IDENTIFICATION OF LEUKOCYTES IN HEALTHY RAT GINGIVAE BY ENDOGENOUS PEROXIDASE

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Using the endogenous peroxidase reaction with the diaminobenzidine, I was possible to identify various types of leukocyte populations in the intercellular spaces of the junctional epithelium (JE) and adjacent connective tissue of healthy rat gingivae. Namely, neutrophils predominated, but monocytes and lymphocytes were few and eosinophils were very rare. No basophils were observed. Each leukocyte was identified by the number and shape of the peroxidase-positive and -negative granules at the electron microscopic level. Peroxidase was localized only in the azurophil granules of mature neutrophils and monocytes. The mature eosinophils also contained peroxidase only in the granules, which consisted of a peroxidase-positive matrix and a peroxidase-negative single discoid core in an equatorial position. No organellae of lymphocytes showed peroxidase-positive activity. In the inhibitory experiments for peroxidase of the identified leukocytes, 0.1 and 1 M KCN inhibited the peroxidase moderately and almost completely, but 0.01 M KCN and 0.01, 0.1 and 1 M 3-amino-1,2,4-triazole (AMT) had no inhibitory effect. The endogenous peroxidase in the granules of the leukocytes, mainly neutrophils, was sensitive to KCN but insensitive to AMT. Although I found no evidence of a direct effect of the peroxidase on foreign stimuli, my findings suggest that the various leukocytes always patrol the JE and adjacent connective tissue of the healthy rat gingivae to protect against foreign stimuli.

Whether leukocytes are located in the junctional epithelium of the healthy gingivae to form a defensive barrier against the inflammatory stimuli such as immunological damage and micro-organisms has not been made clear. Egelberg (8) detected neutrophils, monocytes and lymphocytes in smears from both clinically healthy and inflamed gingivae. On the other hand, Theilade et al. (19) did not find any leukocytes in smears from healthy gingivae. Also, Rovin et al. (17), using germ-free rats, postulated that neutrophils were not inflammatory constituents but a normal histologic component of the gingival tissue. Furthermore, Yamasaki et al. (20) observed the presence of neutrophils at the electron microscopic level in the junctional epithelium of germ-free rat gingiva. Thus, it seems likely that only neutrophils are located in the junctional epithelium of the healthy gingivae, but it is still unclear whether or not leukocytes other than neutrophils are present. On the other hand, Attström et al. (2) found neutrophils, lymphocytes and monocytes in the crevices of healthy gingivae, but ultrastructural and cytochemical details of the leukocytes have not yet been described because they provided only morpho-
logical evidence at the light microscopic level. Therefore, the present study using the cytochemical diaminobenzidine method (5, 11), which is specific for each leukocyte, was designed to 1) examine whether a variety of leukocytes are localized in the junctional epithelium of healthy gingivae, and 2) elucidate ultrastructural and cytochemical natures of the leukocytes.

**MATERIALS AND METHODS**

**Animals**

I used 32- to 34-day-old Sprague-Dawley rats of both sexes. This age was chosen because it was 7 to 10 days after the upper first molar teeth had erupted, and I was able to obtain healthy and sufficiently mature first molar gingivae at this time.

**Cytochemistry**

1) Peroxidase reaction: The animals were killed, and the upper first molar teeth were removed together with the attached gingival tissues. They were then fixed for 3 hr at 4°C in 2% paraformaldehyde-2.5% glutaraldehyde (0.1 M sodium cacodylate buffer, pH 7.4, with 2% sucrose) used in the cytochemical experiment of human myelogenous leukemia cells by Himori et al. (12). After the teeth with attached gingival tissues were decalcified in a 10% EDTA solution (pH 7.4, 0-4°C) with 4% sucrose for 3 to 4 weeks, they were cut bucco-lingually into 50 μm-thick frozen sections parallel to the longitudinal axis of the tooth with an electron-freezing microtome. One of the sections was incubated in a medium (11) containing 12.5 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Dojindo Laboratories, Kumamoto, Japan) in 25 ml 0.1 M tris buffer (pH 7.6) per incubation vial for 90 min at room temperature. Immediately before use, 0.1 ml of 0.5% H2O2 was added, and some sections as histochemical controls were incubated in a medium without H2O2.

2) Inhibitors: I used the method of Fahimi (9) to check the inhibitory effect of two chemicals on the endogenous peroxidase in the leukocytes. The method was slightly modified as follows: The remaining 50 μm thick sections were pre-incubated for 2 hr at room temperature in the presence of (0.01, 0.1 or 1 M) KCN or (0.01, 0.1 or 1 M) 3-amino-1,2,4-triazole and were then further incubated in the above DAB medium with or without H2O2 for 90 min at room temperature.

After incubation, all sections in 1) and 2) were washed two or three times in 0.1 M cacodylate buffer and were then post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 2 hr at room temperature. The sections were then dehydrated in a graded series of ethanol and embedded in Spurr epon. For light microscopy, 1-μm sections were either not counterstained, or were stained with toluidine blue. Most ultrathin sections were either not counterstained, or were stained with only uranyl acetate. They were examined in a Hitachi 600 electron microscope.
RESULTS

General observations

As shown in Fig. 1, rat gingivae consist of oral epithelium (OE), oral sulcular epithelium (OS), junctional epithelium (JE) and adjacent connective tissue (CT). The OE and OS are keratinized epithelium, but only JE is a non-keratinized epithelium. The JE consists of several layers of flattened cells aligned parallel to the enamel surface, and is also attached to the enamel surface (Figs. 2, 3). Wide intercellular spaces and numerous intracytoplasmic vacuoles, which Yamasaki et al. (20) described as the most characteristic findings, were also observed within the JE of healthy rat gingivae (cf. Figs. 11, 12).

Peroxidase reaction

In the healthy rat gingivae incubated in H$_2$O$_2$-containing DAB medium, many brown granules representing endogenous peroxidase-positive products were detected in the leukocytes of the JE (Fig. 2) and adjacent connective tissue. On the other hand, in the control gingivae incubated in a DAB medium without H$_2$O$_2$, no brown granules were detected in the leukocytes, which were recognized by their characteristic polymorphonuclear shape consisting of two or more lobes (Fig. 3). At the electron microscopic level, many leukocytes were also observed in the intercellular spaces of the JE and adjacent connective tissue. One is shown in Fig. 4.

Fig. 1. A diagram of the rat gingivae. The gingival epithelium is divided into two parts: oral epithelium (OE) and oral sulcular epithelium (OS) by an approximate line (a dot line). Adjacent connective tissue (CT), enamel (E), junctional epithelium (JE), stratum corneum (SC), stratum granulosum (SG).
Figs. 2, 3. Light micrographs of a part of healthy rat gingivae incubated in DAB medium with H$_2$O$_2$ and without H$_2$O$_2$, respectively.

Fig. 2. Many leukocytes (arrows) with endogenous peroxidase-positive granules are detected in the JE. Decalcified enamel (DE), keratohyalin granules (K). Not counterstained ×1,300
The leukocyte had many round or elongated peroxidase-positive and -negative granules, and its nucleus consisted of two lobes. The peroxidase was localized only in the granules (Fig. 4). These findings agreed with those of the mature neutrophils described by others (1, 5, 12). In these cells, the peroxidase-positive and -negative granules corresponded to the azurophil granules and specific granules, respectively.

I could also identify very few mature eosinophils (Fig. 5) because 1) the eosinophilic granules consisted of a peroxidase-positive matrix and a peroxidase-negative single discoid core in an equatorial plate, and 2) no peroxidase reaction was detected in the other organelles. A few lymphocytes and monocytes were also detected. The lymphocytes had a round nucleus and a thin rim of cytoplasm, and were also peroxidase-negative (Fig. 6) as described by Bentfeld et al. (5). The monocytes...
Fig. 4. An electron micrograph of a neutrophil in the intercellular spaces of the junctional epithelial cells from healthy rat gingivae incubated in DAB medium with H$_2$O$_2$.

The neutrophil contains azurophil granules (A) as peroxidase-positive round or elongated granules and specific granules (S) as peroxidase-negative granules. Nucleus (N). Not counterstained. ×34,000
Sakano had peroxidase-positive and -negative granules and a kidney or horseshoe shaped nucleus. The peroxidase-positive granules (azurophil granules) in the monocytes (6, 16) were fewer and smaller than those in the neutrophils, but their content was homogeneous (Fig. 7, see Fig. 4).

**Inhibitors**

3-amino-1,2,4-triazole (AMT) did not inhibit peroxidase activity in the leukocytes.

**Fig. 8.** An electron micrograph of a part of the JE preincubated with 0.01 M AMT and further incubated in DAB medium with H_2O_2.

Generally, leukocytes are not sensitive to 0.01 M AMT because peroxidase activity in the neutrophil (Ne) is not inhibited by the AMT (compare Fig. 4 with Fig. 8). Furthermore, a typical eosinophilic granule of an eosinophil (arrow) like those of eosinophils in Fig. 5 is located in the cytoplasmic fragments in the intercellular spaces of the junctional epithelial cells (J). Not counterstained. \( \times 19,000 \)
FIG. 9. A part of the JE preincubated with 1 M AMT and further incubated in DAB medium with H$_2$O$_2$. The AMT does not inhibit the peroxidase activity in the azurophil granules (arrows) of the intact neutrophil (Ne) and neutrophil cytoplasmic fragments. Peroxidase-positive activity (arrowheads) is further detected in the junctional epithelial cells. Not counterstained. ×10,000
For example, Figs. 8 and 9 showed a part of the gingivae preincubated with 0.01 or 1 M AMT and then incubated in DAB medium with H₂O₂. The neutrophils in Figs. 8 and 9 show the peroxidase-positive activity in the azurophil granules like those in the neutrophils in Fig. 4 incubated in only DAB medium with H₂O₂. In addition, numerous granules with a cytoplasmic organellae as part of the disrupted

![Image](image_url)

**Fig. 10.** A part of the JE and adjacent connective tissue preincubated by 0.01 M KCN and further incubated in DAB medium with H₂O₂. Three neutrophils (Ne) and a monocyte (M) are not inhibited by the KCN. The monocyte has peroxidase-positive granules and a kidney-shaped nucleus. The peroxidase-positive granules are few in number compared with those of the neutrophils. Adjacent connective tissue (CT). Not counterstained. ×5,300
neutrophils and eosinophils were released into the intercellular spaces of the junctional epithelial cells. The released granules also showed peroxidase activity and retained morphological shape with no extrusion of the granular contents into the intercellular spaces. An additional noteworthy finding was that the peroxidase-

![Fig. 11](image-url)

**Fig. 11.** A part of the JE preincubated with 0.1 M KCN and incubated in DAB medium with H$_2$O$_2$. One of neutrophils (Ne) has the same peroxidase-positive granules as those of the neutrophil in Fig. 4, but the peroxidase activity of other neutrophils (Ne') is inhibited by the KCN. Therefore, their peroxidase-positive granules are very few in number. Two neutrophils have undergone degenerative changes (arrows). Not counterstained. ×7,500
Fig. 12. A part of the JE preincubated with 1 M KCN and further incubated in DAB medium with H$_2$O$_2$.
Most peroxidase activity is inhibited by the 1 M KCN, but the arrows indicate very faint peroxidase-positive granules in the neutrophils. Not counterstained. $\times$5,000
positive reaction was also seen in the junctional epithelial cells. This phenomenon suggests the uptake of the peroxidase-positive granules by the junctional epithelial cells. Thus, AMT did not have an inhibitory effect on peroxidase.

KCN

KCN at a concentration of 0.01 M did not affect the peroxidase reaction in the leukocytes, because the same neutrophils as shown in Fig. 4 were detected in the intercellular spaces of the junctional cells and in the adjacent connective tissue. Furthermore, the electron microscopic image of monocytes incubated in 0.01 M KCN and DAB medium with $H_2O_2$ was similar to that of the monocytes incubated in only DAB medium with $H_2O_2$ (Figs. 7 and 10).

The gingivae incubated in 0.1 M KCN and DAB medium with $H_2O_2$ had numerous neutrophils in the intercellular spaces of the junctional epithelial cells. Some cells had numerous peroxidase-positive granules, and others had very few granules. In particular, the number of peroxidase-positive granules varied considerably from cell to cell after this incubation (Fig. 11). Thus, 0.1 M KCN seemed to inhibit peroxidase activity in only some of the azurophil granules of the neutrophils. Unfortunately, I could not determine whether 0.1 M KCN inhibited the granular peroxidase activity in the eosinophils or in the monocytes.

In the leukocytes of the gingivae incubated in 1 M KCN and DAB medium with $H_2O_2$, no peroxidase-positive granules were detected at the light microscopic level (not shown). However, at the electron microscopic level (Fig. 12), a few granules with very faint peroxidase activity were detected in the neutrophils and monocytes of the gingivae (compare Fig. 10 with Fig. 12). The KCN inhibited the peroxidase activity almost completely.

DISCUSSION

Several kinds of leukocytes were detected in the JE and adjacent connective tissue of healthy rat gingivae. They seem to readily transude from the blood vessels in the gingivae into the JE, because the proportions of populations in the JE closely reflect the differential counts of leukocytes in the blood. All leukocytes other than the lymphocytes were peroxidase-positive. Generally, the peroxidase-positive reaction in the mature leukocytes is confined to granules, whereas, the activity in the immature cells is also found in the ER cisternae, nuclear envelope and Golgi complex (1, 5, 12). Since I also detected peroxidase reaction only in the granules of various leukocytes, the leukocytes were identified as being mature.

The detection of eosinophils in the JE has not yet been reported. In addition, the exact distribution of monocytes is hard to determine, because, as stated by Lange et al. (14), the ultrastructural differentiation between monocytes and large lymphocytes (macrophages) within the JE is very difficult due to the similarity in their cytoplasmic organelle pattern. In the present study, I could easily differentiate monocytes from lymphocytes and macrophages, namely, the monocytes (mature type) have peroxidase activity only in a minority of the cytoplasmic granules (6, 16) (Figs. 7, 10). On the other hand, macrophages have peroxidase activity in not only the granules but also the rough ER (6), and the lymphocytes were negative for peroxidase (Fig. 6). Thus, the peroxidase-DAB reaction is characteristic in that
I could easily detect monocytes and eosinophils in the JE of the healthy rat gingivae. Neutrophils were most predominant in the JE and adjacent connective tissue. These cells contained two types of granules as demonstrated by peroxidase cytochemistry in the previous study (3, 4, 7). The primary granules (azurophil granules) show a peroxidase-positive reaction and contain acid hydrolases and myeloperoxidase, and the secondary granules (specific granules) are peroxidase-negative and contain lactoferrin. Both types of granules discharge their contents into the phagosomes and the external environment. According to Leffell et al. (15), specific granule degranulation is mainly directed to the external environment whereas azurophil granule degranulation is primarily confined to the phagosomes. The specific granule degranulation investigated by Hoffstein et al. (13) seems to be based on exocytosis. After incorporation of the membrane from specific granules into the plasma membrane, only granular contents are discharged into the external environment. However, in the present study, there was no evidence of the discharge of either type of granule into the phagosomes or external environment. As a consequence of the disruption of the plasma membrane of neutrophils (monocytes) and eosinophils, the spontaneous release of various cytoplasmic organelles such as peroxidase-positive granules were observed in the intercellular spaces of the junctional epithelial cells. It is reasonable to believe that phagocytosis and exocytosis of these leukocytes (mainly neutrophils) do not function in healthy rat gingivae, but become active only by acute infection or some other stimuli. Therefore, the release of peroxidase-positive granules (lysosomes) into the extracellular spaces seems to mean the end of the leukocyte life as a physiological phenomenon rather than an abnormal phenomenon against foreign stimuli. The release of the granules does not induce tissue damage, because most of the released granules are enveloped by an intact limiting membrane that does not allow extrusion of the lysosome enzymes as granular contents through a rupture of the membrane (10). Furthermore, junctional epithelial cells take up the released granules (see Fig. 9) and might ingest them. The junctional epithelial cells also seem to be related to the protection of the tissue from various enzymes by the uptake of the leukocyte granules (18).

In the present study, 0.01 M KCN did not inhibit the peroxidase in the granules of the leukocytes. This finding is in agreement with that of previous studies (3–6). KCN concentration-dependently inhibited peroxidase activity. For example, 0.1 M KCN inhibited partly the peroxidase in the azurophil granules of the neutrophils, and 1 M KCN inhibited the peroxidase of various leukocytes almost completely. Unfortunately, I could not determine whether 0.1 M KCN inhibits the peroxidase activity of the eosinophils and monocytes, because the total number of both leukocytes in healthy rat gingivae were very few. On the other hand, AMT did not inhibit the peroxidase activity of the leukocytes even at higher concentrations. Thus, of interest is that KCN acted as a strong inhibitor for the peroxidase of various leukocytes in the healthy rat gingivae, but AMT did not.

In conclusion, I identified neutrophils, monocytes, lymphocytes and eosinophils in healthy rat gingivae. The leukocytes other than the lymphocytes possessed peroxidase-positive granules, which were sensitive to KCN but were insensitive to AMT. Although I could not observe the direct action of the peroxidase-positive
Leukocytes in Healthy Gingiva by Peroxidase

granules for foreign factors such as bacteria and toxic materials, it seems likely that various leukocytes always patrol the JE and adjacent connective tissue to protect healthy gingivae from foreign factors.

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REFERENCES

