The induction of lysosomal enzyme release from leucocytes of normal and emphysematous subjects and the effects of tobacco smoke upon phagocytosis

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Summary

1. The lysosomal enzymes of circulating polymorphonuclear leucocytes contain a potent elastase; release of this enzyme within the lung is thought to be responsible for the destruction of elastic tissue in pulmonary emphysema.

2. The release of lysosomal enzymes from blood leucocytes of normal and emphysematous subjects during phagocytosis of particulate material was studied in vitro. Acid phosphatase and acid ribonuclease were used as markers of lysosomal enzyme release, no sufficiently sensitive assay for elastase being available. Cigarette smoke was separated into ‘particulate’ and ‘soluble’ fractions. In a preliminary study, the particulate fraction stimulated enzyme release; in the experiments reported here, latex particles were used to produce this effect.

3. Approximately one-third of the total lysosomal enzyme content was released to the exterior of the cell during phagocytosis of latex particles. In this respect there was no difference between normal and emphysematous subjects.

4. The effects of the non-particulate soluble fraction of cigarette smoke on phagocytosis-induced enzyme release were studied. This fraction inhibited enzyme release from polymorphonuclear leucocytes of normal subjects but not from those of emphysematous patients. When the ‘cigarette-smoke solution’ was replaced by the respiratory inhibitor, antimycin A, a similar inhibition of enzyme release occurred. The inhibition of phagocytosis in cells of normal subjects is presumed to be due to a respiratory inhibitor such as carbon monoxide in the soluble fraction of the smoke. We postulate that the polymorphonuclear leucocytes of emphysematous patients are adapted to hypoxic conditions so that inhibition of enzyme release does not occur.

Key words: leucocytes, lysosomal enzymes, phagocytosis, pulmonary emphysema, tobacco smoke.

Introduction

The lysosomes of neutrophil leucocytes contain a potent elastase (Janoff & Scherer, 1968). This has been purified (Feinstein & Janoff, 1975) and has been shown to induce lesions similar to human emphysema when administered experimentally into the trachea (Janoff, Sloan, Weinbaum, Damiano, Sandhaus, Elias & Kimbel, 1977). It is thought that lysosomal elastase may also be responsible for the lung tissue destruction seen in the human disease, although its action would normally be opposed by protease inhibitors, of which α1-antitrypsin is one of the most important. Hereditary α1-antitrypsin deficiency is strongly associated with pulmonary emphysema (Eriksson, 1962) and it was suggested that the predominant destruction of the lower zones of the lungs could be related to the greater blood flow per unit volume in that region. The severity of emphysema in α1-antitrypsin deficiency at a given
age is extremely variable; non-smokers in general develop the disease later in life than smokers and have a chance of avoiding it altogether (Hutchison, Cook, Barter, Harris & Hugh-Jones, 1971; Kueppers & Black, 1974). Likewise in emphysematous patients who are not of the ZZ phenotype there is a strong association with smoking.

Sequestration of neutrophil leucocytes is a normal occurrence within the pulmonary vascular bed, a process that can be much enhanced in the various forms of ‘shock-lung’ and can result in alveolar destruction (Wittels, Coalson, Welch & Guenter, 1974). It has, furthermore, been observed that the total lysosomal elastolytic activity of circulating polymorphonuclear leucocytes is greater than normal in emphysematous patients, both with and without $\alpha_1$-antitrypsin deficiency (Galdston, Melnick, Goldring, Levytska, Curasi & Davis, 1977; Kidokoro, Kravis, Moser, Taylor & Crawford, 1977; Rodriguez, Seals, Radin, Lin, Mandl & Turino, 1979). It has been suggested that the increased lysosomal enzyme activity represents a predisposition towards lung disease rather than a response to it. If, however, those with the higher activities of neutrophil elastase were more prone to lung damage one would also have to postulate that the amount of enzyme actually released in vivo is proportional to the total enzyme content of the individual cells.

Certainly, lysosomal enzymes can be released during phagocytosis of inert particles without there being any serious damage to the phagocytosing cell (Weissmann, Zurier & Hoffstein, 1972). Cigarette smoke contains a proportion of particulate material and we considered that elastase released during ingestion of this material might be responsible for the lung damage of emphysema. Preliminary experiments indicated that lysosomal enzymes could indeed be released in this way, but difficulties in quantification of smoke particles led us to conduct controlled experiments with known numbers of latex particles. The results have already been reported in abstract (Desai, Baum, Bellamy & Hutchison, 1978). As we also reported, and will discuss more extensively in this paper, the non-particulate components of cigarette smoke inhibit enzyme release from cells of healthy subjects in response to a standard phagocytic stimulus. Significantly, no such inhibition was observed with cells from emphysematous patients.

Acid phosphatase and acid ribonuclease were used as markers of lysosomal enzyme release in this study as the available elastase assays were not sufficiently sensitive or reproducible for routine determinations under the conditions of our experiments; we cannot necessarily assume therefore that elastase was always released in parallel with the other enzymes. Nevertheless a significant difference was demonstrated between the behaviour of leucocytes of normal and emphysematous subjects.

**Methods**

**Subjects**

Patients with evidence of pulmonary emphysema were selected according to the radiological criteria of Laws & Heard (1962). All were in a stable clinical condition and free from acute infection. The control subjects were healthy male non-smoking members of the hospital staff. $\alpha_1$-Antitrypsin phenotypes were determined by starch-gel electrophoresis (Cook, 1975). Venous blood (50 ml) was taken from patients and control subjects into lithium/heparin-treated tubes and coded samples were analysed within 2 h of venesection. The patients gave informed consent and the study was approved by the Hospital Ethical Committee.

**Pulmonary function tests**

Forced respiratory volume in 1 s and vital capacity were measured with a Bernstein spirometer. Vital capacity was performed as a slow manœuvre (Hutchison, Barter & Martelli, 1973). Carbon monoxide-transfer factor was measured by the single-breath method (Ogilvie, Forster, Blakemore & Morton, 1957). Arterial oxygen tension was measured with a Corning analyser (type 165). Predicted values were obtained from tables prepared by Cotes (1975).

**Isolation of leucocytes**

Total and differential leucocyte counts were obtained. A mixed leucocyte population was obtained by sedimentation of the erythrocytes with 6% dextran of molecular weight 70 000 in sodium chloride solution (150 mmol/l: saline) (Lomodex 70, Fisons Pharmaceuticals Ltd, Loughborough, U.K.) and any erythrocytes remaining in the supernatant were lysed by suspension in distilled water for 30 s. The leucocytes were washed twice with minimal essential medium (Gibco Biocult Ltd, Paisley, Scotland), buffered to pH 7·2 with HEPES (British Drug Houses Ltd, Poole, Dorset, U.K.) and counted with a haemocytometer. No further fractionation of the leucocytes was undertaken.
**Preparation of aqueous extract of cigarette smoke**

Smoke from three medium-tar filter-tip cigarettes was drawn through 5 ml of saline and the particulate components in the resulting suspension were separated by passage through a 0.2 \( \mu m \) membrane filter. The resulting filtrate was designated the 'cigarette-smoke solution'.

**Lysosomal enzyme release**

All estimations were performed in duplicate and samples from normal and emphysematous subjects were analysed in parallel.

The mixed leucocyte population (1–3 \( \times 10^4 \) cells in 1 ml of minimal essential medium) was equally divided between three reaction vessels and adjusted for volume: (A) vessel 1: leucocytes alone (control); (B) vessel 2: leucocytes mixed with latex particles (approximately 10/cell). The latex particles (diameter 1-1 \( \mu m \)) were opsonized by incubation with calf serum (Flow Laboratories Ltd, Irvine, Scotland) for 30 min before use; (C) vessel 3: as vessel 2, plus 0.5 ml of the cigarette-smoke solution.

The three vessels were incubated at 37°C for 2 h and centrifuged; the cell pellets and supernatant fluids were then assayed for lactate dehydrogenase (EC 1.1.1.27) and for the lysosomal enzymes, acid phosphatase (EC 3.1.3.2) and acid ribonuclease (EC 3.1.27.1). The amount of enzyme released during phagocytosis was expressed as a percentage of the total cellular enzyme content determined after lysis of the cells at the close of each experiment. Cigarette-smoke solution alone had no significant effect on the release of lactate dehydrogenase or lysosomal enzymes so that A was a sufficient control for C as well as for B.

**Experiments with antimycin A**

Methods were the same as described above except that antimycin A (0-01 mg) was substituted for the cigarette-smoke solution in vessel 3. Release of acid phosphatase was assayed in three control subjects and three patients with emphysema.

**Enzyme assay**

Acid phosphatase was determined by measuring the amount of phosphate liberated from \( \beta \)-glycerophosphate (Vaes & Jacques, 1965) and acid ribonuclease by the method of Josefsson & Lagerstedt (1962). Lactate dehydrogenase was assayed spectrophotometrically by following the oxidation of NADH at 340 nm with pyruvate as substrate.

**Results**

Blood samples were obtained from 32 patients with radiological evidence of pulmonary emphysema. Their mean age was 59 years (range 28–71 years). Results of lung-function tests as a percentage of predicted (mean \( \pm 1 \) SD) values were:

- Forced expiratory volume in 1 s 37.5 \( \pm 20 \) 0%
- Vital capacity 79.3 \( \pm 20-9 \) %
- Single breath CO transfer factor 48.2 \( \pm 28-2 \) %
- Arterial O\(_2\) tension was 8.6 \( \pm 1-7 \) kPa (normal range: 11-0–12.5 kPa)

Fourteen patients were current cigarette smokers and the remaining 18 were ex-smokers. Only one of the patients was female and two had \( \alpha \)-antitrypsin deficiency (homozygous for Pi type Z). Blood samples (35 in all) were also obtained from seven healthy non-smoking subjects (mean age 34 \( \pm 6 \) years).

**Leucocyte counts**

In the emphysematous subjects, the mean leucocyte count was 7.3 \( \times 10^9 \) \( \pm 5 \) SD 1.3 \( \times 10^9 \) cells/l. Differential counts were neutrophils, 61 \( \pm 12 \) %, lymphocytes, 31 \( \pm 10 \) %, monocytes, 5 \( \pm 3 \) %, and eosinophils, 2 \( \pm 1 \) %. These results do not differ significantly from normal values.

**Enzyme release**

Duplicate enzyme assays agreed within 5%. Repeatability of assays was assessed in two of the normal subjects over the course of several months. Tests were repeated 16 times in one subject and 10 times in the other. Standard deviation for latex-induced release (B — A) of acid phosphatase was 9.5% and for acid ribonuclease 7.8%, as percentage of total cellular enzyme content; for inhibition of release by cigarette-smoke solution (B — C), \( \pm 4 \) 1% and for acid ribonuclease 4.5%.

The quantity of enzyme released under each condition of incubation, expressed as a percentage of the total cellular enzyme content, is shown in Table 1. The amount of the cytoplasmic enzyme lactate dehydrogenase released was a small proportion of the total and did not increase significantly upon addition of the latex particles, or of the cigarette-smoke solution.
Values are expressed as percentage of total cellular enzyme content (mean ± 1 sd). Details of experiments A, B and C are given in the Methods section.

<table>
<thead>
<tr>
<th>Emphysema (n = 32)</th>
<th>Acid phosphatase</th>
<th>Acid ribonuclease</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control: cells alone</td>
<td>8.5 ± 4.6</td>
<td>13.7 ± 3.9</td>
</tr>
<tr>
<td>B Cells + latex</td>
<td>37.3 ± 9.2</td>
<td>45.2 ± 7.4</td>
</tr>
<tr>
<td>C Cells + latex + smoke solution</td>
<td>35.9 ± 8.6</td>
<td>43.9 ± 7.2</td>
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<table>
<thead>
<tr>
<th>Normal (n = 35)</th>
<th>Acid phosphatase</th>
<th>Acid ribonuclease</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control: cells alone</td>
<td>5.6 ± 5.2</td>
<td>13.9 ± 4.1</td>
</tr>
<tr>
<td>B Cells + latex</td>
<td>38.9 ± 9.4</td>
<td>44.3 ± 7.7</td>
</tr>
<tr>
<td>C Cells + latex + smoke solution</td>
<td>28.5 ± 7.1</td>
<td>34.6 ± 7.3</td>
</tr>
</tbody>
</table>

**Lysosomal and cytoplasmic enzyme release from leucocytes of emphysematous and normal subjects**

**Latex-induced enzyme release**

The enzyme release ascribable to phagocytosis, after subtraction of control values for each subject (B – A), is shown in section 1 of Table 2. Release of enzyme increased substantially during phagocytosis, but the mean differences between normal and emphysematous subjects did not reach significance at the 5% level.

**Effects of cigarette-smoke solution on latex-induced enzyme release**

The results of the experiments in which latex particles and cigarette-smoke solution were combined (C – A) are shown in section 2 of Table 2. After subtraction of control values, the enzyme release was significantly greater in emphysematous patients than in normal subjects; this appears to be due to the fact that inhibition of the latex-induced enzyme release by cigarette-smoke solution (B – C) occurred in normal subjects but not in patients with emphysema (Table 2: section 3). The difference between the two groups of subjects was highly significant in this respect (P < 0.001).

There was no correlation between inhibition of enzyme release (B – C) and age of the emphysematous subjects (r = 0.16; P > 0.05). There was no significant difference in the mean values of B – C between current smokers and ex-smokers by unpaired t-test (P > 0.3). There was no correlation between (B – C) and arterial oxygen tension in the emphysematous patients (r = –0.16; P > 0.05).

**Effects of antimony A**

The results of the experiments in which latex and antimony A were combined are shown in Table 3. As with the cigarette-smoke solution, the inhibitory effects of antimony A on latex-induced enzyme release were combined are shown in Table 3. As with the cigarette-smoke solution, the inhibitory effects of antimony A on latex-induced enzyme release were combined (B – C) and age of the emphysematous subjects (r = 0.16; P > 0.05). There was no significant difference in the mean values of B – C between current smokers and ex-smokers by unpaired t-test (P > 0.3). There was no correlation between (B – C) and arterial oxygen tension in the emphysematous patients (r = –0.16; P > 0.05).

**Table 1. Lysosomal and cytoplasmic enzyme release from leucocytes of emphysematous and normal subjects**

**Table 2. Effects of latex particles and cigarette-smoke solution on lysosomal enzyme release**

**Table 3. Effects of antimony A on latex-induced release of acid phosphatase**
release occurred in normal subjects but not in patients with emphysema \((P < 0.02)\).

Discussion

The destruction of the pulmonary elastic tissue in emphysema appears to be due to a disturbance of the normal balance between proteolytic enzymes and their inhibitors. In \(\alpha_1\)-antitrypsin deficiency, the disease is quite plainly related to the reduction in the main serum protease inhibitor. In non-deficient cases of emphysema the clinical and physiological features are similar in many respects and it is reasonable to suppose that a similar biochemical imbalance exists. This might arise either from an excessive release of proteolytic enzymes or from some failure of inhibitory activity even though the inhibitors are apparently present in normal quantities. In this last context, Janoff & Carp (1977) have observed that cigarette smoke can block the action of \(\alpha_1\)-antitrypsin \textit{in vitro}.

Investigators have, however, paid more attention to factors which might promote release of proteolytic enzymes and obvious sources of enzyme are the lysosomes of circulating polymorphonuclear leucocytes or of the pulmonary alveolar macrophages. A comparison of macrophages obtained by bronchial lavage from smokers and non-smokers revealed clear differences (Pratt, Finley, Smith & Ladman, 1969; Harris, Olsen, Castle & Maloney, 1975); many more macrophages could be obtained from the smokers, the lysosomal bodies were greater in size and number and the elastase activity of each cell was twice as great.

No parallel morphological abnormalities can be observed in the circulating polymorphonuclear leucocytes, but there is ample evidence that lysosomal enzymes can be released from these cells during phagocytosis (Dingle, 1968; Weissmann \textit{et al.}, 1972). During normal intracellular digestion, proteolytic enzymes are transported in the lysosomes and released into phagocytic vacuoles ('phagosomes'), which become isolated from the exterior of the cell. This is a self-limiting process, which comes to a close when all digestible material has been degraded. If, however, the cell is allowed to engulf particulate material, the phagosome may fail to close, allowing lysosomal enzymes to leak to the exterior of the cell. During this process there is no release of cytoplasmic enzymes such as lactate dehydrogenase, which, if it occurred, would imply serious damage to the external cell membrane and probable death of the cell itself.

We have conducted some preliminary experiments which suggest that the particulate fraction of cigarette smoke can induce release of lysosomal enzymes from polymorphonuclear leucocytes, though the amount of enzyme released after treatment with whole cigarette smoke was less than that released in response to smoke particles alone; these experiments were, however, of poor reproducibility and have not been reported in detail here. Our more detailed studies show that latex particles of similar size can act in the same way as the cigarette-smoke particles. There is no direct evidence that this chain of events can occur in the human smoker, though some lysosomal enzyme release must take place during the removal of cell debris and coagulation products from the pulmonary vascular bed; enzyme release must also accompany the continuous process of breakdown and repair of the structural elements of the lung (Fulmer & Crystal, 1976). We have no definite evidence that elastase is always released in parallel with the other lysosomal enzymes, though acid phosphatase behaved in a similar manner to acid ribonuclease in our experiments. It would be reasonable, however, to postulate that enzyme release is enhanced in smokers and there is no doubt that in \(\alpha_1\)-antitrypsin deficiency smoking has very serious consequences. On the other hand, an explanation is still required for the fact that a certain number of \(\alpha_1\)-antitrypsin-deficient subjects develop emphysema at a surprisingly early age, even though they have never smoked.

It was therefore suggested (Galdston, Janoff & Davis, 1973) that variations in the severity of emphysema could be related to the total quantity of lysosomal protease within the polymorphonuclear leucocytes, individuals with the lower enzyme content thus having a better chance of avoiding the disease. They observed wide variations in polymorphonuclear leucocyte protease activities in the families of \(\alpha_1\)-antitrypsin-deficient subjects and similar variations were seen by Kidokoro \textit{et al.} (1977). The latter authors concluded that the abnormalities of lung function in nine subjects with \(\alpha_1\)-antitrypsin deficiency were more closely related to lysosomal protease concentration than to smoking history. In subjects with MM, MZ or ZZ phenotypes, polymorphonuclear leucocyte lysosomal protease was found to be significantly greater in subjects with chronic airflow obstruction than in healthy controls (Galdston \textit{et al.}, 1977; Rodriguez \textit{et al.}, 1979). Klayton, Fallat & Cohen (1975), however, could find no relationship between lysosomal enzyme activity (including elastase) and the severity of emphysema in heterozygotes of type
MZ. It is difficult therefore to draw a definite conclusion on the importance of total polymorph 
lymphocytic protease, though all studies are agreed on the wide variations that exist from subject to 
subject.

With regard to the mechanism of lymphocytic enzyme release from the polymorphonuclear leukocytes, we have postulated that this occurs during phagocytosis of inert material and have inferred that the particulate fraction of cigarette smoke could provide the necessary stimulus. We have also attempted to find an explanation for the fact that many heavy smokers never develop severe emphysema and have sought for differences be-
tween emphysematous patients and healthy controls in respect of phagocytosis-induced enzyme release. In fact, the experiments in which latex particles alone were used revealed no such differences, although on average about one-third of the total 
lysosomal enzyme could be released from the cell by this method. This seems to be a possible mechanism for the lung damage in emphysema; we have no definite evidence, however, on whether this operates in vivo and, if so, in which site particulate material comes into contact with the circulating polymorphonuclear leukocytes.

The experiments in which the latex-induced enzyme release was modified by the cigarette-smoke soluble fraction did, however, demonstrate that the net enzyme release was substantially greater in emphysematous subjects than in controls. A large proportion of the energy required by leucocytes, particularly during active phagocytosis, is supplied by the glycolytic pathway. In normal subjects, however, the inhibition of phagocytosis-induced enzyme release by the smoke soluble fraction (containing the potent respiratory inhibitor, carbon monoxide, of which cigarette smoke may contain as much as 5%) indicates that aerobic metabolism also contributes to the total energy requirement of the phagocytosing leukocyte fraction. The leucocytes of emphysematous patients, on the other hand, are apparently adapted to hypoxic conditions so that inhibition of enzyme release does not occur.

The supplementary experiments with antimycin A lend considerable support to this hypothesis. Antimycin A is a specific inhibitor of the mitochondrial oxidative pathway of energy metabolism, but not of anaerobic glycolysis. As with the cigarette-smoke soluble fraction, phagocytosis-induced enzyme release was suppressed by this inhibitor in cells from normal subjects but was not so affected in cells from patients with emphysema.

A possible criticism of these conclusions lies in the fact that we have studied a mixed cell population, so that the observed differences in enzyme release could result from changes in the proportion of the cell types. In fact this seems unlikely as the total and differential leucocyte counts of the emphysematous patients were quite normal. Again, enzyme release from monocytes could differ from that of the polymorphonuclear leukocytes but, even if this is so, it cannot alter our basic observation that there is an obvious difference between the two sets of blood samples.

So far as enzyme release is concerned, therefore, the soluble and particulate fractions of the cigarette smoke seem to work in opposition, but only in normal subjects. The net enzyme release induced by whole smoke may thus be greater in emphysema. This phenomenon might possibly be the basis for a predisposition to emphysema, but equally likely is an adaptation to the hypoxic conditions produced either by the disease itself or by chronic exposure to the carbon monoxide of cigarette smoke. In these respects, however, we could find no correlation between the depression of enzyme release and the arterial oxygen tension or indeed any other index of the severity of the disease. Nor was there any difference between smokers and ex-smokers in the amount of enzyme released; the matter thus remains an open question.

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References


Lysosomal enzyme release in emphysema


