Formation of Charcot-Leyden crystals by human basophils in sputum and peripheral blood.

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Abstract

To confirm the formation of Charcot-Leyden crystals (CLC) in basophils, we observed basophils in sputum and peripheral blood. Sealed slide and suspension culture methods were used to observe the process of CLC formation in peripheral blood basophils and eosinophils under electron microscopy. CLC formation was observed in basophils and eosinophils, and was found to be augmented by sealed slide method. A temperature of 4 degrees C was better than 37 degrees C for promoting the formation of crystals. There was no correlation between the degranulation of these cells and the formation of CLC after stimulation with anti-IgE or anti-IgG antibodies. CLC were initially detected in the cytoplasmic granules of basophils where they continued to enlarge. No CLC were identified in mast cells under any conditions studied. These findings confirm that CLC in sputum are not exclusive to eosinophils and that CLC appear to be present in basophil-rich sites under the cell damage.

KEYWORDS: Charcot-Leyden crystals, basphils, electron microscopy

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Formation of Charcot-Leyden Crystals by Human Basophils in Sputum and Peripheral Blood

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To confirm the formation of Charcot-Leyden crystals (CLC) in basophils, we observed basophils in sputum and peripheral blood. Sealed slide and suspension culture methods were used to observe the process of CLC formation in peripheral blood basophils and eosinophils under electron microscopy. CLC formation was observed in basophils and eosinophils, and was found to be augmented by sealed slide method. A temperature of 4°C was better than 37°C for promoting the formation of crystals. There was no correlation between the degranulation of these cells and the formation of CLC after stimulation with anti-IgE or anti-IgG antibodies. CLC were initially detected in the cytoplasmic granules of basophils where they continued to enlarge. No CLC were identified in mast cells under any conditions studied. These findings confirm that CLC in sputum are not exclusive to eosinophils and that CLC appear to be present in basophil-rich sites under the cell damage.

Key words: Charcot-Leyden crystals, basophils, electron microscopy

Charcot-Leyden crystals (CLC) (1-3) are hexagonal, bipyramidal structures, which were originally described by Charcot and Robin (4) in 1853 as well as by Leyden (5) in 1872. Ackerman et al., using radioimmunoassay, indicated that CLC protein was distinct from other eosinophil proteins (6). Weller et al. found that CLC protein had a molecular weight of 17,400 and possessed lysophospholipase activity (7-9). It was thought that CLC were found only in eosinophils until 1965, when Archer and Blackwood reported that basophils also formed similar crystals (10). They obtained CLC from basophil extracts and from intact basophils suspended in hypotonic sodium chloride. The presence of CLC protein was also demonstrated by the immunofluorescence method in both basophils and eosinophils purified by a fluorescence-activated cell sorter (11). Recently, the ultrastructural localization of CLC protein has been reported in human eosinophils (12) and basophils (13). However, the process of CLC formation in these cells has not been elucidated.

CLC are often detected in the sputum of patients with bronchial asthma and they are thought to be formed by the degeneration of eosinophils. In this study, we tried to stimulate the formation of these crystals in sputum from patients with bronchial asthma. After determining the conditions for CLC formation, we next observed the process in peripheral blood basophils. Additionally we observed the differences between the CLC formation in basophils and that in eosinophils and mast cells.

Materials and Methods

Patients. Sputum samples were obtained from asthma patients who had repeated acute episodes but were not steroid-dependent. Basophils were obtained from the peripheral blood of 9 patients with chronic myelocytic leukemia (CML) in acute transformation (14-41 % basophils) and 9 patients with asthma (1-3 % basophils). Eosinophils were obtained from the peripheral blood of 4 asthma patients (7-24 % eosinophils). Mast cells were isolated from healthy lung tissues that were taken from 4 patients with lung cancer.

Isolation of cells. Basophils: Heparinized peripheral blood samples were subjected to 9 % Ficoll-Conray density gradient centrifugation to obtain a mononuclear cell layer containing basophils (14). The final purity of the basophils was 2-5 % in the
asthma patients and 25–77% in the CML patients. Eosinophils: Sampled peripheral blood was mixed with EDTA, and then 0.1 ml of $10^{-5}$M formyl-L-methionyl-L-leucyl-L-phenylalanine was added to each 10 ml of blood (15). The eosinophil-rich fraction was obtained by density-gradient centrifugation using 65% and 75% Percoll discontinuous gradients, and the final purity of the eosinophils was 90–96%. Mast cells: Human lung tissues were sliced according to a modification of Schulman’s method (16, 17) and treated with pronase-chymopapain and collagenase-elastase (18). The dispersed cells were isolated by Ficoll-Conray density gradient centrifugation to obtain mast cells, and their final purity was 1–16%.

**CLC formation in sputum.** Sputum samples were refrigerated overnight at 4°C (a common method of observing CLC formation in the sputum). CLC were then observed by light microscopy with or without staining of eosinophils and basophils using Hansel’s stain (Eosinostain®, Torii, Japan).

**CLC formation by cells.** The formation of CLC by basophils and eosinophils in response to nonphysiological stimuli was observed by a) sealed slide (19) and b) suspension culture methods. In the sealed slide method, a drop of each cell suspension in 0.9% NaCl was placed on a slide glass, covered with a cover slip, and sealed with paraffin. The specimen was observed by light microscopy after incubation at 4°C or 37°C for 1, 2, 6, 12, and 24 h. In the case of the suspension culture method, cells were suspended in 0.9% NaCl and incubated at 4°C or 37°C in the same manner as the sealed slide method. Specimens were then observed by both light and electron microscopy at specified intervals up to 48 h.

We also observed the formation of CLC in basophils from asthma patients and in mast cells after immunologic stimulation. In brief, 0.1 ml of anti-human IgE or anti-human IgG rabbit antisera (Behring Werke AG, Germany), diluted to 1:10, was added to 2 ml of each cell suspension (1 × 10^6 cells/ml), and incubation was performed for 1 h at 37°C. Specimens for transmission electron microscopy were fixed in 1% OsO₄ solution using a modification of Karnovsky’s method and were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate. The sections were examined under a Hitachi H-300 electron microscope (Hitachi, Japan).

**Results**

Light microscopic observation revealed numerous CLC in the sputum of the asthmatics. These specimens were stained with Hansel’s staining method to reveal the cells that contained CLC. CLC were frequently found in the few basophils, which were stained metachromatically, and detected among the numerous eosinophils (Fig. 1).

We observed the process of CLC formation by basophils and eosinophils under several sets of conditions. Regular hexagonal and bipyramidal crystals which were morphologically identified as CLC were seen in both eosinophils and basophils. In basophils, most of CLC were detected in the cytoplasm, but in the cultures of eosinophils, most of CLC were seen extra-cellularly (Fig. 2). There were some differences in the process of CLC formation under the various conditions (Fig. 3). CLC formation was observed at 6 h in basophils and at 12 h in eosinophils in the sealed slide specimens. CLC were formed earlier at 4°C than at 37°C in both cell types. CLC were formed slower in the suspension culture method than in the sealed slide method; 12 h for basophils and 24 h for eosinophils. As with the sealed slide method, CLC formation occurred earlier at 4°C than at 37°C.

In basophils and mast cells, after immunologic stimulation with anti-IgE or anti-IgG antibodies, CLC were not detected in either of these cell types by light microscopy.
Fig. 2  Formation of CLC in eosinophils (A) and basophils (B) as demonstrated by the sealed slide method incubated at 4°C for 48 h. In the cultures of eosinophils, most of CLC are seen extracellularly, whereas in basophils, CLC are seen inside the cytoplasm. Arrows indicate CLC. × 1,000.
CLC: See Fig. 1.

Fig. 3  CLC formation by the sealed slide method (A) and the suspension culture method (B). The mean number of CLC per 1,000 eosinophils (4 patients) and per 1,000 basophils (9 patients) are shown. •: 4°C, •: 37°C. CLC: See Fig. 1.
Fig. 4 Changes of basophil granules following stimulation with anti-IgE or anti-IgG antibody. In each experiment, 500 basophil granules from 3 patients were observed.

- granules including crystalline structures;
- isolated, swollen and vacuolated granules;
- normal granules.

Fig. 5 Formation of Charcot-Leyden crystals (CLC) in basophils during suspension culture at 4°C for 24h. CLC are observed inside the granules (A, B), and in the cytoplasm outside the granules (C, D). Arrows indicate CLC. Bar = 1μm.
Transmission electron microscopy, however revealed CLC-like crystalline structures in the basophils. These crystals did not increase in number after stimulation with the anti-IgE antibody although the proportion of isolated, swollen and vacuolated granules increased. Degranulation and CLC formation were not increased by stimulation with the anti-IgG antibody (Fig. 4). In these cultures, all the crystalline structures were observed inside the granules. In the mast cells, changes suggestive of degranulation; the formation of labyrinths or vacuoles, were observed after stimulation with anti-IgE. These changes were observed in 24% of granules of control cells, and in 57% and 26%, respectively after anti-IgE and anti-IgG stimulation. No crystalline structures suggestive of CLC were observed in mast cells after stimulation with either anti-IgE or anti-IgG.

To clarify the localization of CLC in basophils, the process of CLC formation by the suspension culture method was observed using electron microscopy. Irregular or hexagonal crystalline structures were observed in the cytoplasmic granules (Fig. 5, A and B). In addition, large hexagonal bipyramidal crystals typical of CLC were present in the cytoplasm outside the granules (Fig. 5, C and D). CLC, especially the larger crystals, were rarely observed outside the basophils. There appeared to be no differences in the process of basophil CLC formation between the asthmatics and the CML patients. In the case of mast cells, no crystalline structures suggestive of CLC were observed during any of these experiments.

Discussion

Gleich et al. recently reported that desquamative eosinophilic bronchitis was caused by major basic protein (MBP) and was associated with perennial and severe asthma (10, 21). CLC protein as well as MBP is present in eosinophils and CLC are observed together with numerous eosinophils in the sputum of asthma patients. However, the clinical and pathological role of CLC in asthma is still unknown.

CLC were once thought to be exclusive to eosinophils, and it was believed that the CLC in sputum were produced only by eosinophils. In this study, however, we detected CLC in basophils in the sputum. These findings have not been reported before, and it is suggested that the sputum CLC are derived not only from eosinophils but also from basophils.

We also conducted studies to determine the conditions promoting CLC formation and the localization of CLC in basophils. CLC formation was increased by non-physiological stimuli, and a temperature of 4°C was better than 37°C for promoting the formation of crystals. By the sealed slide method, CLC were seen inside the cytoplasm of basophils, whereas in eosinophils, many CLC were seen extracellularly. It was suggested that CLC protein in eosinophils were easily released extracellularly under this condition. Following immunological stimulation with anti-IgE antibody, the morphologic changes of degranulation were observed, but there was no relationship noted between CLC formation and degranulation. Therefore, it was concluded that CLC formation might be related to cell damage. CLC protein is a major eosinophil protein and constitutes 7-10% of total cellular protein (9). The number of CLC in basophils seemed to be almost identical to that in eosinophils, suggesting that a large CLC protein pool is present in basophils. Accordingly it is also necessary to consider the pathological role of CLC protein in basophils.

The present study clarified the very early process of CLC formation in basophils which began in the cytoplasmic granules. In the first stage, many of crystalline structures were irregular and not hexagonal. They then appeared to increase in size and form the hexagonal, bipyramidal structures which are typical of CLC. The crystals grew large and were released outside the granules. Using immunofluorescence and an anti-CLC antibody, Ackerman et al. (11) have detected CLC protein in the cytoplasm of basophils. Our electron microscopic study also demonstrated that CLC were formed in the granules of basophils. While the presence of crystalline structures in basophils has been reported by several investigators (10, 11, 13, 22), this study provides the first observation of the process of CLC formation in the cytoplasmic granules of basophils under the conditions of non-physiologic and immunologic stimulation.

Basophils and mast cells both possess metachromatic granules containing histamine, which are demonstrated by staining with toluidine blue. However, many differences between these two cells have been pointed out by investigators (23, 24). In this study, we attempted to induce CLC formation in mast cells as well as in basophils. However, no crystalline structures were observed in the mast cells under any conditions tested. These findings strongly suggest that mast cells do not contain CLC protein. Confirmation of the presence or absence of CLC
protein in basophils and mast cells may help to elucidate the origin and functional characteristics of these two types of histamine-containing cells.

References


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