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ELABORATION OF LYMPHOTOXIN BY CULTURED HUMAN PERIPHERAL BLOOD LEUCOCYTES STIMULATED WITH DENTAL-PLAQUE DEPOSITS

J. E. HORTON, J. J. OPPENHEIM
AND S. E. MERGENHAGEN

Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland, U.S.A.

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SUMMARY

Leucocyte cultures from subjects with periodontal disease when stimulated by human dental-plaque deposit material, or phytohaemagglutinin, produce a soluble factor, lymphotoxin, which is cytotoxic for fibroblasts *in vitro*. The cytotoxic effect was determined from the degree of inhibition of incorporation of ^{14}C-labelled L-leucine by *in vitro* cultures of human gingival or mouse L-fibroblasts exposed to supernatants from such cultures. Inhibition of protein synthesis by the fibroblasts was not due to either depletion of nutrients or direct toxicity of the antigenic dental-plaque material. Both plaque-stimulated leucocyte culture supernatants from clinically normal subjects and unstimulated leucocyte culture supernatants from subjects with periodontal disease were significantly less inhibitory than supernatants of plaque-stimulated leucocyte cultures from subjects with periodontal disease. This production of lymphotoxin by leucocytes stimulated with antigen(s) present in dental plaque-deposits may reflect a mechanism of tissue destruction by sensitized lymphocytes present in the tissues of subjects with periodontal disease.

INTRODUCTION

Following *in vitro* lymphocyte activation, soluble factors with various biological activities appear in the supernatants of stimulated leucocyte cultures (Pick & Turk, 1972). These products are elaborated in response to antigens only by leucocytes from sensitized donors and by normal leucocytes when stimulated by non-specific mitogens. These mediators have various biological activities, such as inhibition of macrophage migration (MIF) (Bloom & Bennet, 1966), toxicity for homologous and heterologous-cultured cells (lymphotoxin) (Lawrence & Landy, 1969), monocyte chemotactic factor (Ward, Remold & David, 1969; Snyderman *et al.*, 1972), and activation of osteoclasts which results in bone resorption (OAF) (Horton *et al.*, 1972a). The appearance of these substances in supernatants of stimulated leucocyte cultures is believed to reflect the *in vivo* role of mediators which are elaborated

Correspondence: Dr John E. Horton, Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014, U.S.A.

by activated sensitized lymphocytes participating in cellular immune reactions, such as delayed hypersensitivity.

We have investigated the relationship of one of these mediators, lymphotoxin, to the pathogenesis of periodontal disease. The present report establishes that a cytotoxic factor, which behaves like lymphotoxin, appears in supernatants of cultures of peripheral blood leucocytes from subjects with periodontal disease when stimulated by human dental-plaque deposits. These supernatants were cytotoxic for cultured human gingival fibroblasts as well as mouse-L-cell fibroblasts.

MATERIALS AND METHODS

Human dental-plaque deposits (Ag) were collected at various clinical facilities during routine scaling procedures and placed in sterile tubes containing 0·02 M phosphate buffered saline (PBS, with 100 units penicillin/ml and 100 μg streptomycin/ml). The collected material was pooled and then sedimented by centrifugation (2000 ***g*** for 20 min at 4°C). The resultant supernatant was discarded, and the sediment frozen at −20°C for up to 6 mth. An aliquot of the frozen material was resuspended in sterile PBS (10 mg/10 ml), homogenized and ultrasonicated, as previously reported (Horton, Leikin & Oppenheim, 1972b). The supernatant of the ultrasonicate was filtered through a 0·45-μm Millipore filter (Millipore Corp., Bedford, Mass.) and stored at −20°C prior to use.

Replicate 1 ml leucocyte suspensions were established from the peripheral blood of selected 19–45-yr old male subjects, grouped on the basis of their degree of periodontal disease (PD) (Horton *et al.*, 1972b; Russell, 1956; Mobley & Smith, 1963). Experimental cultures received additions of phytohaemagglutinin (PHA, 10 μg/ml, Burroughs Wellcome, Tuckahoe, N.Y.) or an ultrasonicated solubilized fraction of Ag (10 μg of wet frozen weight of starting material/ml in PBS). No test substance was added to control cultures. The cultures were incubated for 6 days at 37°C in an humidified atmosphere of 5% CO_2 in air.

Supernatants of leucocyte cultures (SLC) were obtained at the end of the incubation period. After sedimenting the cultures by centrifugation (200 ***g*** at 20°C), the supernatants were aspirated and appropriately pooled, filtered through a 0·45-μm pore-size Millipore filter, and stored for up to 45 days at −20°C. One μCi of tritiated thymidine (TdR^3H. sp.act. 6·0 μCi/mM, Schwarz Biochemical, Rockville, Md) was added to each residual 0·15 ml of sedimented leucocytes, which were then re-incubated for 4·5 hr. The counts per min (cpm) of radioactivity in trichloracetic acid-precipitated material from the cell cultures were assayed in a Packard Tri-Carb scintillation spectrometer (Model 3375) (Oppenheim, Wohlstencroft & Gell, 1967). The degree of lymphocyte transformation was expressed as the mean ratio of TdR^3H incorporated by replicate (ten or more) test cultures to that incorporated by control cultures.

The assay to test for lymphotoxin was modified from a previous reported method (Granger & Kolb, 1968). Target cell suspensions of normal human gingival (passage number 6 to 8)* and mouse L-cell fibroblasts† were obtained from existing lines. After a 10 min treatment with Puck's saline A containing 0·25% trypsin (NIH Media Unit) at 37°C, the cells

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were washed twice and resuspended at 250×10^3 cells/ml in RPMI 1640 with 10% foetal calf serum (GIBCO, Grand Island, N.Y.). Replicate 1-ml vol. of this cell suspension were cultured in 1 dram loosely-capped vials at 37°C for 18 hr in an humidified atmosphere of 5% CO_2 in air. Cultures were then examined by phase microscopy (40×, Nikon inverted microscope, Nippon Logaku, K.K., Tokyo, Japan) and used only if they consisted of a uniform monolayer. The medium in each vial was aspirated, replaced with 1 ml of a 1:1 mixture of SLC and L-leucine-free RPMI 1640, and re-incubated for an additional 48 hr. The morphology of the monolayers was evaluated at the end of this 48-hr period by phase microscopy.

Cytotoxic effects of SLC on the cultured fibroblasts were determined from the inhibition of incorporation of radio-labelled L-leucine by the target cell monolayers. After 46 hr of incubation with the SLC mixture, 0·5 μCi of [^{14}C]L-leucine (^{14}C, sp.act. 312 mCi/mM, Schwarz Biochemical) was added to the fibroblast cultures and they were re-incubated for an additional 2 hr. The fibroblast cultures were harvested by the same method as the leucocyte cultures to determine the [^{14}C]L-leucine content of the trichloracetic-acid precipitated fraction (omitting the final methanol wash) of the fibroblast cultures. The mean cpm of triplicate cultures were calculated and the effect of the SLC determined from the ratio of cpm of fibroblasts cultured with SLC from Ag-stimulated or PHA-stimulated cultures to those incubated with SLC from cultures of control leucocytes from the same donor.

The data were grouped on the basis of the degree of the leucocyte donor's periodontal disease and tested for significance by analysis of variance. The difference in effect on fibroblastic monolayers of SLC from stimulated and unstimulated leucocyte cultures was analysed by Tukey's method for multiple comparisons (Ostle, 1963).

RESULTS

Ag-stimulated *in vitro* transformation of lymphocytes from subjects with varying degrees of PD was determined (see Tables 1 and 2). Consistent with our previous findings (Horton *et al.*, 1972b), none of the leucocyte cultures from subjects in the minimally-affected Group I responded significantly to Ag stimulation. Leucocyte cultures from Group III subjects with established destructive PD exhibited considerable incorporation of TdR^{3}H.

SLC from Ag-stimulated leucocytes of subjects with PD had significant inhibitory effects on the protein synthesis of cultured mouse L-fibroblasts (Table 1). SLC from Ag-stimulated leucocyte cultures from Group I subjects, which did not incorporate TdR^{3}H, did not inhibit protein synthesis of fibroblasts to any greater degree than their control SLC. Inhibition of protein synthesis was produced by SLC of Ag-stimulated leucocyte cultures from subjects with PD as well as by SLC of PHA-stimulated leucocyte cultures from all of the subjects. SLC from Ag-stimulated leucocyte cultures from Group III subjects who exhibited the destructive form of PD significantly produced greater inhibition of protein synthesis by mouse L-fibroblasts than did SLC from Ag-stimulated leucocytes from Group II subjects.

In order to confirm the biological relevance of a cytotoxic soluble mediator produced by Ag-stimulated leucocyte cultures from subjects with PD, SLC from such cultures were also tested on cultured human gingival fibroblasts (Table 2). Similarly, incorporation of [^{14}C]L-leucine by human gingival fibroblasts was inhibited by SLC of Ag-stimulated leucocyte cultures from PD subjects (Group II < Group III) as well as by SLC of PHA-stimulated lymphocytes from subjects in all three groups. Fibroblast monolayers cultured in the presence

TABLE 1. The effect of supernatants of leucocyte cultures stimulated with phytohaemagglutinin and human dental-plaque deposits on the incorporation of (^{14}C)L-leucine by mouse L-fibroblasts

P.I. of subjects†	No. (*n*) of subjects	Leucocyte culture stimulant	Incorporation of TdR^{3}H by cultured leucocytes: Mean cpm‡	Incorporation of TdR^{3}H by cultured leucocytes: Mean ratio (stimulated/unstimulated)	Incorporation of (^{14}C)L-leucine by mouse L-fibroblasts: Mean cpm‡	Incorporation of (^{14}C)L-leucine by mouse L-fibroblasts: Mean %inhibition (unstimulated/stimulated × 100)
Group I (<0·5)	7	None	1,147 ± 242	–	66,242 ± 9,234	–
		PHA	110,789 ± 40,146	96*	28,948 ± 7,945	56·30*
		Plaque-antigen	1,750 ± 496	1·5	67,352 ± 9,693	0
Group II (0·5–1·5)	2	None	690 ± 191	–	57,495 ± 17,901	–
		PHA	43,226 ± 2,650	63*	11,174 ± 1,671	80·57*
		Plaque-antigen	2,910 ± 964	4·2*	54,448 ± 17,395	5·30
Group III (1·5–4·0)	10	None	721 ± 294	–	105,681 ± 20,071	–
		PHA	99,255 ± 49,436	137*	22,151 ± 4,136	79·04*
		Plaque-antigen	17,111 ± 5,529	24*	47,294 ± 9,239	55·25*

* Significant by analysis of variance and Tukey's method for multiple comparisons, $P<0·01$.

† Russell's periodontal index, where <0·5 exhibits minimal gingivitis; 0·5–1·5 exhibits moderate gingivitis; and 1·5–4·0 exhibits destructive periodontal disease.

‡ Mean and S.E. of the mean cpm.

TABLE 2. The effect of supernatants of leucocyte cultures stimulated with phytohaemagglutinin and human dental-plaque deposits on the incorporation of (^{14}C)L-leucine by human gingival fibroblasts

P.I. of subjects†	No. (*n*) of subjects	Leucocyte culture stimulant	Incorporation of TdR^{3}H by cultured leucocytes: Mean cpm‡	Incorporation of TdR^{3}H by cultured leucocytes: Mean ratio (stimulated/unstimulated)	Incorporation of (^{14}C)L-leucine by human gingival fibroblasts: Mean cpm‡	Incorporation of (^{14}C)L-leucine by human gingival fibroblasts: Mean %inhibition (unstimulated/stimulated × 100)
Group I (<0·5)	4	None	1,412± 357	–	3,660± 877	–
		PHA	150,551±63,877	106*	1,766± 512	51·75*
		Plaque-antigen	2,358± 705	1·6	3,825± 947	0
		Plaque-antigen only (without leucocytes)	ND§	ND	4,083± 356	−11·56
Group II (0·5–1·5)	2	None	690± 191	–	10,836± 738	–
		PHA	43,226± 2,650	63*	7,239± 460	33·19*
		Plaque-antigen	2,910± 964	4·2*	10,636± 734	1·85
		Plaque-antigen only (without leucocytes)	ND	ND	11,487± 344	—6·01
Group III (1·5–4·0)	6	None	693± 398	–	11,415± 842	–
		PHA	72,569±37,586	105*	6,092± 799	46·63*
		Plaque-antigen	21,901± 8,109	31·6*	7,350± 904	35·61*
		Plaque-antigen only (without leucocytes)	ND	ND	12,070±1,123	−5·65

* Significant by analysis of variance and Tukey's method for multiple comparisons, $P<0·01$.

† Russell's periodontal index, where<0·5 exhibits minimal gingivitis; 0·5–1·5 exhibits moderate gingivitis; and 1·5–4·0 exhibits destructive periodontal disease.

‡ Mean and S.E. of the mean cpm.

§ No determination.

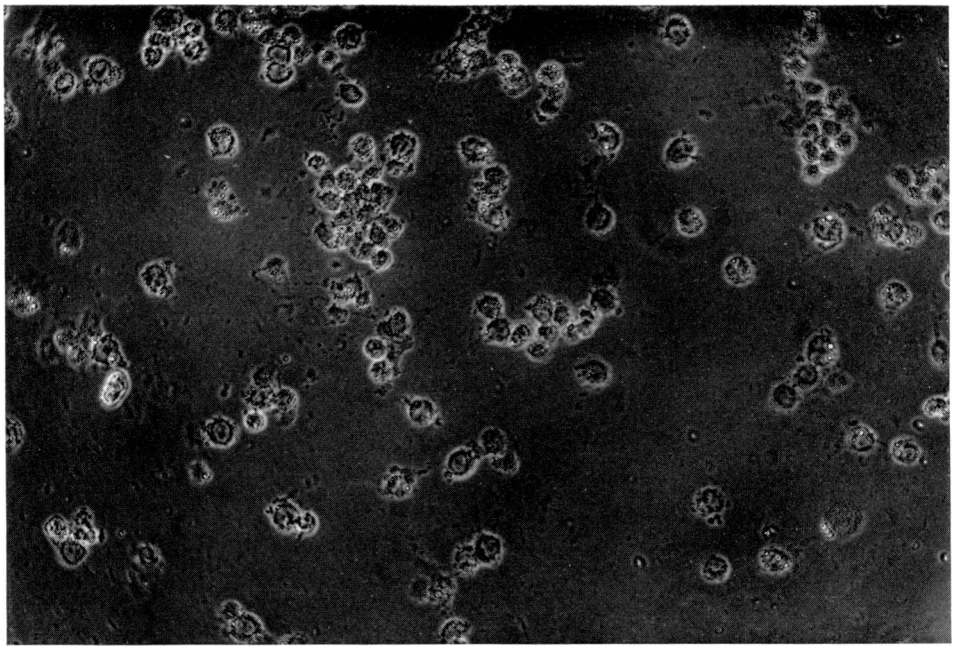

FIG. 1. Representative example of the cytotoxic appearance of a monolayer of human gingival fibroblasts cultured 48 hr with supernatant from plaque-antigen reactive leucocyte cultures established from a subject with destructive periodontal disease. Phase photomicrograph, ×120 magnification.

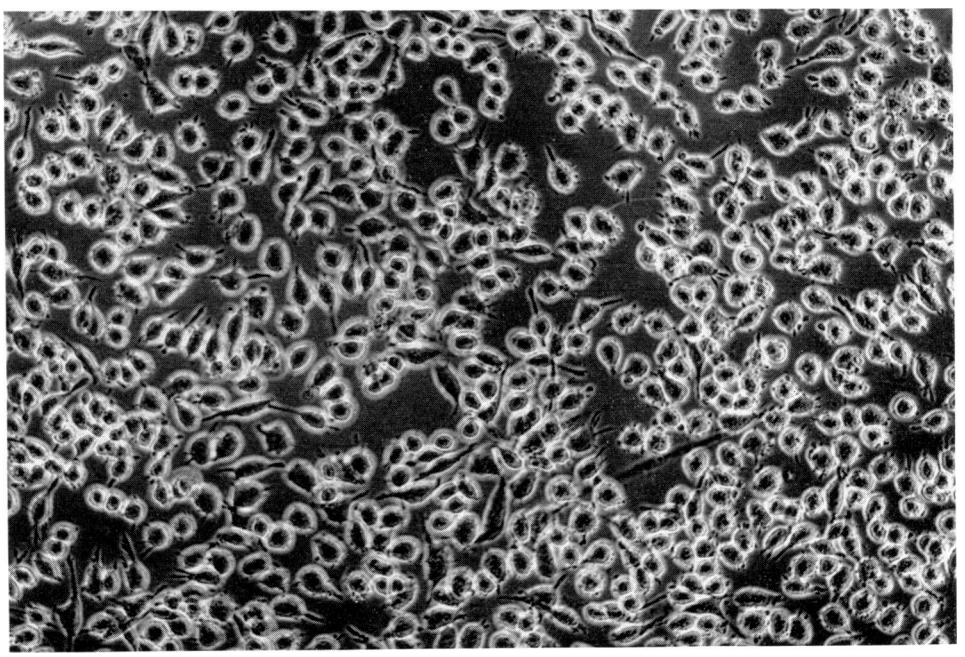

FIG. 2. Representative example of a normal-appearing monolayer of human gingival fibroblasts cultured 48 hr with supernatant from plaque-antigen non-reactive leucocyte cultures established from a subject minimally-affected with periodontal disease. Phase photomicrograph, ×120 magnification.

of such SLC were disrupted and no longer confluent or intact (Fig. 1). In contrast, monolayers of human gingival fibroblasts cultured 48 hr either in the presence of control SLC or SLC from Group I subjects that were not stimulated by Ag revealed no inhibition of [^{14}C]L-leucine uptake and consisted of confluent sheets of normal-appearing cells (Fig. 2).

The ratio of TdR^{3}H incorporated by PHA- and Ag-stimulated to unstimulated leucocyte cultures correlated directly with the per cent inhibition of [^{14}C]L-leucine uptake by mouse L-cell and human gingival fibroblasts produced by the resultant SLC (Figs 3 and 4). However, SLC from Ag-stimulated leucocyte cultures exhibited less stimulation of TdR^{3}H uptake than those stimulated by PHA but produced only slightly less lymphotoxin than

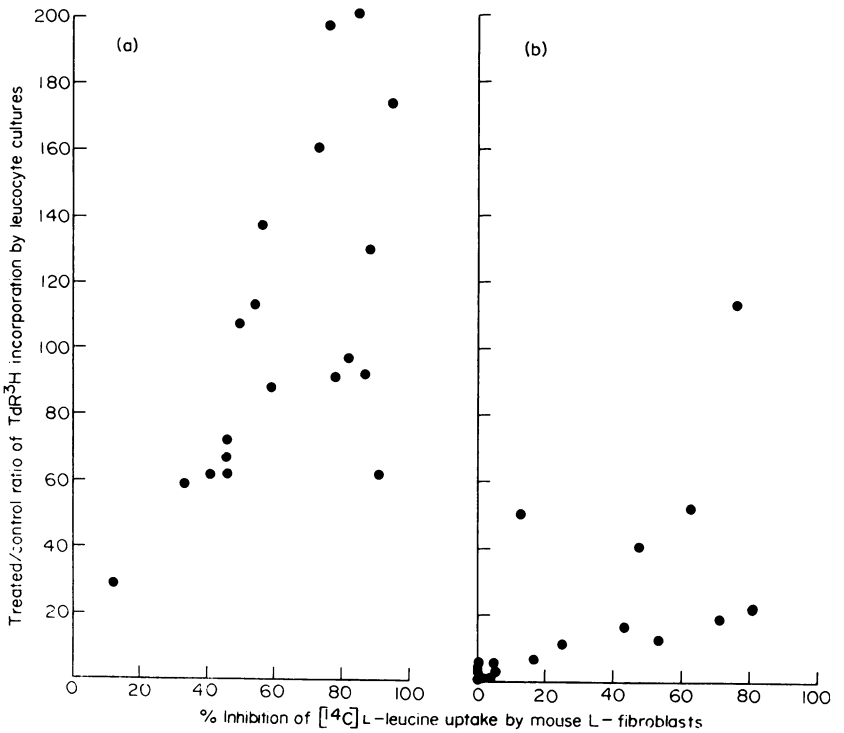

FIG. 3. Scattergram representing the correlation between the ratio of TdR^{3}H incorporated by (a) phytohaemagglutinin, and (b) plaque-antigen stimulated to control cultures with the per cent inhibition of radio-labelled L-leucine by mouse L-cell fibroblasts.

PHA-stimulated cultures. Calculation of the correlation coefficients of the capacity of SLC from PHA- and Ag-stimulated cultures to inhibit mouse L-cell fibroblasts were 0·69 and 0·78, respectively. This was statistically significant with a lower 95% confidence limit of 0·35 and 0·50, respectively (Fig. 3a and b). Correlation coefficients of the inhibitory effect of SLC from PHA- and Ag-stimulated cultures on human gingival fibroblasts were 0·22 and 0·94, respectively (Fig. 4a and b). Thus the lower 95% confidence limit was not significantly different from 0 in the case of SLC from PHA-stimulated cultures, but was highly significant for SLC from Ag-stimulated cultures with a lower 95% confidence limit of 0·79.

The inhibitory effect of SLC from Ag-stimulated cultures was not attributable to the

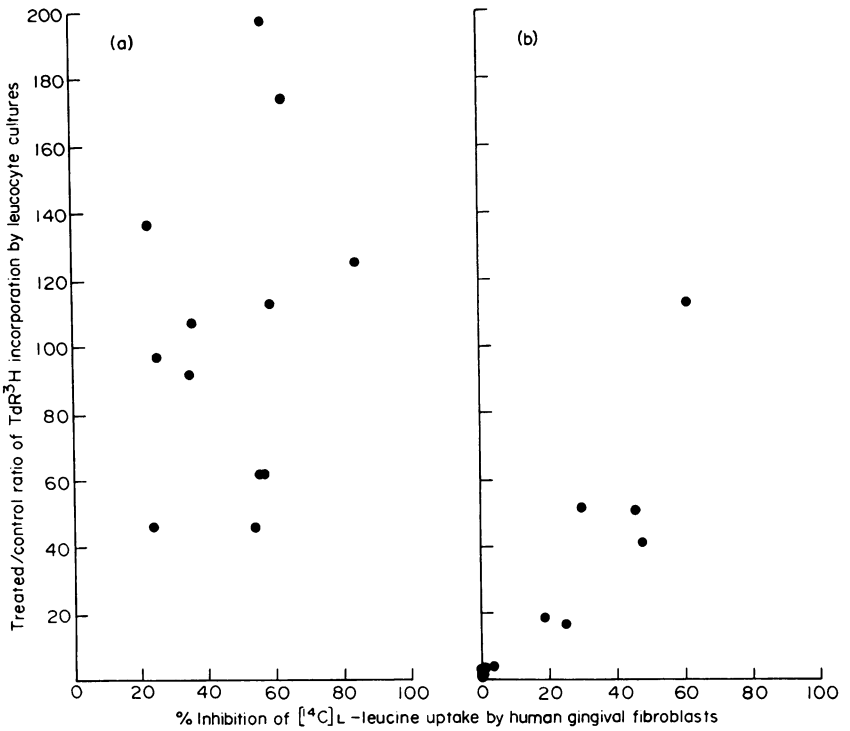

FIG. 4. Scattergram representing the correlation between the ratio of TdR^{3}H incorporated by (a) phytohaemagglutinin, and (b) plaque-antigen stimulated to control cultures with the per cent inhibition of radio-labelled L-leucine by human gingival fibroblasts.

presence of Ag in the SLC. Culture medium incubated with Ag in the absence of leucocytes, when mixed 1:1 with L-leucine-free RPMI 1640, resulted in slightly increased incorporation of [^{14}C]L-leucine by human gingival fibroblasts in comparison with the effect of control SLC (Table 2).

Changes in pH would not seem to be responsible for the inhibition of [^{14}C]L-leucine uptake by the fibroblasts. At the onset of the addition of SLC to the 18-hr established fibroblast monolayer, the mean pH was $7{\cdot}63 \pm 0{\cdot}15$, whereas the addition of media exhibited a mean pH of $7{\cdot}33 \pm 0{\cdot}13$. However, within 1·5 hr of incubation following this addition of either SLC or media, all cultures registered a pH of $7{\cdot}13 \pm 0{\cdot}03$.

The presence of human plasma in the SLC non-specifically inhibited protein synthesis by fibroblast cultures as measured by [^{14}C]L-leucine uptake, but did not produce any monolayer disruption. The mean cpm of [^{14}C]L-leucine incorporated by both types of fibroblasts cultured with media containing 20% human plasma mixed 1:1 with L-leucine-free RPMI 1640 was much less than when cultured with L-leucine-free RPMI 1640 media depleted 1:1 with Dulbecco's isotonic saline (Table 3). Therefore the cytotoxic activity of SLC from stimulated cultures was determined in comparison with the [^{14}C]L-leucine uptake in the presence of control SLC. This inhibition was above the marked non-specific inhibition due to the presence of human plasma in SLC.

The possibility that depletion of the culture nutrients in the SLC was responsible for the

cytotoxic effects of SLC on cultured fibroblasts was also ruled out (Table 4). Monolayers of mouse-L-cells were cultured successfully for 48 hr with varying dilutions of L-leucine-free RPMI 1640 and Dulbecco's phosphate buffered saline with added Ca and Mg. The incorporation of [^{14}C]L-leucine was not decreased as the culture medium was diluted to 1:4 with Dulbecco's saline. A marked decrease in incorporation of [^{14}C]L-leucine was seen only when the cells were cultured in undiluted saline. The same results were obtained when human gingival rather than mouse L-fibroblasts were used.

TABLE 3. The effect of various media on the incorporation of ^{14}C-labelled L-leucine by mouse L-cell and human gingival fibroblasts

RPMI 1640 mixed 1:1 with:	Mouse L-fibroblasts		Human gingival fibroblasts	
	Mean cpm*	% Change	Mean cpm	% Change
Dulbecco's saline	248,979	-	17,809	-
RPMI 1640 with 20% human plasma	97,039	−64·3	9,841	−48·3

* Mean cpm of triplicate cultures.

TABLE 4. The effect of dilution of media with Dulbecco's phosphate buffered saline on the incorporation of ^{14}C-labelled L-leucine by mouse L-cell and human gingival fibroblasts

Per cent RPMI 1640 in medium (%)	Mouse L-fibroblasts		Human gingival fibroblasts	
	Mean cpm*	% Change	Mean cpm	% Change
100	192,491	-	14,931	-
75	221,736	+15·19	15,701	+ 5·16
50	248,979	+29·35	17,809	+19·28
25	274,603	+42·66	17,434	+16·76
0	142,239	−26·11	8,533	−42·85

*Mean cpm of triplicate cultures.

DISCUSSION

Cytotoxic lymphotoxin is detected in the supernatants of leucocyte cultures incubated with non-specific mitogenic stimulants (Granger & Kolb, 1968). Lymphotoxin can also be produced by activation of sensitized lymphocytes with specific antigens to which the lymphocytes' donor has been sensitized (Ruddle & Waksman, 1968). It has been shown to be a product of stimulated lymphocytes (Williams & Granger, 1969). The precise mechanism of the cytotoxic effect of this heat-labile, soluble (mol. wt ~ 80,000) lymphocyte mediator is unknown. However, lymphotoxin has been observed to be non-specifically cytotoxic for a wide range of cultured cells *in vitro* (Lawrence & Landy, 1969).

The degree of cell lysis may be assayed by ^{51}Cr release (Peter, 1971), by determining direct total cell counts in a monolayer (Kramer & Granger, 1972), or by the inhibition of [^{14}C]-amino acid precursors into protein (Granger & Kolb, 1968). The degree of inhibition of [^{14}C]amino acid uptake by lymphotoxin-containing SLC correlates with the degree of target cell death (Peter, 1971). Our findings that supernatants of both PHA- and Ag-stimulated leucocyte cultures have a similar degree of non-specific cytotoxic effect on target cells is consistent with these previous reports. In addition, there was a direct correlation between the degree of PD of the leucocyte donor and the amount of lymphotoxin elaborated by his lymphocytes. In contrast, control cultures or Ag incubated in medium with or without leucocytes from non-reactive Group I periodontally-diseased subjects failed to produce lymphotoxin. The Ag preparation was not directly toxic. Other experiments ruled out nutrient depletion as a cause for our findings.

It is surprising that while the antigenic ultrasonicate of plaque-deposit stimulated considerably less lymphoproliferation than the phytomitogen, almost as much lymphotoxin was produced when it was used as a stimulant. It has been our experience that antigens such as Streptolysin O and *Candida albicans* stimulate small proportions of lymphocytes to proliferate and result in considerably less lymphotoxin production than non-specific mitogens, such as phytohaemagglutinin, Staphylococcal filtrate, and pokeweed mitogen (Oppenheim *et al.*, 1972). One possible explanation for the greater efficacy of plaque-deposit material in stimulating lymphotoxin production is that the nature of the antigen is an important determinant of the type of response it elicits. For example, a carbohydrate-rich, protein-poor purified protein derivative of tuberculin can stimulate MIF production but not lymphocyte transformation (Chaparas *et al.*, 1970). Alternatively, the persistent deposition of plaque on the gingival tissues may, by continued stimulation of the immune response, favour the generation of lymphotoxin-producing cells.

A number of recent findings indicate that cellular immunity may play an important role in PD. First, culture filtrates of micro-organisms present in the oral cavity induce transformation of cultured lymphocytes from patients with gingivitis and moderate periodontitis (Ivanyi & Lehner, 1970). Second, the degree of lymphocyte transformation stimulated by dental-plaque material can be directly correlated with the degree of the leucocyte donor's PD (Horton *et al.*, 1972b). Also, lymphocytes stimulated with ultrasonicates of an oral micro-organism are cytotoxic for ^{51}Cr-labelled chicken red cells, and SLC from such stimulated leucocyte cultures inhibit migration of guinea-pig peritoneal macrophages (Ivanyi, Wilton & Lehner, 1972). In addition, we have recently reported the detection of a new biological activity, osteoclast activation factor (OAF), present in SLC from Ag-stimulated lymphocyte cultures from PD subjects which activates osteoclasts to resorb bone (Horton *et al.*, 1972a). Finally, we have established in this study that SLC from plaque-antigen stimulated lymphocyte cultures from PD subjects are cytotoxic to mouse L-cells and to human gingival fibroblasts.

In vivo clinical and histopathological tissue changes present in PD reflect these *in vitro* findings (Schroeder, Munzel-Pedrazzoli & Page, 1972). The disease characteristically consists of a slow chronic inflammatory process, in which damage to fibroblastic connective tissue is associated with cell death and disruption of collagen fibres, and eventually culminates in alveolar bone resorption. Thus, the pathogenesis of the chronic inflammatory component of PD may well be due to plaque-antigen stimulation of lymphocytes to produce mediators with deleterious effects on periodontal tissues and bone.

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