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Accumulation of Plasma Cells in Inflamed Sites: Effects of Antigen, Nonspecific Microbial Activators, and Chronic Inflammation

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Plasma cells are common in chronically inflamed sites, including periodontal lesions. The aim of this study was to determine which factors contribute to this local accumulation of plasma cells. Specifically, we sought to evaluate the effects of specific antigen and nonspecific activators from an infectious agent associated with chronic inflammation (*Fusobacterium nucleatum*, an organism prominent in chronic periodontal lesions) and the effect of the chronic inflammation itself. Chronic inflammation (14 to 17 days) was induced in horseradish peroxidase (HRP)-immune rabbits by subcutaneous injection of 50 μl of sterile alum in several sites in their backs. Controls included sites injected with saline or more acute sites examined after 3 days of alum inflammation. Sites were challenged with HRP (the antigen), sonicated *F. nucleatum* (the nonspecific activator), or both together to see whether *F. nucleatum* has an adjuvant effect. Three days after challenge, HRP-specific antibody-forming cells (AFC) were enumerated after peroxidase histochemistry. In noninflamed sites or sites with acute inflammation, virtually no HRP-specific AFC were evident. In contrast, chronic inflammation alone was sufficient to elicit a specific AFC response (≈10 cells per mm^2). Addition of either *F. nucleatum* or HRP to the chronic lesion about doubled the number of HRP-specific AFC. However, a dramatic 8- to 15-fold (80 to 150/mm^2) increase was seen in chronically inflamed sites challenged with antigen and activator together. Interestingly, the activator did not have this adjuvant effect in the acute sites or in normal skin. In short, accumulation of plasma cells in inflamed sites is promoted by chronic inflammation, activators of microbial origin, and specific antigen. This milieu can be expected to develop in some periodontal lesions and could help explain why gingival crevicular fluid from some sites may contain extraordinary levels of locally produced specific antibodies for certain antigens.

Periodontitis is an example of a chronic inflammatory disease in which the predominant cells infiltrating the lesion are of B-cell lineage (31). Development of periodontitis typically depends on the accumulation of certain oral bacteria in gingival crevices. Studies with gingival crevicular fluids show that local specific humoral responses may be mounted against antigens from certain periodontitis-associated bacteria (6, 32). In addition to bacterium-specific antigens, these bacteria are known to contain potent polyclonal B-cell activators (PBA) (1–4, 7, 17–19, 27, 31). We have suggested that local nonspecific or polyclonal responses may be an important factor in the periodontal disease process (31). We have also shown that PBA may have a dramatic adjuvant effect and greatly enhance antigen-induced B-lymphocyte responses in vitro (33). Interestingly, recently stimulated B lymphocytes must be present for PBA to have an optimal effect including their adjuvant effect (22, 31, 33).

A recent in vivo study with rabbits indicated that PBA-stimulated responses may occur in the local chronic inflammatory milieu created by ligature-induced periodontitis (16). However, neither the effect of the chronic inflammatory process alone nor the effect of local microorganisms on the PBA response was studied. The present in vivo study was designed to determine the effects of chronic inflammation, nonspecific activator, and specific antigen alone and in combination on the local accumulation of antibody-forming cells (AFC). To do this, sterile lesions were induced in rabbit skin such that the amount of polyclonal activator, inflammation, and antigen in a site could be controlled. The results showed that, in recently primed rabbits, chronic inflammation, antigen, or nonspecific activator alone or in any combination could induce local accumulations of AFC. However, by far the most potent recruiter of antigen-specific AFC was the combination of antigen, activator, and chronic inflammation. The fact that combinations of antigen, nonspecific activator, and inflammation are present in periodontal lesions may explain the extraordinary amount of antibody synthesis that occurs in some periodontal lesions (6, 25, 32).

MATERIALS AND METHODS

Animals. New Zealand White male rabbits (2 kg) were obtained from Blue and Grey Rabbitry (Ayllett, Va.). The animals were kept in standard cages, with free access to food and water.

Antigen and activator. Horseradish peroxidase (HRP) (type VI; Sigma Chemical Co., St. Louis, Mo.) was used as the antigen in this study. HRP was chosen as the antigen in this study because it is histochemically detectable and its enzymatic activity is not neutralized by either specific antibody (29, 30) or by histochemical blocking agents such as milk and normal goat, rabbit, or bovine serum. *Fusobacterium nucleatum* was chosen as the primary activator for this study because it is one of the most common periodontitis-associated organisms and has potent PBA activity (1). *F. nucleatum* D42-1 was kindly provided by W. E. C. Moore and L. V. Moore of Virginia Polytechnic Institute and State University, Blacksburg, Va. *F. nucleatum* was isolated from a subgingival sample from a patient with periodontitis. The strain was grown in mass cultures at Virginia Polytechnic Institute, centrifuged, frozen, and shipped to the Medical

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College of Virginia. Prior to use, *F. nucleatum* samples were sonicated and the protein concentration was determined as described previously (1). *Actinobacillus actinomycetemcomitans* N-27, known to be a good PBA, and *Selenomonas sputigena* D28M-16, a weak PBA, were also used as activators. These bacteria were isolated, cultured, and treated like *F. nucleatum*.

Immunizations. Rabbits were immunized in both hind feet with 0.5 mg of HRP (no. P8250; Sigma Chemical Co.) in Freund's complete adjuvant (Difco, Detroit, Mich.). Two weeks later, secondary, or booster, immunizations were given by injection of 0.5 mg of HRP in phosphate-buffered saline (PBS) (pH 7.2) into both hind feet.

Induction of inflammation. Four weeks after the booster immunization, multiple inflammatory sites were induced by injecting 50 μl of alum per site (Maalox-TC; Rorer Pharmaceutical Corp., Fort Washington, Pa.) subcutaneously in the backs of the HRP-immune rabbits. Alum will remain in a site for months, inducing a cellular infiltrate (11). After 10 days of alum-induced inflammation, the rabbits were given a booster immunization via subcutaneous injection with 0.5 mg of HRP in PBS into both hind feet. The immunization provides recently stimulated cells in the circulation, which are needed to optimize the polyclonal response (16). Four days after the booster immunization, individual inflammatory sites were injected with 5 μg of *F. nucleatum*, 5 μg of HRP, or a combination of both in a volume of 50 μl of sterile PBS. Also at this time, new (acute) inflammatory sites were established on the contralateral sides of the rabbits' backs. These acute sites and the chronic sites on the rabbits were injected on the same day with the same combinations of *F. nucleatum* and HRP. Three to four days later, all sites were surgically removed and fixed with a solution of 1% paraformaldehyde–0.9% glutaraldehyde in cacodylate buffer (pH 7.4). Controls included inflamed sites without *F. nucleatum* or HRP injections and noninflamed skin without injections or with injections of HRP, *F. nucleatum*, or a combination of the two.

Histochemistry. In preparation for vibratome sectioning, all sites were fixed for 1 h and then cut into 1.0-mm-thick sagittal pieces with a razor blade. The 1.0-mm-thick pieces were then fixed for an additional 5 h, washed overnight in cacodylate buffer, embedded in 8.5% Noble agar (Difco), and sectioned at a 50-μm-thickness setting with an Oxford vibratome. To demonstrate cells producing antibody specific for HRP, the vibratome sections were first incubated in methanol with 0.02% H_2O_2 to reduce endogenous peroxidase activity (28). This treatment was highly effective (see Fig. 2E). After three washes in cacodylate buffer, the tissue was incubated in a solution of 0.005% HRP in cacodylate buffer for 24 h to allow ample time for HRP to penetrate the sections. The sections were washed for 24 h in buffer and then incubated with a solution of 3,3-diaminobenzidine and 0.01% H_2O_2 to visualize the bound HRP. After three washes in cacodylate buffer, the sections were mounted on slides in cacodylate buffer, covered with coverslips, sealed with clear nail polish, and evaluated with an Olympus BH-2 microscope. HRP-binding cells were identified as plasma cells by their histological appearance (see Fig. 2D). In a previous study using the same histochemical system (16), electron microscopy confirmed that the HRP-binding cells were plasma cells. In that study, specificity controls for antigen binding, using histochemically detectable antigens HRP and glucose oxidase, indicated serologic specificity. Furthermore, antigen-specific plasma cells were not found in nonimmune rabbits. By using an ocular micrometer calibrated in millimeters, the number of AFC per square millimeter was determined. Only HRP-binding AFC located in connective tissue lateral and deep to the alum, at a distance of at least 1.0 mm but no more than 3.0 mm distal from the alum deposit, were counted. The distribution of AFC in this area tended to be quite uniform. One to four sites for each treatment of antigen and activator were examined. HRP-specific AFC in 5 to 8 sections per site were counted. The data are expressed as the mean number of HRP-specific AFC of all sections counted.

RESULTS

Histology of the chronic lesion. After 2 weeks of alum-induced inflammation, the tissue had features typical of chronic inflammation characterized by a mononuclear cell infiltrate (plasma cells, lymphocytes, and macrophages) (23) in hematoxylin and eosin sections. The alum appeared to be surrounded by a fibroblastic proliferation rimmed by the infiltrating plasma cells and lymphocytes.

Accumulation of specific AFC in chronic lesions. A dose-response study was done by using HRP or *F. nucleatum* injected directly into the inflamed sites to establish the activity of each stimulant. The chronically inflamed site contained approximately 10 HRP-specific AFC per mm^2 in the absence of antigen or activator (Fig. 1). However, both the specific antigen and the nonspecific activator had the ability to increase the number of HRP-specific AFC above the background of the chronically inflamed sites alone. The maximum response induced by either antigen or activator alone was approximately 30 to 40 AFC per mm^2 (Fig. 1) (the histological appearance of AFC is illustrated in Fig. 2B to D; note that most AFC were large and had eccentric nuclei typical of plasma cells). The dose-response curves with activator tended to be irregular and somewhat unpredictable, although activator replicated well within an experiment. Abscess formation was sometimes associated with higher doses of antigen. The 5-μg amount of HRP and *F. nucleatum* was close to the maximum stimulating dose for either agent alone. Therefore, the 5-μg amounts of HRP and *F. nucleatum* were used in subsequent studies to avoid abscess formation.

Adjuvant effect of *F. nucleatum*. HRP and *F. nucleatum* were injected together into chronically inflamed sites to determine whether *F. nucleatum* exhibited an adjuvant effect on HRP-specific AFC accumulations. Figure 2 is a light micrograph illustrating HRP-specific AFC in tissue sections from such an experiment. Note the abundant accumulation of HRP-specific AFC in sites where HRP and *F. nucleatum* were used in combination (Fig. 2C and D). The dramatic nature of the adjuvant effect is presented in Table 1. Addition of either specific antigen or nonspecific activator into a chronically inflamed site approximately doubled the number of HRP-specific AFC over that of chronically inflamed sites alone. However, the number of HRP-binding AFC observed in sites challenged with the combination of HRP plus *F. nucleatum* was more than five times larger than in sites challenged with HRP alone (100 versus 17 AFC per mm^2). Control antigen-activator combinations not listed in Table 1 included the following: (i) replacing HRP with the irrelevant antigen bovine serum albumin, which had no effect on HRP-specific AFC accumulations; (ii) replacing *F. nucleatum* with the well-known, less complex PBA, pokeweed mitogen, giving an adjuvant effect almost identical to that of *F. nucleatum*; and (iii) replacing *F. nucleatum* with other periodontal organisms with known polyclonal activity, such as *A. actinomycetemcomitans* (a good PBA) (1) and *S.*

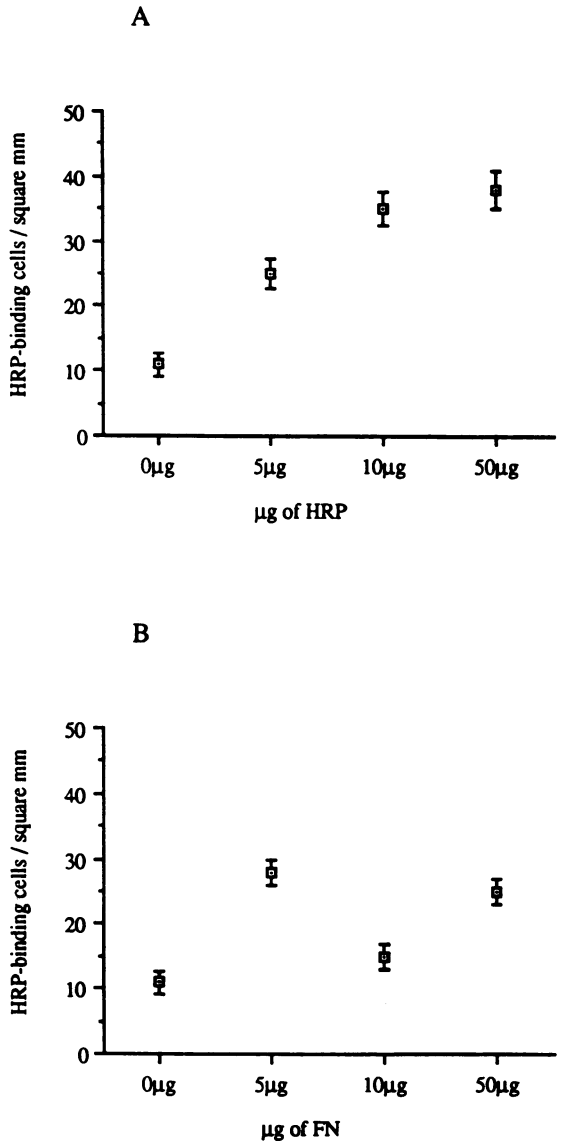

FIG. 1. Effects of antigen (HRP) and nonspecific activator (*F. nucleatum* [FN]) on the accumulation of HRP-specific AFC in chronically inflamed skin. The dose-response curve for HRP is in panel A, and the dose-response curve for *F. nucleatum* is in panel B. The bars represent standard errors of the means.

sputigena (a weak PBA) (4). *A. actinomycetemcomitans* had good adjuvant effect, with 121 AFC per mm^2, and *S. sputigena* in the same experiment was active but much weaker, with 51 AFC per mm^2.

Acute versus chronic inflammation. In view of the adjuvant effect of *F. nucleatum* in chronic sites but not in normal skin, we sought to determine whether a chronic inflammatory environment was necessary or whether a more acute inflammatory environment was adequate to facilitate plasma cell accumulations. To do this, the influence of a 3-day (acute) inflammatory environment was compared with that of 17-day chronically inflamed sites. Multiple (4 to 8) chronic and acute sites were assessed on each of three rabbits. The results shown in Fig. 3 are mean responses of the three replicates. In acute inflammatory sites (alum alone), HRP-specific AFC were rarely observed, whereas, in chronic inflammatory sites on the same animal, HRP-binding cells were observed in typical numbers (about 10/mm^2). Injection of combined HRP and *F. nucleatum* into acute sites increased the number of HRP-specific plasma-binding cells to about 10 cells per mm^2, which is similar to results obtained by injecting HRP or *F. nucleatum* into normal skin (Table 1). In contrast, injection of both HRP and *F. nucleatum* into chronic sites induced the typical dramatic response, with over 100 HRP-specific AFC per mm^2.

DISCUSSION

The results of this study support the concept that something in the environment associated with chronic inflammation is essential for optimal local accumulation of antigen-specific plasma cells. A clear distinction was apparent between acute inflammatory sites (3 days of irritation) which generally lacked detectable antigen-specific AFC and chronic sites on the same animals which contained about 10 specific AFC per mm^2. However, the distinction between chronic inflammatory sites and normal or acute sites was most apparent when the adjuvant activity of the activator was being tested. In chronic sites, the activator synergized with antigen to produce an extraordinary accumulation of antigen-specific AFC (Table 1 and Fig. 3). In contrast, in normal tissue or acute inflammatory sites, it did not appear that the effects of antigen and activator were even additive (Table 1 and Fig. 3).

Many in vitro studies have shown that periodontitis-associated bacteria contain potent PBA (1–4, 7, 17–19, 27, 31). Results from our laboratory show that antigen and PBA together are manyfold-more-powerful activators of antigen-specific B cells in vitro than either one alone (33). The results from this study confirm and extend these in vitro findings (33) in vivo. The in vivo phenomenon of high local immune responses in certain periodontal sites (6, 15, 32) is probably attributable to a combination of local factors which include (i) recent antigen-induced activation of antigen-specific T and B cells with increased sensitivity to nonspecific activators (16, 22); (ii) specific antigen in the local site (Fig. 3 and Table 1); (iii) nonspecific activators in the local site (Fig. 3 and Table 1); and (iv) the local milieu of chronic inflammation (Table 1 and Fig. 3). Most important, the data show that it is crucial that all four of these factors are concurrently present to attain optimal local plasma cell accumulation. The omission of just one of these four factors invariably results in greatly diminished plasma cell accumulation.

We were surprised that the acute sites did not demonstrate even a modest enhancement of plasma cell accumulation. This may relate to the lack of lymphocyte homing receptors or cell adhesion molecules. There is evidence which suggests that at least 1 week is required before effective lymphocyte extravasation into inflamed tissues can occur and that the route of lymphocyte entry into chronically inflamed tissue is through high endothelial venules (HEV) (8, 26). It has been reported that, after 1 week of *Mycobacterium bovis* BCG-induced chronic inflammation, lesions in rat skin demonstrate venules with morphological and histochemical characteristics identical to those of HEV (9). Furthermore, HEV-like surface antigens identified by the HEV-specific monoclonal antibody MECA-325 are expressed by endothelia of inflammation-associated microvasculature only after at least 1 week of inflammation (5). The expression of such vascular addressins by HEV is important for lymphocyte extravasation, and thus the expression of addressins by inflamed tissues may be dependent on chronic inflammation.

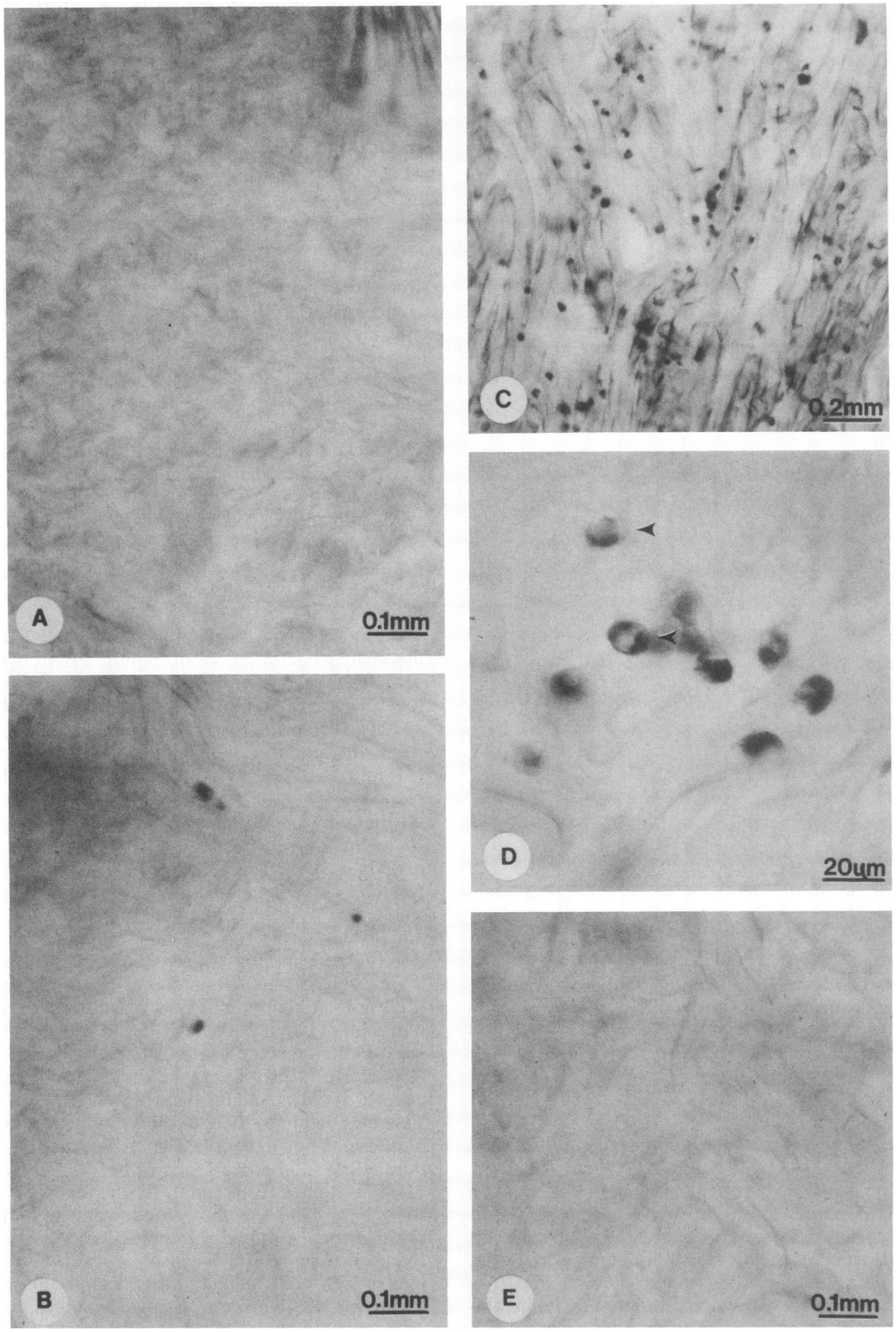

FIG. 2. The histochemical identification of HRP-specific AFC in rabbit skin. The tissues used in panels A to D were treated to reveal HRP-binding plasma cells. (A) Tissue was prepared from healthy skin, and no HRP-specific AFC are apparent. (B) Tissue was prepared from a chronically inflamed site, and a few HRP-specific AFC are apparent. (C and D) Tissues were prepared from a chronically inflamed site injected with both *F. nucleatum* and HRP. Note the hundreds of HRP-binding cells in these sections. The arrowheads in panel D point to cells with typical plasma cell morphology. Note the eccentric unstained nuclei and the cytoplasm darkened with the HRP reaction product. (E) Control section which was adjacent to the section in panel D. Tissue in panel E was treated as that in panel D except that it was not incubated in HRP. Note the lack of any endogenous peroxidase activity in this histochemical control.

TABLE 1. The adjuvant effect of *F. nucleatum* on HRP-specific AFC accumulation in chronically inflamed skin

Rabbit	No. of HRP-specific AFC/mm² after injection in[a]:							
	Skin with chronic alum-induced inflammation				Healthy skin			
	Alum[b]	HRP (5.0 μg)	*F. nucleatum* (5.0 μg)	HRP + *F. nucleatum* (5.0 μg)	PBS[c]	HRP	*F. nucleatum*	HRP + *F. nucleatum*
1	10 ± 1	19 ± 1	ND	106 ± 9	ND	ND	ND	ND
2	10 ± 1	14 ± 1	17 ± 1	89 ± 7	ND	ND	ND	ND
3	6 ± 1	6 ± 1	ND	96 ± 3	0.1	11 ± 1	15 ± 4	24 ± 4
4	10 ± 2	28 ± 5	21 ± 3	108 ± 5	0	10 ± 1	12 ± 2	14 ± 2
Mean ± SE	9 ± 1	17 ± 5	19	100 ± 5	0.05	10.05	13.5	19

[a] Values represent the means ± the standard errors of the means of HRP-specific AFC observed in at least five tissue sections. ND, not done.
[b] Alum alone (50 μl) injected into several sites on one side of the rabbits' backs.
[c] Control sites were injected with 50 μl of PBS.

In conjunction with the appearance of HEV in chronically inflamed tissues, it is possible that B lymphocytes, like T lymphocytes, express very late activation antigens. As reviewed by Yokoyama et al. (35), very late activation antigens are only expressed on T lymphocytes 14 days after antigen activation. Very late activation antigens are thought to be of the integrin family of cell surface adhesion antigens which may facilitate extravasation into local inflammatory sites (12). However, to date, very late activation antigens have not been reported to be expressed on B lymphocytes.

Recruitment of lymphocytes from the HEV into chronically inflamed tissues may be promoted by chemoattractants. As a result of inflammation, lymphocyte chemotactic agents such as denatured proteins (24), IL-1 (21), C5a (13a), and LCF-a (13, 20) are known to be released by inflammatory tissues. In addition, extracts of *F. nucleatum* have been shown to have chemokinetic influence on B lymphocytes (10). It has also been shown that, once lymphocytes have migrated into the inflamed tissue, antigen in the inflamed sites can selectively retain antigen-specific T lymphocytes (14). The requirement for antigen in the inflamed sites in this study suggests that antigen-specific B lymphocytes may also be selectively retained. The requirement of recently stimulated lymphocytes to obtain optimal PBA responses is supported by work by us and others (22, 33). Thursh and Emeson (34) showed in adoptive transfer experiments that recently stimulated lymphocytes but not lymphocytes which have reverted to a resting state are able to localize to

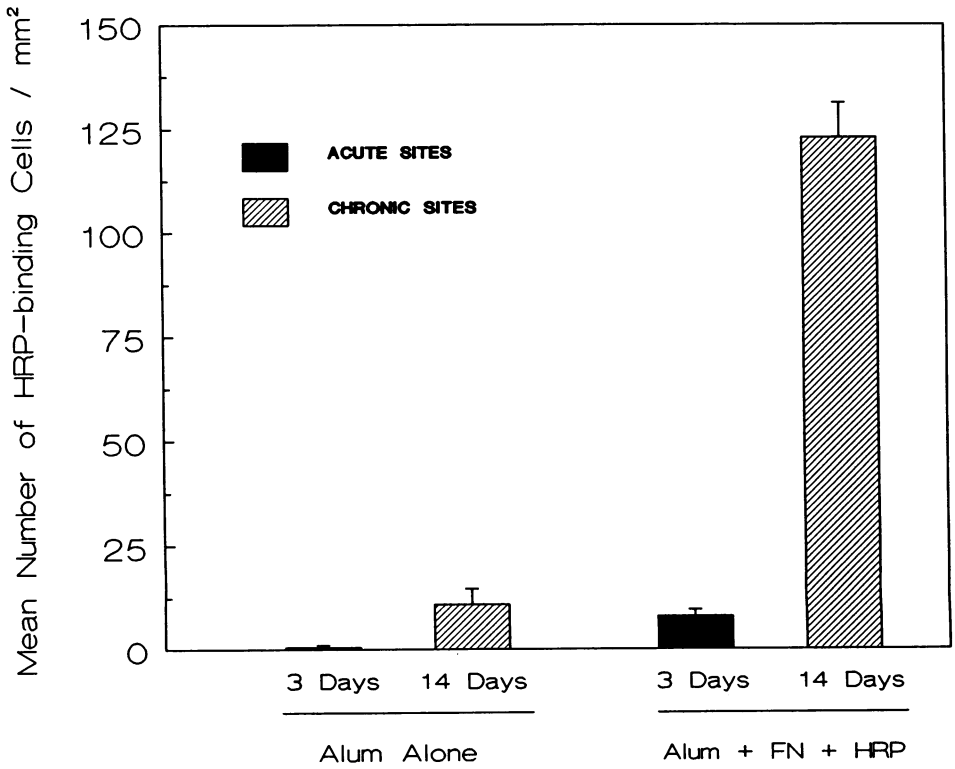

FIG. 3. Comparison of chronic inflammation and acute inflammation on local HRP-specific AFC accumulations. Three HRP-immunized rabbits were used, and the data represent the means and standard errors of the means from these three replicates. Each rabbit had eight chronic and four acute alum sites. The HRP-specific AFC in at least five tissue sections from each site were counted. FN, *F. nucleatum*.

antigen-stimulated lymph nodes. Furthermore, recently stimulated B lymphocytes are also more responsive to activation by PBA (22, 33). Clearly, immunoregulatory effects which may occur in inflamed gingival tissue could be complex.

In conclusion, the implications are that B-cell recruitment and AFC maturation are markedly influenced by events occurring during the chronic inflammatory process. The mechanisms are unknown, but vascular addressins or local increases in certain cytokines could play a role. Either antigen or nonspecific activators of microbial origin may markedly increase the total plasma cell population in chronically inflamed sites, and the effect of the combination of these factors may be dramatic. The large number of plasma cells that bind periodontitis-associated bacteria in some human gingival tissue (25) and the extraordinary titers of antibody specific for oral bacteria found in the gingival crevicular fluid of some periodontal lesions (6, 32) may be due to the combined effects of microbial antigen activation, nonspecific microbial activators, and recently stimulated microbe-specific B lymphocytes in the milieu. The accumulation of a large amount of local specific antibody could be helpful in controlling the microorganism in the local site. Alternatively, the large numbers of metabolically active AFC may contribute to the pathological process associated with periodontal disease. We look forward with interest to studies addressing these issues.

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