INFECTION AND IMMUNITY, Oct. 1981, p. 43-49
0019-9567/81/100043-07$02.00/0

Vol. 34, No. 1

Polyclonal B-Cell Activation Induced by Extracts of Gram-Negative Bacteria Isolated from Periodontally Diseased Sites

PETER H. BICK,[1]* A. BETTS CARPENTER,[1] LILLIAN V. HOLDEMAN,[2] GLENN A. MILLER,[1] RICHARD R. RANNEY,[1] KENT G. PALCANIS,[1] AND JOHN G. TEW[1]

Clinical Research Center for Periodontal Disease, Virginia Commonwealth University, Richmond, Virginia 23298,[1] and Virginia Polytechnic Institute and State University,[2] Blacksburg, Virginia 24061[2]

Received 25 March 1981/Accepted 30 June 1981

The objective of this research was to determine whether gram-negative bacteria frequently isolated from periodontally diseased sites contained polyclonal B-cell activators. Polyclonal B-cell activation, which results in nonspecific activation of multiple B-cell clones, was analyzed by a hemolysis-in-gel assay designed to detect a broad range of antibody specificities. Extracts from numerous bacterial strains, including *Bacteroides gingivalis*, *Bacteroides melaninogenicus* subsp. *melaninogenicus*, *B. melaninogenicus* subsp. *intermedius*, *Fusobacterium nucleatum*, *Selenomonas sputigena*, *Capnocytophaga ochracea*, and *Actinobacillus actinomycetemcomitans*, were tested. Extracts of the above organisms were found to stimulate polyclonal antibody responses in cultures of normal human peripheral blood lymphocytes, although the magnitude of stimulation varied among the extracts. Optimal antibody-forming cell responses were found at stimulator doses between 5 and 1,000 μg/ml. We conclude that the resident gram-negative subgingival flora associated with periodontal lesions possesses potent polyclonal B-cell activators. These activators may contribute to disease pathogenesis by inducing B lymphocytes to produce antibody, osteolytic factors, or both and possibly other mediators of inflammation.

Periodontal disease can be characterized by gingival inflammation, soft tissue destruction, loss of attachment, and loss of bone. The presence of an immunological component of the disease has long been suspected, and extensive studies have been made to delineate the nature of this component. Several lines of evidence argue for a significant role of polyclonally stimulated B lymphocytes in the pathology of the disease. Among these are (i) previous reports with in vitro blastogenesis assays do not yield data regarding the specificity of the lymphocyte response and may well reflect a nonspecific polyclonal response (1, 10-12, 13), (ii) both T and B lymphocytes from periodontal patients have been demonstrated to proliferate in response to oral microorganisms and to produce lymphokines in response to dental plaque antigens (11, 16), and (iii) the predominating cell types in the periodontal lesion are of the B-cell series, small lymphocytes, B lymphoblasts, and plasma cells (17, 25). These observations are consistent with the hypothesis that severe periodontal disease pathology is B cell mediated. Furthermore, many microorganisms can induce polyclonal antibody synthesis and blastogenesis in cultures of human peripheral blood lymphocytes (PBLs) (2, 22, 24). Few periodontally associated species, however, have been shown to stimulate human PBLs (28).

In our initial studies, we demonstrated the capability of certain oral microorganisms to function as polyclonal B-cell activators of antibody synthesis in cultures of normal human PBLs. We previously reported that the oral organisms *Actinomyces viscosus* and *Actinomyces israelii* and nonoral isolates of *Bacteroides melaninogenicus*, *Escherichia coli*, and *Actinomyces naeslundii* were all potent polyclonal activators (28).

The microflora associated with periodontal disease is a complex, mixed flora in which more than 150 species have been detected (W. E. C. Moore, L. V. Holdeman, and K. G. Palcanis, J. Dent. Res., Spec. Issue A, abstr. 220, p. 322, 1980). In contrast to the flora in health, the flora in disease has been reported to have a predominance of gram-negative species (27, 29). Therefore, we wished to determine whether representative gram-negative species isolated from the microflora associated with periodontal disease were capable of functioning as polyclonal activators.

The results presented here confirm and extend

the previous findings. We present data demonstrating the polyclonal B-cell-activating ability of several gram-negative oral organisms isolated from the subgingival areas of periodontally diseased sites. Organisms of the genera *Actinobacillus*, *Bacteroides*, *Capnocytophaga*, *Fusobacterium*, and *Selenomonas* (and several yet unnamed organisms) were tested. We concluded that the resident flora associated with periodontal disease possesses potent polyclonal activators which may contribute to pathogenesis.

MATERIALS AND METHODS

Bacterial strains. The strains tested were either selected as representative of the more frequently occurring gram-negative species in samples characterized in our own laboratories or reported as prominent by others. These included *Fusobacterium nucleatum*, *B. melaninogenicus* subsp. *melaninogenicus*, *B. melaninogenicus* subsp. *intermedius*, *Bacteroides gingivalis* (formerly included in *Bacteroides asaccharolyticus* [*B. melaninogenicus* subsp. *asaccharolyticus*]), *Selenomonas sputigena*, *Capnocytophaga ochracea*, and *Actinobacillus actinomycetemcomitans*. Because of known phenotypic or genetic heterogeneities within some of the species, several strains were tested.

In our samples to date, *F. nucleatum* is the most common species in the subgingival flora of periodontally diseased sites. It comprises about 9% of the total cultivable flora and has been detected in 67% of 76 samples analyzed. In addition to two of our isolates (D17B17 and D18B11), we tested two strains (327, previously recognized as *Fusobacterium polymorphum*, and 325 from S. S. Socransky, Forsyth Dental Center, Boston, Mass.). *B. melaninogenicus* subsp. *intermedius* is the second most frequent gram-negative species in our subgingival samples, accounting for nearly 5% of the total cultivable flora. Our strains D16B12A and D10D1 were tested. The saccharolytic selenomonads, represented by *S. sputigena* (our isolate D28M16 was used), account for nearly 2% of the flora, and *B. melaninogenicus* subsp. *melaninogenicus* accounts for 1% (our strains D2B18 and D26B18 were tested). These species also are reported to be prominent constitutents of the flora by others (26, 30, 31). *B. gingivalis* recently has been reported to be associated with severely inflamed sites (30). One of our isolates (D11B26) and one isolate obtained from S. S. Socransky (K32) were tested. *Capnocytophaga* species and *A. actinomycetemcomitans* have been associated with juvenile periodontitis (periodontosis) (19, 30). *C. ochracea* D28B7 was our isolate; *A. actinomycetemcomitans* Y4 and N27 were obtained from A. Tanner, Forsyth Dental Center.

Additionally, three strains (VPI D19F6B, D16B17, and D3A6) of anaerobic gram-negative bacilli with characteristics unlike those of any described species were tested (previously unnamed species account for approximately 40% of all species detected thus far in our periodontal samples). D19F6B belongs to a new species to be named *Bacteroides gracilis* by Tanner et al. (A. Tanner, personal communication). D16B17 represents a group of non-saccharolytic bacilli with polar flagella. D3A6 represents a group of saccharolytic bacteroides, distinct from *B. oralis*, that we have called *Bacteroides* D8 (W. E. C. Moore and L. V. Holdeman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, C41, p. 281).

Bacterial extract preparation. The bacterial strains were harvested from broth cultures in peptone-yeast extract broth with appropriate additives to support growth; they were then washed and suspended in 1 part glycerol to 2 parts phosphate-buffered saline at approximately 0.1 g of cells per ml. The suspensions were stored frozen (−20°C) until sonicated for use.

Cell wall sonicates from the various bacterial isolates were prepared by the method of Baker et al. (1). Bacterial suspensions were thawed, and the cells were washed three times in phosphate-buffered saline. Cells were resuspended in phosphate-buffered saline and sonicated (Biosonik sonicator, Bromwill Scientific Inc., Rochester, N.Y.) for maximum disruption. The degree of disruption was assessed by phase-contrast microscopy. The sediment, consisting largely of cell wall fragments, was the extract used to stimulate PBL cultures in the polyclonal antibody assay. Protein concentrations were determined by the method of Lang (14).

Polyclonal antibody assay. Normal human PBLs were obtained, by Ficoll-Hypaque separation, from the venous blood of individuals who were from 19 to 29 years of age and who did not have periodontitis as defined by loss of attachment. PBLs were cultured in RPMI 1640 supplemented with 2.0 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml (all components from GIBCO Laboratories, Grand Island, N.Y.), and 10% heat-inactivated A^+ or AB^+ serum. The human serum was absorbed twice with sheep erythrocytes before use to remove natural antibody (6, 20). One million PBLs were cultured in 0.9-ml cultures in plastic tubes (12 by 75 mm; Falcon Plastics, Oxnard, Calif.), to which was added 0.1 ml of medium (control) or 0.1 ml of bacterial cell wall extract (experimental) or pokeweed mitogen (positive control; GIBCO) to yield the appropriate final concentrations of activator. Each group was cultured in duplicate or triplicate for 5 to 7 days at 37°C, without being rocked, in a humidified atmosphere of 5% CO_2 in air. For the determination of polyclonal plaque-forming cell (PFC) activation, PBL cultures were centrifuged, washed, and suspended in serum-free RPMI 1640 at a concentration suitable for detecting significant numbers of PFCs. PFCs were determined by using flourescein isothiocyanate-coupled sheep erythrocyte target cells in a thin-layer localized hemolysis-in-gel assay as modified by Fauci and Pratt (8). This assay method detects polyclonal antibody synthesis, because these heavily haptenated target cells detect a broad range of antibody specificities (20). The detected PFCs produce antibody which is not specific for the stimulating bacterial components. This is in contrast to the clonally restricted specific response which would be stimulated by an antigen. Each PBL culture was assayed individually, and viability was determined for each group from pooled samples. The data are expressed as PFCs/10^6 viable recovered cells. Due to the sensitivity of the assay system and the resulting inherent variability, it was difficult to statistically analyze each in-

dividual experiment, as has been observed by others (7; A. S. Fauci, personal communication). Therefore, the mean PFC response for each group was calculated, and the corresponding group means from several experiments were pooled and analyzed statistically by Student's *t* test.

RESULTS

To determine the polyclonal activating ability of periodontally associated bacteria, extracts of the isolated organisms were added to cultures of PBLs from normal volunteers judged to be free of periodontitis. Various doses of the bacterial extracts were added to 10^6 PBLs in 1 ml of RPMI 1640 and cultured for 6 days. After 6 days, the cultures were harvested and assayed for polyclonal antibody synthesis by determination of PFC response of the cultured cells to a heavily haptenated erythrocyte target as described previously (28). Representative experiments for polyclonal antibody responses are presented in Table 1. Each experiment included a positive-control group stimulated by pokeweed mitogen and an unstimulated negative-control group. Three separate experiments are shown. Each experiment was performed with PBLs from a separate normal donor. These experiments are representative of a large series of experiments and illustrate several important points: (i) nearly all of the extracts tested were found to stimulate a polyclonal antibody response, but the magnitude of stimulation varied from organism to organism; (ii) the intensity of the lymphocyte response to a given extract varied among cells from different individuals, suggesting that there exist high and low responders for these bacterial extracts; (iii) the optimal stimulatory dose of the bacterial extracts and pokeweed mitogen varied among the experiments, supporting the concept that individual patterns of responsiveness to these activators exist in the normal population; and (iv) although the degree of responsiveness to the positive control, pokeweed mitogen, may not vary greatly between two experiments, the response to a selected bacterial extract may be very different. These data suggest that these extracts may be affecting different B-lymphocyte subpopulations and that the responsiveness of the B-cell populations, or their regulation, varies among the individuals tested.

Because of the observations outlined above, it is imperative that each bacterial extract be tested at several doses and with PBLs from several normal donors. Furthermore, due to the

TABLE 1. *Representative experiments for polyclonal antibody responses induced by B. melaninogenicus subsp. intermedius, B. melaninogenicus subsp. melaninogenicus, and F. nucleatum*

Group	Dose (μg/ml)	Anti-FITC-SRBC PFCs[a] per 10^6 viable recovered cells[b]		
		Expt 1	Expt 2	Expt 3
Extract tested[c]				
B. melaninogenicus subsp. *melaninogenicus* (D26B18)	50	785 ± 522	723 ± 370	98 ± 37
	100	187 ± 105	400 ± 364	500 ± 419
	200	1,159 ± 705	227 ± 150	417 ± 145
B. melaninogenicus subsp. *intermedius* (D10D1)	50		18 ± 12	17 ± 9
	100		49 ± 28	73 ± 37
	200		114 ± 23	12 ± 10
F. nucleatum (325)	50	2,091 ± 817	166 ± 84	94 ± 30
	100	4,273 ± 2,106	420 ± 209	951 ± 209
	200	92 ± 61	572 ± 168	377 ± 207
F. nucleatum (327)	50	5,583 ± 1,344	98 ± 76	329 ± 140
	100	459 ± 219	19 ± 10	462 ± 197
	200	0	1 ± 1	10 ± 5
Controls				
Unstimulated		6 ± 5	36 ± 27	0
Pokeweed mitogen	1:100[d]	215 ± 10	416 ± 94	334 ± 87
	1:200[d]	461 ± 64	1,515 ± 407	1,207 ± 494
	1:500[d]	538 ± 197	1,606 ± 498	672 ± 53

[a] PFCs against fluorescein isothiocyanate-coupled sheep erythrocytes.

[b] Values are expressed as means ± standard error.

[c] One million PBLs from normal donors were cultured in triplicate with various doses of the organism extracts for 6 days and assayed for polyclonal antibody synthesis.

[d] Dilution.

inherent variability of this system with human cells, it becomes necessary to pool data from several experiments to facilitate statistical evaluation. The maximal responses at the optimum stimulatory dose were pooled from several experiments ($n = 3$ to 17) for each bacterial extract and evaluated. Extracts from all but two of the strains tested stimulated a polyclonal antibody response, but the magnitude varied considerably, even when strains from organisms of the same species were used, e.g., *B. melaninogenicus* subsp. *intermedius* D16B12A versus D10D1 (Fig. 1). Also, extracts of certain organisms were, overall, more stimulatory than others. Extracts of *B. melaninogenicus* subsp. *intermedius* D16B12A were weak activators compared with extracts of *F. nucleatum*, *B. melaninogenicus* subsp. *melaninogenicus* D2B18, or the unnamed gram-negative rod D3A6.

Overall, all of the oral bacterial isolates demonstrated the ability to polyclonally stimulate PBLs to produce antibody. The bacterial extracts varied in the potency of their activity, and the PBL populations of normal individuals are heterogeneous in their degree of responsiveness to the extracts.

DISCUSSION

The present study establishes that gram-negative organisms isolated from periodontal lesions possess potent polyclonal activating properties for human B lymphocytes. We recently proposed a model of periodontal disease that focused on B-cell reactivity as a causative component (28) because of the following observations: (i) the vast numbers of lymphocytes of the B-cell lineage found in periodontal lesions (17, 25), (ii) the capability of B cells to function in cell-mediated phenomena and to produce osteolytic factors (4, 15, 23), (iii) hyperresponsiveness to the B-cell polyclonal activator staphylococcal protein A by PBLs from young adults with severe periodontal disease (28), and (iv) the potent polyclonal B-cell-activating properties of microorganisms associated with the disease. Claggett and Engel recently also proposed a role for polyclonal activation in periodontal disease (5).

In our model of young adult severe periodontal destruction, a hyperreactive B-cell state would exist in the disease-susceptible individual. This could result as a failure of T-lymphocyte- or macrophage-regulatory components or as a primary B-cell lesion. Polyclonal stimulation of the susceptible B-cell population by the polyclonal activators in the resident subgingival flora would result in any of several B-cell responses, including proliferation, polyclonal antibody production, and the release of osteolytic lymphokines (including osteoclast-activating factor). These responses would lead to increased inflammation in the disease site, activation of bone resorption, and loss of periodontal support for the teeth.

The data presented here support the possibility for such a mechanism and also suggest that polyclonal activation could be a pathogenic mechanism in less dramatic instances of periodontal disease. For example, periodontitis in an individual with normal B-cell responsiveness could result from the sequelae of polyclonal activation if activators are present in sufficient quantity. We noted that nearly all of the bacterial extracts stimulated polyclonal B-cell antibody production in cultures of normal PBLs. However, different individuals demonstrated highly varied response patterns to the panel of extracts tested, indicating that there exist high and low responders for a given organism. Indeed, among periodontally diseased patient populations, we may find a skewed pattern of responsiveness. Differences in the blastogenic responses of periodontal patients to oral microorganisms have been reported (1, 10, 12, 13, 16, 21). In our studies, separate individuals displayed a different dose-response curve, suggesting differential susceptibilities to activation. In addition, differential age-dependent responses to polyclonal activation have been observed among inbred strains of mice, suggesting a genetic control of the level of responsiveness (18).

One possible interpretation of the data is that the bacterial extracts affect different B-cell subpopulations and their susceptibility to stimulation varies among individuals. Previous reports in human and mouse systems indicate that this is the case (8, 9). One might expect that suboptimal doses of organisms that affect different B-cell subpopulations may synergistically give an elevated response. One ligand may drive an immature B-cell population to differentiate and enlarge the pool of relatively mature B cells which are sensitive to a second ligand. In support of this hypothesis, we have observed that extracts prepared from whole dental plaque are much more potent polyclonal activators than are several of our extracts from pure cultures. The optimal activating dose of the dental plaque extracts is much lower than that of the single bacterial extracts, suggesting that synergistic stimulation may occur in vivo (unpublished data). This finding correlates well with the observation that severity of disease is directly related to the amount of dental plaque present. Previous studies indicate that extracts of *A. viscosus* stimulate B lymphocytes from germfree rats and thymusless "nude" mice (3). The patho-

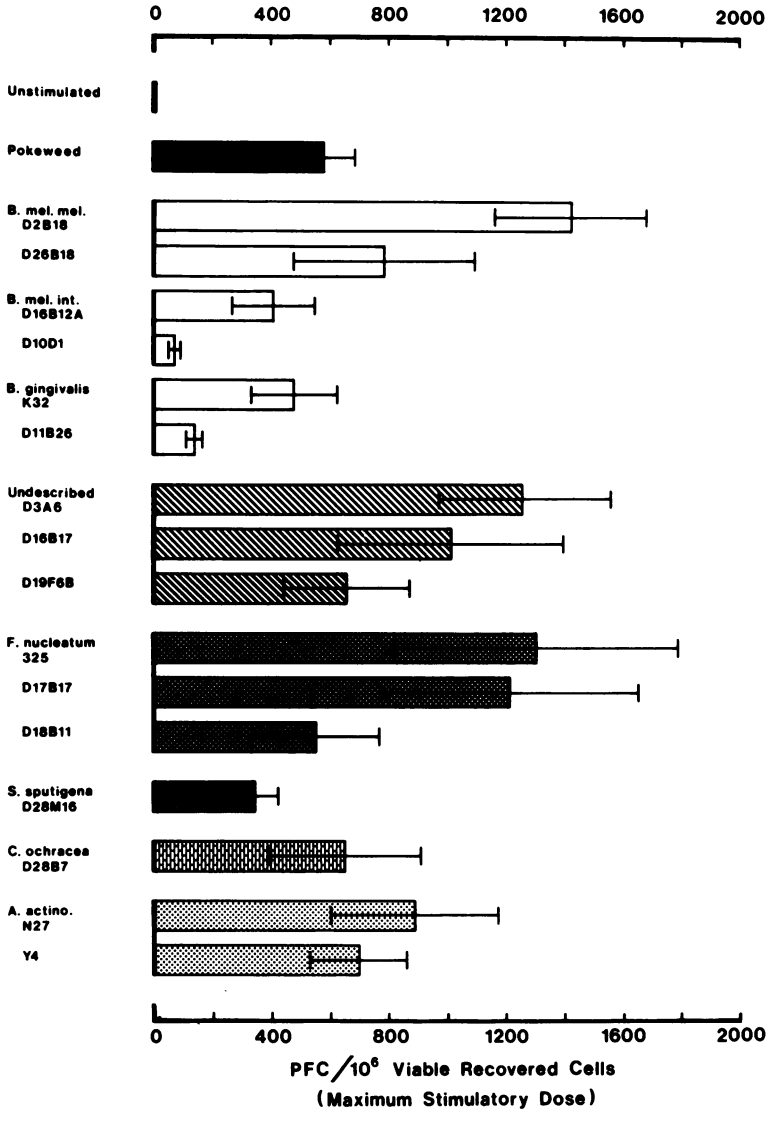

FIG. 1. *Summary of polyclonal antibody responses stimulated by extracts of gram-negative organisms isolated from periodontally diseased sites. PBLs were cultured alone (unstimulated) or with various doses (5 to 1,000 μg/ml) of a bacterial extract. After 5 to 7 days, the cultures were assayed for polyclonal PFCs. For statistical comparison, the optimal PFC response values were pooled from individual experiments (regardless of dose), and the mean ± standard error of each bacterial extract group and the unstimulated group was compared by Student's t test. The number of experiments included in the various groups was: unstimulated, 50; pokeweed mitogen, 50; and bacterial extract, 3 to 20. Three of the tested extracts were from previously undescribed anaerobic gram-negative bacilli (D3A6, D16B17, and D18B11). All of the bacterial extract-stimulated PFC responses were significantly different from the unstimulated control group ($P < 0.001$) with the exception of B. gingivalis (D11B26) and B. melaninogenicus subsp. intermedius (D10D1), which were not significant. Pokeweed mitogen-stimulated responses were different from control responses ($P < 0.001$).*

logical consequences of having localized sites infected with organisms capable of such strong stimulatory effects as demonstrated here must be considered in models that attempt to explain the mechanisms of periodontal disease.

Another facet of our proposed model of B-cell reactivity in periodontal disease is the production of osteolytic factors and osteoclast-activat-

ing factors after polyclonal activation. Recently, we have observed the generation of osteolytic factors or osteoclast-activating factors in the same PBL cultures in which bacterial extracts generated a strong polyclonal antibody response (J. G. Tew et al., manuscript in preparation). The osteolytic activity could not be attributed to the bacterial extracts themselves and is therefore a product of the responding PBL population. We are now attempting to define the cellular site of production and regulation of the osteolytic factor.

Experiments are currently under way to define the subsets of B cells responding to the bacterial extracts and the regulatory pathways of these responses in normal individuals and in patients with severe periodontal disease.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant DE-05139 from the National Institute of Dental Research.

The fine technical assistance of E. Sully, P. Dickinson, and P. L. Rice is gratefully acknowledged.

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