Immunohistological Demonstration of Lysozyme in Human Leucocytes, Salivary Corpuscles and Nasal Discharge Cells after Blockade of Myeloperoxidase Activity

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Summary: Using a peroxidase-antiperoxidase method, the intracellular lysozyme (LZM) content of human blood cells, salivary corpuscles and nasal discharge cells was estimated after blockade of myeloperoxidase activity. The endogenous myeloperoxidase activity was entirely blocked by 0.3% hydrogen peroxide-methanol treatment in the polymorphonuclear leucocytes.

In the peripheral blood of healthy subjects and various leukaemic patients, mature neutrophilic polymorphonuclear leucocytes and immature ones from promyelocytes onwards were distinctly observed with the immuno-specific reaction. Occasionally, monocytes were also found to contain LZM intracellularly. There was no discernible enzyme in the other blood cell elements.

In salivary corpuscles and nasal discharge cells, neutrophilic granulocytes only demonstrated LZM immunopositively.

The presence of lysozyme (LZM) in human leucocytes was first noted by Fleming (1922). The morphological appearance of the enzyme in tissues, cells and secretions, has been demonstrated in various ways: by overlaying tissues with a suspension of *M. lysodeikticus* and observing the microscopic areas of lysis around LZM-containing cells (Speece 1964); by similar observations on stained mixtures of *M. lysodeikticus* with peripheral or bone marrow blood cells (Briggs et al. 1966;
Ghoos and Vantrappen 1970); by examining unstained agarose preparations with isolated polymorphonuclear leucocytes or alveolar macrophages (Mestecky 1967); by the immunofluorescent antibody method using an antibody against specific human LZM in various tissues and cells (Asamer et al. 1969; Kraus et al. 1969; Kraus and Mestecky 1971; Asamer et al. 1974; Pryzwansky et al. 1979); and by the immunoperoxidase antibody method in histochemical and cytochemical studies of various gland cells (Mason and Taylor 1975; Klockars and Reitamo 1975; Montero and Erlandsen 1978; Kami et al. 1979; Ogawa et al. 1979).

The detection of LZM in peripheral and bone marrow blood cells by employing an immunoperoxidase antibody technique, however, was not possible due to the presence of myeloperoxidase in granulated leucocytes (Kami et al. 1979). Mitsui and his co-workers (Sasaki 1952; Tsukamoto 1952; Mitsui et al. 1979) found that methanol inhibited the myeloperoxidase staining of leucocytes more intensely than other fixatives such as ethanol, acetone, etc. The peroxidase staining of leucocytes ended in failure on treatment with methanol for only 3 minutes. Recently, Mitsui (1978) and his co-workers (Ochi et al. 1978) indicated that methanol exerts a stronger inhibiting influence on the purified myeloperoxidase activity by estimation of the enzyme reaction using a rate assay method.

These latter findings were evaluated for the detection of immunoreactive LZM in polymorphonuclear leucocytes containing myeloperoxidase. In the present study, the authors examined the appearance of LZM in human blood cells, salivary corpuscles and nasal discharge cells using an unlabelled peroxidase-antiperoxidase (PAP) method.

Materials and Methods

Specimen smears of peripheral blood, saliva and nasal discharge, taken from healthy adult volunteers and various types of leukaemic patients, were studied. Venous blood mixed with heparin (25 i. u./ml) was centrifuged at 250 g for 7 minutes after which the buffy coat layers were smeared on methanol-cleaned slide glasses, and air dried. Salivary corpuscle and nasal discharge cell smears were prepared on several clean slides after swabbing the throat (soft palate) and/or concha nasalis inferior. All three kinds of smears were stained routinely with May-Giemsa solution in the usual manner.

**Antisera:** Anti-human lysozyme (LZM) rabbit serum was purchased from Behringwerke Co. (Batch No. 7101 F, West Germany) and its immunological monospecificity was tested by Laurell's rocket immunoelectrophoresis using 1% agar gel which contained 1.5% antiserum and the normal human serum and saliva (Fig. 1).

**Immunocytochemical procedures:** Antigenic LZM was demonstrated by the unlabelled antibody peroxidase-antiperoxidase (PAP) complex method (Sternberger et al. 1970).

1. Air dried smear specimens were fixed-and-blocked for endogenous myeloperoxidase in methanol containing 0.3% hydrogen peroxide (H$_2$O$_2$) (Tsukamoto 1952; Vacca et al. 1978) at 4°C for 2 to 20 minutes.

2. They were treated with 3% normal goat serum in Tris-HCl saline (pH 7.4) for 30 minutes, followed by application of specific rabbit antiserum to human LZM (dilution 1: 800-1: 1,200) at 3-5°C for 48 hr, returning to room temperature for the last 2 hr. The slides reacted with the antisera were washed in 3 changes of Tris-HCl saline containing 1% bovine serum albumin (BSA) for 2 minutes each.
(3) They were then treated for 30 minutes in succession with goat anti-rabbit IgG (dilution 1:10), and PAP (dilution 1:50). After each antiserum treatment, the slides washed in the same manner as described above. Next, a freshly prepared 0.05% 3,3'-diaminobenzidine-4HCl (DAB, Dotaito Co., Japan) and 0.001% hydrogen peroxide (H₂O₂) substrate in 0.05 M Tris-HCl buffer at pH 7.4 was applied until the specific staining reaction occurred. The period was not longer than 4 minutes.

(4) After stopping the reaction with distilled water, counterstaining was carried out with May-Giemsa solution for a few minutes. For immunospecific controls, the slides were treated with DAB-H₂O₂ solution alone and/or diluted normal rabbit serum in place of the specific antiserum in step 2 mentioned above.

(5) An indirect immunofluorescent technique was also applied for the detection of LZM in various leucocytes, and the results were compared with those obtained by the PAP technique.

Results

Peripheral venous blood cells: The endogenous myeloperoxidase activity was entirely blocked by 0.3% hydrogen peroxide-methanol treatment in the polymorphonuclear leucocytes (Fig. 2). Neutrophilic and basophilic peroxidase could not be demonstrated after the above-mentioned inhibitory treatment for 4 minutes. The eosinophilic one, however, was slightly more stable to methanol containing H₂O₂ fixation than the other two kinds of granulocytes. With every donor material, the PAP antibody method was applied for the detection of LZM following blockade of the eosinophilic peroxidase stainability. Although the blockade time was different in case, the average was 9 minutes (5-13 minutes).

Mature neutrophilic leucocytes (Fig. 3) and occasional monocytes (Fig. 5) consistently revealed a large amount of immunoreactive LZM. A few basophils also showed a very weak immunopositive reaction, but most yielded no reaction. There was no discernible LZM in the large and small lymphocytes (Fig. 4), eosinophils, platelets and erythrocytes. In some cases, antigenic LZM was identified in promyelocytes and later myeloid precursors. The other erythrocyte precursors (Fig. 6) and basophilic cells gave no immunoreactive findings of LZM. In the fluorescent antibody method, the reaction pattern generally ran parallel to the immunoreactivity obtained by the PAP method (Fig. 3b and c).

Salivary corpuscles and nasal discharge cells: Most of the salivary corpuscles could be stained with the peroxidase reaction immuno-specific against their endogenous LZM, since they were myelogenous leucocytes (Fig. 7). Neutrophils can be identified by May-Giemsa stain unless they are degenerated in the saliva, but it is very difficult to distinguish them from the lymphocytic series when the neutrophils are degenerated. In proportion to the degree of degeneration by saliva (Fig. 7b), the granules become sparse, not uniform in size, fewer in number, and not well-defined, until no reacting granules appear in the protoplasm although they are myelogenous. Neutrophils and monocytic cells both immunoreacted LZM positive in the nasal discharge cells (Fig. 8). With the eosinophils in the nasal discharge, however, the PAP reaction demonstrated exactly similar results to blood eosinophils. Both the protoplasm of the oral and nasal epithelial cells were always LZM negative.
Discussion

It is interesting to note that the granules in leucocytes which were made entirely myeloperoxidase negative by the inhibitor, methanol containing 0.3% hydrogen peroxide, still stained very finely with May-Giemsa stain (Kami et al. 1979). The hydrogen peroxide \( (H_2O_2) \) solution represents the most important component of the peroxidase staining reagent. The myeloperoxidase granules, according to the theory proposed previously, catalyze the reduction of \( H_2O_2 \) releasing an oxygen molecule which in turn oxidizes the hydrogen donor. The peroxidase staining capacity of blood cells changes by varying degrees according to the amount of \( H_2O_2 \) present in the peroxidase stain (Vacca et al. 1978). The results of many investigators (Tsukamoto 1952; Vacca et al. 1978) indicated that excessive \( H_2O_2 \) solution in the reagent inhibits the peroxidase reaction of all polymorphonuclear leucocytes as in the peroxidase reaction by the biochemical method (Ochi et al. 1978). The optimal level of \( H_2O_2 \) concentration for blocking endogenous myeloperoxidae in both neutrophils and eosinophils is that of 0.3% hydrogen peroxide solution in methanol due to the strong inhibiting influence on the peroxidase reaction of the granulocytes (Tsukamoto 1952). When optimal blocking procedures are employed, good preservation of the antigenic reactivity of LzM is combined with excellent cell morphology, so that both antigen negative and antigen positive cells can be identified with confidence.

The above results for blood smears provide evidence that mature neutrophilic leucocytes (Fig. 3 and 4) and monocytes (Fig. 5) contain LzM in all peripheral blood elements. In materials from patients with myeloproliferative diseases, antigenic LzM was identified as a component of promyelocytes and later myeloid precursors, but it was absent from myeloblasts. This enzyme is thought to be present in the primary or azurophilic granules of neutrophils (Bretz and Baggioni 1974; Spitznagel et al. 1974; West et al. 1974; Pryzwansky et al. 1979) as has been suggested by the present demonstration of LzM in neutrophilic precursors from the promyelocyte stage series. Monocytes demonstrated LzM in the present study but the reaction activity was consistently weaker than that in polymorphonuclear neutrophils (Figs. 5). Most investigators agree that there is a definitely higher LzM activity than normal in the serum and urine of monocytic and myelocytic and myelocytic leukaemic patients, and less in lymphatic leukaemic ones (Briggs et al. 1966; Osserman and Lawlor 1966). Immunocytologically demonstrable LzM in normal monocytes, however, is less prominent. By contrast, only the neutrophilic granulocytes under normal conditions at the maturation stage of the myelocytes series reveal a distinct presence of LzM. It may be that monocyte LzM is much more efficiently released by these procedures than is neutrophilic granulocyte LzM. Support exists for this interpretation in the report by Asamer et al. (1969) that monocytes contain considerably less LzM than do polymorphonuclear granulocytes. There is good evidence that the two cell types handle LzM in azurophilic granules differently \textit{in vivo}: granulocytes only release LzM at degranulation following phagocytosis, whereas monocytes liberate the enzyme continuously into their environment (Osserman and Lawlor 1966; Mason et al. 1975).

On the other hand, most of the salivary corpuscles (Fig. 7) and nasal discharge cells (Fig. 8) could be stained with the LzM–PAP stain, since they were myelogenous leucocytes (Ikeda 1951; Maruyama 1954). The secretions on the various
mucosae generally represent an unsuitable environment for the survival of blood cells. When leucocytes become mixed in saliva and nasal secretion in the oral and/or nasal cavity, respectively, they are gradually inactivated. The morphological alteration of the above-mentioned cells is characterized by loss of segmentation in the nucleus, vacuolation of the protoplasm, disappearance of distinct granules (both azurophilic and secondary), and finally they undergo fragmentation terminating in autolysis (Maruyama 1954). However, saliva and nasal secretions contain LZM which originates from the parotid and lingual serous glands (Kraus and Mestecxy 1971; Mason and Taylor 1975), in addition to the nasal mucosal glands (Ogawa et al. 1979). The immunoreaction in background of the slide glasses or surrounding cells was found to be very weak or absent immunohistochemically (Kami et al. 1979). It is generally believed that neutrophils are always present in saliva, but other blood cells such as lymphocytes, monocytes, eosinophilic and basophilic leucocytes are also found on precise examination (Ikeda 1951; Maruyama 1954), although all blood cell elements except only neutrophils immunoreacted LZM negative. Of course, the epithelial cell proliferations were always LZM negative. In these results of the present study on the presence of LZM in human blood cell elements generally run parallel with the data obtained from chicken blood cell LZM (Kami et al. 1979b).

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References


PLATE
Explanation of Figures

Plate I

Fig. 1. Monospecificity of anti-human lysozyme (anti-LZM) rabbit serum was tested by the Laurell's rocket immunoelectrophoresis using 1% agar gel which contained 1.5% antiserum (1a). Both normal human serum (HSE) and saliva (HSA) migrated toward the cathode (left) at alkaline pH. The presence of antigenic LZW was indicated by arrows, respectively. An antigenic LZM of normal human serum could not be shown by the ordinary immunoelectrophoresis (1b, below). RSE: normal rabbit serum.

Fig. 2. Immunospecific control experiment. Human blood smear was fixed-and-blocked for endogenous myeloperoxidase in methanol containing 0.3% H$_2$O$_2$, then, it was applied with diluted normal rabbit serum in place of the specific antiserum (see Materials and Methods). Endogenous myeloperoxidase and LZM in both neutrophil (n) and eosinophil (eo) were not demonstrated even after the treatment with PAP-DAB-H$_2$O$_2$. ×1,000

Fig. 3. Immunospecific experiment of human blood smear. Neutrophils (n) contained antigenic LZM. In the fluorescent antibody method, the reaction pattern (b and c) ran parallel to the immunoreactivity obtained by the PAP method (a). ×1,150

Fig. 4. Immunospecific experiment of human blood smear. Note immuno-negative large and small lymphocytes (L). n: neutrophilic leucocyte. ×1,000

Fig. 5. Immunospecific experiment of human blood smear. Note immuno-positive monocyte (m) that was less prominent in the reactivity than neutrophilic leucocyte (5a). 5b: immuno-negative monocyte. ×1,000

Fig. 6. Immunospecific experiment of blood smear of leukaemic patient. Note immuno-negative erythroblast (eb). n: mature neutrophilic leucocyte. ×1,100

Fig. 7. Immunospecific experiment of human salivary corpuscle. Note immuno-positive neutrophilic salivary corpuscles (nsc). An inactivated neutrophilic salivary corpuscle (7b) was characterized by disappearance of granules and less stainability of the nucleus. ×1,100

Fig. 8. Immunospecific experiment of human nasal discharge cells. Note immuno-positive neutrophilic nasal discharge cells (nndc). ×1,000