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IN-VITRO EVALUATION IN MAN OF IMMUNO-STIMULATION BY SUBFRACTIONS OF *ACTINOMYCES VISCOSUS*

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Summary—*Actinomyces viscosus* was fractionated into cell wall (PEL) and intracellular supernatant (SUP) fractions following ultrasonication. *In vitro* lymphocyte transformation induced by each fraction was assessed using the lymphocytes obtained from subjects with minimal periodontal disease. In kinetic experiments, a detectable blastogenic response to both fractions was measured by the fourth day, with a peak at day 7. Nuclease treatment enhanced the immunostimulatory activity of the PEL fraction 5.5-fold over untreated PEL; it had no effect on the activity of SUP. Although pronase treatment had no effect on the PEL fractions, it abrogated the ability of SUP to activate lymphocytes. Fractions of nuclease-treated and untreated SUP were batch-eluted from DEAE-sepharose columns with increasing concentrations of NaCl, producing 3 major stimulatory subfractions which accounted for 75 per cent of the activity of unfractionated SUP. Comparisons of lymphocyte responses to the DEAE-fractionated SUP indicated that not all individuals responded equally to each subfraction. As there was a differential responsiveness to the various antigenic components, detailed evaluation of the antigens of *A. viscosus* is warranted to define differences in antigen responsiveness in individuals with differing severities of periodontal disease.

INTRODUCTION

Periodontal disease research supports the hypothesis that immune mechanisms may be associated with the pathogenesis of the disease (Ivanyi and Lehner, 1971; Ivanyi, Wilton and Lehner, 1972; Baker *et al.*, 1976). These findings indicate sensitization of immunological mechanisms following contact between oral micro-organisms in the dental plaque and the gingiva. *Actinomyces viscosus* induces significant *in vitro* lymphocyte blastogenesis in periodontally diseased individuals (Baker *et al.*, 1976; Ivanyi and Lehner, 1971), stimulates neutrophil degranulation (Taichman and McArthur, 1976), is associated with gingival inflammation in healthy volunteers refraining from oral hygiene for periods of several weeks (Syed and Loesche, 1978; Loesche and Syed, 1978) and causes periodontal disease in experimental animals (Guggenheim and Schroeder, 1974; Jordan, Keyes and Bellack, 1972).

Antigenic preparations of *A. viscosus*, as well as preparations of other oral microbes used to assess *in vitro* lymphocyte reactivity, have been most commonly prepared from the soluble supernatant of ultrasonicates of the microbes. These preparations, while highly stimulatory, are mostly protein (Reed *et al.*, 1976) and contain little or none of the carbohydrate shown to be significant in the antigenicity of the cell wall (Bowden, Hardie and Fillery, 1976). Studies in our laboratory indicate that cell-wall fractions markedly stimulate peripheral blood lymphocytes obtained from healthy volunteers, as well as periodontitis.

To further characterize the nature of *A. viscosus* antigen, we fractionated supernatants obtained from *A. viscosus* cell suspension after ultrasonication.

MATERIALS AND METHODS

Preparation of Actinomyces viscosus *antigens*

(i) *Cultural conditions.* An isolate of *Actinomyces viscosus* strain GA, a catalase-positive, filamentous rod, characterized according to criteria described by Holmberg and Hallander (1973), was obtained from a naturally-occurring gingivitis site and cultivated in a broth composed of: tryptone, 10 g; yeast extract, 5 g; glucose, 5 g; sucrose, 1 g; sodium chloride, 5 g; potassium nitrate, 1 g; mineral salt solutions 1 and 2 (Loesche, Hockett and Syed, 1972), 75 ml each; menadione (0.1 per cent), 1 ml; sodium carbonate, 0.45 g; dithiothreitol, 0.2 g; haemin (0.1 per cent), 1 ml; distilled H_2O, 850 ml. Screw-cap flasks containing 500 ml of pre-reduced broth were inoculated with agar-grown cultures and incubated at 37°C inside an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.). The gaseous environment of the chamber was 5 per cent hydrogen, 10 per cent carbon dioxide and 85 per cent nitrogen. The cultures were incubated for 60–72 h and harvested by centrifugation at 12,000 ***g*** for 30 min. The cell pellets were extensively washed in sterile phosphate-buffered saline (PBS, 0.05 M PO_4, 0.15 M NaCl, pH 7.4), resuspended in sterile distilled water and lyophilized. The cell harvest was approximately 100 mg dry weight per 1000 ml culture medium.

(ii) *Ultrasonication.* Washed cells, resuspended to 20 mg/ml in distilled water, were quick-frozen and thawed 3 times in an ethanol–dry-ice bath and subjected to a total of 40 min of ultrasonic disruption (Heat Systems-Ultrasonics, Inc., Model W185D, 85 watts) delivered at 5-min intervals with alternating periods of cooling in an ice bath. The temperature of

the cell suspension was monitored after each sonication cycle and did not exceed 15°C. Cell disruption, evaluated by Gram-staining, phase-contrast microscopy and transmission electron microscopy, was greater than 95 per cent. Following disruption, the sonicate was centrifuged at 12,000 **g** for 30 min. The supernatant (SUP) fraction was removed and the disrupted cell wall material (PEL) was recovered by 6 cycles of differential centrifugation. The cell wall/whole cell mixture was centrifuged at low speed (800 **g** for 5 min) followed by a high-speed centrifugation of the supernatant (12,000 **g** for 30 min) to pellet the cell-wall fragments. This was repeated 6 times and followed by a final resuspension of the cell walls in PBS (0.05 M PO_4, 0.15 M NaCl, pH 7.4).

(iii) *Enzymic digestion.* The PEL preparations (50 mg in 5 ml tris–HCl, 0.05 M, pH 8.0; 0.5 ml 0.1 M $MgCl_2$) were treated with 5 mg ribonuclease A (Sigma Chemical Co., type I-A, 71 μ/mg), and 5 mg deoxyribonuclease I (Aldrich Chemical Co., 1651 μ/mg), for 16 h at 37°C. The nuclease-treated cell walls were centrifuged at 12,000 **g**, washed and resuspended in the tris–HCl buffer, and digested with pronase (50 mg, K + K Laboratories, Inc.) for 16 h at 37°C.

Ultrasonicate supernatants were also treated with nucleases and pronase. However, because of difficulties associated with their removal from completed digestions, soluble enzymes were linked to sepharose 4B (Pharmacia Fine Chemicals) activated with cyanogen bromide by the method of Cuatrecasas (1970). Enzymic activity of the washed beads was determined and ultrasonicate supernatants were then treated. Following digestion, beads were removed by low-speed centrifugation and supernatants were dialysed against distilled water at 4°C and lyophilized. To determine if the enzymes were leaching from the beads, mock incubations in PBS were carried out and processed as described above. These preparations were also tested in lymphocyte culture in dilutions equivalent to those used in the actual experiment. In addition, unconjugated, as well as heat-inactivated, enzyme-conjugated sepharose beads were incubated with antigen preparations to determine if an active component was being absorbed from the reaction mixture.

(iv) *DEAE-sepharose chromatography.* Fractionation of *A. viscosus* supernatants was performed on DEAE-sepharose CL (Pharmacia Fine Chemicals) ion-exchange columns (16 × 1.5 cm, bed volume). SUP (500 mg) was dissolved in 10 ml 0.10 M tris–HCl, pH 8.0 and applied to the column equilibrated with the same buffer. The columns were batch-eluted with increasing concentrations of NaCl (0.1–1.0 M) in tris–HCl at a flow rate of 90 ml/h. Fractions (50 drops/tube) were monitored at 280 nm. Appropriate fractions were pooled and dialysed against distilled water at 4°C to remove all salt and lyophilized.

(v) *Chemical analysis.* All PEL and SUP fractions were analysed for protein (Lowry *et al.*, 1951) using bovine serum albumin as a standard, for total hexose (Roe, 1955) using glucose as a standard, for methylpentose (Spiro, 1966) using fucose as a standard and for RNA (Dische, 1953) using ribose nucleic acid (Nutritional Biochemical Corp.) as a standard.

In vitro *lymphocyte blastogenesis*

(i) *Experimental subjects.* Systemically healthy volunteer subjects, aged 21–40 years, were evaluated for periodontal health using a modification of the Periodontal Disease Index (PDI) (Ramfjord, 1959; 1967). The average scores and ranges for each subject were found to be: average gingivitis score, 0.35 (0.05–0.56); average pocket depth, 2.22 mm (2.0–2.5); average loss of attachment, 0.25 mm (0.0–0.6).

(ii) *Lymphocyte preparation.* Mononuclear leukocytes were isolated from heparinized venous blood, drawn from the volunteers. Briefly, the buffy coat layer from whole blood was centrifuged at 350 **g** for 30 min, diluted in sterile PBS and layered on Ficoll-Hypaque (Boyum, 1968) (Ficoll-Paque, Pharmacia Fine Chemicals, Piscataway, N.J.). The tubes were centrifuged at 500 **g** for 30 min at 23°C. The cells banded at the interface were aspirated, washed 3 times in PBS and resuspended to a final concentration of 1 × 10^6 cells/ml in RPMI 1640 culture medium (Grand Island Biological Co., Grand Island, N.Y.). The cells were then supplemented with 40 mM hepes buffer, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin.

(iii) *Microculture.* Lymphocyte blastogenesis was assessed in a microtitre plate system containing 1 × 10^5 mononuclear leukocytes in a final volume of 0.20 ml RPMI 1640 medium containing 10 per cent autologous plasma. Dilutions of PEL and SUP fractions were added to each well and incubated for 168 h. During the last 16 h of culture, 2 μCi [methyl-^{3}H]-thymidine (Amersham/Searle, TRA 120, 5 Ci/mmol) was added to each well. All cultures were harvested with a multiple-automated sample harvester (MASH II, Microbiological Associates) onto

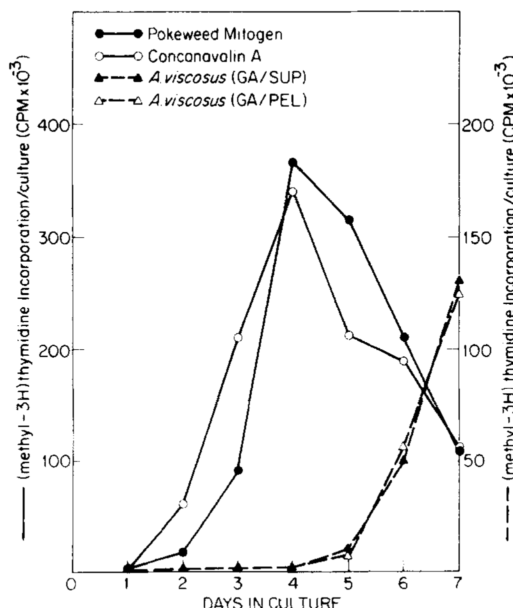

Fig. 1. Kinetics of blastogenic response to pokeweed mitogen, Concanavalin A, *A. viscosus* PEL, and *A. viscosus* SUP, by lymphocytes of one subject. 1 × 10^5 lymphocytes were cultured with stimulants for 1–7 days. Six hours before harvest [methyl-^{3}H]thymidine was added to each culture. Counts/min shown occurred at optimal concentration of subfractions tested between 1 and 1000 μg/ml. Unstimulated [methyl-^{3}H]thymidine incorporation was subtracted at each time point.

glass fibre filters. The filters were dried, placed in vials (Bio-Vial, Beckman Inst. Co.), filled with 3 cc scintillation cocktail (OCS, Amersham/Searle), and counted in a Packard Model 3320 liquid scintillation spectrometer.

RESULTS

Kinetics of the blastogenic response to A. viscosus *antigen*

As the kinetic response of human lymphocytes to mitogens and antigens differs, i.e., peaking at 3 and 5 to 7 days, respectively (Oppenheim and Schechter, 1976), the kinetics of response to the two main subfractions, PEL and SUP, were evaluated as a preliminary characterization. The experiment was carried out with a [^{3}H]-thymidine labelling period of 6 h, rather than 16 h, to permit greater resolution of intervals. The blastogenic response to both fractions was identical (Fig. 1): detectable by days 4–5, maximum at day 7, and beginning to subside by day 8 (not shown). The responses to the mitogens (pokeweed mitogen and Concanavalin A), however, were detectable by day 2, reaching a maximum by day 4. Throughout this period, the unstimulated baseline incorporation remained between 80 counts/min at the start of the culture, and 1400 counts/min at its conclusion. The lower baseline incorporation and maximum response is mainly a function of a reduced labelling period.

Enzymic treatment of A. viscosus *subfractions*

Commonly employed lymphocyte blastogenic assays use radioactive nucleic acid precursors to measure the rate of DNA synthesis as a function of lymphocyte stimulation. As our preparations of *A. viscosus* probably contained significant amounts of DNA and RNA, bacterial nucleic acids may have diluted the radioactive thymidine label. To assess this, PEL and SUP fractions were treated with ribonuclease and deoxyribonuclease, followed by extensive washing or dialysis to remove potential nucleic acid contamination of cultures. As the SUP preparations contained high levels of protein, aliquots of the nuclease-treated fractions were also treated with

Table 1. Biochemical analysis of *A. viscosus* ultrasonicate fractions*

Fraction†	Protein	Hexose	Methyl-pentose	Total nucleic acids
Whole-sonicate	584	96.5	212	430
PEL	328	106.6	772	56
PEL–N	420	225.0	536	58
PEL–N/P	166	148.0	388	48
SUP	1099	54.0	70	300
SUP–N	430	52.0	45	<20
SUP–N/P	295	51.1	40	<20

* μg/mg dry weight. Because of limitations of standards utilized, the sum of assayed components may exceed 100 per cent.

† PEL, washed insoluble cell-wall material; SUP, soluble material; PEL–N, nuclease-treated PEL; PEL–N/P, nuclease/protease-treated PEL.

Table 2. Effect of nuclease and pronase treatment of *A. viscosus* cell wall and supernate fractions on *in vitro* lymphocyte blastogenesis

	Maximum [methyl-^{3}H]thymidine incorporation (CPM)*						
	Cell walls†			Supernate‡			
Subjects	Untreated	Nuclease¶ treated	Nuclease-‖ pronase treated	Untreated	Nuclease treated	Nuclease-pronase treated	Baseline§
1	3829	43,494	41,382	9971	8723	1042	518
2	3302	37,504	39,270	18,931	19,483	948	1030
3	3907	12,303	11,483	10,796	9625	1232	1090
4	8872	16,454	17,127	13,659	14,807	1426	1113
Average	4977	27,439	27,315	13,339	13,159	1162	938

* 1 × 10^5 lymphocytes cultured with dilutions of *A. viscosus* subfractions for 7 days. Sixteen hours before harvest, 2 μCi [methyl-^{3}H]thymidine added to each culture. Max CPM represents maximum CPM incorporation at optimal antigen concentration (100 μg/ml, dry weight).

† Insoluble fraction obtained following ultrasonication of whole *A. viscosus* cells.

‡ Soluble fraction obtained following ultrasonication of whole *A. viscosus* cells.

§ CPM of unstimulated lymphocyte culture at 7 days.

¶ RNAse and DNAse.

‖ RNAse, DNAse, and pronase.

pronase to determine if the antigen was protein in nature. The untreated SUP fraction contained significant levels of protein with trace amounts of hexose and methyl-pentose (Table 1). Nucleic acids represented 30 per cent of the total dry weight of the SUP. Nuclease and pronase treatments of SUP significantly diminished both the protein and nucleic acid content of the preparations. The untreated PEL fraction contained significantly less protein and nucleic acids than did the SUP; however, it contained significantly more methyl-pentose (~ 77 per cent by dry weight). Treatment with enzymes had little effect on measurable nucleic acid, but it reduced the protein content by approximately 50 per cent.

Immunostimulatory activity of these fractions was evaluated by *in vitro* lymphocyte blastogenic assay using the lymphocytes of 4 healthy individuals (Table 2). Untreated PEL produced a 4 to 9-fold maximum stimulation over unstimulated controls (4977 counts/min vs a baseline of 938 counts/min). Nuclease treatment enhanced the antigenicity of the PEL fraction with an increase of 27,438 counts/min over the baseline response, or about a 5.5-fold increase over that of the non-nuclease-treated fraction. Subsequent pronase treatment had no effect on the PEL fraction. The average maximum blastogenic response induced by the SUP fraction in the 4 subjects averaged 13,339 counts/min over the baseline response. Although treatment with nuclease alone had no effect on the immunostimulatory activity of the SUP fraction, a combined treatment of nuclease and pronase reduced stimulation to baseline levels. Controls for enzyme leaching from beads or absorption of active stimulatory components were negative and indicated that all treatment effects resulted from enzymic activity.

Similar comparisons in a total group of 20 additional individuals gave identical results (not shown). Variations between individuals occurred only in the magnitude of the blastogenic response to untreated preparations, not in relative changes after enzyme treatment.

Subfractionation of SUP antigens

DEAE-sepharose chromatography of the *A. viscosus* SUP yielded 8 major fractions (i.e. A–H) as shown in Fig. 2 (upper panel). Fractions A–E were eluted with the starting buffer (0.1 M tris–HCl, pH 8.0); F with 0.2 M NaCl; G with 0.4 M NaCl; and H with 0.6 M NaCl. No additional material could be eluted at concentrations up to 2.0 M NaCl. Similarly nuclease-treated SUP was fractionated on the same column after regeneration, resulting in the profile shown in Fig. 2 (lower panel). Fractions B–D were pooled because of the lack of significant resolution between them. All fractions were dialysed against distilled water, lyophilized and subjected to biochemical analysis for protein, hexose, methyl-pentose, and nucleic acids (Table 3). Following dialysis of the nuclease-digested SUP, approximately 20 per cent of the total dry weight became insoluble at pH 8.0, but soluble at pH 6.0. This material, designated precipitate (PPT), was also evaluated.

The DEAE fractions were treated in *in vitro* lymphocyte culture and assessed in log dilutions between 1 and 1000 μg/ml as shown in Fig. 3. The maximum counts/min obtained at the optimal antigen concentrations (100 μg/ml) is also shown. Although all of the major DEAE fractions obtained from untreated *A. viscosus* SUP were stimulatory, only 6 fractions (i.e. A, E, F, G, H and PPT) were immunostimulatory following nuclease treatment. As each fraction obtained from SUP was tested at its optimal dilution (100 μg/ml) its true activity relative to its concentration in unfractionated SUP was evaluated. The stimulatory activity of each fraction was normalized to its percentage (dry weight) of the unfractionated SUP from which it was obtained (Fig. 4). This figure depicts such an evaluation of the lymphocyte response of one individual: Fractions F, G and PPT possessed most antigenic activity as well as representing the more highly charged material within the *A. viscosus* cell sap.

Table 4 shows normalized data from an additional 4 volunteers, indicating that although F, G and PPT

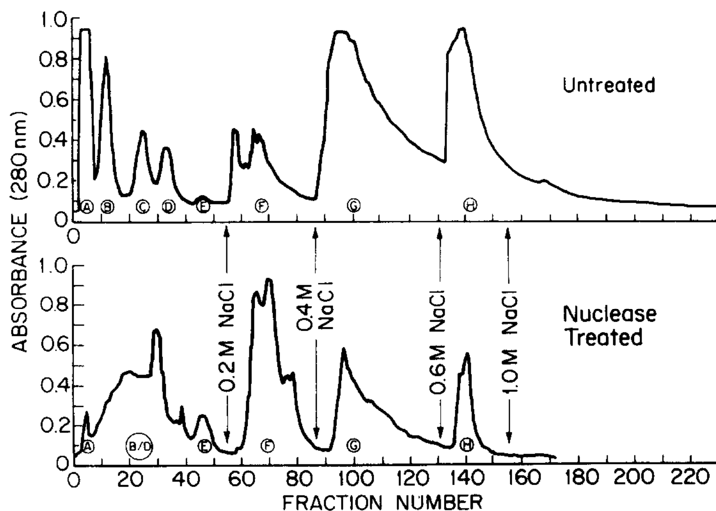

Fig. 2. DEAE-sepharose elution profile of *A. viscosus* SUP (upper) and nuclease digested SUP (lower). The column was equilibrated in 0.1 M tris–HCl buffer (pH 8.0) and batch-eluted with increasing concentrations of NaCl (0.2–1.0 M). Letters (A–H) indicate major fractions.

Table 3. Biochemical analysis* of *A. viscosus* supernate DEAE-sepharose fractions

Fraction	% of Total sample	Protein	Hexose	Methyl-pentose	Total nucleic acids
SUP	100.0	988	64	65	300
SUP/N†	100.0	430	52	45	<20
A	19.3	724	36	38	<50
A/N	0.3	nd‡	nd	nd	nd
B	1.4	336	55	58	<50
B/N	<0.1	nd	nd	nd	nd
C	1.2	128	34	9	<20
C/N	<0.1	nd	nd	nd	nd
D	0.6	nd	nd	nd	nd
D/N	<0.1	nd	nd	nd	nd
E	3.2	70	14	12	<20
E/N	0.4	nd	nd	nd	nd
F	12.3	575	27	30	<20
F/N	10.4	604	94	86	<20
G	19.9	726	35	47	200
G/N	5.6	594	94	31	132
H	6.7	54	9	29	200
H/N	<0.1	nd	nd	nd	nd
PPT	16.0	1080	50	46	≤20

* μg/mg dry weight. Because of limitations of standards used, the sum of assayed components may exceed 100 per cent.

† Nuclease treated before fractionation.

‡ Not done because of insufficient material.

were the most stimulatory fractions, the magnitude of the lymphocyte response to each was not identical. Different individuals varied in their responses to these fractions. The magnitude of response to the fractions was variable when tested over a period of 3 months; however, the relative stimulatory activities of each fraction was similar during this interval.

DISCUSSION

Our purpose was to document the *in vitro* lymphocyte responses of donors with minimal periodontal disease to *A. viscosus* cell wall and intracellular antigens. Reports by Engel *et al.* (1977) and Burckhardt, Guggenheim and Hefti (1977) indicate the presence of

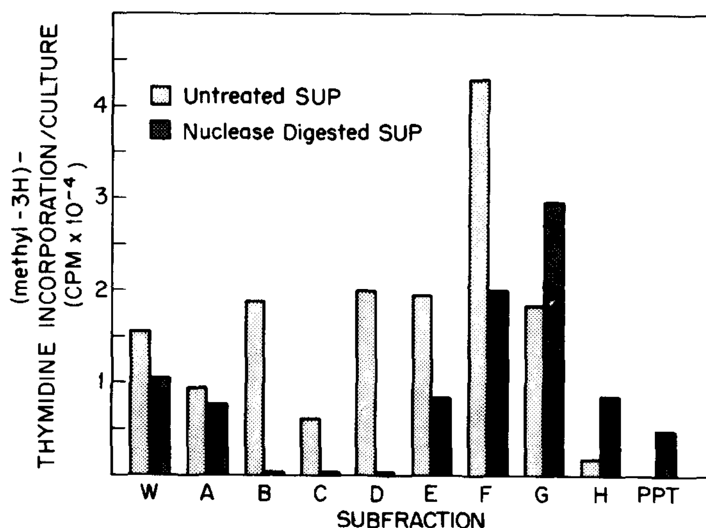

Fig. 3. *In vitro* blastogenic response of lymphocytes (obtained from a healthy donor) induced by DEAE-sepharose subfractions of *A. viscosus* SUP tested at 100 μg/ml (dry weight) in culture. W, whole, unfractionated SUP; A–H, DEAE-sepharose fractions; PPT, precipitate obtained following nuclease digestion, insoluble at pH 8.0, soluble at pH 6.0. The lymphocyte cultures were incubated for 7 days. [Methyl-^{3}H]-thymidine was added 16 h before termination of the culture period.

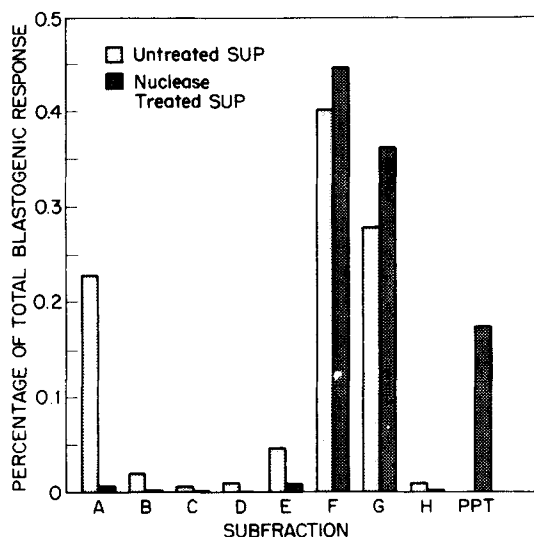

Fig. 4. *In vitro* blastogenic response of healthy donor lymphocytes to DEAE-sepharose fractionated *A. viscosus* SUP. Normalized by the percentage represented by that subfraction in unfractionated SUP.

B-cell polyclonal activators in ultrasonicates and extracts of *A. viscosus*. These activators stimulate mouse lymphocytes, but not human lymphoid cultures. Therefore, we stimulated human lymphocytes with the *A. viscosus* cell fractions, SUP and PEL, and with 2 known mitogens, Concanavalin A and pokeweed mitogen, and compared their *in vitro* kinetic responses. The results showed that the *A. viscosus* stimulants produced kinetic responses consistent with the typical antigen response (Oppenheim and Schechter, 1976) (i.e., detectable between days 3 and 4, maximal between days 5 and 7).

The importance of comparing cell-wall antigenicity with that of SUP was documented by Bowden *et al.* (1976) who revealed the complex antigenic structure of *A. viscosus*, including neutral cell-wall carbohydrates and charged polypeptides, found in extracts and supernatant culture fluids. By serological analysis, many of the cell-wall carbohydrates and charged polypeptides were shown to be species-specific, whereas others were cross-reactive. Careful documentation of the contribution of each major component to total antigenicity may be important in evaluating the role of a microorganism in a disease with potential immunological aetiology. Our studies confirm the importance of pronase-sensitive, charged antigens to the antigenicity of *A. viscosus*. Although several major immunostimulatory fractions can be identified in SUP, there was an indication of a varying lymphocyte responsiveness to them by different individuals (Table 4). Such differential responsiveness may be a result of individual variations (e.g. immunogenicity, tolerance) in immune reactivity to major antigens of *A. viscosus* strains. Differential response may also be due to response to or cross-reaction with antigens resident in the specific flora to which the subject has been sensitized. Our study does not resolve this problem, but shows that these differences may exist. The most obvious differences in immunostimulatory activity appeared to be associated with the PPT fraction which is probably tightly complexed to nucleic acid in untreated supernates, and made up 20 to 60 per cent of the total immunostimulatory activity of unfractionated SUP.

The *A. viscosus* cell-wall is a potent immunostimulant. Its major component was carbohydrate, primarily methyl-pentose (Table 1) and its immunostimulatory activity was resistant to pronase degradation (Table 2). The increase in stimulatory activity of the cell wall after nuclease treatment, indicated that nucleic acids may mask the immunodominant moieties, and perhaps act as an artifact of cell disruption.

Although nuclease digestion did not enhance [^{3}H]-thymidine incorporation stimulated by SUP fractions, it had a significant effect on the immunostimulatory activity of the PEL fraction. This enhancement could not be due to the removal of unlabelled

Table 4. *In vitro* lymphocyte blastogenic response to *A. viscosus* supernate fractions*

A. viscosus subfraction	Individual							
	A		B		C		D	
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated
A	0.30†	0.01	0.25	0.01	0.23	0.01	0.26	0.06
B	0.03	0.00	0.03	0.00	0.02	0.00	0.03	0.05
C	0.01	0.00	0.01	0.00	0.01	0.00	0.10	0.00
D	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00
E	0.02	0.01	0.05	0.02	0.05	0.01	0.07	0.03
F	0.34	0.22	0.41	0.31	0.40	0.45	0.49	0.24
G	0.28	0.16	0.20	0.19	0.28	0.36	0.12	0.07
H	0.02	0.00	0.04	0.00	0.01	0.00	0.02	0.00
PPT§	—	0.56	—	0.42	—	0.17	—	0.56

* Each fraction normalized to its percentage of the total dry weight of the unfractionated sample.

† Nuclease digested.

‡ For example, 0.30 indicates that 30 per cent of the antigen stimulating capacity can be accounted for by that particular fraction.

§ Insoluble at pH 8.0 after nuclease digestion.

thymidine, as the nucleic acid content of the PEL fraction was significantly lower than the SUP fraction, the activity of which was unaffected by nuclease treatment.

Because of difficulties in obtaining biologically active, soluble PEL antigens, characterization of individual PEL antigens was difficult. Further study is needed to determine if cell-wall antigens can be solubilized and still retain biological activity; however, stabilization in a rigid matrix may be required to produce this activity (Unanue, 1972; Unanue and Askonas, 1968).

The correlation between lymphocyte responsiveness and the ultimate determination of a etiological association with periodontal disease, must be considered in conjunction with the total antigenic matrix of a microorganism. The cell wall of *A. viscosus* is rich in pronase-resistant, probably carbohydrate, immunostimulatory moeities, and its intracellular supernatant contains several major classes of highly charged, pronase, sensitive antigens. Although the subjects in our study responded immunologically to all of these major antigenic fractions, there was a differential sensitivity to various fractions.

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