EXTRACTS OF AIRBORNE GRAIN DUSTS ACTIVATE ALTERNATIVE AND CLASSICAL COMPLEMENT PATHWAYS

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This study investigated the action of aqueous extracts of airborne grain dusts on the human alternative and classical complement pathways. Extracts were shown to consume hemolytic complement in vitro in a dose-dependent manner. No relationship was determined between complement activity ranking and either protein or endotoxin levels. Differential serum chelation with EDTA or EGTA showed that the alternative pathways were activated, while hemolytic titers of the early complement components (C1, C4, C2 and C3) showed that the classical pathway was also involved. Extract-treated sera were chemotactic for human polymorphonuclear leukocytes and complement was required. The in vitro data suggest the potential for the in vivo contribution of the activation of the complement cascade (by either pathway) in eliciting pulmonary pathophysiology after the inhalation of airborne grain dusts.

Introduction

SEVERAL STUDIES HAVE DEMONSTRATED that organic dusts which are associated with occupational exposures can activate the complement cascade by the alternative pathway.[1,2,3] Those studies suggested a potential contribution of the activated complement mechanism in eliciting respiratory pathophysiology after exposure. Our laboratories have since shown that airborne grain dusts can activate the alternative pathway of complement *in vitro*.[4,5] Inhalation of airborne grain dust results in dust contacting moist mucosal surfaces by impaction, sedimentation or diffusion (Brownian movement), based on the aerodynamic mass of the particles.[6] In addition, the pulmonary macrophage ingests and processes the inhaled organic material. Both direct mucosal contact and indirect macrophage processing could result in rapid aqueous extraction of the airborne grain dusts. Our preliminary study showed that rapid aqueous extracts of airborne oat and spring wheat dusts activate the third component of complement (C3) by the alternative pathway and consume total hemolytic complement (CH_{50} u/ml) in the absence of specific antibody.[7] While the data provide evidence of alternative pathway activation, they do not negate the potential involvement of the classical pathway.

It is the purpose of this presentation to expand the preliminary studies of airborne grain dust extracts and complement activation via the alternative pathway and to examine the possible activation of the classical pathway as well. In addition, generation of chemotactic factors will be analyzed with human polymorphonuclear leukocytes as a functional and practical consequence of *in vitro* complement activation by airborne grain dust extracts.

Materials and Methods

Airborne Grain Dust Extracts. Airborne dusts of barley, corn, oat, rye, sunflower seed and spring wheat were collected by a previously described method[4] from active port grain terminals in the Duluth-Superior areas of the United States. One gram of each dust sample was added to 10 ml sterile, nonpyrogenic water (Travenol Laboratories, Inc., Deerfield, IL). The mixtures were

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gently rocked for five minutes at room temperature, centrifuged at 1000 g for 10 minutes and the supernatant fluids were filtered (0.45 μm). All extracts were used fresh or stored for short periods at -88°C. No differences in assay activity were noted between fresh or frozen extracts.

Characterization of Extracts. The airborne dust extracts were characterized in terms of protein nitrogen by a modification of the method of Lowry[8] and gram-negative bacterial endotoxin contamination by a spectrophotometric modification of the *Limulus* amebocyte lysate gel test (Pyrostat, Worthington Biochemical Corp., Freehold, NJ). Sample results were compared with a standard curve and analyzed by linear regression.

Quantification of Hemolytic Complement Activities. Aliquots (10, 50, 100, and 150 μl) of each extract were added to 0.5 ml pooled normal human serum (NHS) which was demonstrated by counterimmunoelectrophoresis to be without detectable precipitating antibody to the extracts and by radioallergosorbent testing to be without detectable IgE to barley, oat, rye and wheat (Pharmacia Diagnostics, Piscataway, NJ). Sera from the members of the pool were tested individually before mixing together. To avoid quantifiable differences due to dilution by the added volume of extract, all test samples, including the untreated control NHS, were diluted with sterile, nonpyrogenic saline (Travenol Laboratories, Inc., Deerfield, IL) to a final volume of 650 μl. Treated and control NHS were incubated at 37°C for 60 minutes in a shaking water bath (125 strokes/min). Aliquots of the reaction mixtures were then assayed for hemolytic complement activity (CH_{50} u/ml) by the tube method of Mayer.[9]

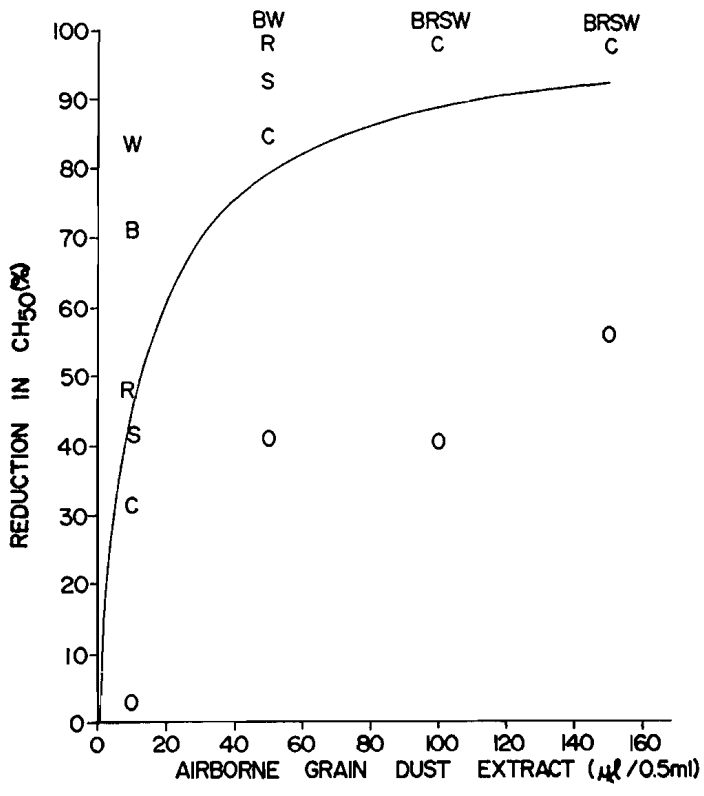

Figure 1. Composite dose-response curves of percentage reduction of CH_{50} in pooled normal human serum which was treated with increasing amounts of airborne grain dust extracts. Solid line depicts the least squares dose-response curve over all the extracts. B, barley dust extract; C, corn; O, oat; R, rye; S, sunflower seed; and W, spring wheat.

The least squares dose-response curve over all the dusts was computed by the method of Finney[10] and the median dose levels for relative extract anticomplementary activity ranking were defined by probit analysis.[10]

Conversion of C3 in the samples was assayed by two-dimensional electrophoresis[11] with the use of barbital buffer pH 8.4, which contained 0.01 M ethylenediaminetetraacetate (EDTA, Fisher Scientific Co., Pittsburgh, PA) to avoid any non-specific activation of C3 by the agarose. Agarose which was used in the second electrophoresis contained goat anti-human C3 (β_{1C}/β_{1A}, Cappel Laboratories, Inc., Cochranville, PA).

Aliquots from the same reaction mixtures (50 μl/0.5 ml) used for the CH_{50} and C3 assays were evaluated in duplicate for functional hemolytic levels of the early complement components C1, C4, C2 and C3 by only minor modification of the procedures described by the manufacturer (Cordis Laboratories, Miami, FL). All results were compared with those obtained from NHS which was treated with 50 μl sterile, nonpyrogenic saline and analyzed by linear regression.

Conversion of C3 in Chelated NHS. Normal human serum (0.5 ml) was chelated with 25 μl of 100 mM EDTA or ethylene-glycol-bis-(β-amino-ethyl ether)N, N'-tetraacetic acid (EGTA, Sigma Chemical Co., St. Louis, MO) as previously described[12] prior to reaction with 50 μl of grain dust extract. Conversion of C3 was then monitored by two-dimensional electrophoresis after incubation.

Chemotaxis of Polymorphonuclear Leukocytes. Grain extract generated complement directed chemotaxis of purified human polymorphonuclear leukocytes under agarose was examined by adaptation and modification of the methods of Nelson, et al.[13,14] Grain dust extracts which consumed the greatest amounts of hemolytic complement were chosen to examine the generation of chemotactic factors. A toxicity study indicated that 10 μl and 50 μl were the least toxic and therefore extracts were added to 0.5 ml NHS in those quantities. After 1 h incubation at 37°C, the treated sera were studied. Negative controls included untreated NHS, growth medium with and without extract and heat inactivated NHS with and without extract. To test for true gradient formation, PMN was added six hours after the addition of NHS, a period during which random diffusion of the NHS resulted in the destruction of any chemotactic gradient. Positive chemotactic controls include 0.5 ml NHS which were treated with 1.5 mg

of purified *Salmonella typhimurium* L-3629 lipopolysaccharide (Sigma Chemical Co., St. Louis, MO). The migration distances of replicate plates ($\bar{N}=5$) were determined after an incubation of 18 h at 37°C in a humidified incubator with 5% CO_2. All data are expressed as net chemotactic migrations (chemotactic migration — spontaneous migration).

Results

Consumption of CH_{50}. Extracts of each airborne grain dust were shown to consume human complement (CH_{50}) in a dose-dependent manner and Figure 1 illustrates the composite dose-response curve of percentage CH_{50} reduction. In addition the least squares dose-response curve over all the extracts is shown. It can be seen that the extracts differed in their anticomplementary activities but showed a composite dose-dependent activity. Table I further explores this difference in activities by examining the relative anticomplementary activity ranking of the extracts in terms of the concentrations which consume 50% of the available serum CH_{50} u/ml. An 11-fold difference in anticomplementary activity was observed between the most active extracts, barley and spring wheat, and the least active, oat extract.

Characterization of Extracts. Protein nitrogen levels and endotoxin contamination of the six extracts studied are shown in Table I. The extracts ranged in protein composition from 0.32-3.28 mg N/ml with a mean and standard deviation of 1.95 ± 1.01 mg N/ml. Gram-negative bacterial endotoxin activities of the extracts ranged from 33.4-44.0 ng FDA *Klebsiella* endotoxin equivalents per ml with a mean and standard deviation of 39.8 ± 4.9 ng/ml. It should be noted that a purified lipopolysaccharide which was extracted from a *Klebsiella* sp. is used as an endotoxin standard by the Bureau of Biologics of the U.S. Food and Drug

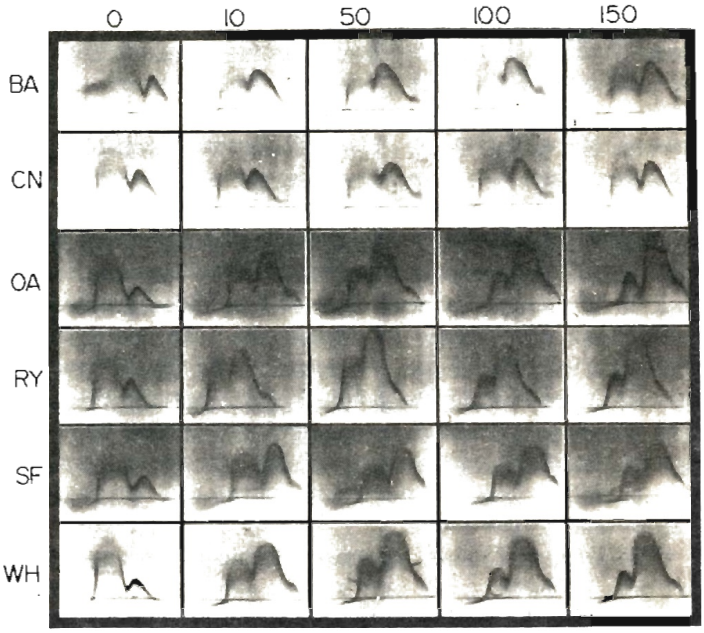

Figure 2. Two-dimensional immunoelectrophoresis of C3 from pooled normal human serum (NHS) which was treated with increasing amounts (0-150 μl/0.5 ml NHS) of airborne grain dust extracts. In each frame the left arc is C3 and the right arc is C3b. BA, barley dust extract; CN, corn; OA, oat; RY, rye; SF, sunflower seed; WH, spring wheat.

Table I. Comparison of the Concentration of Airborne Grain Dust Extracts Which Consume 50% of Human Serum CH_{50} (Relative Activity Ranking) with Protein Concentrations and Endotoxin Contaminations.

Extract	50% CH_{50} Concentration (ul/0.5 ml)	Protein (mg N/ml)	Endotoxin * (ng/ml)
Barley	< 10.0	1.72	44.0
Wheat, spring	< 10.0	2.39	33.7
Rye	10.5	3.28	33.4
Sunflower seed	12.1	2.43	43.4
Corn	16.4	0.30	41.9
Oat	108.9	1.55	42.5

* Assayed as nanograms FDA *Klebsiella* endotoxin equivalents per ml.

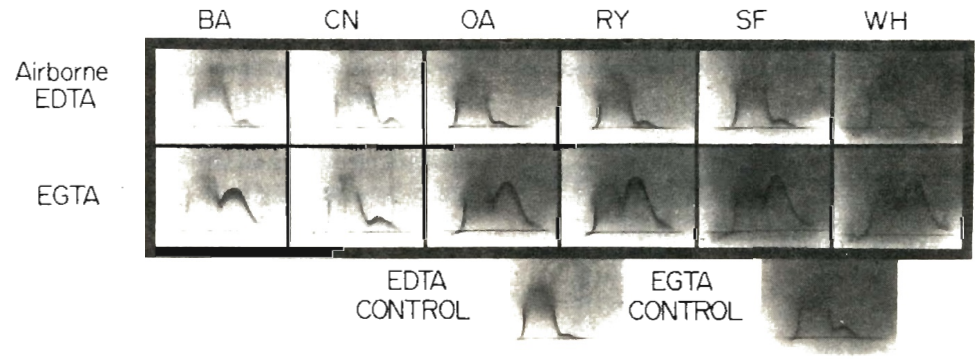

Figure 3. Two-dimensional immunoelectrophoresis of C3 from pooled normal human serum (NHS) which was chelated with EDTA or EGTA prior to treatment with 50 μl of airborne grain dust extracts. In each frame the left arc is C3 and right arc is C3b. BA, barley dust extract; CN, corn; OA, oat; RY, rye; SF, sunflower seed; WH, spring wheat; CONTROLS, NHS with chelator alone.

Administation (FDA).[15]

Conversion of C3 in NHS and Chelated NHS. Examination of C3 by two-dimensional electrophoresis revealed that C3 was converted by airborne dust extracts to the active fragment C3b in a dose-dependent manner (Figure 2). When NHS was first chelated with EDTA, which blocks both classical and alternative complement pathway activation, no conversion of C3 to C3b could be observed (Figure 3). However, prior chelation with EGTA, which blocks the classical pathway, resulted in conversion of C3 by the alternative complement pathway.

Hemolytic Levels of Early Complement Components. In order to examine any potential involvement of classical complement pathway components, the functional hemolytic activities of C1, C4, C2 and C3 were assayed. Table II illustrates the percentage reduction in complement components and CH_{50} when 0.5 ml NHS were treated with 50 μl of the various extracts. The standard errors of the control saline-treated NHS are expressed as percentages of the means for ease of interpretation and comparison with the percentage reductions of the tests.

Chemotaxis Under Agarose. Table III shows that extracts of barley, rye and spring wheat were capable of generating chemotactic factors in NHS. Those factors were complement-dependent and a true migration gradient was formed. Rye extract was the most potent ($P<0.01$) and treatment with 10 μl/0.5 ml generated greater migration than did 50 μl/0.5 ml ($P<0.01$). The positive control studies with lipopolysaccharide yielded a mean (± standard error) net chemotactic migration of 2.84 ± 0.13 mm.

Table II. Percentage Reduction in Hemolytic Activity of Individual Complement Components and CH_{50} in Normal Human Serum after Treatment with Airborne Grain Dust Extracts.

Extract (50 μl/0.5 ml)	$C1H_{50}$ (%)	$C4H_{50}$ (%)	$C2H_{50}$ (%)	$C3H_{50}$ (%)	CH_{50} (%)
Barley	17.2*	53.3	71.8	50.6	100.0
Corn	15.2	75.5	68.1	30.2	84.2
Oat	17.0	33.4	39.8	54.4	41.1
Rye	8.6	44.0	50.3	39.0	98.7
Sunflower seed	6.7	50.7	65.1	36.2	93.3
Wheat, spring	57.6	36.9	36.8	47.7	100.0
Controls†	4.9	3.1	1.5	2.9	0.8
	N=5	N=11	N=11	N=6	N=12

* Average percentage decrease of duplicate tests.
† Saline treated controls. Standard error expressed as percentage of mean.

Table III. Net Chemotactic Migrations of Human PMN Toward Serum Treated with Airborne Grain Dust Extracts.

Extract	10 μl/0.5 ml NHS (mm)	50 μl/0.5 ml NHS (mm)
Barley	6.52 ± 0.36*	4.85 ± 0.13
Rye	6.84 ± 0.27†	6.03 ± 0.12
Wheat, spring	5.48 ± 0.13	3.71 ± 0.20
LPS **	2.84 ± 0.29	

* Mean ± SEM, $\bar{N} = 5$.
† Rye > Barley > Wheat ($P<0.01$). 10 μl > 50 μl ($P<0.01$).
** Commercially purified *Salmonella typhimurium* lipopolysaccharide (1.5 mg/0.5 ml).

Discussion

This study demonstrated that rapid aqueous extracts of airborne grain dust consumed human hemolytic complement in a dose-dependent fashion. Like the parent airborne dusts, not only did the extracts differ in complement consumption with increasing dose but the extracts differed from each other at each dose. There was good correlation between the relative CH_{50} activity rankings of the extracts and the rankings of the parent airborne dusts. Rye, barley and spring wheat were the most active dusts against human CH_{50} in a previous study[5] and extracts of those dusts ranked as the top three most active in this study (barley, spring wheat and rye). Therefore, one might conclude that the active anti-complementary substance(s) is (are) readily soluble. These data substantiate the concept that the complement activation which is observed *in vitro* is not a function of the surfaces of the particles since the study was performed with particle-free extracts. In our study we observed little difference in complement consumption between freshly filtered (0.45 μm) extracts and those which were frozen. This confirms the work of Marx and Flaherty[2] which showed complement activation by extracts of organisms which are associated with hypersensitivity pneumonitis. On the other hand, our study may not completely address the conclusions of Edwards[16] which state that extract "microprecipitates" are required for complement activation via the alternative pathway. Our extracts were filtered through 0.45 μm filters whereas his filters were of 0.22 μm pore size. However, a study of commercial grain extracts which were filtered with 0.22 μm pore size filters also demonstrated large decreases in complement components and total hemolytic activity (manuscript in preparation).

Characterization of the extracts in terms of protein concentration and endotoxin activity showed no correlation between either of those parameters and CH_{50} activity ranking. The amount of endotoxin contamination is relatively small when compared with the amount of purified endotoxin which is required to induce the level of complement activation reported in this study. Our own experiences as well as those reported by others[17,18] show that microgram rather than nanogram quantities of purified endotoxins are required. We do not totally dismiss the potential contribution of endotoxin in the observed complement consumption but rather suggest that another unidentified substance(s) may account for the major reduction of hemolytic complement.

Differential chelation of NHS with EDTA or EGTA defined the activation of the alternative pathway of complement by each airborne grain dust extract tested, which thereby expands our preliminary study.[7] Complement component C3 was converted to the active

form, C3b, in the presence of EGTA but not EDTA, which showed that the calcium-independent alternative pathway was utilized. This study, as well as our previous studies which used Factor B conversion and C4-deficient guinea pig serum to further illustrate the activation of the alternative pathway,[5,12] does not negate the potential activation of the classical pathway in the non-chelated, natural NHS. We therefore examined the levels of the early complement components (C1, C4, C2 and C3) with functional, hemolytic assays. As expected by the electrophoretic analysis of C3 activation, C3 was decreased after NHS was incubated with each airborne grain dust extract. In addition, large decreases in hemolytic C1, C4 and C2 were observed. Components of the classical complement pathway, therefore, are consumed by extracts of airborne grain dust. The mechanism by which this activation occurs is not defined by the present study. It is possible, although unlikely, that specific antibody is present in the NHS at a level below that which is detectable by counterimmunoelectrophoresis. It is also possible that the antibody-independent lipid A moiety of bacterial lipopolysaccharide may activate the classical complement pathway.[18,19] Although the potential contribution of this mechanism has not been disproved, the small amount of endotoxin activity is unlikely to account for all of the observed decreases. Marx, et al.[20] suggest C1 esterase activity as a possible explanation for the *in vitro* decrease in C4 observed after NHS is treated with a purified pigeon-dropping extract. Edwards[21] has further shown that soluble material from moldy hay dust activated C1 directly and also demonstrated enzyme activity. Enzyme activity may be present in our grain dust extracts. In any case, both alternative and classical complement pathways are activated but it is not yet proved whether the same antigens or substrates are responsible for both activation sequences.

To evidence the possible *in vivo* ramifications of activation of the complement cascade (regardless of pathway) this study demonstrated the complement-dependent directional migration of purified human polymorphonuclear leukocytes (PMN) toward NHS which was treated with airborne grain dust extracts. Extremely small amounts (10 μl) of extract induced significant migration of PMN ($P<0.01$) in this sensitive and reproducible assay. The difference in this study between 10 μl and 50 μl of extract ($P<0.01$) is presumably due to increased intrinsic toxicity of the extract at the larger dose. Rye extract induced the greatest response, as in a previous study, rye dust demonstrated the highest CH_{50} activity ranking of all the airborne dusts.[5]

In conclusion, we have reported that rapid, aqueous extracts of airborne grain dusts can activate both the human alternative and classical complement pathways *in vitro* and thereby generate complement-dependent directional migration of human PMN. Components of both the alternative and classical pathways are present in human airways.[22,23,24] The potential for direct activation of both pathways after inhalation of grain dust therefore exists, either directly by the dust or as rapidly extracted material. Studies with organic particulate antigens instilled intratracheally showed that soluble antigens were identified in the serum within one minute after instillation, presumably due to direct solubilization by bronchoalveolar material.[25] This would expose the material also to serum complement. In addition, the pulmonary macrophage is expected to rapidly ingest organic material, a process which would be facilitated by complement-mediated opsonization.[26] After ingestion, degradation and/or transport would result in additional soluble material in the circulation[25] and the release of complement activating enzymes from the macrophages.[27]

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"There is a time in every man's education when he arrives at the conviction that envy is ignorance; that imitation is suicide; that he must take himself for better or for worse as his portion; that though the wide universe is full of good, no kernel of nourishing corn can come to him but through his toil bestowed on that plot of ground which is given to till. The power which resides in him is new in nature, and none but he knows what that is which he can do, nor does he know until he has tried."

Robert Waldo Emerson