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ACTIVITY OF SOME HYDROLYTIC ENZYMES IN THE CELLS OF SKIN EXUDATE AND IN BLOOD AFTER PYROGEN INJECTION*

By

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The investigations presented here are concerned with the influence of the intravenously injected pyrogen upon the cells of skin exudate and white blood count in patients suffering from bronchial asthma and atopic dermatitis.

The injected pyrogen brings about the disintegration of granulocytes thereby liberating fever provoking agents Wood (3).

Changes in the osmotic resistance of granulocytes and in their enzymic activity may give indications as to the mechanism of this disintegration. Another problem presented here is the differentiation of monuclear elements in the sterile skin exudate.

MATERIAL AND METHODS

Skin exudate obtained by the Rebeck Crowley method (10) from 20 patients suffering from bronchial asthma and atopic dermatitis were examined. Examinations were made every hour for a period of 24 hours. The same procedure was repeated after the pyrogen administration. At the same time body temperature was taken. Blood smears were made before the administration of pyrogen, and while the temperature reached its peak after the injection. At this moment the osmotic fragility of white blood cells was calculated acc. to *Giedosz et al.*

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(3). Counts were repeated 4 times during 3 hours and the lysis rate of white blood cells was put down on a diagram. The results were statistically analyzed.

Blood preparations obtained from 12 patients were stained according to the Giemsa method in order to discover the changes in the percentage number of the types of the cell exudate and their morphological features.

Histochemical investigations performed on the material of 8 remaining patients included: acid phosphatase detected by the method of *Barka & Andersen* (1) using naphthol phosphate AS-CL as a substrate and hexasonium pararosaniline as a coupler at pH 4.7. The incubation time was 75 minutes.

Beta-glucuronidase shown by the method of *Hayashi* (4) using naphthol AS-BI glucuronide as a substrate. The product of hydrolysis was coupled with hexasonium pararosaniline at pH 4.9. The incubation time was 3 hours.

The alkaline phosphatase was shown by the method of *Kaplow* (7) modified by *Cichocki* using alpha naphthol sodium phosphate as a substrate and diazo salt Fast Blue RR as a coupler. The pH of the incubating medium was 9.6 and the time of incubation was 20 minutes.

The intensity of histochemical reactions in blood was evaluated by the score technique whereas in exudates, apart from the intensity of the reaction, its localization and character were also described. In order to compare the histochemical reactions from the same case as well as exudates from different persons, beta-glucuronidase was shown in the exudate obtained during the 5th hour, acid phosphatase during the 6th and alkaline phosphatase during the 7th hour of the given cycle.

In a few cases treated by increasing doses of pyrogen the skin exudates were examined several times in order to establish possible relations between the behaviour of the exudate cells and the patients' reactions to pyrogen.

RESULTS

Before the administration of pyrogen the differential count of cells in the skin exudate as well as the time of its appearance were found to be in normal range. During the first hours of the experiment the exudates consisted mainly of granulocytes, in the 6th hour and in some cases in the 4th or 5th hour mononuclear cells began to predominate. About the 24th hour the whole process resulted in the fibroblastic transformation. Eosinophils were found only sporadically (up to 4 per cent of cell population) in the early hours of the cycle, their count rose however to 10 per cent in patients with exacerbation of atopic dermatitis. Vacuolization of mononuclear cells and not so pronounced of granulocytes were observed. The remnants of disintegrated cells were also present.

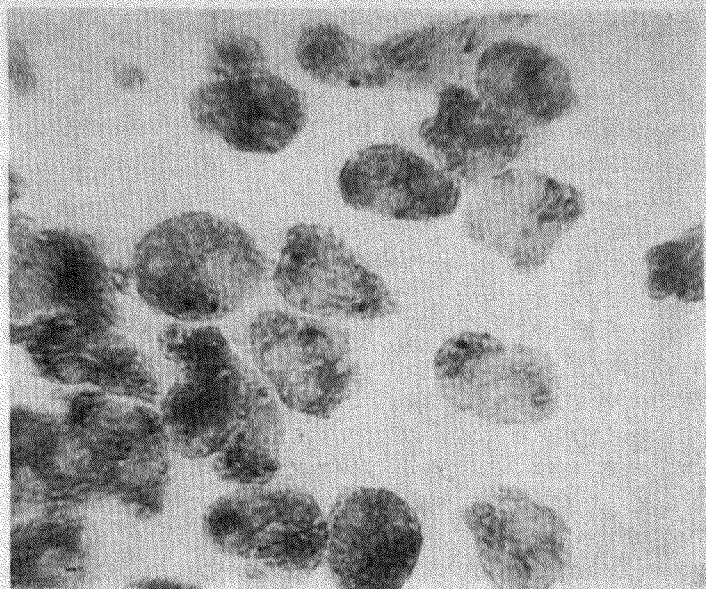


Fig. 1.

The cells of control Rebuck exudate. Barka and Anderson method for acid phosphatase. Reaction within granulocytes in fine granular and in the form of large grains that react very strongly.

The reaction for acid phosphatase in granulocytes showed a varying intensity. This depended on how the cell were spread out. Granulocytes with a tendency to disintegrate were usually negative, but the granulocytes which were submerged in protein exudate tended to react vere strongly. The reaction occured mostly in the whole cytoplasm, was fine granular (Fig. 1), while coarse granules occured very seldom. The formation of separated vesicles, which were pinched off from the cells, was observed. These were strongly active for acid phosphatase, while the rest of cytoplasm reacted much more weakly or was even completely negative.

Mononuclear cells did not behave uniformly. The majority of them resembled blood monocytes with a strong diffuse reaction in the whole cytoplasm. Also much smaller cells rounder in the shape were encountered. They resembled lympho-

cytes and displayed the reaction in form of granules well delimited from the negative cytoplasm. In other mononuclear cells both the diffusive reaction and the granular one occurred. The granules were of two types *i. e.* some of them spherical stained intensively, the others larger with blurred outlines. The active area was located in a concavity of the nucleus and corresponded to the localization of Golgi zone.

Beta-glucuronidase reaction behaved similarly to acid phosphatase, however granulocytes on the whole reacted less strongly. Some of the pinched off vesicles showed a strong enzymatic activity, other were negative. In mononuclears the reaction was somewhat weaker than that for acid phosphatase. The localization was similar, though the number of positively reacting granules was smaller.

The eosinophiles observed in the exudate reacted more strongly than the neutrophils. The reactions for beta-glucuronidase and acid phosphatase were localized in the whole cytoplasm and were more marked near to the surrounding and/or on the surface of specific granules.

The reaction for alkaline phosphatase was positive in the majority of granulocytes in the exudate. The precipitate was fine granular and diffused. The disintegrating granulocytes reacted very weakly. The vesicles observed during the process of separation from the granulocytes were generally active, but among the free vesicles both active and negative were observed. Mononuclear cells were mostly negative, but in two cases a very slight diffuse reaction located in the whole cytoplasm was observed. The vesicles pinched off from them were mostly negative. No reaction for alkaline phosphatase was observed within eosinophils.

After intravenous injection of pyrogen the cell exudate changed considerably and differed markedly from the picture described above. The cell exudate appeared also 2-3 hours after the skin abrasion, but the cell were fewer in number. The exudates consisted mainly of granulocytes approaching 90 per cent of all cell population. The domination of granulocytes was observed during the whole 24 h. Rebeck cycle. Their



Fig. 2.

The cells of Rebuck exudate after pyrogen injection. Reaction for acid phosphatase. Mononuclear cells show very strong reaction and vacuolization of their cytoplasm.

percentage number rose correspondingly to body temperature. During first 2–5 hours of the Rebuck cycle the disintegration of granulocytes and the presence of vacuoles in the cytoplasm and in the nucleus was observed. A few monocytes present in the exudate showed considerable vacuolization, (Fig. 2) and in sporadic cases their disintegration was observed.

Eosinophiles completely disappeared from the exudate. These changes are in agreement with that observed by *Weiss* (11). The reaction for acid phosphatase in granulocytes was definitely weaker than before the pyrogen injection, especially in the disintegrating cells. It seems that the disintegration proceeds in two ways: some of the granules seemed to have melted completely and showed negligible enzymatic activity, other formed the above described vesicles that contained the

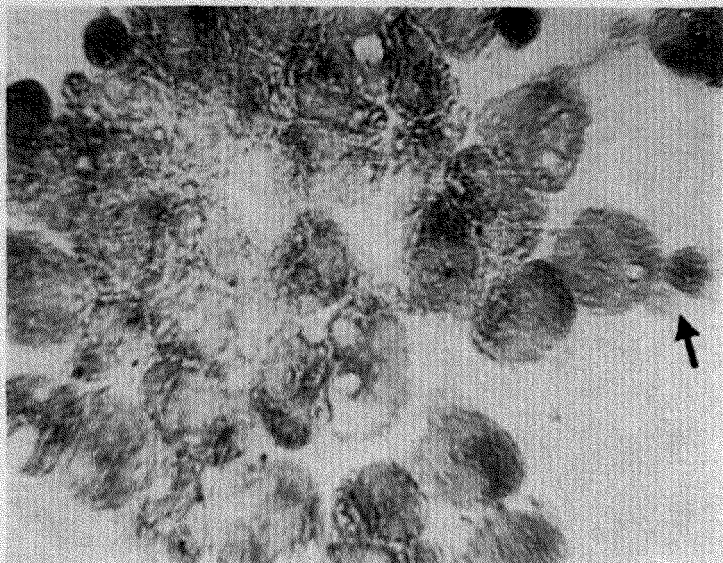


Fig. 3.

The cells of Re buck exudate after pyrogen injection. The cluster of granulocytes. Some of them are vacuolized and disintegrating. An arrow points to the granulocyte from which two vesicles one positive and the other negative are being pinched off. The free vesicles—both positive and negative are also visible.

Reaction for acid phosphatase.

whole active enzyme leaving the rest of the cell inactive. It would seem that the latter process is likely to proceed the former. The separated vesicles were present in the exudate and were both active and negative (Fig. 3). The extracellular activity of acid phosphatase in the form of granular masses was also observed. Mononuclear cells sometimes reacted more strongly than in the control group *i. e.* before pyrogen injection, except the disintegrating cells which were less active. More frequently than in the control group the concentration of enzymatic activity within wide pseudopodia which subsequently get pinched off was observed. Vacuoles present in great number in the mononuclear cells sometimes were surrounded by a zone of highly active cytoplasm (Fig. 2). Similarly as in the control group, mononuclear cells which cor-

responded to blood monocytes displayed signs of phagocytosis of lymphocytes. These digestive vacuoles were highly active.

Beta-glucuronidase gave similar localization as the acid phosphatase.

The activity of alkaline phosphatase in granulocytes after the administration of pyrogen was much more weaker than in the control group. Many granulocytes were completely negative. The extracellular activity was observed in the form of granules but their amount was distinctly less than those reacting for acid phosphatase.

In histochemical analysis of blood smears, reactions to acid and alkaline phosphatases seemed to be stronger in granulocytes of blood than in those derived from the skin exudate. When comparing blood smears before and after the administration of pyrogen no morphological changes in granulocytes were found, but the tendency towards lowering all investigated enzymes activity after the injection of pyrogen was noted. The differences seemed to be more pronounced than in the granulocytes in the skin exudate, although the changes occurred parallelly. The osmotic resistances of granulocytes did not change characteristically when measured before the pyrogen injection and at the time of the highest body temperature when the leucocytosis after the initial fall did exceed the control values.

DISCUSSION

The pyrogenic action of intravenously injected endotoxin from *Escherichia coli* is well known. The fever provoking mechanism is complex, and has been so far not fully understood. Disintegration of granulocytes and their increased adhesion to the walls of blood vessels due to the pyrogenic factor may explain the decrease in the number of circulating white blood cells (*Besson* quot. after *Wood* 13). Endogenous pyrogenic substances are liberated from the disintegrated granulocytes and act upon the thermoregulating centers. The substances mentioned above are probably contained in specific granules of granulocytes. They correspond to lysosomes but

do not contain acid proteases (*Janoff & Zweifach* (5)). The protein fraction responsible for setting in motion the pyrogenic mechanism is isolated from lysosomes by weak solutions of acids and precipitated by 20 per cent ethyl alcohol. A group of those proteins, polycation in character in addition to the pyrexial reaction causes the local inflammation author which referred *Seegers and Janoff* (11) and does not show any peroxydase activity in contrast to the fraction liberated from eosinophiles and acting in a similar way *Janoff et al.* (6).

Changes observed by us in the structure of cells forming the exudate as well as in the intracellular enzyme activity support the opinion that granulocytes are involved in the process of fever provoking and in some local phenomena of inflammation. The decrease in the amount of cell elements in the exudate observed after the injection of pyrogen could be explained by slower migration of granulocytes adhering to the blood walls. The percentage decrease in the number of mononuclear cells present in the exudate can be therefore regarded as a result of the general retardation in the Rebeck cycle.

The disintegration of granulocytes caused by pyrogen, as described above, is often preceded by accumulation of acid hydrolases at the pole of the cell which was later pinched off, forming a separate vesicle. It is possible that this mechanism serves the purpose of saving some substances active in later stages of inflammation. The free vesicles described also in the skin exudate by *Kowalewski* (8) may derive from two sources: from disintegrating granulocytes and from disintegrating mononuclear cells. It is probable that the activity of alkaline phosphatase exists only in the vesicles pinched off from granulocytes, while the activity of acid phosphatase and beta-glucuronidase can be by found in the vesicles originating from both sources. Negative vesicles may contain an enzyme which not detectable or some other nonenzymatic substances which may be active in certain inflammation stages *i.e.* the factor increasing the permeability of the vessels described by *Janoff et al.* (6) which probably acts by liberating histamine from

mastocytes. The vacuolization of cells especially of mononuclears, the presence of large lysosomes (digestive vacuoles acc. to *de Duve* (2) in granulocytes and the active zone observed around the vacuoles in mononuclear cells indicate the changes in inhibition and enhancement of phagocytosis following the pyrogen injection. The phagocytosis is particularly intensive in mononuclear cells and according to *Oppenheim et al.* (9) these cells are responsible for phagocytosis of the induced pyrogen.

Transformation of lymphocytes in to large mononuclear cells which is observed sporadically may be regarded as a further reaction to the endotoxin.

The decrease in the number of eosinophiles is probably due to their disintegration, bringing about the liberation of peroxidase-positive fraction acting as a liberator of histamine. The changes in the number of eosinophiles and neutrophil granulocytes may also be influenced by the function of adrenal cortex.

The modifications in the blood were parallel to the changes in the skin exudate. A decrease in the activity of alkaline and acid phosphatases following the pyrogen injection suggests that the cells of higher enzymatic activity are eliminated from the circulation. The results obtained by the osmotic resistance test indicate that the disintegration of granulocytes was due rather to the chemical action of pyrogen on lysosomal membranes than to the physical changes in the osmotic resistance of white blood cells.

The results presented here were impaired by the fact that the blood was examined at the time of highest body temperature when leucocytosis exceeded normal values. It is therefore difficult to draw any conclusions concerning the mechanism of the initial decrease in white blood count.

CONCLUSIONS

Intravenous injection of pyrogen causes changes in the cell composition of the skin exudate. Injection of pyrogen causes

a marked decrease of enzymatic activity of granulocytes in the exudate. This fact suggests that the lysosomal material has been utilized extracellularly probably as a pyrogenic factor with a liberation of histamine from mastocytes.

Mononuclear cells which mostly corresponded to monocytes after pyrogen injection showed a considerable vacuolization and rise in phagocytic activity manifested by increased activity of lysosomal enzymes. Lymphocytes were scarce in skin exudate and showed transitional forms towards large mononuclear cells.

In the granulocytes of the blood a decrease in activity of alkaline and acid phosphatase following the injection of pyrogen was observed. Disintegration of granulocytes observed in the skin exudate after the pyrogen injection resulted in the formation of vesicles containing substances active in further stages of inflammation.

Pyrogen for intravenous injections from *Escherichia Coli* was produced by Serum and Vaccine Laboratory Cracow, Poland.

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