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Antibody-Independent Complement Activation by Cardroom Cotton Dust[1]

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The *in vitro* activities of cardroom cotton dust (before treatment, BCD) and treated cotton dust (TCD) on the human complement cascade were examined. While both dusts consumed hemolytic complement and reduced C3, quantifiable differences were observed. Untreated BCD was much more active than was TCD. Complement was activated via the alternative pathway, and although endotoxin was 58 times greater in BCD than TCD, the endotoxin content of BCD could not account for the total complement consumed. Activation of the complement cascade *in vivo* may provide one mechanism to account for the symptoms experienced by byssinotic subjects after exposure to cotton dust.

INTRODUCTION

The occurrence of respiratory disease in persons exposed to cotton dust has been recognized for almost 150 years, but the mechanism by which byssinosis is produced is uncertain. One mechanism which has gained popularity with researchers concerned with other vegetable dusts and disease is complement activation via the alternative pathway (Edwards *et al.*, 1974). It is therefore the purpose of this paper to examine cardroom cotton dust and dust treated in a manner which is alleged to remove the byssinotic agent(s) for their potential to activate *in vitro* the complement cascade in serum from nonsensitized humans and guinea pigs.

MATERIALS AND METHODS

Collection and preparation of cardroom cotton dust. Settled cardroom cotton dust was collected from the surfaces of carding machines belonging to the Sudan Textile Industry, Limited in Khartoum North, Democratic Republic of the Sudan. After collection, the cardroom cotton dust was placed in sealed plastic bags, transported at ambient temperatures, and stored at room temperature and humidity.

In preparation for testing, the settled cardroom cotton dust was first ground in a Wiley Mill to a particle size corresponding to 60 mesh. Sixty-mesh aliquots were then refined four times in a liquid nitrogen freezer mill (Spex Freezer/Mill; Spex

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Industries, Inc., Metuchen, N.J.). The samples were then stored in capped jars at room temperature and humidity.

Preparation of aqueous extracts of cardroom cotton dust. Twenty grams of 60-mesh cardroom cotton dust were placed in 1000 ml of distilled water and stirred vigorously for 18 hr at 4°C. The particles settled and the supernatant fluid was filtered through Whatman No. 4 filter paper. The volume of the filtrate recovered after extraction was always approximately 850 to 900 ml. The extract was filtered through a sterile 0.45-μm membrane filter and then pervaporated in sterile cellulose dialysis tubing at 4°C for 12 days at which time the volume of the extract had been reduced to approximately 25 ml. The extracts were collected in sterile capped vials and stored at −20°C.

Preparation of treated cotton dust. Fifteen grams of freeze-milled cardroom cotton dust were extracted with 300 ml of absolute ethanol three times during a 1-hr period. This preliminary treatment removed chlorophyll and reduced the lipid content of the dust so that subsequent grinding was facilitated. The alcohol was decanted, and the dust was resuspended in 150 ml of distilled water and autoclaved for 30 min at 15 psi and 126°C. The supernatant fluid was removed and 500 ml of 0.1 N NaOH were added. This mixture was stirred for 1 hr at 95°C, and neutralized to pH 7.0 with 12 N HCl. Aliquots of the dust were placed in separate sterile, pyrogen-free tubes and repeatedly washed with sterile, pyrogen-free distilled water until the supernatant wash water was colorless. The dust was then lyophilized, and later freeze-milled and stored in sterile capped pyrogen-free vials at room temperature and humidity. The extraction methods were chosen since they parallel the preparation of medical grade cotton which is reputed to be unrelated to byssinosis in Egypt (El-Batawi and El-Din Shash, 1962).

Particle size analyses. Dust particle diameter sizes were measured using light microscopy and a Porton graticule (BGI, Inc., Waltham, Mass.).

Endotoxin content of cardroom cotton dust and treated cotton dust extracts. Quantitative determination of the endotoxin content of an extract of dust was performed using a spectrophotometric modification of the *Limulus* amebocyte lysate assay capable of detecting 0.1 ng *Klebsiella* endotoxin equivalents per milliliter (Pyrostat; Worthington Biochemical Corp., Freehold, N.J.). The extract was prepared by mixing 5 ml of sterile, pyrogen-free distilled water with 100 mg of cardroom cotton dust or treated cotton dust for 15 min at room temperature. The mixture was then centrifuged at 900*g* for 7 min, and the supernatant fluid filtered through a 0.45-μm membrane filter. The standard curve was plotted by linear regression using various dilutions of *Escherichia coli* W (0127:B 8) endotoxin, and the results were analyzed by linear regression and expressed as nanograms of FDA *Klebsiella* endotoxin equivalents per gram of dust (Selzer, 1970).

Collection of sera. Sera were collected and pooled from normal volunteers who had no known previous respiratory exposure to cotton or other vegetable dusts. The pool was used immediately in the complement activation assays.

Sera were also collected, pooled, and stored at −86°C from NIH Strain 13 guinea pigs and guinea pigs genetically deficient in the fourth component of complement.

Both human and guinea pig sera were examined for immune precipitins to the

aqueous extract of cardroom cotton dust using both gel diffusion and counterimmunoelectrophoresis (Gordon *et al.*, 1971). Sera were judged to be precipitin negative if no immune arcs were noted after 48-hr incubation at 4°C.

In Vitro complement activation studies. Cardroom cotton dust and treated cotton dust activation of the complement system was studied *in vitro* using a protocol established for grain dusts (Olenchock *et al.*, 1979). This procedure included: (a) assays for conversion of Factor B, (b) quantification of hemolytic complement consumption (CH_{50}), and (c) two-dimensional immunoelectrophoresis for detection of C3 conversion to C3a and C3b.

Varying quantities of cardroom cotton dust or treated cotton dust, ranging from 0.1 to 20 mg, were incubated in 0.5-ml aliquots of precipitin-negative pooled human serum (NHS) for 60 min at 37°C. These mixtures were then centrifuged at 900*g* for 7 min and the supernatant fluids analyzed. Negative controls consisted of 0.5 ml of normal human serum with no dust added and positive controls included 0.5 ml of normal human sera to which were added 5 μg of *Escherichia coli* endotoxin (Worthington Biochemical Corp., Freehold, N.J.). Both of these controls were treated in the same manner as the aliquots of serum that contained dust.

(a) Conversion of Factor B to Bb was demonstrated by immunoelectrophoresis (Götze and Müller-Eberhard, 1971) of 8 μl of the supernatant fluid for 45 min. The slides were developed with rabbit anti-human C3 activator (Behring Diagnostics, Somerville, N.J.) and photographed. The presence of Bb was confirmed by the change in electrophoretic mobility of the Factor B arc (positive to negative).

Differential cation chelators were included with this assay in an attempt to differentiate between alternative and classical complement pathway activation. Normal human serum was treated with 25 μl of 100 mM EDTA (disodium ethylenediaminetetraacetic acid, Fisher Scientific Co., Fair Lawn, N.J.) or with 25 μl of 100 mM EGTA (ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid, Sigma Chemical Co., St. Louis, Mo.). These sera were mixed gently for 5 min and then 1.0 mg of dust was added, and the mixtures were treated as previously described. Negative controls consisting of the chelator and serum were included to rule out activation of Factor B by these compounds.

(b) Hemolytic complement assays were performed on the human supernatant fluids recovered after incubation with either cardroom cotton dust or treated cotton dust. Results are expressed as CH_{50} u/ml of serum and were performed according to the method of Mayer (1961).

Normal NIH strain 13 guinea pig sera and NIH C4-deficient (C4D) guinea pig sera were used to differentiate between activation of the alternative and classical pathways of complement. The supernatant fluids recovered after incubation with the two dusts were assayed for hemolytic complement using sheep red blood cells coated with guinea pig C1 and C4 (EAC1gp4gp, Cordis Laboratories, Miami, Fla.). One hundred units of purified guinea pig C2 (Cordis Laboratories) were added to each sample prior to beginning the assay since Frank *et al.* (1971) demonstrated consistently lower C2 levels in C4D than in normal guinea pigs.

(c) Two-dimensional crossed immunoelectrophoresis was performed by the method of Laurell (1965) using agarose containing 0.01 M EDTA and goat anti-human C3 (β_1C/β_1A, Cappel Laboratories, Cochranville, Pa.). Conversion of C3

TABLE 1
COMPARISON OF PARTICLE SIZE DISTRIBUTIONS OF CARDROOM COTTON DUST AND TREATED COTTON DUST

Particle size (μm)	Cardroom cotton dust (%)	Treated cotton dust (%)
<5	64	79
5–10	16	9
10–20	9	6
20–40	5	5
>40	6	1
Total	100	100

to C3b was quantified by triangulation of the respective arcs. Percentage conversion of C3 to and C3b was calculated according to the following equation:

$$\text{Percentage cleavage of C3 to C3b} = \frac{\text{Area of C3b peak}}{\text{Area of C3 peak + area of C3b peak}} \times 100.$$

RESULTS

Particle Size Analysis of Cardroom Cotton Dust and Treated Cotton Dust

Particle size analysis of the freeze-milled cardroom cotton dust and the treated cotton dust showed both dusts to be of similar particle size distribution (Table 1).

Examination of Sera for Immune Precipitins to an Extract of Cardroom Cotton Dust

All human and guinea pig sera used in the complement activation assays were uniformly negative for immune precipitins to an extract of cardroom cotton dust using both gel diffusion and counterimmunoelectrophoresis techniques. However, during the course of the examination of these sera, it was noted that the cardroom cotton dust extract complexed and precipitated at least two serum proteins in the agarose gel (Kutz *et al.*, manuscript in preparation). Since this interaction was found in all of the sera that were examined, they were generally considered to be nonimmunological in nature, and are usually referred to as "pseudo-immune" reactions (Edwards and Jones, 1973). All of the sera used in the complement activation assays were therefore considered to be void of specific antibodies to any component of the cardroom cotton dust.

Hemolytic Complement Consumption and C3 Activation

Figure 1 demonstrates percentage consumption of hemolytic complement (CH_{50} u/ml) in human sera treated with either cardroom cotton dust (before treatment, BCD) or treated cotton dust (TCD). Equal amounts of BCD consumed greater quantities of available complement than did the TCD. One-half milligram of BCD consumed 55% of the available CH_{50} u/ml whereas the same amount of TCD consumed only 17%.

Both BCD and TCD caused the conversion of C3 to C3b as demonstrated by two-dimensional electrophoresis (Fig. 2). As expected from the CH_{50} results, a greater amount of C3 was converted by the BCD than the TCD as seen by the larger C3b arc with 5 mg BCD. This difference was quantifiable in that 5 mg of BCD converted 66% of the available C3 while the same amount of TCD converted

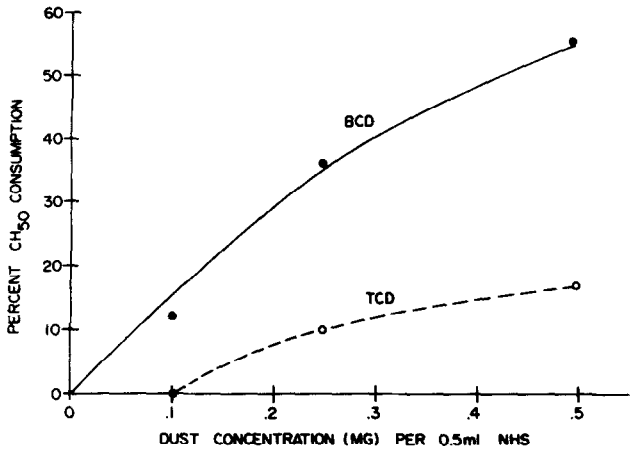

FIG. 1. Percentage consumption of hemolytic complement (CH_{50} u/ml) in pooled normal human serum (NHS) by increasing concentrations of cardroom cotton dust (BCD) and treated cotton dust (TCD).

only 33% (Table 2). BCD and TCD were statistically different ($P < 0.01$) at both dust levels tested.

Alternative Pathway Activation by Untreated and Treated Cotton Dusts

Activation of the human complement cascade by the alternative pathway was examined by monitoring Factor B conversion by BCD and TCD. Figure 3 illus-

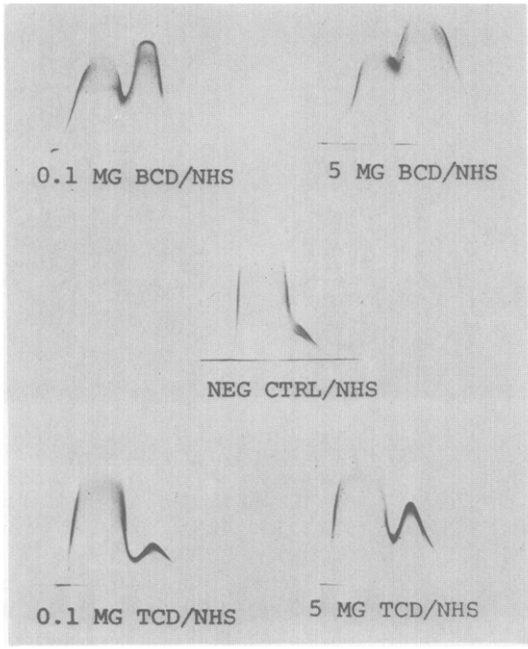

FIG. 2. Two-dimensional electrophoresis of C3 in pooled normal human serum (NHS) treated with cardroom cotton dust (BCD) or treated cotton dust (TCD). No dust was added to the negative control serum (NEG CTRL/NHS). C3 arc is to the left and C3b is to the right in each frame.

TABLE 2
PERCENTAGE CONVERSION OF HUMAN C3 BY CARDROOM COTTON DUST AND TREATED COTTON DUST

Dust added (mg/0.5 ml)	Cardroom cotton dust (%)	Treated cotton dust (%)	
0	0	0	
0.1	48.0[a]	19.7	$P < 0.01$
	(3.9)	(1.4)	
5.0	65.5	33.3	$P < 0.01$
	(1.3)	(6.0)	

[a] Mean of triplicate samples, standard deviation in parentheses.

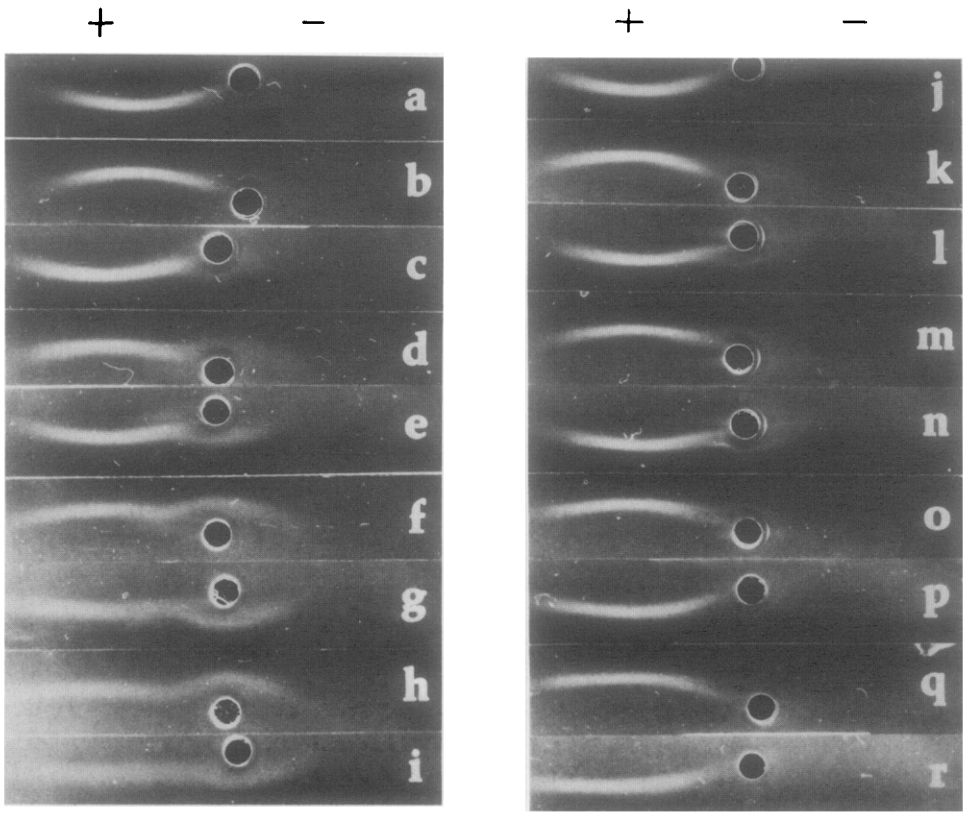

FIG. 3. Electrophoresis of human Factor B from pooled normal serum treated with increasing amounts of cardroom cotton dust (BCD) or treated cotton dust (TCD).

a. Control, no dust added
b. 0.1 mg BCD
c. 0.25 mg BCD
d. 0.5 mg BCD
e. 1.0 mg BCD
f. 5.0 mg BCD
g. 10.0 mg BCD
h. 15.0 mg BCD
i. 20.0 mg BCD
j. Control, no dust added
k. 0.1 mg TCD
l. 0.25 mg TCD
m. 0.5 mg TCD
n. 1.0 mg TCD
o. 5.0 mg TCD
p. 10.0 mg TCD
q. 15.0 mg TCD
r. 20.0 mg TCD

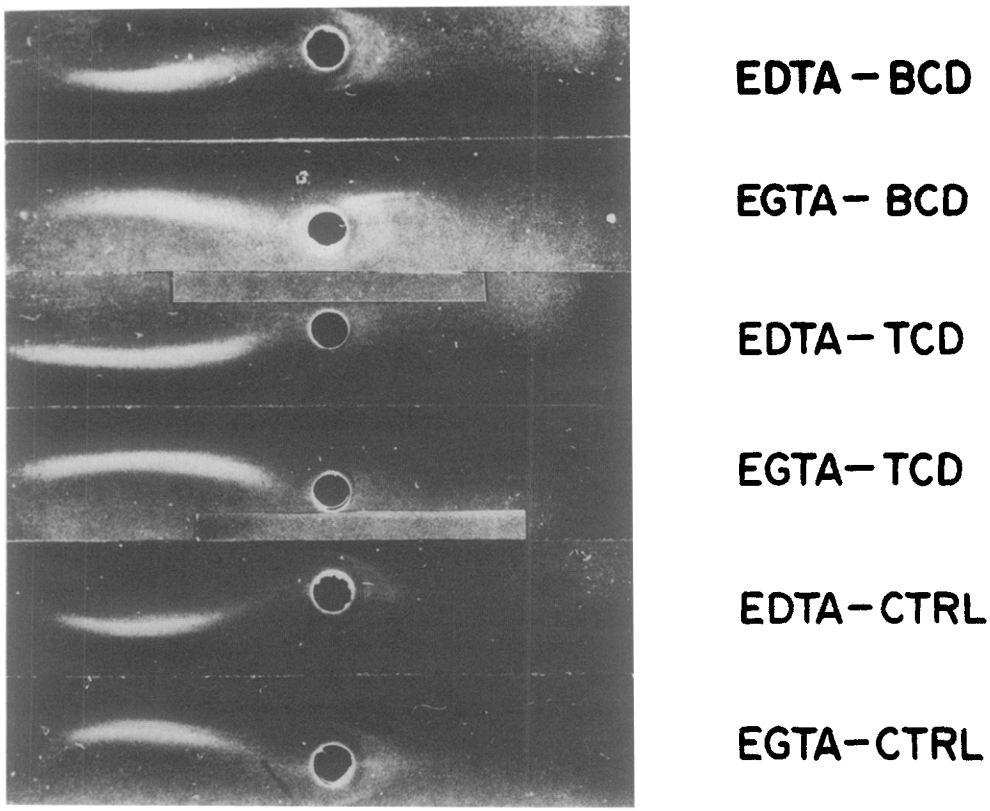

FIG. 4. Electrophoresis of human Factor B from pooled normal serum which was chelated with EDTA or EGTA before reaction with cardroom cotton dust (BCD) or treated cotton dust (TCD). Serum plus EDTA or EGTA alone is marked CTRL. Factor B arc to left of wells; Bb to right of well in EGTA–BCD frame only.

trates electrophoretically the dose–response conversion of Factor B to Bb by increasing amounts of BCD. Factor B conversion could not be demonstrated with TCD at any dose tested (0.1–20.0 mg/0.5 ml of serum).

Factor B conversion by BCD was inhibited by EDTA which strongly chelates magnesium and calcium ions while conversion of Factor B was observed in the presence of EGTA which chelates magnesium less effectively (Fig. 4). This evidence strongly suggests that BCD can activate the human alternative complement pathway *in vitro* (Fine *et al.*, 1972; Des Prez *et al.*, 1975).

To confirm the activation of the alternative pathway by BCD, sera from guinea pigs genetically deficient in C4 (C4D), and therefore with a nonfunctional classical pathway, were treated with dust. Table 3 shows that complement was consumed by the functional alternative pathway in C4D guinea pigs. Of interest, TCD also consumed hemolytic complement in C4D but to a lesser extent ($P < 0.01$) than did the BCD. Both BCD and TCD reduced the hemolytic complement in Strain 13

TABLE 3
COTTON DUST CONSUMPTION OF HEMOLYTIC COMPLEMENT IN SERA FROM C4-DEFICIENT AND STRAIN 13 GUINEA PIGS

Serum[a]	Cardroom cotton dust[b] (% reduction)	Treated cotton dust[b] (% reduction)	
C4 deficient	26.2[c]	17.2	$P < 0.01$
Strain 13	35.6	23.0	$P < 0.01$

[a] CH_{50} performed using EAClgp4gp cells immediately following addition of guinea pig C2.
[b] 1.0 mg dust/0.5 ml serum.
[c] Average of duplicate samples.

guinea pig sera, but again the BCD reduced the CH_{50} u/ml by a statistically greater amount ($P < 0.01$) than did the TCD.

Quantification of Endotoxin Content of Untreated and Treated Cotton Dusts

The gram-negative bacterial endotoxin contents of both BCD and TCD were assayed by the *Limulus* amebocyte lysate method and the results are shown in Table 4. The treatment used to prepare TCD destroyed over 98% of the endotoxin activity in the BCD.

DISCUSSION

Cardroom cotton dust was compared with dust which we treated in a manner parallel to the preparation of medical grade cotton in Egypt. It is reported that byssinosis is not found in the medical grade cotton workers whereas byssinosis is found in those workers exposed to the untreated cardroom cotton dust (El-Batawi and El-Din Shash, 1962).

We have shown that human complement (CH_{50} u/ml) is consumed *in vitro* by both cardroom cotton dust (before treatment, BCD) and by treated cotton dust (TCD), although a large quantifiable difference in activity between the two dusts was found. The third component of complement was shown electrophoretically to be converted greater by BCD than by TCD ($P < 0.01$). Factor B conversion and evidence from the differential chelation studies with EDTA and EGTA suggested that BCD, and not TCD, consumed complement by the calcium-independent alternative pathway. A much more sensitive technique, CH_{50} analysis of sera from guinea pigs genetically deficient in C4, demonstrated that BCD and TCD activate complement via the alternative pathway, although BCD was again more active ($P < 0.01$). Augmented complement consumption was evidenced by the greater reduction in CH_{50} u/ml when guinea pig Strain 13 sera were tested. This strain

TABLE 4
ENDOTOXIN CONTENT OF CARDROOM COTTON DUST AND TREATED COTTON DUST

Dust	Endotoxin[a] (ng/g dust)
Cardroom cotton dust	438.4
Treated cotton dust	7.6

[a] Results are expressed as nanograms FDA *Klebsiella* endotoxin equivalents per gram of dust. Average of duplicate samples.

maintains a functional classical pathway as well as a functional alternative pathway. One may conclude that the greater reduction in hemolytic complement was attributed to the activation of both pathways simultaneously. Alternatively, the differences observed between reduction in C4D CH_{50} u/ml and Strain 13 CH_{50} u/ml may be strain differences in the sensitivities of their respective complement cascades.

In every experimental system tested, BCD consumed greater amounts of complement or complement components than did TCD. This difference cannot be based solely on differences in particle surface area since particle size distribution studies reflected similar size patterns. In fact, the TCD tended towards the smaller particle size range with the greater percentage of particles less than 5 μm in size. These data suggest the presence of a biologically active mediator(s) in the BCD and reduced after treatment (TCD). One such mediator could be gram-negative bacterial endotoxin. Our studies showed a 58-fold decrease in the amount of endotoxin present in the BCD after treatment. Activation of both the alternative and classical complement pathways by endotoxin is well documented (Morrison and Kline, 1977; Marcus *et al.*, 1971; Gewurz *et al.*, 1968) but our own experiences with purified endotoxins as well as the work of others (Gewurz *et al.*, 1968; Morrison and Kline, 1977) have shown that microgram rather than nanogram quantities of purified endotoxin are required to induce the degree of complement consumption observed with BCD. Moreover, since other substances in addition to endotoxin are known also to cause positive results in the *Limulus* amebocyte lysate assay (Elin and Wolff, 1973; Brunson and Watson, 1976), it is highly unlikely that the observed complement activation could be attributed to endotoxin alone. Other as yet unidentified hemolytically active substances were probably removed by the physical and chemical protocol used to convert BCD to TCD.

In summary, we consider the possibility that the results of these *in vitro* data suggest the potential for *in vivo* airways changes mediated by complement activation, to follow cotton dust inhalation. The complement cascade is functionally intact within the secretions of the respiratory tract (Robertson *et al.*, 1976; Newhouse *et al.*, 1976). Generation of C3a and C5a by alternative pathway activation by cotton dust, either directly or upon processing by macrophages, would be expected to induce airways changes via release of histamine and other pharmacologically active mediators from mast cells. In addition, polymorphonuclear leukocytes could accumulate locally due to the generation of chemotactic factors from the complement cascade. However, it must be recognized that it is very difficult to correlate *in vitro* quantifiable results with *in vivo* response. The exact interaction of the alternative pathway of complement and cotton dust *in vivo* should be examined further since no such relationship has been documented.

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