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Blastogenesis and Lymphokine Synthesis by T and B Lymphocytes from Patients with Periodontal Disease

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Thymus-derived (T) and bone marrow-derived (B) lymphocytes were isolated from human peripheral blood and cultured with various mitogens and antigens. Purified protein derivative of tuberculin stimulated both purified T and B cells from patients with positive skin reactivity to purified protein derivative but did not stimulate nonimmune lymphocytes. Similarly, both T and B lymphocytes from patients with periodontal disease were stimulated to proliferate when incubated with dental plaque, whereas cells from normal individuals without gingivitis were unresponsive. In contrast, one component of plaque, bacterial endotoxins (lipopolysaccharide), minimally stimulated B lymphocytes from both normal or gingivitis patients. T lymphocytes from patients with periodontal disease were also stimulated by plaque antigen to produce chemotactic lymphokine activity (CTX) for human monocytes. B cells purified by the EAC rosetting method nonspecifically produced CTX without concomitant blastogenesis; however, after dissociation of adherent EAC these immune B cells did not spontaneously produce CTX. Lymphokine synthesis by B cells was not dependent on concomitant blastogenesis. Dissociated B cells from periodontitis patients also produced CTX activity after stimulation with dental plaque antigen. Therefore, both T and B lymphocytes, after stimulation with nonendotoxin antigenic components of plaque, proliferated and produced lymphokines, which are presumed to contribute to the pathogenesis of periodontal disease.

The clinical severity of gingival inflammation, tissue destruction, and bone resorption characteristic of human periodontal disease has been directly correlated with the degree of in vitro lymphocyte blastogenic responses to microbial and dental plaque antigens (3, 7-9). In constrast, lymphocytes from normal donors with negligible plaque accumulation and gingival inflammation were not stimulated by plaque (3, 7-9). Stimulation of immune lymphocytes with plaque has also been found to generate pharmacologically active lympokines (B. F. Mackler, L. Altman, S. Wahl, D. L. Rosenstreich, J. J. Oppenheim, and S. E. Mergenhagen, J. Dent. Res. **53**:225, 1974; 4, 5, 9) which are presumed to contribute to chronic inflammation and the tissue destruction associated with this disease. These observations, together with the lack of any correlation with the presence of serum antibodies, led to the view that periodontal disease was predominantly based on the immune thymus-derived (T) lymphocyte reactions of blastogenesis and lymphokine production. However, there were several observations that did not fit this model for periodontal disease such as (i) the preponderance of bone marrow (B)-derived plasma cells in chronic inflamed gingiva (12), and (ii) the finding of immunoglobulin M-like "antigen-specific" serum inhibitory and enhancing factors (6).

We therefore assessed the blastogenic responses of purified human T and B lymphocytes from normal and immune patients to bacterial endotoxins, purified protein derivative (PPD) of tuberculin, and dental plaque. Secondly, we investigated whether immune T and B lymphocytes also generated mononuclear cell chemotactic lymphokines after antigen stimulation. We have found that B lymphocytes as well as T lymphocytes from periodontitis patients can proliferate and produce lymphokines in response to dental plaque antigen.

MATERIALS AND METHODS

Patients. Peripheral blood was obtained from patients, age 20 to 45 years, with various degrees of

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periodontal disease as characterized by the Russell Periodontal Index (PI) (11); group I included patients with negligible gingival inflammation and plaque accumulation (PI <0.1), and group II included patients with moderate to severe destructive periodontal disease who were clinically characterized by abundant plaque deposits and acute gingival inflammation (PI >0.8 to 2.5). Patients within these groups were also skin tested for tuberculin sensitivity and divided into skin test positive reactors, who had been previously vaccinated with BCG, and skin test negatives, or normals.

Purification of human T and B lymphocyte populations. T and B lymphocyte populations were isolated from whole blood by a modified sequential rosetting technique (W. West, J. McCoy, C. Alford, J. Doering, and R. Herberman, manuscript in preparation; 10). Mononuclear cells were separated from diluted (1:4) whole blood by Ficoll-hypaque (FH) gradient centrifugation, washed, and incubated with fresh sheep erythrocytes (E) plus 2.5% absorbed fetal calf serum for 60 min at 3 C. Lymphocytes with adherent E rosettes were partitioned from non-E rosette-forming cells by centrifugation through two consecutive FH gradients. The non-E rosette-forming cells were washed, mixed with EAC (sheep erythrocytes [E] coated with 19*S* anti-sheep erythrocyte antibodies [A] and components of mouse complement [C]) and incubated for 30 min at 37 C with constant agitation. Lymphocytes with EAC bound to their surfaces by their C3 receptors were separated from the remaining non-EAC rosette-forming cells on two additional FH gradients. Prior studies have demonstrated that E rosette-forming cells are thymus-derived (T) lymphocytes (16), and that EAC rosette-forming cells are bone marrow-derived (B) cells (1). The dissociation of EAC from B cells was achieved by treating the EAC-B cell rosettes with rabbit anti-mouse C3 globulin (1 mg of immunoglobulin G per 1×10^7 cells; Cappel Laboratories, Downingtown, Pa.). B cells dissociated from EAC were then isolated on a FH gradient and viability determined by trypan blue dye exclusion.

The purity of T and B populations was assessed by immunofluorescent staining of membrane-associated immunoglobulin, a characteristic of B cells. Rosetted cells (1×10^7 cells) were treated with isotonic ammonium chloride lysing buffer, washed, and kept at 3 C. Fluorescein-conjugated anti-human immunoglobulin sera (C20F-1341, Meloy Laboratories, Springfield, Va.) were added to the packed cells, the mixtures were incubated for 30 min at 3 C and washed 3× in cold azide-bovine serum albumin buffer, and wet mounts were prepared.

Dental plaque antigen preparation. Dental plaque was collected from numerous patients with clinically defined periodontal disease who had abundant plaque deposits (PI 0.4 to 8). These individual specimens were immediately frozen in 0.85% saline. A soluble antigen fraction was prepared from the above pooled plaque specimens by the method of Horton et al. (3). The pooled crude plaque was sonically treated at 145 W (Ultrasonics, Inc., Plainview, N.Y.) for 10 min, and the insoluble material was removed by high-speed centrifugation. An overnight dialysis of the soluble material against saline was used to remove low-molecular-weight materials which suppressed blastogenesis (B. F. Mackler, submitted for publication). The nondialyzable, solubilized plaque antigen was sterilized by ultrafiltration through a 0.2-μm membrane filter (Millipore Corp.) and stored in portions at −10 C until use. This pooled plaque antigenic preparation was used for all experiments, since Horton et al. (3) found that plaque from diseased donors stimulated sensitized allogeneic lymphocytes from other equally diseased donors, as well as autologous cells.

Bacterial endotoxin lipopolysaccharide and PPD tuberculin preparations. The *Veillonella alcalescens* and *Leptotrichia buccalis* endotoxin preparations were gifts from Mark Wilton (Guys Hospital, London, England) and Howard Creamer (University of Oregon Dental School, Portland, Ore.); *Salmonella typhimurium* was purchased from Difco Laboratories, Detroit, Mich. The PPD tuberculin extract was obtained from Connaught Medical Research Laboratories, Toronto, Canada (lot A12).

Lymphocyte blastogenesis assay. Human T and B lymphocytes with their adherent rosettes and dissociated B cells were diluted to 2×10^6 cells/ml in RPMI-1640 supplemented with penicillin-streptomycin, glutamine, and 0.5% human AB sera from donors without clinical signs of periodontal disease (10). In each well of a microtiter tray (Cooke Engineering Co., Alexandra, Va.) 0.1 ml of cells (2×10^5 cells) was placed, and 0.1 ml of either saline, lipopolysaccharide, PPD, or dental plaque antigen was added. Each variable was tested in triplicate. The covered trays were cultured for 72 h in 5% CO_2-95% air at 37 C; 3.0 μCi of tritiated thymidine (6 Ci/mmol, Schwarz/Mann, Orangeburg, N.J.) was added to each well for the last 18 h of culture. Cultures were processed with an automatic harvestor, and the total radioactivity incorporated into deoxyribonucleic acid was determined in a liquid scintillation counter. Results are expressed as the ratio of the mean counts per minute in triplicate stimulated cultures versus the mean counts per minute in triplicate control cultures.

Assay for lymphocyte-derived chemotactic lymphokines. Culture supernatants were tested for chemotactic lymphokine activity (CTX) by the method of Snyderman et al. (13). Mononuclear cells and lymphocyte culture supernatants were placed in modified Boyden chemotactic chambers separated by 5-μm polycarbonate filters (Nuclepore, Wallabs, Inc., San Rafael, Calif.). The chambers were incubated for 90 min at 37 C in air. The filters were then removed, the cells were stained with Giemsa, and cell migration was quantified as previously reported (13). Results are expressed as the mean number of migrating cells per oil immersion field ±1 standard error. All samples were tested in triplicate.

RESULTS

Purity and recovery of T and B lymphocyte populations. Immunofluorescent studies of lymphocytes separated into T and B populations in four experiments showed that 95 ± 1%

standard error of the E rosetting cells had no surface immunoglobulin, whereas the EAC rosette-forming cells were 95 ± 2% immunoglobulin positive. Both populations had <0.5% monocyte or macrophage contamination or both. From 45 to 75% of the original peripheral blood lymphocyte population was recovered as E rosette-forming cells and 5 to 35% as EAC rosette-forming cells. However, the percentages of T and B cell recoveries in replicate experiments for each patient were consistent and normally averaged about 60% of peripheral blood lymphocytes as T cell and 5 to 10% as B cells. These percentages varied between patients, with some showing higher percentages of recoveries. The high rates of recovery indicate that the subsequent in vitro reactions may well be representative of T and B lymphocyte immune effector functions.

Activation of human T and B lymphocytes by bacterial components. To assess the ability of bacterial endotoxins and other antigens to activate human lymphocytes, purified T and B subpopulations were isolated from patients with and without periodontal disease and then cultured with these stimulants. The tritiated thymidine incorporation levels at optimal stimulant concentrations are shown in Table 1; lower concentrations induced dose-dependent responses. Several endotoxin preparations induced low but detectable levels of T and B cell activation, irrespective of whether normal cells or cells from diseased patients were used. In contrast, PPD induced significant T and B lymphocyte stimulation (13- to 25-fold) in cultures of immune lymphocytes (reactors), but not in cultures from normal (nonreactors) patients. All of the PPD reactors had previously received BCG vaccination and gave positive skin reactivity. Of particular interest is the observation that B as well as T lymphocytes from normal patients lacking any skin reactivity were completely unresponsive at all PPD concentrations (0.1 to 300 μg).

Dental plaque-induced blastogenesis of T and B lymphocytes. Dental plaque antigen, an extract of the bacterial matt on teeth and gingiva, was found not to stimulate blastogenesis of cells from patients without any clinical signs of periodontal disease (acute inflammation and plaque accumulation). To ascertain whether T and B lymphocytes from patients with periodontal disease would respond to dental plaque antigens, lymphocyte subpopulations from five patients with gingival inflammation were purified and cultured in vitro with dental plaque antigen. The blastogenic response of T cells from these patients to a range of plaque antigen concentrations is shown in Fig. 1; Fig. 2 depicts the corresponding response of B cells from the same patients. Both lymphoid subpopulations from each diseased donor showed significant blastogenic dose-dependent responses. The magnitude of the in vitro response at optimal stimulating dental plaque concentrations and dose-dependent responses varied

TABLE 1. *Activation of human T and B lymphocytes by bacteria preparations, PPD, and plaque*

Determination	Concn (μg)	Activity					
		T lymphocytes			B lymphocytes		
		N[a]	Mean ± SE[b]	E/C	N	Mean ± SE	E/C
Control			127 ± 25	1.0		205 ± 38	1.0
Endotoxins							
Leptotrichia buccalis	100	11	402 ± 105	3.2	11	835 ± 182	4.1
Veillonella alcalescens	100	6	292 ± 88	2.3	5	296 ± 81	1.4
Salmonella typhimurium	100	2	349 ± 190	2.8	3	252 ±115	1.2
Antigens							
PPD							
Normals	100	8	199 ± 34	1.6	9	157 ± 28	0.8
Reactors	100	6	1,688 ± 433	13.3	3	5,191 ± 1,313	25.3
Dental plaque							
Normals	5–50	5	179 ± 15[c]	1.4	5	218 ± 25[c]	1.1
Reactors	5–50	11	1,042 ± 225[c]	8.2	11	1,692 ± 707[c]	8.3

[a] N, Number of experiments.
[b] Mean counts per minute ± standard error (SE) of mean tritiated thymidine incorporated by 2×10^5 lymphocytes.
[c] Means are from the highest stimulation levels obtained in dose-response experiments (5 to 50 μg).

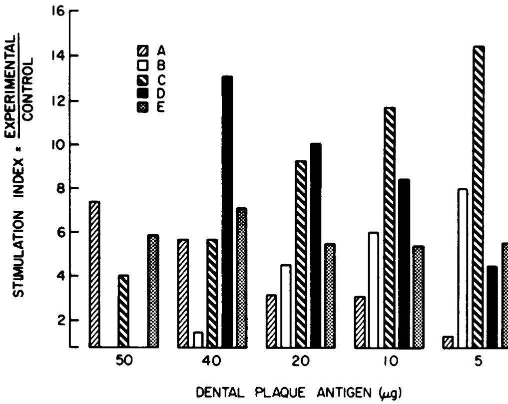

FIG. 1. *Plaque-induced blastogenesis of immune human T lymphocytes. The dose-dependent T lymphocyte responses to plaque of five patients with periodontal disease (PI 0.8 to 2.8) are shown.*

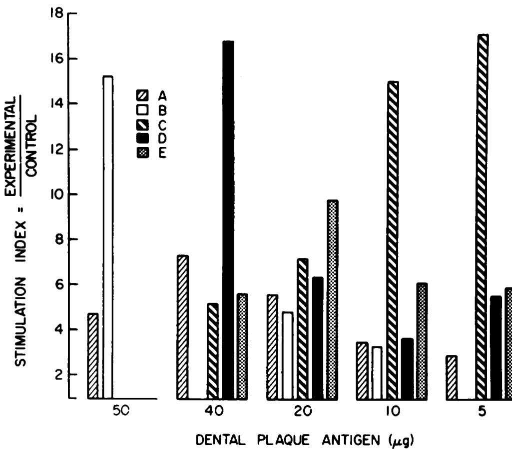

FIG. 2. *Plaque-induced blastogenesis of immune human B lymphocytes. The dose-dependent responses of B lymphocytes from five patients with periodontal disease (PI 0.8 to 2.8) are depicted.*

between patients, an indication of differences in the in vivo hypersensitivity levels that each patient possessed toward the pooled plaque antigen preparation. These results indicate that both T and B cells from periodontal disease patients responded to dental plaque antigen.

Production of chemotactic lymphokines by human T and B lymphocytes. To determine if T and B lymphocyte subpopulations could produce lymphokines, supernatants from plaque-stimulated T and B cell cultures were assessed for mononuclear CTX. Each plaque-stimulated culture supernatant was assayed along with a paired control supernatant to which an equivalent amount of plaque was added after incubation. These reconstituted supernatants served as controls for the measurement of any inherent chemotactic activity in dental plaque antigen extracts as well as for conditioned medium effects.

Significant chemotactic activity was found in the supernatants of plaque-stimulated T lymphocyte cultures from seven patients with varying degrees of periodontal disease (Table 2). In contrast, supernatants of T cells from clinically defined normal individuals stimulated with plaque had no chemotactic activity. There was, however, no correlation between the magnitude of T cell blastogenesis (E/C ratios) and the amount of lymphokine activity produced in these cultures.

B lymphocytes cultured with their adherent EAC rosettes had chemotactic activity irrespective of whether they were incubated in the presence or absence of dental plaque (Table 3). The nonspecific production of chemotactic activity was so high that plaque did not cause any measurable increase in the amount of lymphokine production. To further investigate the effect of plaque on B cell lymphokine synthesis, B cells were dissociated from their adherent EAC rosettes with rabbit anti-C3 globulin and separated on Ficoll-hypaque gradients. Dissociated B cells without adherent EAC cultured in the absence of any stimulant generated low levels of chemotactic activity. These findings indicated that the binding of EAC to the membranes of B cells induced chemotactic lymphokine production in the absence of concomitant blastogenesis. Dissociate B cells from patients with periodontal disease, when cultured in the presence of plaque, produced significant levels of chemotactic activity in contrast to nonimmune B cells. These experiments indicated that B cells, like T cells, can produce lymphokines after appropriate antigenic stimulation.

DISCUSSION

We have shown that both T and B lymphocytes from sensitized patients cultured in vitro with antigens undergo blastogenic responses. Both dental plaque and PPD, which were used as positive controls in our experiments, behaved as specific antigens in that they activated only lymphocytes from sensitized donors. T and B lymphocytes from normal donors, having negligible dental plaque deposits or PPD tuberculin sensitivity, were unresponsive. This is in contrast with findings in mice, in which PPD is a nonspecific B cell mitogen (14). Endotoxins (lipopolysaccharide) derived from oral bacteria were able to induce only minimal human B cell proliferation in cell cultures from patients and normal subjects, indicating that these plaque components in dental plaque were not responsi-

TABLE 2. *Chemotactic lymphokine production by human T lymphocytes*

Cell source (PI)[a]	N[b]	Stimulant	Blastogenesis (E/C)[c]	Chemotaxis[d]		
				Experimental	Reconstituted control	E/C
Normal (<0.1)	5	None	1.0		9 ± 2	
Normal (<0.1)	2	Plaque	1.2	10 ± 2	8 ± 2	1.2
Periodenal disease patients						
C.O. (2.8)		Plaque	2.9	67 ± 5	26 ± 1	2.6
M.I. (1.5)		Plaque	6.0	26 ± 3	10 ± 1	2.7
S.D. (1.5)		Plaque	13.1	51 ± 7	17 ± 4	3.0
M.P. (1.3)		Plaque	8.5	60 ± 9	24 ± 5	2.5
M.J. (1.3)		Plaque	11.7	84 ± 9	19 ± 4	4.7
R.M. (0.8)		Plaque		29 ± 6	12 ± 1	2.4
B.M. (0.8)		Plaque	6.9	67 ± 5	26 ± 1	2.5

[a] Periodontal index (PI) assessed by the method of Russell (11).
[b] N, Number of experiments.
[c] E/C obtained from dose-response experiments.
[d] Mean number of migrating cells per oil immersion field ± 1 standard error.

TABLE 3. *Chemotactic lymphokine production by human B lymphocytes*

Cell population	Cell source (PI)[a]	N[b]	Stimulant	Blastogenesis (E/C)	Chemotaxis[c]		
					Experimental	Reconstituted control	E/C
B-EAC rosettes	Normal (<0.1)	6	None	1.0		93 ± 7	
	Normal (<0.1)	2	Plaque	1.3	127 ± 25	128 ± 20	1.0
Dissociated B cells[d]	Normal (<0.1)	6	None	1.1		22 ± 6	
	Normal (<0.1)	1	Plaque	1.1	25 ± 5	23 ± 3	1.1
	P.D. patients (1.5-1.3)	2	Plaque	5.3	101 ± 6	6 ± 2	12.6

[a] PI, Periodontal index as assessed by the method of Russell (11).
[b] N, Number of experiments.
[c] Mean number of migrating cells per oil immersion field ± 1 standard error.
[d] EAC disassociated with anti-C3 globulin at 37 C for 30 min.

ble for the considerable and specific B cell stimulation by plaque. However, the antigens in dental plaque which stimulated sensitized lymphocytes from periodontitis patients are reported to be derived from the oral bacteria which comprise plaque (7, 8). In our studies, the use of pooled plaque preparations in large dose-response experiments assured us of the widest possible presentation of such antigens to immune T and B lymphocytes.

The results indicated that both T and B lymphocyte subpopulations from periodontitis patients (PI 0.8 to 2.5) were stimulated to undergo blastogenesis by pooled plaque antigen. It was unlikely that the 3 ± 2% immunoglobulin-negative cells in immune B cell preparations stimulated by plaque or PPD were responsible for the responses, since equivalent blastogenesis levels were obtained with both T and B cell populations. The difference between patients in their magnitude of and dose-dependent blastogenic responses to these two subpopulations may have represented either variations of the in vivo hypersensitivity to one or multiple plaque antigens. Irrespective of this, the results were consistent with plaque stimulation levels obtained in unseparated leukocyte cultures reported by other investigators (3, 7). These data suggested that the in vitro blastogenic responses of unseparated leukocyte cultures to pooled plaque were actually due to a combination of cellular (T cell) and humoral (B cell) mediated immune blastogenic responses to plaque. One must conclude from these studies that in vitro antigen-induced lymphocyte blastogenic responses of leukocyte cultures actually reflect both immune T and B lymphocyte responses.

The data obtained also demonstrated that human B lymphocytes were capable of producing lymphokines after appropriate activation by nonspecific and specific stimuli. Lymphokine synthesis induced by EAC binding to human B cells was shown not to require concomitant blastogenesis. This observation suggested that lymphokine synthesis and blastogenesis are either functions of different lymphocyte subpopulations or require different degrees of membrane stimulation. In contrast to EAC binding, plaque antigen stimulated both T and B lymphocytes to undergo blastogenesis and produce CTX. Antigens, like mitogens (10), may have either stimulated different subpopulations (T_1, T_2, ... and B_1, B_2, ...) or provided multiple membrane stimulation events for their initiation.

B lymphocytes were found, in other studies, to produce a variety of lymphokine activities which include mitogenic (10) and human bone marrow macrophage and granulocyte colony stimulating (J. Prival, submitted for publication) factors. There were a number of compelling reasons which indicated that B cells, rather than any contaminating T cells, were the source of these lymphokines. (i) EAC binding via the C3 receptors was specific for only B cells. (ii) Only after dissociation of EAC and B cells be specifically stimulated by plaque and mitogens (10) to produce CTX. (iii) B cell populations generated quantitatively more lymphokine activity per culture volume than equivalent numbers of T cells. (iv) Reconstitution experiments (10) with T cells neither increased nor decreased EAC-induced B cell production of CTX. But it is quite possible, in view of the observations of Geha et al. (2), that contaminating T cells were directly stimulated or necessary helpers for B cell blastogenesis; however, B cell lymphokine production was found to occur in the absence of blastogenesis (10).

If we accept the concept that lymphokines induced by plaque stimulation of immune lymphocytes contribute to the pathogenesis of periodontal disease, then both T and B cells may be presumably contributing to gingival inflammation, tissue destruction, and bone resorption via lymphokine production. This view is supported by the preponderance of infiltrating plasma (B) cells observed in the gingiva of periodontal patients (12). Furthermore, B cell production of lymphokines may also explain the apparent dichotomy reported by Ivanyi et al. (9), in which lymphocytes from severe, chronic-diseased periodontal patients (PI >4) are blastogenically unresponsive to plaque, but still induce migration inhibition factor lymphokines. The blastogenic inhibitory agents were found to be immunoglobulin M-like antibodies which bound microbial-plaque antigens (6). It is suggested that the binding of plaque antigen-antiplaque antibody aggregates to B cells via their Fc and/or plaque antigen receptors may be sufficient to trigger B cell lymphokine production, but not blastogenesis. Further evidence for this explanation comes from the reports that EA (7*S* antibody) binding to human B cell Fc receptors (10) and anti-gammaglobulin to guinea pig B cell membrane-bound immunoglobulin (15) induced lymphokine production. We therefore propose, on the basis of our investigations, that B cells, as well as T cells, can be stimulated by dental plaque to produce lymphokines whose pharmacological activities are capable of inducing many of the pathogenic effects associated with periodontal disease. Furthermore, these studies indicate that human B cells possess three immunologic capabilities—lymphokine production and antigen-specific blastogenesis as well as antibody synthesis.

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