Deposits of immunoglobulins, complement, and immune complexes in inflamed human gingiva

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Gingival biopsy specimens from 20 patients with moderate to advanced periodontitis were obtained from inflamed sites with pockets of 5 mm or more. Sections were studied by an immunofluorescence technique, using polyclonal rabbit or goat anti-IgG, anti-IgM, anti-C1q, anti-C3a, and anti-C3c and mouse monoclonal anti-C9. Prewashed ethanol-fixed and nonwashed ethanol-fixed or frozen specimens showed many plasma cells staining for IgG or C3a, suggesting the possible occurrence of a receptor for C3a in plasma cells. Plasma cells containing IgM were also seen. Deposits of IgG and IgM with C1q, C3a, and C3c, suggesting immune complexes, were demonstrated by a double staining technique, combining fluorescein (FITC) or rhodamine (TRITC)-labeled anti-immunoglobulins with TRITC- or FITC-conjugated antibody to C3a, C3c, and C1q. The complexes were located mainly within or around vessel walls. Deposits of C3a and C1q were found in vessel walls, in the basement membrane zone of oral gingival epithelium, or diffusely distributed in the tissues. Deposits of C3c were found to a lesser extent and only in vessel walls. Mouse monoclonal anti-C9, visualized with FITC-labeled rabbit anti-mouse and swine anti-rabbit antiserum, showed granular deposits of C9, mainly in the basement membrane zone of oral gingival epithelium. The study indicates the involvement of immune complex vasculitis in inflammatory periodontal lesions. Also, our observations of the occurrence of deposits of complement factors support the hypothesis that complement factors play an important role in the immunopathology of the periodontal lesion.
□ *Complement; immune complexes; immunoglobulins; periodontitis*

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Attström et al. (1) and Schenkein & Genco (2) have demonstrated local activation of complement via the alternative pathway as evidenced by complement factor C3 and factor B cleavage in adult periodontitis and in localized juvenile periodontitis patients. The marked depression of complement factor C4 levels in juvenile periodontitis (2) also indicated complement activation via the classical pathway.

The discussion about the presence of immune complexes (antigen and antibody bound together) and their contribution to the immunopathology of periodontal lesions has been going on for many years. Early reports by Genco & Krygier (3) and Toto et al. (4), using specific fluorescein-conjugated antibodies, indicated the occurrence of small deposits of immune complexes containing complement components in diseased human gingiva. Platt et al. (5) and Genco et al. (6), using a direct fluorescent antibody technique, sought to localize immune complexes and complement factor C3 in gingival tissue. They demonstrated that occasional gingival samples from subjects with gingivitis or periodontitis showed immunoglobulins or complement deposits. Using fluorescein-conjugated antisera, Genco et al. (6) showed that extensive washing of gingival tissues reduces the amount of detectable immunoglobulins and that complement factors C3 and C4 are occasionally fixed to the tissue. They concluded that most of the proteins are present

in the interstitial fluid in soluble form rather than as immune complexes fixed to tissue components.

These findings were confirmed by Clagett & Page (7), who failed to extract insoluble immune complexes from diseased gingival tissues taken from either humans or dogs. When they reapplied fluoresceinated eluates to diseased gingival tissues taken from the same and from other individuals, the frequency and intensity of binding of immunoglobulins and complement factor C3 were very low. In a recent study Pecović & Fillery (8), using a direct and indirect double-staining immunofluorescence technique, claimed to have demonstrated the occurrence of antigen–antibody–complement complexes in inflamed human gingiva.

The conclusion from the above studies is that, at least in gingivitis and in adult chronic marginal periodontitis, deposits of immune complexes with complement are not a major component of periodontal immunopathologic changes (9).

Recently, more refined fixation methods have been developed, preserving the antigenicity of the immunoglobulins and complement. Also, specific antisera to several complement factors have become commercially available. It has been possible to demonstrate, by a double-staining immunofluorescence technique, both immunoglobulins and various complement components at the same site in dental periapical granulomas (10).

The aim of this investigation was to study the presence and location of deposits of complement factors C1q, C3a, C3c, and C9 in sections of washed and nonwashed specimens of inflamed gingival tissues, and the concomitant occurrence of deposits of these complement factors and immunoglobulins (IgG or IgM) at the same location in such tissue sections.

Materials and methods

Human biopsy specimens were obtained from 20 individuals with moderate to severe chronic periodontitis who were scheduled to undergo gingivectomy as part of their treatment plan. Specimens were taken from sites that showed clinically overt inflammation corresponding to a Gingival Index (11) score of 1 or 2 and which 1 week earlier had shown pockets of 5 mm or more.

The specimens were collected in cold phosphate-buffered saline (PBS) (4°C), pH 7.2. They were cut axially in several pieces 2–3 mm long and oriented in such a manner that the pocket epithelium (PE), the connective tissue, and the oral gingival epithelium (OGE) were present in the same section. Each piece was oriented by the use of pins, so as later to recognize the correct way of embedding after fixation. If the biopsy was large enough, an equal number of pieces were treated by three different methods:

a) The specimens were washed with gentle agitation in PBS at 4°C for 48 h before fixation, to eliminate diffusable immunoglobulins and complement factors (12). During that period the PBS was changed once. The specimens were then oriented and fixed in ethanol at 4°C by the method of Sainte-Marie (13), embedded in paraffin, and stored at 4°C until sectioning (prewashed specimens).

b) Other specimens were immediately immersed and fixed in precooled ethanol (13), embedded in paraffin, and stored at 4°C until sectioning.

c) Some specimens were placed on copper blocks in Tissue Tek mounting medium, snap-frozen in isopentate, prechilled in liquid nitrogen to −140°C, and stored at −80°C until sectioning.

Tissue sectioning

Paraffin-embedded tissue was sectioned at 4–5 μm and collected on gelatin-coated glass slides. Sections were deparaffinized in toluene, dehydrated, and rinsed in PBS before incubation with the appropriate antibodies.

The frozen tissues were cut in a cryostat at 4 μm, air-dried, rinsed in PBS, and immediately incubated with the appropriate antibodies.

Histologic examination

To study the morphology of the inflamed gingival specimens, parallel sections from all

specimens were stained with hematoxylin, eosin, and safranin (HES). The presence and the extent of the chronic inflammatory infiltrate were recorded, and specimens showing advanced periodontal disease (14) with massive cell infiltrates, were selected for incubation and further examination.

Incubation with polyclonal (monospecific) antibodies

Sections from paraffin-embedded and frozen tissue were pretreated with 25% bovine serum albumin (BSA) (Armour Pharmaceutical Co. Ltd., Eastbourne, England) for 30 min to reduce non-specific background staining from the antisera. The sections were then incubated for 18 h at room temperature, using a direct immunofluorescent double-staining technique, with the following rabbit antisera: fluorescein isothiocyanate (FITC)-conjugated anti-human IgG or IgM (codes OSEB 04-05 and OSED 04-05; Behringwerke AG, Marburg, FRG), combined with tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-human C3c or C3a (codes OSEN 04-05 and R1103; Behringwerke AG). The following goat antisera were also used: TRITC-conjugated anti-human IgG and IgM (codes 627115 and 627515; Zymed Laboratories Inc., San Francisco, Calif., USA) combined with FITC-conjugated (rabbit) anti-human C1q (code F254; Dakopatts A/S, Denmark).

The antisera were diluted in PBS containing 12.5% BSA, and the working dilutions had previously been tested separately. For control, polyclonal antibodies were replaced by PBS.

Incubation with monoclonal antibody

Cryostat sections were incubated for 18 h at room temperature (15, 16) with previously determined optimal dilutions in PBS of a mouse monoclonal antibody to human C9. The antibody was a gift from Dr. Moldnes, Ullevål Hospital, Oslo, Norway. After being washed in PBS, all sections were incubated with FITC-conjugated rabbit anti-mouse Ig (RAMIg) (code 232; Dakopatts) diluted 1:20 in PBS containing 50% pooled human serum, to reduce background staining. Before use, RAMIg was absorbed with human proteins insolubilized by glutaraldehyde prepared as described by Avrameas & Ternynck (17) and centrifuged at 100,000 *g* for 1 h.

To enhance the fluorescent staining, sections were incubated with FITC-conjugated swine anti-rabbit Ig (SARIg) (Dakopatts A/S) diluted 1:20 in PBS as the third step of incubation.

Microscopy

The sections were studied in a Leitz Orthoplan microscope equipped with a Ploemopak and a Philips CS 200-W mercury lamp for incident light fluorescence. Filters for FITC and TRITC fluorescence (Leitz K2, N2-filters) were used. Photomicrographs were taken of selected sections, using 35-mm black and white (Kodak Tri-X, 400 ASA) or color film (Kodak Ectachrome, 400 ASA).

Results

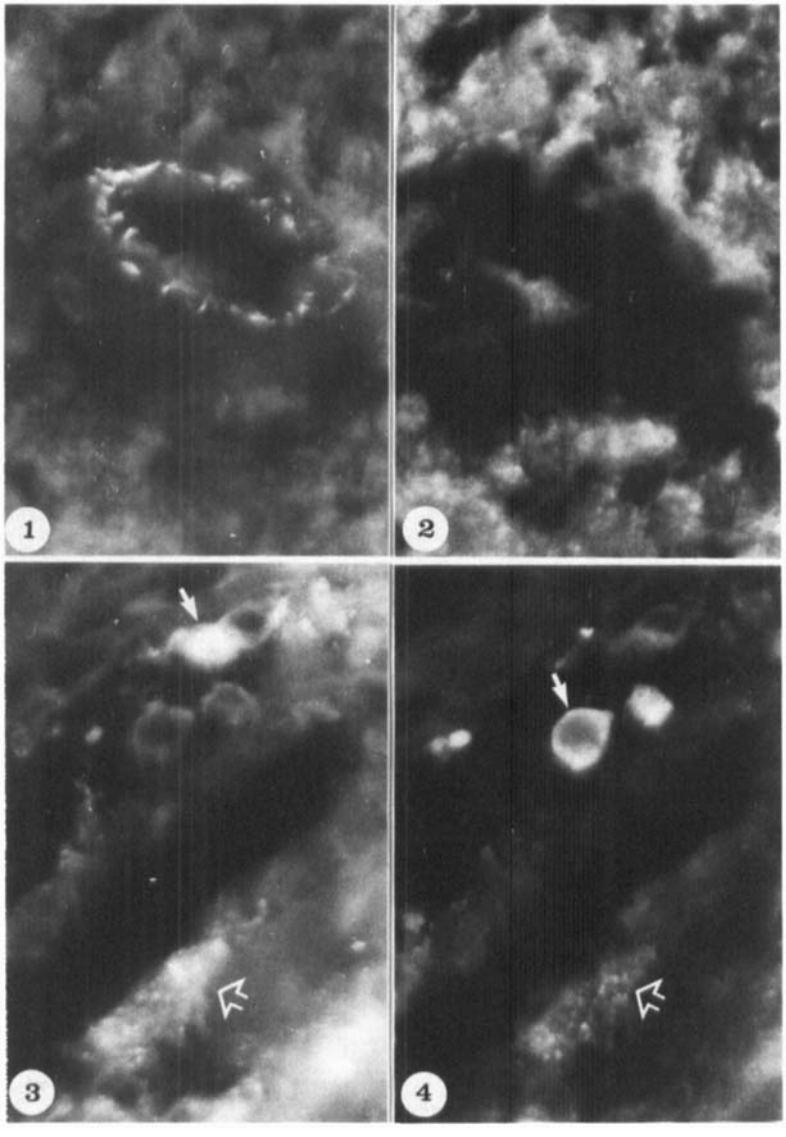
Histologic examination of sections stained with HES demonstrated characteristic advanced periodontal lesions. Most of the sections showed a massive inflammatory infiltrate underneath the pocket epithelium, containing mainly lymphocytes, plasma cells, and monocytes/macrophages. Some of the lesions showed considerable peripheral fibrosis and consisted mainly of connective tissue.

Polyclonal antibodies

Immunofluorescent examination of the specimens showed that the prewashed tissue displayed better cell morphology and less background staining than the directly fixed or frozen tissue.

In all specimens deposits of IgG were found interstitially within the inflamed gingiva, both in prewashed and particularly in nonwashed tissue. It was found within vessel walls or diffusely distributed around the vessel.

IgM was also found within or adjacent to vessel walls and diffusely distributed in the



Figs. 1 and 2. Deposits of C3c (Fig. 1, TRITC) and IgG (Fig. 2, FITC) in a vessel wall. Prewashed and ethanol-fixed section from inflamed human gingiva. (Magnification, ×900.)

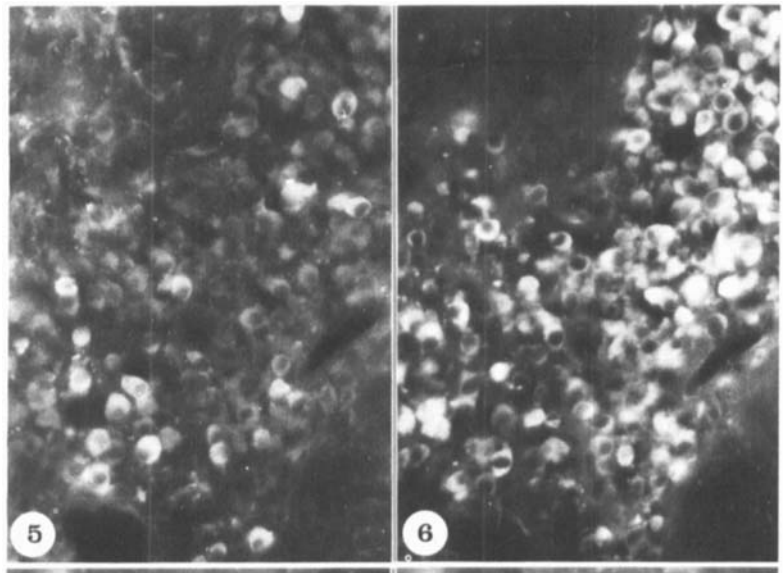
Figs. 3 and 4. C3a (Fig. 3, TRITC) and IgG (Fig. 4, FITC) in plasma cells (small arrows) and extracellularly in a vessel wall (open arrows). Prewashed and ethanol-fixed section. (Magnification, ×900.)

basement membrane zone of the OGE. This immunoglobulin was found bound to the cell membrane of the lymphocytes.

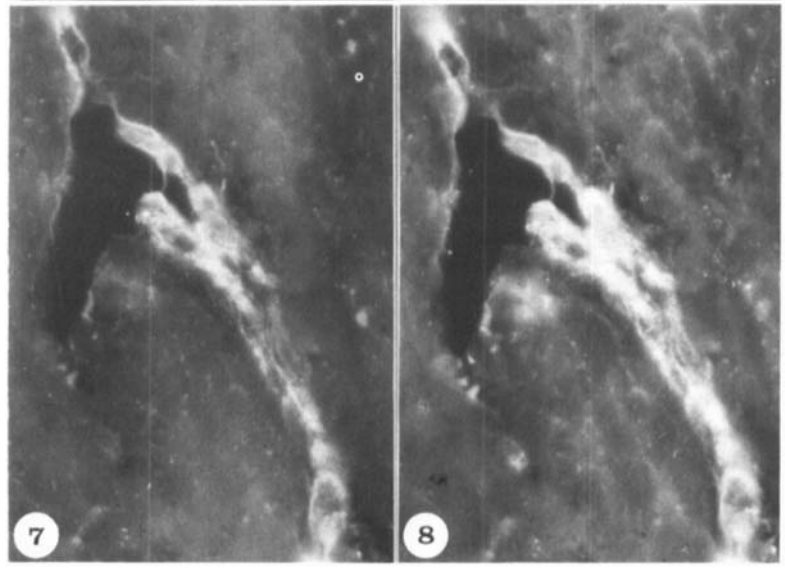
There were very few deposits of C3c in prewashed tissue, and they were observed only in vessel walls (Fig. 1), partly or completely coinciding with deposits of IgG (Fig. 2) or IgM (not shown). Such deposits were more frequently seen in nonwashed tissue.

C3a deposits were found mainly within the vessel walls. They coincided partly or completely with IgG or IgM deposits (Figs. 3 and 5), especially in vessels of the connective tissue papillae subjacent to OGE. Deposits of C3a were also found along the basement membrane zone of OGE. Staining for C3a was also frequently seen in the cytoplasm of plasma cells but generally not in the same cells as those that were positive for IgG or IgM (Figs. 4 and 6).

C1q deposits were found diffusely distributed interstitially or in vessel walls (Fig. 7), partly or completely coinciding with IgG (Fig. 8) or IgM deposits. Also, there was a



Figs. 5 and 6. C1q (Fig. 5, FITC) and IgG (Fig. 6, TRITC) in plasma cells of inflamed human gingiva. Prewashed and ethanol-fixed section. (Magnification, ×500.)



Figs. 7 and 8. Deposits of C1q (Fig. 7, FITC) and IgG (Fig. 8, TRITC) in a vessel wall. Prewashed and ethanol-fixed section. (Magnification, ×700.)

weak staining along the basement membrane zone of the OGE.

For C3a and C1q there was little difference between nonwashed and prewashed sections with regard to the occurrence of detectable deposits.

Monoclonal antibody

In cryostat sections from some specimens C9 was found diffusely distributed interstitially subjacent to the PE area. Deposits of C9 also showed strong fluorescence, observed as a granular pattern along the basement membrane zone of the OGE (Fig. 9). It could not be established with certainty that the location of these deposits corresponded to the deposits of immunoglobulins.

Discussion

Thorough washing of the tissue before fixation reduced the staining of diffusible

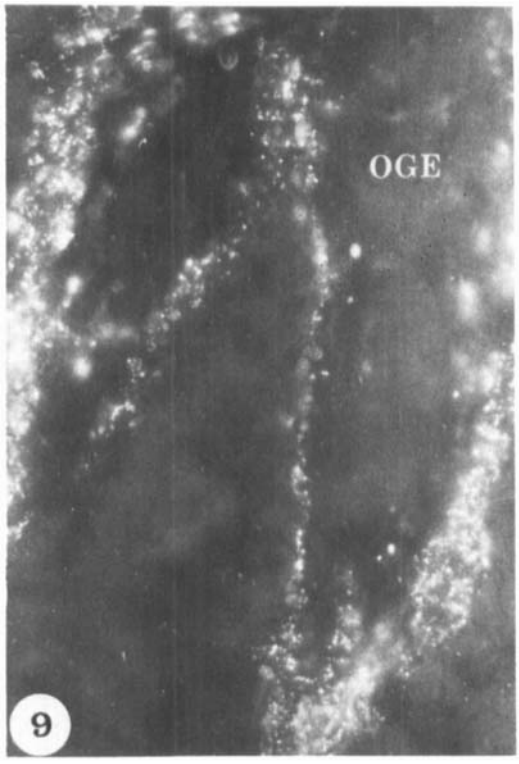


Fig. 9. Cryostat section incubated with monoclonal antibody against C39 and FITC-conjugated rabbit anti-mouse Ig. Granular deposits subjacent to oral gingival epithelium (OGE). (Magnification, ×448.)

immunoglobulins and complement factors, making possible the detection of in vivo-bound deposits. In future experiments more emphasis will be placed on the study of prewashed sections.

A direct comparison of our findings with those of Genco & Krygier (3), Genco et al. (6), or Toto et al. (4) with regard to the distribution of complement factors cannot be made, since they used antibody to whole human C3. The use of a double-staining technique made possible the detection of both immunoglobulins and complement components at the same site. Although it cannot be proved, our findings strongly suggest that immune complex formation had occurred at such sites. The immune complexes may activate the complement cascade via the classical pathway (C1 to C9), leading to lysis of bacteria. Another reaction resulting from complement activation which would primarily be protective is immune adherence, which probably plays a major role in facilitating phagocytosis of microorganisms after coating with antibody and C3b. Moreover, two fragments, C3a and C5a, produced during complement consumption, stimulate two helpful features of the acute inflammatory response. First, they are chemotactic factors for neutrophil polymorphs to the site of complement activation, and, secondly, they have anaphylatoxin activity through histamine release from mast cells, which increases vascular permeability and hence the flow of serum proteins, including antibodies and complement to the infected area.

The demonstration of IgG or IgM coinciding with C1q also suggests activation of the complement cascade via the classical pathway.

The demonstration of immunoglobulins together with C1q, C3a, or C3c within and adjacent to vessel walls indicates that allergic immune complex-mediated vasculitis is involved in the pathogenesis of inflammatory periodontal lesions. The scarcity of deposits of C3c in prewashed tissue and the rather few in nonwashed tissue (ethanol) (fixed or frozen) and only within vessel walls were expected, since C3c is shed in the fluid phase during the complement cascade (18) and is found in abundance in the gingival fluid (2). It also indicates that the complement cascade has been activated via either the classical or the alternative pathway.

The presence of cytoplasmic bound C3a in plasma cells suggests the possibility of a receptor for C3a in plasma cells. Until recently, cellular receptors for C3a have not been widely investigated. Indirect evidence suggests that mast cells have receptors for C3a, but studies of the binding of C3a to various cell types are incomplete (19), and the significance of the presence of C3a in plasma cells is unknown.

C3a deposits were found in great numbers both in nonwashed and in prewashed tissues, coinciding with IgG or IgM, as deposits in vessel walls, or diffusely distributed in the tissue. This indicates that C3a has an affinity for tissue components that might contribute to chemotactic and anaphylatoxin activity.

To our knowledge, deposits of C9 have not previously been demonstrated in inflamed gingival tissue. The occurrence of such deposits in the tissues may indicate that the complement cascade has proceeded to its end point. Further studies are needed to study more extensively this phenomenon and its significance.

Earlier studies have emphasized mainly the scarcity of deposits of immunoglobulins associated with complement factors in chronic marginal periodontitis (for review, see Genco & Slots (9), Schenkein (18)). In our study, although the presence of such deposits was not a predominant feature of the lesions, discrete but quite widely distributed deposits were observed. Deposits of complement factors, which would not be expected to be found in association with immune complexes but which are known to have important biological activities, such as C3a, were also observed.

These observations provide support for the hypothesis that complement factors may play a significant role in the immunopathology of the periodontal lesion.

References

1. Attström R, Laurel A-B, Larsson U, Sjöholm A. Complement factors in gingival crevice material from healthy and inflamed gingiva in humans. J Periodont Res 1975;10:19–27.
2. Schenkein HA, Genco RJ. Gingival fluid and serum in periodontal disease. II. Evidence for cleavage of complement component C3, C3 proactivator (factor B), and C4 in gingival fluid. J Periodontol 1977;48:778–84.
3. Genco RJ, Krygier G. Localization of immunoglobulins, immune cells and complement in human gingiva. J Periodont Res 1972(suppl 10):30–1.
4. Toto PD, Lin LM, Gargiulo AW. Immunoglobulins and complement in human periodontitis. J Periodontol 1978;49:631–4.
5. Platt D, Crosby RG, Dalbow MH. Evidence for the presence of immunoglobulin and antibody in inflamed periodontal tissues. J Periodontol 1970; 41:215–22.
6. Genco RJ, Mashimo PA, Krygier G, Ellison SA. Antibody mediated effects on the periodontium. J Periodontol 1974;45:330–7.
7. Clagett JA, Page RC. Insoluble immune complexes in chronic periodontal diseases in man and the dog. Arch Oral Biol 1978;23:153–65.
8. Pecović DD, Fillery ED. Identification of bacteria in immunopathological mechanisms of human periodontal disease. J Periodont Res 1984;19:329–51.
9. Genco RJ, Slots J. Host responses in periodontal diseases. J Dent Res 1984;63:441–51.
10. Johannessen AC, Nilsen R, Skaug N. Deposits of immunoglobulins and complement factor C3 in human dental periapical inflammatory lesions. Scand J Dent Res 1983;91:191–9.
11. Løe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. Acta Odontol Scand 1963;21:533–51.
12. Brandtzaeg P, Kraus FW. Autoimmunity and periodontal disease. Odontol Tidsskr 1965;73:281–393.
13. Sainte-Marie G. A paraffin embedding technique for studies employing immunofluorescence. J Histochem Cytochem 1962;10:250–6.
14. Page RC, Schroeder HE. Pathogenesis of inflammatory periodontal disease. A summary of current work. Lab Invest 1976;33:235–49.
15. Brandtzaeg P. Prolonged incubation time in immunohistochemistry: effects on fluorescence staining of immunoglobulins and epithelial components in ethanol- and formaldehyde-fixed paraffin-embedded tissues. J Histochem Cytochem 1981;29:1302–15.
16. Nilsen R, Johannessen AC, Skaug N, Matre R. In situ characterization of mononuclear cells in human dental periapical inflammatory lesions using monoclonal antibodies. Oral Surg 1984;58:160–5.
17. Avrameas S, Ternynck T. The crosslinking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochem 1969;6:53–66.
18. Schenkein HA. The complement system in periodontal diseases. In: Genco RJ, Mergenhagen SE, eds. Host-parasite interactions in periodontal diseases. Washington: American Society for Microbiology, 1982:299–308.
19. Fearon DT, Wong WW. Complement ligand-receptor interactions that mediate biological responses. Ann Rev Immunol 1983;1:243–7.

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