

Complement activation by commercial allergen extracts of cereal grains*

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Summary

Commercial allergen extract preparations of whole cereal grains were examined *in vitro* for their reactivity with the human haemolytic complement cascade. Total haemolytic complement was consumed in a dose-dependent manner which did not correlate with the protein nitrogen content or bacterial endotoxin contamination of the extracts. Alternative pathway activation was shown by C3 conversion in the presence of EGTA while classical complement components C1, C4 and C2 were also much decreased in serum which was free of detectable specific antibody. Very small amounts of allergen extract initiated the formation of complement-dependent factors which were chemotactic for human polymorphonuclear leucocytes. These data suggest a potential for complement activation *in vivo* by ingestion, inhalation, or injection of cereal grain products.

Introduction

Various studies have indicated a relationship between complement activation by allergens and subsequent involvement of immediate hypersensitivity. Commercial or laboratory-purified house dust extracts have been used as typical representatives of inhalant allergens (Berrens, 1974; Berrens, Van Rijswijk-Verbeek & Guikers, 1976; Weemaes, Stoop & Berrens, 1977; Brady, Glovsky & Hugli, 1978). Complement activation by extracts of allergens associated with occupational (Marx & Flaherty, 1976; Olenchok, Mull & Major, 1979) as well as avocational exposures (Marx, Boren & Moore, 1977) have also been reported. Recently, complement activation has been implicated as a possible disease mechanism following ingestion of allergens (Massey, Capner & Mowbray, 1977; Arroyave *et al.*, 1977; Strunk *et al.*, 1978; Allen *et al.*, 1979). It is felt by some investigators that certain undigested (soluble) grain antigens may

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cross the gastrointestinal tract, enter the peripheral circulation, and thereby result in complement activation *in vivo* (Massey *et al.*, 1977; Strunk *et al.*, 1978). While the majority of studies suggested the alternative pathway as the mechanism of complement consumption, activation of classical pathway components, independent of specific antibody, has been shown both *in vivo* (Arroyave *et al.*, 1977; Weemaes *et al.*, 1977; Strunk *et al.*, 1978) and *in vitro* (Berrens *et al.*, 1976; Marx *et al.*, 1977; Brady *et al.*, 1978). It is to this last point that the study reported herein was directed.

During a study of occupational exposures to grain, our laboratories demonstrated that the cereal grain itself, ground whole rye, could activate the alternative pathway of complement *in vitro* (Olenchock *et al.*, 1978). Subsequently, the anti-complementary activity was found in a 15 min aqueous extract of the crudely ground grain (Olenchock & Major, 1979). Commercial grain antigens are used in skin test evaluations of occupationally exposed workers (do Pico *et al.*, 1977; Klaustermeyer, Bardana & Hale, 1977; Chan-Yeung, Wong & MacLean, 1979) as well as patients with food hypersensitivities (Bock *et al.*, 1978). It is therefore the purpose of this paper to explore both alternative and classical complement activation by commercially prepared allergen extracts of six cereal grains.

Materials and methods

Grain extracts

Standard allergenic preparations of whole ground barley, maize, oat, rye, sunflower seed, and wheat were obtained commercially from a U.S. licensed manufacturer in filtered (0.22 μ m) single lot samples. Oat, rye, sunflower seed, and wheat extracts were used at 15 000 PNU/ml while maize and barley extracts were used at 9000 PNU/ml based on supplier's labelled concentrations (rye, sunflower seed, and wheat were received at a concentration of 20 000 PNU/ml and were diluted with sterile, non-pyrogenic saline. Maize was received at 10 000 PNU/ml and diluted similarly). All extracts were stored at 4°C as per the supplier's directions and were used prior to the expiration dates.

Endotoxin and protein assays

All extract preparations were assayed with a spectrophotometric modification of the Limulus amebocyte lysate gel test (Pyrostat; Worthington Biochemical Corp., Freehold, NJ) which was capable of detecting as little as 0.1 ng FDA Klebsiella endotoxin equivalents per ml. Protein nitrogen concentrations were determined by the Oyama & Eagle (1956) modification of the method of Lowry.

Normal human serum

Normal human serum was pooled from members of our laboratory and used throughout this study. The pooled serum was negative by counterimmunoelectrophoresis for specific precipitating antibodies to the extracts and also negative for specific IgE to barley, oat, rye, and wheat as defined by the radioallergosorbent test (RAST; Pharmacia Diagnostics, Piscataway, NJ). Aliquots of normal human serum were used immediately or stored for short periods at -88°C.

C3 activation studies

Normal human serum (0.5 ml) was reacted with aliquots (10, 50, 100, and 150 μ l) of

each extract or sterile non-pyrogenic saline. All test and control samples including untreated normal human serum were diluted to a standard final volume of 650 μ l with sterile, non-pyrogenic saline in order to avoid quantifiable differences due to dilution by the increasing volumes of extract. After 60 min incubation in a shaking water bath at 37°C, 5.0 μ l aliquots of the samples were assayed for C3 conversion by two-dimensional electrophoresis (Laurell, 1965) in the presence of barbital buffer, pH 8.4 which contained 0.01 M ethylenediaminetetraacetate (EDTA; Fisher Scientific Co., Pittsburgh, PA). The agarose used during the second electrophoresis contained goat anti-human C3 (β_{1C}/β_{1A} ; Cappel Laboratories, Inc., Cochranville, PA). Conversion of C3 was interpreted as an increase in the C3b arc.

C3 activation in chelated serum

Normal human serum (0.5 ml) was first chelated with 0.025 ml 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 (Olenchock *et al.*, 1978) before the addition of 50 μ l of grain extract. Conversion of C3 was monitored by two-dimensional electrophoresis as above.

Quantification of C1, C4, C2 and C3

Total haemolytic complement consumption was determined in terms of CH₅₀ u/ml by the method of Mayer (1961) for each sample used in the C3 conversion studies.

Table 1. Assay systems for monitoring chemotaxis under agarose

Internal well	Centre well	External well
—	PMN	—
Heated NHS*	PMN	Heated NHS
Heated NHS + extract (10 or 50 μ l)	PMN	Heated NHS
NHS + extract (10 or 50 μ l)	PMN	NHS
NHS + extract (10 μ l)	PMN	NHS + MEM† (10 μ l)
NHS + extract (10 μ l)	PMN	MEM
MEM + extract (10 μ l)	PMN	MEM
MEM + extract (10 μ l)	PMN	NHS
MEM + extract (10 μ l)	PMN	NHS + extract (10 μ l)
NHS + extract (10 or 50 μ l)	PMN‡	Heated NHS
NHS + extract (10 or 50 μ l)	PMN‡	NHS + extract (10 or 50 μ l)

* Normal human serum heated at 56°C for 30 min.

† Minimum essential medium.

‡ PMN added 6 hours after addition of NHS.

The reaction mixtures which contained 50 μ l/0.5 ml were evaluated in duplicate for functional haemolytic levels of the individual complement components C1, C4, C2 and C3 by minor modification of the procedure of the supplier (Cordis Laboratories, Miami, FL). All results were compared with those obtained from normal human serum which was reacted with 50 μ l sterile, non-pyrogenic saline and the results were analysed by linear regression.

Chemotaxis of human neutrophils

As a functional monitor of complement activation, the generation of chemotactic factors in normal human serum by the grain extracts was assayed by combination and modification of the methods of Nelson, Quie & Simmons (1975) and Nelson, Fiegel & Simmons (1976) which observe migration of purified human neutrophilic polymorphonuclear leucocytes (PMN) under agarose (Mull, Olenchok & Major, 1979). Assay systems which are indicated in Table 1 included controls for complement activation, extract toxicity, and true chemotactic gradient formation. As a positive chemotactic control 1.5 mg of purified *Salmonella typhimurium* L-3629 lipopolysaccharide (Sigma Chemical Co., St Louis, MO) were added to 0.5 ml of normal human serum. A dose-response study of inherent extract toxicity to the PMN indicated that a dose of 10 μ l was the optimum and 50 μ l only slightly toxic to the PMN. Therefore the three extracts which consumed the greatest amount of haemolytic complement (oat, sunflower seed and wheat) were added to 0.5 ml of normal human serum in quantities of 10 or 50 μ l.

For quantification of chemotaxis, the cell migrations were magnified and projected onto paper by a Tri-Simplex Microprojector (Bausch & Lomb, Rochester, NY) equipped with a $\times 10$ objective. The linear distance the cells moved from the margin of the well toward the chemotactic factor (chemotaxis) and the linear distance the cells moved from the margin of the well toward the control medium (spontaneous migration) were measured. The net chemotactic migration, chemotaxis-spontaneous migration, was determined and means were calculated for each replicate ($\bar{n}=5$).

Results

Endotoxin and protein levels

All six commercial extract preparations contained measureable gram-negative bacterial endotoxin-like activity as shown in Table 2. The range of activity was 2.2–38.7 ng/ml and the mean $\pm$ s.e.mean was 22.0 ± 7.1 ng/ml. Protein nitrogen levels ranged from 0.6 to 31.2 mg N/ml with a mean level of 10.2 ± 5.6 mg N/ml. Although maize and barley extracts were tested at lower PNU/ml than the other extracts, no differences in endotoxin activity or protein nitrogen were noted since barley was one of the highest in endotoxin and maize was one of the highest in protein nitrogen. Furthermore, no associations between endotoxin activity and protein nitrogen content were observed over all of the extracts.

C3 activation

Complement component C3 was converted to the active form, C3b, by the grain extracts (Fig. 1). This activation was dose-dependent since increasing the amount of extract decreased the native C3 arc. The dose-dependent nature of this activation was confirmed by quantification of CH₅₀ u/ml (data not shown). Although the commercial

Table 2. Quantification of endotoxin contamination and protein nitrogen content of grain extracts.

Extract*	Endotoxin† (ng/ml)	Protein‡ (ng N/ml)
Barley	37.4	0.6
Maize	2.2	25.2
Oat	9.4	1.9
Rye	38.7	1.0
Sunflower seed	38.0	31.2
Wheat	6.2	1.3

* Barley and maize extracts used at 9000 PNU/ml while all others used at 15 000 PNU/ml (supplier's label concentration).

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

‡ Assayed by Oyama & Eagle (1956) modification of Lowry.

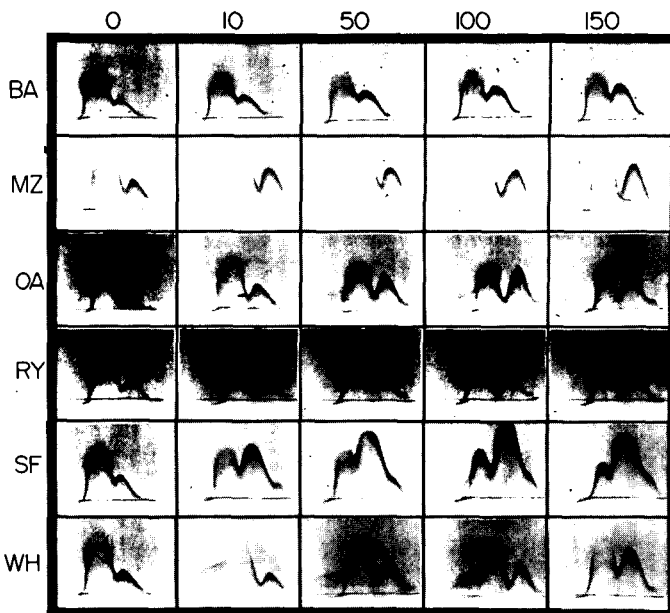


Fig. 1. Two-dimensional electrophoresis of C3 in 0.5 ml of normal human serum after treatment with increasing amounts (10–150 μ l) of commercial grain allergen extracts. In each frame, left arc is C3; right arc is C3b. BA, barley; MZ, maize; OA, oat; RY, rye; SF, sunflower seed; and WH, wheat.

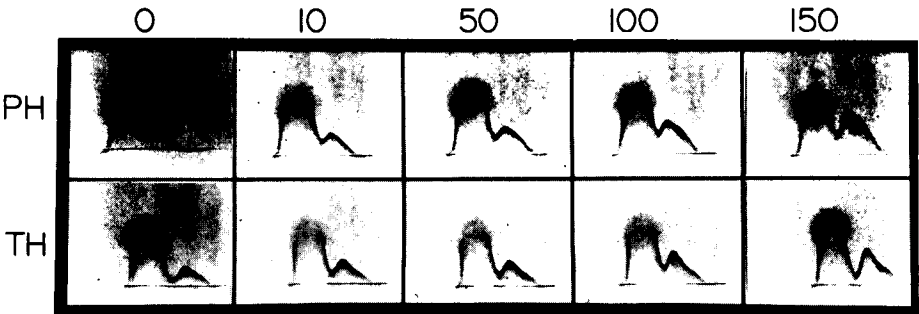


Fig. 2. Two-dimensional electrophoresis of C3 in 0.5 ml of normal human serum after treatment with increasing amounts (10–150 μ l) of 0.4% phenol (PH) or 0.01% thimerosal (TH).

extracts contained a preservative (either 0.4% phenol or 0.01% thimerosal), very little effect on the C3 arc was observed when laboratory solutions of 0.4% phenol or 0.01% thimerosal were added to normal human serum (Fig. 2).

Activation of C3 was totally prevented when normal serum was first chelated with EDTA (Fig. 3). On the other hand, with prior chelation with EGTA, which permits alternative pathway activation to occur suboptimally, C3 was converted to C3b. It should be noted that sunflower seed extract showed only minor, if any, arc conversion at the level tested. Likewise maize extract converted C3 only weakly.

Quantification of C1, C4, C2 and C3

Percentage decreases in the functional haemolytic activities of the early complement components in normal human serum after reaction with 50 μ l of the grain extracts are shown in Table 3. As an index of classical complement pathway activation, decreases in C1, C4, and C2 were observed. As expected from the two-dimensional electrophoretic studies of C3, decreases in haemolytic C3 were also noted. Sunflower seed extract showed only small decreases in C3 and C2 levels. Maize extract also induced relatively low consumption of C3 and C2.

For ease of interpretation, the results of replicate tests of normal serum which was reacted with 50 μ l sterile non-pyrogenic saline are expressed in both the mean titre $\pm$ s.e.mean and the standard error as a percentage of the mean.

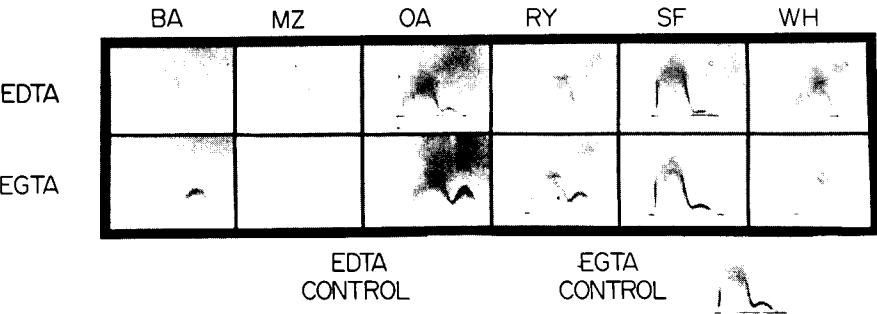


Fig. 3. Two-dimensional electrophoresis of C3 in 0.5 ml of normal human serum which was chelated with either EDTA or EGTA before treatment with 50 μ l of commercial grain allergen extracts. BA, barley; MZ, maize; OA, oat; RY, rye; SF, sunflower seed; WH, wheat. Controls are normal human serum + chelator only.

Table 3. Decrease in functional activity of early complement components in normal human serum after reaction with grain extracts.

Extract* (50 μ l/0.5 ml)	C1H ₅₀ (%)	C4H ₅₀ (%)	C2H ₅₀ (%)	C3H ₅₀ (%)
Barley	18.1†	21.8	13.4	17.4
Maize	30.6	21.2	3.7	15.7
Oat	14.4	20.1	9.3	47.4
Rye	27.3	16.9	18.6	20.3
Sunflower seed	25.3	13.3	6.7	8.5
Wheat	18.2	21.0	9.1	23.7
Controls‡	71 433 ± 3503 (4.9%) n=5	208 800 ± 6525 (3.1%) n=11	18 797 ± 278 (1.5%) n=11	21 015 ± 610 (2.9%) n=6

* Extracts of barley and maize used at 9000 PNU/ml. All others used at 15 000 PNU/ml.

† Average percentage decrease of duplicate tests.

‡ NHS reacted with saline. Mean of actual titre values ± s.e.mean followed in parentheses by standard error expressed as percentage of mean.

Chemotaxis of PMN

Probit analysis of the dose-response CH₅₀ data defined the oat, sunflower seed, and wheat extracts as the most active in consuming the total haemolytic complement activity in normal serum. For that reason, those three extracts were chosen to study the generation of complement-dependent chemotactic factors in the serum. All three extracts induced factors which were chemotactic for purified human PMN (Table 4). At a concentration of 50 μ l/0.5 ml, oat, sunflower seed, and wheat extracts induced the

Table 4. Chemotaxis of purified human PMN induced by grain extract-treated normal human serum.

Extract	10 μ l (mm)	50 μ l (mm)
Oat	5.36 ± 0.16*	5.38 ± 0.39
Sunflower seed	6.43 ± 0.18†	5.20 ± 0.14
Wheat	4.98 ± 0.27	5.72 ± 0.27
LPS (1.5 mg)‡	2.84 ± 0.29	

* Mean of net chemotactic migrations ± s.e.mean; $\bar{n}$ =5.

† Sunflower seed > oat = wheat at 10 μ l (P <0.01). Sunflower seed 10 μ l > 50 μ l (P <0.01).

‡ Purified *Salmonella typhimurium* lipopolysaccharide; mean ± s.e.mean, n =5

same level of chemotaxis. At the 10 μ l/0.5 ml concentration (which was less toxic to the PMN), sunflower seed extract induced a statistically greater chemotactic response than did oat or wheat extract ($P < 0.01$).

Discussion

In this study, pooled normal human serum was used in order to avoid the possibility of using serum which may have a hyperlabile complement system (Berrens, 1974). The pool was free of detectable specific precipitating antibody as well as specific IgE. In addition, a single lot of each allergen was used and all studies were completed well before the expiration date of the product since it has been shown that commercial extracts can vary from lot to lot (Fink *et al.*, 1977) and prolonged storage even under refrigeration may reduce the potency of the extracts (Center, Shuller & Zeleznick, 1974; Ribon & Gavencak, 1978). The protein nitrogen unit (PNU) was chosen as the basis of comparison since the PNU is the concentration standard used most frequently by United States manufacturers (May *et al.*, 1979). It must be recognized, however, that the PNU may not relate to allergen-induced biological responses (Baer *et al.*, 1970). In fact, our data show no relationship between the PNU concentration stated on the label and the protein nitrogen content of our extracts. This discrepancy could be due to a difference in our protein-assay methods (May *et al.*, 1979) or variation in the potency of the extract (Fink *et al.*, 1977). Finally, our extracts were sterilized by 0.22 μ m pore size filtration and not frozen as suggested by the studies of Edwards (1976) in order to avoid the formation of microprecipitates. The commercial extracts in this study did demonstrate endotoxin activity as shown by the *Limulus* amoebocyte lysate assay (LAL). The amounts were, however, relatively small and it must be recognized that many substances in addition to gram-negative bacterial endotoxin can interfere with the LAL and produce positive results (Elin & Wolff, 1973; Brunson & Watson, 1976; Kotani *et al.*, 1977).

All of the commercial allergen extracts which we tested activated C3 as demonstrated by two-dimensional immunoelectrophoresis. This effect could not be attributed to the preservatives used in the extracts since even 150 μ l of 0.4% phenol or 0.01% thimerosal had only minimal activity against C3 as evidenced by the composite effects of Figs 1 and 2. The commercial extracts do activate complement by the alternative pathway as shown by the EDTA/EGTA chelation studies. However, in those studies the maize and sunflower seed preparations showed little conversion of C3 in the presence of EGTA. It should be remembered that maize extract was used at a concentration of 9000 PNU/ml and may not have contained sufficient material to overcome the inhibition of EGTA. Sunflower seeds, on the other hand, have previously been resistant to analysis in chelation tests (Olenchock *et al.*, 1980). For the sake of comparisons, however, all extracts were treated with the same chelation regimen. When the early components of the complement cascade are tested, the haemolytic activities of C1 and C4 are markedly decreased after incubation with the commercial cereal extracts. In the absence of detectable antibody, this apparent activation of the classical pathway may represent enzyme activity which is intrinsically present in the grain comparable to the esterase activity suggested by Marx *et al.* (1977) in purified pigeon dropping extract or enzyme activity shown by Edwards (1979) in soluble material from mouldy hay dust. A result of this complement activation—by both pathways—is the generation of complement-dependent factors which are chemotactic

for human polymorphonuclear leucocytes. Only 10 μ l of allergen extract in 0.5 ml of normal human serum cause significant chemotaxis for PMN.

These results indicate that commercial allergen extracts of cereal grains activate human complement *in vitro* by both the alternative and classical pathways. While these data do not prove the action of allergens on the human complement cascade *in vivo*, they do represent the potential for such activation. It would appear that intact undigested (soluble) grain antigens do possess the potential to activate peripheral complement by both pathways upon crossing the gastrointestinal tract and reaching the circulation as suggested by Massey *et al.* (1977) and Strunk *et al.* (1978). Furthermore, this investigation demonstrates that the actual grain itself contains material which can activate both complement cascades in workers exposed to airborne fragments of whole grains (Olenchock *et al.*, 1978). As the grains are transported on conveyor belts, poured into storage bins, or moved for any reason, the constant rubbing of kernels can cause the release, from the grain itself, of complement activating materials which could contribute to the respiratory insult of grain workers. Finally, the potential exists for false positive or ambiguous skin test reactions as a result of 'non-immune' C1 activation (Edwards, 1979) or later influx of PMN suggested by this study. Whether these materials are enzymic in nature or different antigenic moieties must yet be examined.

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