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Activation of the alternative pathway of complement by grain

I. C3PA conversion and quantification of complement consumption by rye

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Summary

Ground whole rye and airborne rye dust of comparable size distribution were tested for their ability to activate the complement cascade via the alternative pathway. Precipitin-negative pooled normal human serum was incubated with increasing amounts of the two rye dusts. Electrophoresis of the resultant supernatant fluids demonstrated the conversion of the proactivator of the third component of complement to the gamma-migrating activator of the third component. This activation was completely prevented by pre-treating the serum with the chelator EDTA, while pre-treatment with EGTA allowed suboptimal arc conversion, strongly implying that complement was activated via the alternative pathway. Quantification of the supernatant fluids showed dose-dependent complement consumption as defined by both CH_{100} immunodiffusion and CH_{50} tube haemolytic techniques.

Airborne rye dust showed a greater quantitative potential than ground whole rye for activating the alternative pathway. These results indicate the possibility of the direct action of airborne organic dusts on the induction of inflammatory sequelae in the lungs of both sensitized and unsensitized individuals.

Introduction

Since the writings of Ramazzini (1713), diseases caused by grains and grain dusts have been documented, but insight into the aetiological mechanisms underlying these diseases has developed slowly. Using dust analysis, clinical examinations and survey questionnaires, it has been shown that allergic history is possibly important in the development of symptoms from grain dust exposure (Williams, Skoulas & Merriman, 1964; Skoulas, Williams & Merriman, 1964). After examination of environmental, clinical and physiological parameters, Kleinfeld *et al.* (1968) revealed that smoking history was a most important factor in causing the increased incidence of respiratory symptoms seen in the grain industry. Studies which included immunological assess-

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ments of grain workers have concluded that precipitating antibody titres do not correlate with clinical disease (Tse *et al.*, 1973: Warren, Cherniack & Tse, 1974), but positive prick testing indicates immediate hypersensitivity to crude grain dusts (Warren *et al.*, 1974) and fungi isolated from the grain (Darke *et al.*, 1976). That a myriad of fungal elements can be isolated from grain is now well-established (Farant, Moore & Windish, 1973; Darke *et al.*, 1976). These elements, alone or in concert with other contaminants of grain, such as mites, insects, rodent and bird parts and excreta, fumigants and applicants, all might well be expected to elicit altered pulmonary responses upon inhalation. The pulmonary insult is intensified by aflatoxin and other toxic metabolic products from micro-organisms which have been isolated from various grains in different states of deterioration (Trenk & Hartman, 1970; Mirocha *et al.*, 1976; Sommer, Buchanan & Fortlage, 1976; Lillehoj, Fennell & Kwolek, 1976). Many of these same contaminants can be found as incitants in another group of lung diseases, the extrinsic allergic alveolitides (Pepys, 1969).

One of the most extensively studied diseases of the extrinsic allergic alveolitides (EAA) is farmers' lung (Pepys, 1969; Emanuel *et al.*, 1964; Hapke *et al.*, 1968). It has been shown that a high proportion of reactive farmers have circulating precipitating antibodies to one or more of the thermophilic actinomycetes (Pepys & Jenkins, 1965). On the other hand, some individuals have the overt disease yet do not have demonstrable antibodies to the thermophiles (Edwards *et al.*, 1974; Pether & Greatorex, 1976). Although precipitins may also contribute to the pathogenesis of chronic EAA through antigen–antibody–classical complement reactions, they are primarily considered as evidence of exposure (Salvaggio, 1972). To explain part of the physiopathology experienced by precipitin-negative patients with farmers' lung, Edwards, Baker & Davies (1974) have shown that mouldy hay dust can activate the alternative pathway of complement *in vitro*, suggesting that multiple immunological mechanisms may be involved in this classical disease. This involvement of the alternative pathway with subsequent complement consumption can be quantified (Edwards, 1976), using standard haemolytic techniques (Mayer, 1961). It is the purpose of this paper to examine the possible relationships between grain dusts and activation of the alternative pathway of complement.

Materials and methods

Grain samples

Ground rye. Whole grain was obtained from active grain elevators in the Superior–Duluth areas of the United States, while the grain was being transported from elevator to elevator. The grain was frozen in liquid nitrogen and ground through a Wiley Mill (Arthur H. Thomas Co., Philadelphia, U.S.A.) to a 80 mesh size and stored at a 33 cm (Hg) vacuum in a dessiccator charged with anhydrous calcium sulphate (Drierite; W. A. Hammond Drierite Co., Xenia, U.S.A.).

Airborne rye dust. Airborne rye dust was collected using an industrial vacuum cleaner (Multi-Clean Wet-Dry 250, H. B. Fuller Co., St Paul, U.S.A.) equipped with a hose positioned 4–5 m above a conveyor belt on which rye was transported. Airflow through the hose was 3·54 m^3/min and dust was collected in a paper dust filter and a dual bag filter trapping 0·25 μm particles with 99·5% efficiency (Duo-bag filter, H. B. Fuller Co., St Paul, U.S.A.). The collected dust was stored using the procedure described above for ground rye.

Particle measurement. Both ground and airborne rye dust samples were measured using light microscopy and a Porton graticule (BGI Inc., Waltham, U.S.A.).

Reaction of grain with normal human serum. Various weights of the 80 mesh ground or airborne grain dusts were added to 0·5 ml of normal human serum (NHS) pooled from members of our laboratory. This serum was used fresh or frozen at −88°C and was negative for precipitins against rye as defined by both counter-immunoelectrophoresis and double-diffusion in gel. The reaction mixtures were then incubated in a shaking water bath at 125 strokes per min and 37°C for 60 min, after which the samples were centrifuged at 900 *g* for 7 min and the supernatant fluids removed.

8 μl aliquots of each supernatant fluid underwent electrophoresis in 1% agarose with pH 8·4 buffer for 45 min at 200 V in the cold (4°C). Conversion of C3 pro-activator (C3PA) to the active C3 activator (C3A) was observed by developing the electrophoresis slides with rabbit antiserum to human C3A (B_2-glycoprotein II; Behring Diagnostics, Sommerville, U.S.A.) for 24 hr in the cold. After examining the slides for the double arc which is indicative of C3PA conversion (Gotze & Müller-Eberhard, 1971), the slides were photographed.

Cleavage of C3 was assayed by two-dimensional antigen–antibody crossed electrophoresis using goat anti-human C3 (β_1C/β_1A; Cappel Laboratories, Cochranville, U.S.A.) in agarose with barbital buffer, pH 8·2, containing 0·01 M EDTA.

As a point of comparison, NHS without dust was treated in a similar manner.

Chelated serum and rye dust

Aliquots of 0·5 ml NHS were treated with either 0·05 ml of 100 mM EDTA (Fisher Scientific Co., Fair Lawn, U.S.A.) or 0·05 ml of 100 mM EGTA (Sigma Chemical Co., St Louis, U.S.A.) by the method of Fine *et al.* (1972), prior to the addition of 5 mg of ground or airborne rye dust. Electrophoresis of the resultant supernatant fluids reflected the suboptimal conversion of C3PA experienced by Des Prez *et al.* (1975). Altering the reaction mixture to 0·025 ml of 100 mM chelators allowed observation of the weak C3A arc with EGTA while completely blocking the C3PA conversion with EDTA.

Quantification of complement consumption by rye

Total haemolytic complement activity (CH_{100}) titres were defined for the supernatant fluids of NHS treated with ground or airborne rye dust, and untreated NHS, by radial diffusion of serum and subsequent lysis of sensitized sheep erythrocytes in agar gel (Quantiplate; Kallestad, Chaska, U.S.A.). Complement consumption by various quantities of rye dust was determined by comparison with the control NHS.

Haemolytic complement activity (CH_{50}) was quantified by the method of Mayer (1961).

Results

Particle size distribution of both rye samples is shown in Table 1. Both test samples were of the same relative size composition with the predominant particle population within the respirable range.

Both the ground rye and airborne rye samples acted as potent activators of C3PA conversion to C3A as demonstrated by Figs 1 and 2. It can be observed that increasing the amounts of the dusts resulted in greater gamma migration and increasingly

Table 1. Particle size distribution of ground whole rye and airborne rye dust

Particle size (μm)	Ground rye (%)	Airborne rye (%)
< 5	56·7	55·9
5–10	14·2	20·6
10–20	6·0	8·8
20–80	21·6	10·8
> 80	1·5	3·9
Total	100·0	100·0

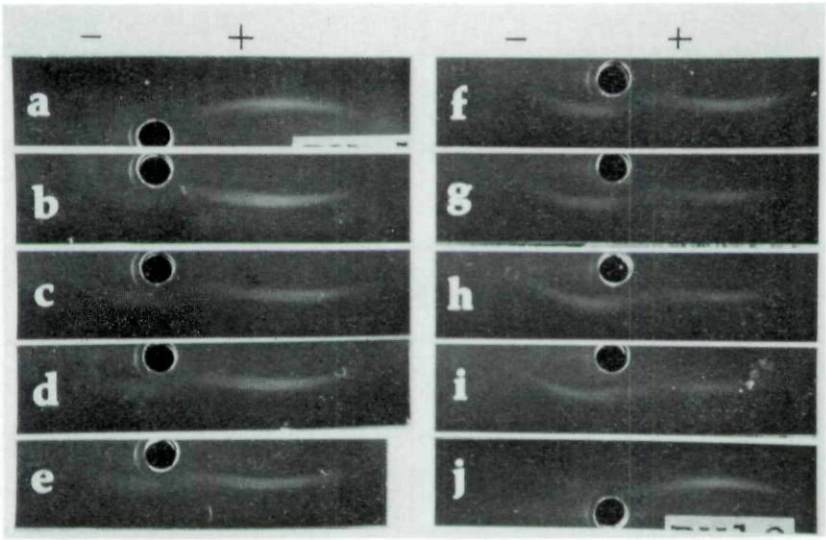

Fig. 1. Immunoelectrophoresis of pooled normal human serum after incubation with increasing amounts of ground whole rye and developed with rabbit anti-human C3 activator. Control serum without dust, (a) and (j). Serum with 0·1 mg dust, (b); 0·25 mg, (c); 0·5 mg, (d); 1 mg, (e); 5 mg, (f); 10 mg, (g); 15 mg, (h); and 20 mg, (i).

intense arcs. Judged by the migration and intensity of the arcs, the dose-dependent conversion of C3PA to C3A was also stronger with the airborne rye than with the ground grain. Conversion of the protein required a higher concentration of ground grain than airborne rye.

Ground rye and airborne rye dust also converted C3 to C3b in a dose-dependent manner. Fig. 3 illustrates the action of 0·5 mg dust on 0·5 ml NHS. 0·1 mg of dust caused a lesser C3b response.

To confirm the assumption that complement is activated via the alternative pathway, NHS was first chelated with EDTA or EGTA. The results of incubation of the chelated serum with ground or airborne rye are shown in Fig. 4. At a concentration of 5·0 mg dust and using 0·025 ml of 100 mM EDTA, the conversion of C3PA to C3A was totally prevented. On the other hand, the same amount of both dusts

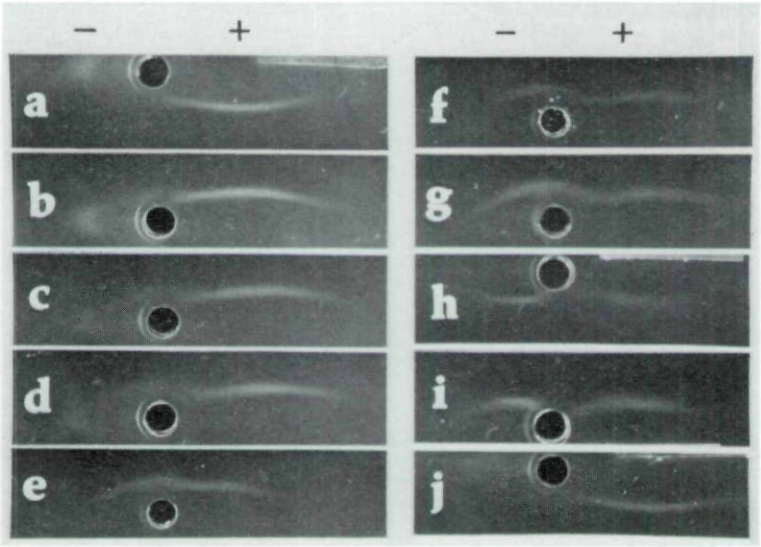

Fig. 2. Immunoelectrophoresis of pooled normal human serum after incubation with increasing amounts of airborne rye dust and developed with rabbit anti-human C3 activator. Control serum without dust, (a) and (j). Serum with 0·1 mg dust, (b); 0·25 mg, (c); 0·5 mg, (d); 1 mg, (e); 5 mg, (f); 10 mg, (g); 15 mg, (h); and 20 mg, (i).

caused C3PA conversion in the presence of a similar concentration of EGTA. These results strongly imply that the alternative pathway of complement was activated.

Figs 1 and 2 imply a dose–response relationship of dust and complement activation. These figures further suggest a functional difference in the ability of ground and airborne rye dusts to convert C3PA to C3A. To test these observations, complement consumption, as defined by percent reduction in CH_{100} u/ml, was assayed on the same supernatant fluids that were used for immunoelectrophoresis in Fig. 1 and 2. In confirmation of the qualitative observations, both ground and airborne rye caused increasing complement consumption with increasing amounts of dust (Fig. 5). The results also indicate a quantifiable difference between ground and airborne rye: 5 mg of the airborne rye consumed 100% of the haemolytic complement, while the same amount of ground rye consumed 47%. This difference between samples held true for every dust concentration tested.

CH_{50} u/ml available in the supernatant fluids were also assayed, in order to provide haemolytic reference levels in absolute numbers for each dust concentration. As expected from the CH_{100} study, there were quantifiable differences between the ground and airborne samples as well as dose–response differences.

Discussion

Examinations of grain workers have concluded that precipitating antibody titres did not correlate with clinical disease (Tse *et al.*, 1973; Warren *et al.*, 1974). These findings are consistent with the role of precipitins in farmers' lung, in which the precipitating antibodies to mouldy hay dust are considered indicative of exposure and not necessarily pathognomonic (Salvaggio, 1972). This concept does not negate the potential for the specific antibody contribution in disease production via an Arthus reaction in the

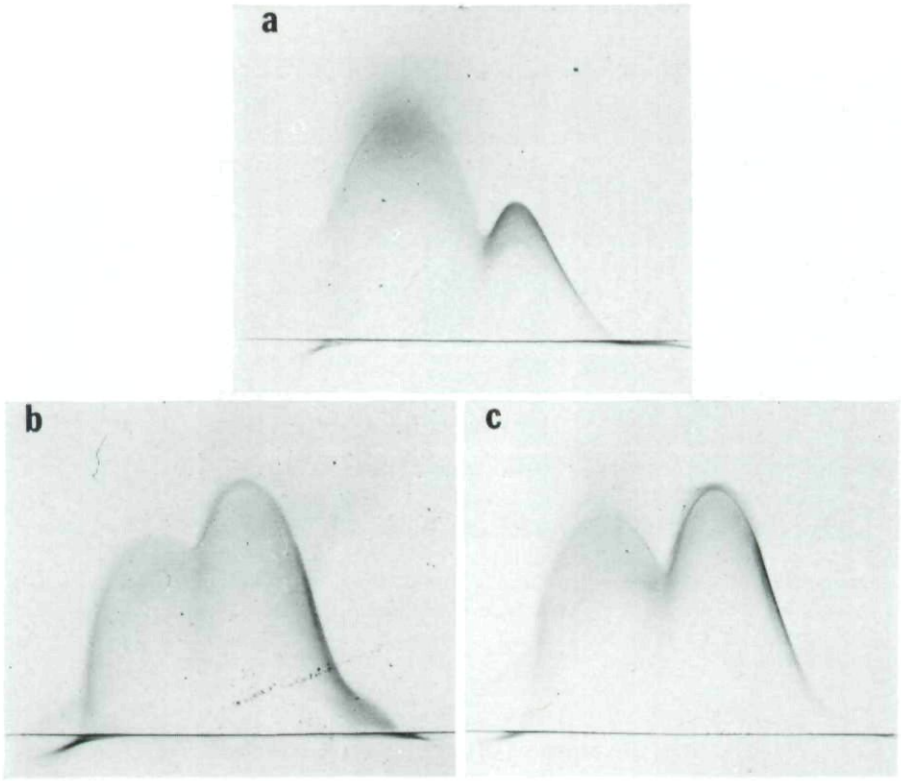

Fig. 3. Two-dimensional crossed electrophoresis using goat anti-human C3 in agarose containing 0·01 M EDTA. Pooled normal human serum without dust, (a); NHS plus ground rye, (b); NHS plus airborne rye, (c). Left arc is C3; right arc is C3b.

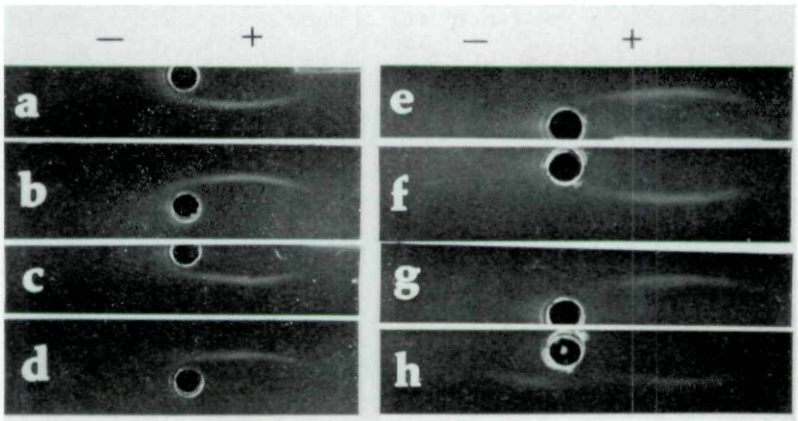

Fig. 4. Immunoelectrophoresis of chelated pooled normal human serum after incubation with ground or airborne rye dust and developed with rabbit anti-human C3 activator. Normal human serum and EDTA controls, (a) and (e); NHS plus EDTA plus ground rye, (b); NHS plus EDTA plus airborne rye, (f); NHS plus EGTA controls, (c) and (g); NHS plus EGTA plus ground rye, (d); NHS plus EGTA plus airborne rye, (h).

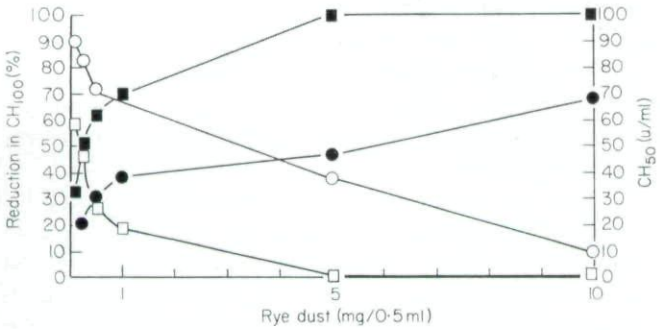

Fig. 5. Percentage reduction in CH_{100} u/ml and quantification of CH_{50} u/ml from pooled normal human serum incubated with increasing amounts of airborne (■, □) or ground (●, ○) rye dust. Closed symbols represent percentage reduction in CH_{100} while open symbols indicate CH_{50} u/ml.

lung (Pepys, 1969), but rather stimulates the search for additional inflammatory mechanisms occurring in the lung in response to the inhalation of organic dusts.

One such mechanism which is gaining popularity as a potential response to dust inhalation is the direct action of organic dusts on the complement cascade, specifically via the alternative pathway. This activity and subsequent tissue damage can occur in the absence of specific antibody (Osler & Sandberg, 1973) and may explain, in part, the clinical responses observed in patients without demonstrable antibody (Edwards *et al.*, 1974).

In the studies presented in this paper, pooled normal human serum, free of precipitating antibody to rye, was used for *in vitro* testing of the action of rye on the alternative pathway of complement. Increasing amounts of dust produced increasing conversion of the proactivator of C3, a hall-mark of alternative pathway activation (Götze & Müller-Eberhard, 1971). This was observed with both the ground whole rye and the airborne rye dust, although to a greater degree with the latter. The dose–response difference between the two samples was not a surface area phenomenon, since both were of similar size distribution. One may speculate then that the compositions of both ground and airborne rye differed. This possibility warrants further investigation.

That the activation of complement was indeed via the alternative pathway was demonstrated by the chelated serum techniques of Fine *et al.* (1972). The calcium-independent conversion of C3PA was demonstrated by the appearance of the C3A arc in serum chelated with EGTA. Because EGTA binds magnesium less effectively than EDTA (which completely prevented C3PA conversion) the arc conversion was suboptimal (Des Prez *et al.*, 1975; Fine *et al.*, 1972).

Qualitative dose–response differences evidenced by increasing gamma mobility and intensity of the C3A arc were also quantified in terms of the amount of complement consumed per dose of dust. This study showed that rye dust, either ground or airborne, produced large decreases of haemolytic complement, and that the difference between the two dusts was quantifiable. As a result, the airborne fraction was found to be an exceedingly potent activator of the complement cascade. In order to provide reference values of haemolytic complement available in each treated serum sample, the CH_{50} u/ml were also examined and the data presented.

Preliminary examination of airborne dusts of barley, corn, durum wheat, oats

and spring wheat indicates similar dose-dependent complement consumption via the alternative pathway. Similarly, early findings from studies of an extract of rye show that it too can activate the alternative pathway of complement. These studies imply, then, that airborne grain dusts have the propensity for inciting an inflammatory response directly upon inhalation, in addition to indirectly via macrophage processing. Details of these studies will be reported later.

With these results in mind, one may conclude that airborne rye dust, to which the grain workers would be most often exposed, has the potential to activate complement in both sensitized and unsensitized individuals. This potential is dose-dependent and might result in inflammatory sequelae in the lung. Although it is difficult to correlate *in vitro* quantifiable results with *in vivo* relevance, results of this study demonstrate that rye dust is potentially toxic and exposure to the airborne dusts should be minimized.

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