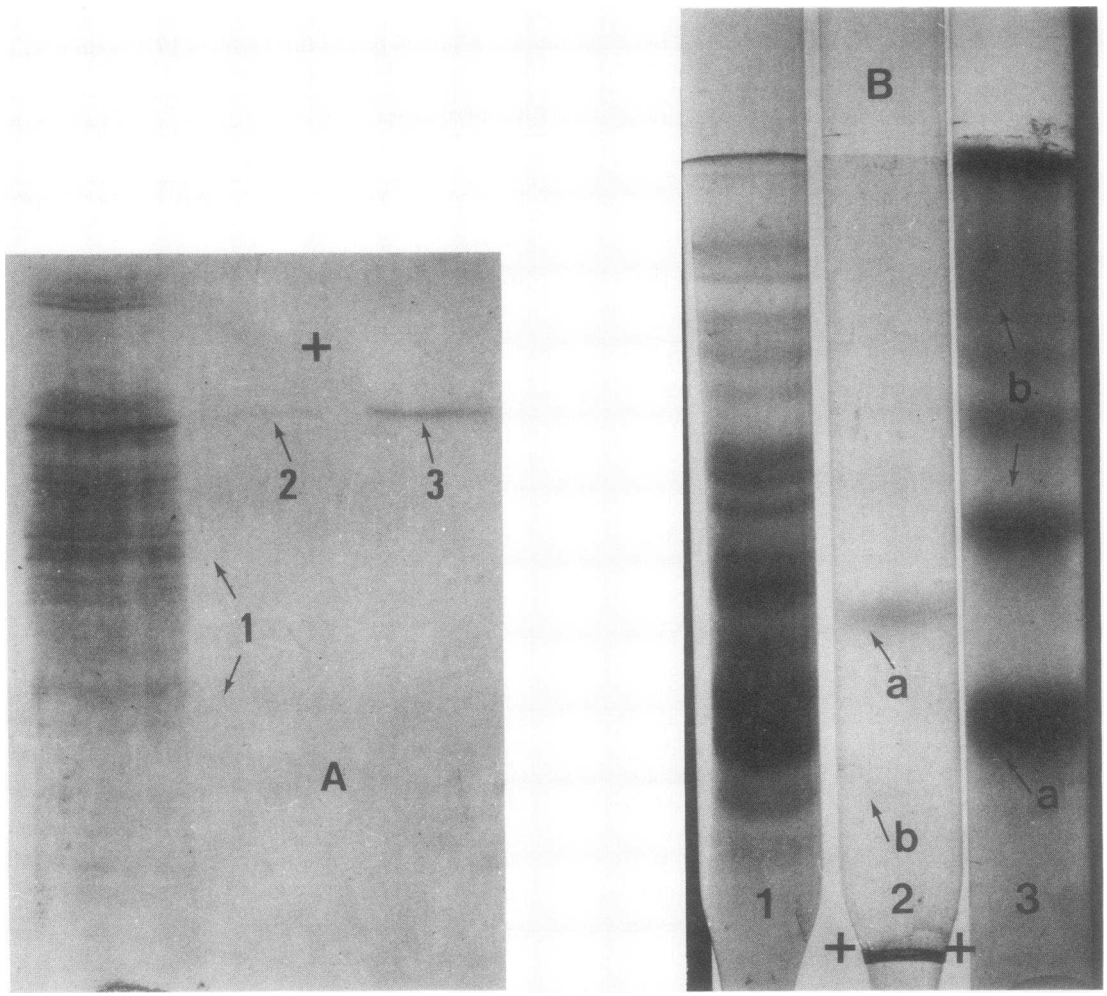

FIGURE 7 Analytical comparison between CEP-Si and F3'EP-Si. (A) Isoelectric focusing with pH gradient of 4–6 of (1) CEP-Si and of (2) F3'EP-Si four and (3) 10 times more concentrated than the original CEP-Si. (B) SDS-PAGE of (1), CEP-Si (2) F3'EP/Si 10 times concentrated on which a is the active fraction and b the inactive fraction of lower molecular weight and controls of known molecular weight (3), which are monomer (a) and polymers (b) of human serum albumin. The anodal portion of both A and B preparations is marked with +.

Molecular weight of the Coomassie-stainable material in F3'EP-Si and results of polyacrylamide disc electrophoresis of F3'EP-Si and CEP-Si. The molecular weight of the Coomassie-stainable material in F3'EP-Si detected by SDS-PAGE was calculated at 80,000 (Fig. 7B). On the other hand, calculations based on the relative mobility in 5 and 7.5% polyacrylamide gels of both the Coomassie-stainable material and its biological activity indicated a 95,000 mol wt. It must be noted, however, that unlike the case of isoelectric focusing, a rather smaller second band of nonactive material of 39,000 mol wt was also detected after a first step of SDS-PAGE using high amounts of F3'EP-Si, i.e., 10 times more concentrated than the original CEP-Si. This band represents most likely, substances with the same isoelectric point as the active product but with different molecular weight as it can be observed in Fig. 7. Unless a prerun of at least 2 h was performed to eliminate excess TEMED, no biological activity was eluted from the gels with either CEP-Si or F3'EP-Si; similarly no Coomassie-stainable material was detectable in F3'EP-Si analyzed on the same gels.

Interestingly, after 7.5% PAGE performed without excess TEMED, the in vitro suppressor activity for the PHA response was distributed from the top down to two-thirds of the total gel, eluting mainly from the first one-sixth (from the top) and progressively less from the lower parts of the gel. When these eluates were pooled together and run again, most of the

biological activity was contained in the eluates corresponding to the fourth one-sixth of the gel. Some activity was also found in the fifth one-sixth of the gel but was detectable only at concentrations as high as 1/40, unlike the eluate of the fourth one-sixth of the gel, which was active up to the dilution of 1/160.

DISCUSSION

The present report confirms our preliminary finding (1) that CEP-Si can be fractionated by isoelectric focusing on an appropriate support. F3′EP-Si, the most purified fraction of CEP-Si containing the immunosuppressor activity and representing only ~1% of the protein in CEP-Si, was as powerful, both in vivo and in vitro as the original preparation and, still more important, their effects correlated excellently, indicating that all the original activity can be recovered in one single fraction by means of two steps of isoelectric focusing. It should be noted, however, that more significant amounts of biological activity can be eluted in FIII than in the present case. In that event, the activity of F3′EP-Si that is obtained exclusively from FV + VI will be obviously reduced. When experimental results, as in the present report, indicate decrease in [^{3}H]thymidine uptake, the possibility of artefact due to the presence of high concentrations of cold thymidine in the cultures must be considered (6, 27). This possibility was exhaustively excluded as a viable explanation for the in vitro effects of CEP-Si (1) and for similar reasons can also be excluded in the case of F3′EP-Si. In addition, the biological effects of F3′EP-Si were reduced after treatment with proteolytic enzymes and abolished by heating at 70°C.

Interestingly, F3′EP-Si was eluted at pH 4.2–4.3 out of F(V + VI)EP-Si, which consistently showed an isoelectric point between pH 4.5 and 5, and all the other protein-containing subfractions of F(V + VI)EP-Si were eluted either below or above the pH values of 4.5 and 5. The most likely explanation for these observations is the "sticky" nature of the biologically active component of CEP-Si. Indeed, the biological activity of CEP-Si was not recovered when preparative isoelectric focusing was performed with Sephadex G-75 as support. The active substance adhered to cellulose acetate strips, as F3′EP-Si remained at the starting point after electrophoresis, and did not penetrate into 5% acrylamide gels when TEMED was used or when the excess of this catalyzer was not removed from the gels by a preliminary electrophoretic run. It is likely that the suppressor component of CEP-Si also binds to other nonactive contaminant substances in the crude preparation, forming complexes that are dissociated by electrophoresis and have electric charges that are the combination of those exhibited by the different components. This would explain why two discontinuous fractions of CEP-Si with biological activity were consistently obtained after the first step of isoelectric focusing, as well as the observation that when CEP-Si was first submitted to polyacrylamide gel electrophoresis, the suppressor activity was eluted from a wide region of the polyacrylamide gel, and when the eluate was pooled and submitted to further electrophoresis, the activity was found in a narrower and more anodal region. This latter observation suggests, indeed, that an original complex containing the suppressor molecule was dissociated by electrophoresis, and thus this molecule after the second run exhibits either a different electric charge, or lower molecular weight, or both.

The absorption maximum of F3′EP-Si was at 260 nm, suggesting the presence of nucleotides, but its activity was resistant to DNase and RNase and it was not stainable by methylene blue. Also, the involvement of polysaccharides in the suppressor molecule was not demonstrated as the activity was resistant to neuraminidase and there was no material stainable by periodic-acid Schiff in the active fraction. We tentatively conclude that the suppressor effects of CEP-Si are due to a protein material, since it was sensitive to chymotrypsin, trypsin, and heating and since the most purified fraction contained Coomassie-stainable material. This fraction focused as a single band on SDS-PAGE and on analytical isoelectric focusing. Phenylalanine is an aromatic amino acid that sticks to Sephadex and cellulose with adhesive properties dependent on electrostatic conditions (28–30), and has an absorbance maximum at 260 nm. Therefore, we assume that the immunosuppressor factor is a protein of roughly 90,000 mol wt, probably with phenylalanine as one of its major constituents and that a unit of PHA biological activity can be equivalent to an amount of this protein as low as 2 ng.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Thomas Higerd for supplying CEP-Si preparations. We thank Dr. Moradas Ferreira for stimulating discussions on the biochemical nature of the immunosuppressor substance, and Charles L. Smith for excellent editorial assistance.

Research supported by the Portuguese Government, in part by the "Junta Nacional de Investigacao Cientifica e Tecnologica," Portugal, and by U. S. Public Health Service grants HD-09938 and CA-25746.

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