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INTERACTIONS OF COMPLEMENT WITH AN EXTRACT OF AIRBORNE SPRING WHEAT DUST

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The inhalation, by grain elevator workers, of airborne grain dusts can lead to pulmonary problems. Complement, which is present in human airways, can interact with various grain dusts, producing activation products that have been shown to participate in the inflammatory reaction. Because of this apparent connection between grain-dust inhalation, complement activation, and respiratory difficulties, we are studying the reaction of an aqueous extract of spring wheat dust (swd) with human complement. The swd extract activates both the classical and alternative pathways; it acts on purified C2 to inhibit it, and it reacts with undiluted serum to consume C4 with kinetics significantly different from those shown by a "typical" antigen-antibody complex (sensitized sheep erythrocytes). Enzyme susceptibility experiments suggest that the alternative and classical pathway activators of swd extract are neither protein nor nucleic acid; periodate oxidation indicates these substances are carbohydrate, and gel filtration suggests they are macromolecular. Enzyme vulnerability also indicates that the C2 inhibitor of swd extract is ribonucleic acid. Although endotoxin is present in swd extract, a gel-filtration experiment showed that a major fraction of the complement reactivity was not associated with this substance.

INTRODUCTION

Since the writings of Ramazzini early in the eighteenth centruy, diseases caused by airborne grain dusts have been documented (Rammazini, 1713). Pulmonary symptoms associated with grain dust are common wherever grain is handled. Because of the deleterious effects of airborne grain dust on lung function, it has been suggested that grain dust should not be regarded as a mere nuisance dust (Chan-Yeung et al., 1981), since these substances pose a potentially serious threat to the health of a sizable population.

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Our laboratories have been engaged in the study of the reaction of grain dusts with the complement system (Olenchock et al., 1978, 1980b, 1983b) because there is good reason to postulate that such a reaction can account for much of the pulmonary pathology that follows respiratory exposure. Airborne grain dusts activate human complement *in vitro* in a dose-dependent fashion (Olenchock et al., 1980a), and both the classical and the alternative pathways have been implicated (Olenchock et al., 1979). The complement-active substances in the dust are rapidly soluble in water (Olenchock et al., 1979), and these substances can generate factors that are chemotactic for human polymorphonuclear leukocytes (Hugli and Müller-Eberhard, 1978). Because complement components are present in human airways (Robertson et al., 1976; Reynolds et al., 1977; Ackerman et al., 1978), the potential exists for direct interaction between the dust, or rapid extract, and complement after inhalation of airborne grain dusts. These studies suggest therefore the contribution of complement-derived active fragments to the generation of respiratory pathophysiologic changes.

In this communication, we present a biochemical study, in terms of complement reactivity, of an aqueous extract of spring wheat dust. Spring wheat dust was chosen for this study because it is one of the most complement-reactive dusts tested (Olenchock et al., 1980a,b).

MATERIALS AND METHODS

Airborne spring wheat dust (swd) was collected as previously described at an active port grain terminal in the Superior-Duluth (Wisconsin, Minnesota) region (Peach et al., 1980). A water extract of swd was prepared at room temperature by adding 5 ml distilled water to 1.0 g swd and shaking in a Spex Mixer Mill, fast setting (Spex Industries, Metuchen, N.J.), for 20 s. The suspension was poured into a centrifuge tube and the mixing vial was rinsed with a 2.5-ml water wash, which was added to the centrifuge tube. The suspension was centrifuged at 5°C for 20 min at 19,000 X g, and the supernatant fluid was collected.

Sheep red blood cells were obtained locally and also from a commercial supplier (GIBCO, Madison, Wis.). IgM anti-forsmann antibody was kindly provided by Thelma Gaither, National Institute of Allergy and Infectious Diseases, Bethesda, Md., and C4-deficient guinea pig serum was a gift of Dr. Eric Brown of the same institution. Human serum was prepared from blood drawn from laboratory personnel and stored at −65°C. Guinea pig serum was obtained from JEM Research, Inc., Kensington, Md., divided into 1-ml aliquots, and stored at −65°C. Functionally pure human complement components were purchased from Cordis Corp., Miami, Fla. The method for the assay of human C3 was that described by Müller-Eberhard (1977). The buffers and other reagents used in the complement assays were prepared, with minor modification, according to Rapp and Borsos (1970); hereafter, VBS refers to veronal buffered saline, pH 7.3,

ionic strength 0.150; 0.065 ionic strength buffer refers to VBS at the indicated ionic strength and pH 7.3 (Rapp and Borsos, 1970). The assays for C1 and C2 were conducted as described by Rapp and Brosos (1970). With minor modification, C4 was assayed according to the procedure of Gaither et al. (1974) and C1 inhibitor (C1-Inh) according to Gigli et al. (1968).

The immobilized enzymes Enzite-Agarose-Chymotrypsin, Enzite-Agarose-Trypsin, and Enzite-Agarose-Protease were purchased from Miles Laboratories, Inc. (Elkhart, Ind.), and were prepared for use by suspending in pH 8.0 VBS. (For complement work, VBS is used at pH 7.3; pH 8.0 VBS was used only for these enzyme studies.) The suspensions were incubated at 37°C for 60 min, after which the beads were washed by centrifugation 4 times in the same buffer; the beads of agarose-immobilized enzymes were then suspended in pH 8.0 VBS so that the settled beads occupied 25% of the volume. Positive controls for the immobilized proteolytic enzymes were included to confirm that the enzymes were active; to this end, the enzyme beads were incubated with either 10 CH50 units of purified human C4 (Cordis Corp.) or 1000 CH50 units of purified human C3 (Cordis Corp.) under the conditions described in the results section. In every case, the complement component suffered a major loss in activity, indicating that the enzymes were active. Bovine pancreas DNAse I (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 0.15 *M* NaCl for a concentration of 108 Kunitz units/ml. The activity of the enzyme was confirmed by a published assay procedure (Decker, 1977). RNAse T_2 was obtained from Sankyo Co. Ltd., Tokyo. It was diluted to 26 units/ml in 0.1 *M* acetate buffer, pH 4.5, containing 2 m*M* ethylenediaminetetraacetic acid (EDTA); the activity was confirmed by a published procedure (Uchida and Egami, 1967). Bovine pancreas RNAse was obtained from Schwartz Mann (Orangeburg, N.Y.) and also from Sigma. Bovine pancrease RNAse samples were prepared at a concentration of 26 units/ml in 0.1 *M* acetate buffer, pH 5.0; the activities were confirmed by a published procedure (Decker, 1977).

RESULTS

Interactions of swd Extract with the Human Complement System

In agreement with earlier work, we found that swd extract reduced the titer of whole complement in human serum (Olenchock et al., 1980b). In the following experiments (Table 1), this was extended to the individual early components.

Experiment 1: 0.4 ml human serum, 0.1 ml VBS, and 50 μl extract were mixed and incubated at 37°C for 30 min; the volume was adjusted to 4.0 ml with VBS and the sample was transferred to an ice bath.

Experiment 2: 50 μl VBS or EDTA (0.05 *M*) or ethyleneglycol-bis-(β-amino-ethyl ether) N, N′-tetraacetic acid (EGTA; Sigma Chemical Co., St. Louis, Mo.) (0.05 *M*) $MgCl_2$ (0.01 *M*) were mixed with 0.1 ml human serum

TABLE 1. Reaction of swd Extract with Human Serum

		Serum titers					
		A		B (EDTA)		C (EGTA)	
Experiment	Component	Control	Extract-treated	Control	Extract-treated	Control	Extract-treated
1	C1	143,000	22,000				
2	C4	137,000	6,400	85,000	75,000	59,000	63,000
2	C2	1,135	186	1,358	1,417	1,302	1,313
2	C3	12,864	4,025	13,863	12,000	13,324	8,000

and 0.1 ml swd extract diluted 1/20. The mixture was incubated at 37°C for 30 min and transferred to an ice bath. The sample volume was adjusted to 5.0 ml with VBS that included 0.25 ml 0.01 *M* $CaCl_2$ for the samples containing EDTA or EGTA.

The treated serums were assayed for the complement components indicated in Table 1. It is apparent from column A that extract-treated serum displays a considerable drop in the levels of C1, C4, C2, and C3, indicating that the classical pathway has been activated by the wheat dust extract. In columns B and C, it can be seen that EDTA prevents consumption of C4, C2, and C3, while in the presence of EGTA, C4 and C2 titers are again stable but C3 undergoes a drop; this indicates that the alternative pathway is activated by swd extract.

Ziccardi (1981) studied the kinetics of C4 inactivation in undiluted serum by various complement activators including sensitized sheep red cells (EA). We carried out a similar experiment in which we compared EA and swd extract. A series of tubes received a suspension of 4×10^8 EA; the suspension was centrifuged and the supernatant fluid discarded. Another series of tubes received 10 μl swd extract diluted 1:6 with VBS. These quantities of EA and extract were chosen because they inactivated approximately equal fractions of the serum C4. The tubes were placed in a 37°C bath and received, in turn, 0.10 ml human serum at 1-min intervals. At the appropriate times, tubes were transferred to an ice bath and the reaction was halted with 4.0 ml of 0°C VBS. This mixture was further diluted and assayed for C4. Figure 1 shows that EA and swd extract differ markedly in the rate at which they promote the same extent of C4 inactivation. The inactivation of C4 caused by EA ceases after about 3 min, while the inactivation caused by swd extract does not stop until about 11 min. In three experiments the extract consistantly displayed slower kinetics when compared to EA. Ziccardi (1981) concluded that the early (3-min) termination of C4 consumption by EA was brought about by the C1 inhibitor (C1-Inh) present in serum. The finding that the C4 consumption caused by swd

extract continued until 11 min prompted us to ask whether a constituent of extract was interfering with C1-Inh. The question was addressed as follows.

C1-Inh obtained from Cordis Corp. was diluted 1/3.2 with VBS to achieve the C1-Inh concentration in human serum [as determined by the assay of Gigli et al. (1968)]; in a series of tubes, 100-μl portions of diluted C1-Inh were treated with 10 μl swd extract. These volumes were chosen to reproduce those used in the experiment shown in Fig. 1; the C1-Inh was thus present at the same concentration shown in Fig. 1 and the swd extract at 6 times the concentration. At various times the reactants were diluted 1/50 by the addition of 5 ml cold 0.065 ionic strength buffer (Rapp and Borsos, 1970); for assay the mixtures were further diluted with 0.065 ionic strength buffer to a final dilution of 1/3250. The mixtures were assayed for C1-Inh by the method of Gigli et al. (1968). The assay consists of subjecting a C1 solution to C1-Inh and measuring the surviving C1. In Fig. 2, it can be seen that the C1-Inh reduced the titer of the C1 preparation from about 85,000 to about 30,000. It is apparent that treatment of a C1-Inh with swd extract did not compromise its ability to inactivate C1. Thus there is no evidence that the change in kinetics shown in Fig. 1 is caused by inhibition of C1-Inh by swd extract.

When purified human C4 (Cordis Crop., diluted 1:10) was treated with an equal volume of 1:8 extract, there was no loss of C4 activity; in the absence of C1, this was not surprising. On the other hand, when purified human C2 (Cordis Corp., 1:10) was treated with an equal volume of 1:8 swd extract for 30 min at 37°C, there was a modest but consistent loss of C2; for example, the C2 titer was observed in one case to drop from 1170 to 735 units/ml after exposure to extract. Thus, wheat dust extract displays at least three activities relative to complement: activation of the classical pathway, activation of the alternative pathway, and inactivation of purified C2. A preliminary characterization of these entities was pursued through gel filtration, periodate oxidation, and enzyme treatment.

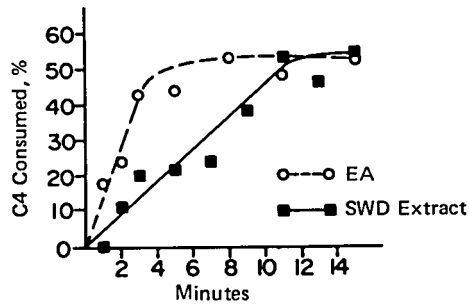

FIGURE 1. Percent of C4 consumed on incubating undiluted human serum with sensitized sheep red cells (EA) or an aqueous extract of spring wheat dust (swd).

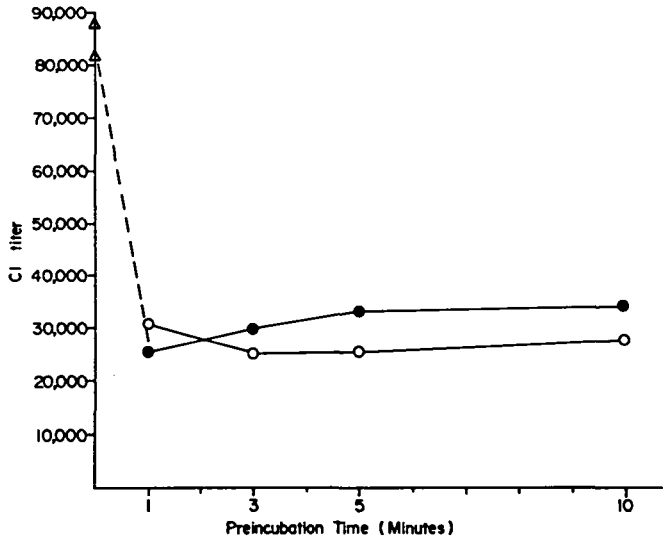

FIGURE 2. C1 inhibitor (C1-Inh) activity as measured by its ability to inactivate C1; open circles—control in which C1-Inh is incubated with buffer, closed circles—C1-Inh incubated with spring wheat dust (swd) extract, and open triangles—C1 and buffer only.

Properties of Complement-Reactive Agents in swd Extract

Gel filtration used a Sephadex G-200 column, 90 X 1.6 cm, prepared using 5 m*M* phosphate buffer, pH 7.5, with 0.03% sodium azide. A 0.75-ml sample of extract was applied to the column. The column was run at about 8 ml/h, and 4-ml samples were collected and assayed for the appropriate activities as follows.

C4 consumption (or inactivation); 100 μl of a human serum sample diluted 1/900 was treated with 10 μl of column fraction. The mixture was incubated at 37°C for 60 min and then used in its entirety for the assay of C4 (Gaither et al., 1974).

C3 consumption: 50 μl of a human serum sample diluted 1/2.5 and containing EGTA and $MgCl_2$ at concentrations of 30 and 6 m*M*, respectively, was treated with 100 μl of column fraction at 37°C for 60 min. Two milliliters of 0.67 m*M* $CaCl_2$ in 0.065 ionic strength buffer was then added and 10μl of this mixture was diluted with 1 ml 0.065 ionic strength buffer; 100 μl was assayed for C3 (Müller-Eberhard, 1977).

C2 inhibitor assay: 50 μl of a Cordis human C2 sample diluted 1/10 with VBS was treated with 50 μl of column fraction at 37°C for 60 min. One milliliter of VBS was added to the mixture and 200 μl removed for C2 assay (Rapp and Borsos, 1970).

The gel filtration behavior of the three activities is shown in Fig. 3. The classical and alternative pathway activators emerged in the void volume.

The C2 inhibitor was eluted as a broad peak, with its maximum corresponding to a molecular weight of about 36,000.

Periodate oxidation. The vulnerability of the swd extract activities to periodate oxidation was examined as follows: 0.3 ml extract was treated with 0.1 ml 0.2 *M* $NaIO_4$ for 2 h at refrigerator temperature (approximately 5°C), after which 0.025 ml ethylene glycol was added to consume the remaining periodate. (In a preliminary experiment, extending treatment to 49 h did not increase the effect of periodate oxidation.) The control consisted of premixing and adding ethylene glycol and periodate to the extract. The results showed that the swd C2 inhibitor has no detectable vulnerability to periodate, while the classical pathway and alternative pathway activators are substantially (although not completely) destroyed by periodate; e.g., in one set of experiments, classical pathway and alternative pathway activators suffered losses of 62 and 57%, respectively. These results are consistent with the classical pathway and alternative pathway activators being carbohydrate in nature.

Enzyme treatment. The effects of several enzymes on the swd extract are summarized in Table 2. The treatments were carried out as follows.

Proteolytic enzymes: 1-ml portions of suspensions of agarose-immobilized chymotrypsin, trypsin, and protease were centrifuged and drained. Extract of swd was diluted 1:2 with VBS (pH 8.0) and the mixture adjusted to pH 8.0. Then 1-ml portions of the adjusted extract were added to the drained agarose enzyme beads and the mixtures agitated briefly. The mixtures were incubated with occasional agitation at 37°C. At 0 and 60 min, 4.0 ml of pH 7.3 VBS were added and the suspensions were mixed and briefly centrifuged; the supernatant fluids, which now had a pH 7.3,

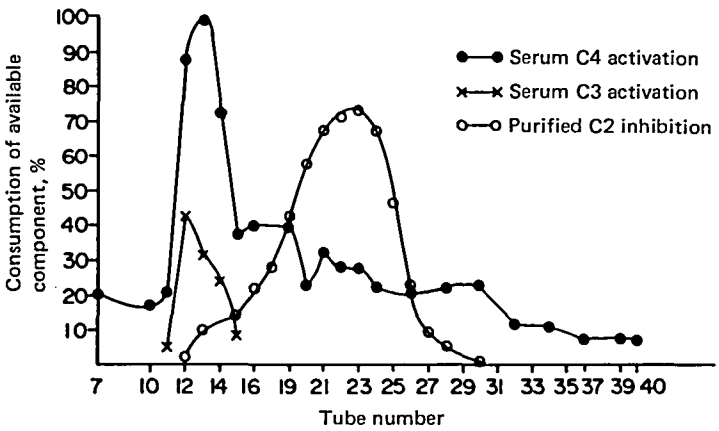

FIGURE 3. Gel filtration of spring wheat dust (swd) on Sephadex G-200. The void volume of this column is represented by tube 12 as indicated by dextran blue. The consumption (or activation) of serum C3 was done in the presence of EGTA, thus proceeding by the alternative pathway. The peak of C2 inhibitor corresponds to a molecular weight of about 36,000, as indicated by calibrating the column with gamma globulin, bovine serum albumin, and cytochrome C.

TABLE 2. Enzyme Treatment of Spring Wheat Dust Extract[a]

	Enzyme	Classical pathway activator	Alternative pathway activator	C2 inhibitor
A	Proteolytic enzymes			
	Chymotrypsin	5% loss	4% gain	11% gain
	Trypsin	5% loss	11% gain	16% gain
	Protease	9% gain	4% gain	20% gain
	DNAse	5% gain	1% gain	5% loss
	RNAse			
	Bovine pancreas (a)	2% loss	3% loss	67% loss
	Bovine pancreas (b)			7% loss
	T_2 (Sankyo)			2% gain
B	Chymotrypsin-RNAse			
	Bovine pancreas (a)			88% loss
	Bovine pancreas (b)			59% loss

[a]Part A: dust extract was treated with the enzymes indicated. Part B: dust extract was pretreated with chymotrypsin and then treated with RNAse.

were removed. Controls, lacking the enzyme beads but otherwise treated as described, were included as 100% extract activity.

DNAse (bovine pancreas): a 0.4-ml sample of 1:2 extract in 0.1 *M* acetate buffer pH 5.0 was treated with 0.1 ml DNAse (108 units/ml) for 30 min at room temperature; 1.5 ml VBS was then added and the pH adjusted to 7.4.

RNAses: a 0.4-ml portion of 1:2 swd extract in 0.1 *M* acetate buffer, pH 5.0, was treated with 0.1 ml bovine pancreas RNAse (26 units/ml) for 20 min at 37°C, after which 1.5 ml VBS were added and the pH was adjusted to 7.4; this experiment was carried out using bovine pancreas RNAses supplied by Schwartz Mann (a) and Sigma (b). In a similar fashion, extract was treated with T_2 RNAse (26 units/ml), except that the buffer was pH 4.5, 0.1 *M* acetate, containing 0.002 *M* EDTA.

The capacities of the enzyme-treated swd extract samples to activate the classical and alternative pathways and inhibit C2 were assayed as usual. In Table 2A it can be seen that chymotrypsin, trypsin, and protease have little effect on the classical and alternative pathway activators. The C2 inhibitor, however, undergoes a small but consistent enhancement by these enzymes; this was seen in three experiments. DNAse has no detectable effect on any of the activities assayed. Bovine pancreas RNAse supplied by Schwartz Mann (a) produced no detectable effect on the classical or alternative pathway activators, but it repeatedly produced a substantial drop in the C2 inhibitor. Neither T_2 RNAse nor the bovine pancreas RNAse supplied by Sigma (b) caused any loss of the C2 inhibitor. The C2 inhibitor became vulnerable to Sigma bovine pancreas RNAse (b) after treatment

with chymotrypsin (T_2 RNAse was not tested); this result is presented in Table 2B. The chymotrypsin treatment protocol was the same one employed in Table 2A. Trypsin pretreatment had the same effect as chymotrypsin: i.e., bovine pancreas RNAse (b) caused substantial loss of C2 inhibitor *after* the extract was pretreated with trypsin.

Endotoxin Content of Spring Wheat Dust Extract

The endotoxin content of three separate swd extracts was assayed by a spectrophotometric modification of the *Limulus* amebocyte lysate method (Olenchock et al., 1983a). The endotoxin concentrations of the three extracts were 8.87, 17.50, and 4.72 μg/ml, respectively. The mean of the three determinations was 10.36 μg/ml with a standard error of ±5.98. It has been known for a number of years that endotoxin (LPS) will activate the alternative pathway (Gewurz et al., 1968). More recently, it was recognized that certain LPS preparations can also, in the absence of specific antibody, activate the classical pathway (Morrison and Kline, 1977). Thus, the occurrence of endotoxin in spring wheat dust raises the question of whether this agent is responsible, in whole or in part, for the complement reactivity of the dust. This question was addressed in the experiment illustrated in Fig. 4: 1.8 ml of swd extract was applied to a Sepharose 4B column (85 cm length X 1.6 cm diameter, 43 ml void volume), and 3-ml fractions were collected and assayed for their ability to consume C4 in diluted normal human serum. Two major peaks are evident (I and II) that will promote the consumption of C4 (presumably by the classical pathway). Peak I emerged at about 44 ml, which is the void volume, suggesting a molecular weight in excess of 5×10^6 (Gel Filtration Theory and Practice, 1979). Peak II emerged at 126 ml, which was the volume at which glucose came

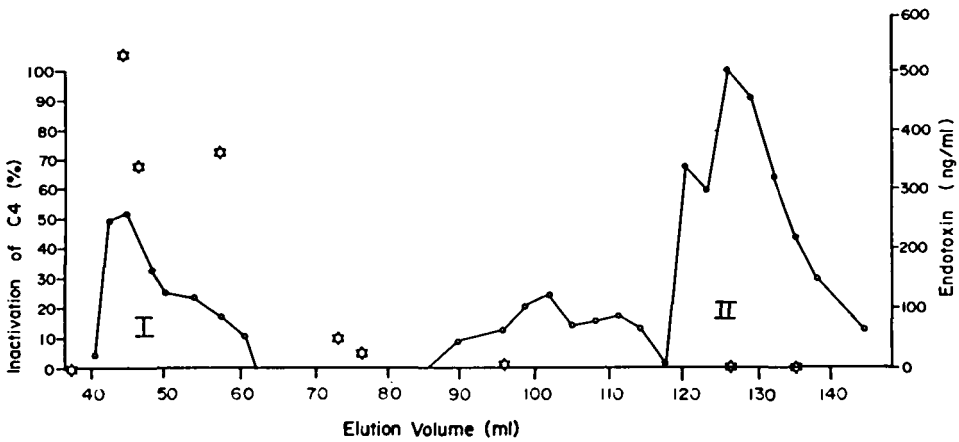

FIGURE 4. Gel filtration of spring wheat dust (swd) extract on Sepharose 4B. The open circles connected by lines represents C4 inactivation (or consumption). Endotoxin (LPS) is indicated by open stars.

off the column; this suggests a molecular weight of 6×10^4 or less. Selected fractions were assayed for endotoxin by the *Limulus* amebocyte lysate assay (indicated in Fig. 4 by stars). A prominent peak of endotoxin coincided with peak I of C4-consuming material. Peak II, on the other hand, is apparently devoid of endotoxin. Thus, a major fraction of the complement reactive material of spring wheat dust extract is probably independent of endotoxin.

DISCUSSION

In this work we used an aqueous extract of spring wheat dust produced by 20 s of rapid mixing in a Spex Mill. This admittedly arbitrary method was chosen to reflect the fact that respiratory symptoms can emerge soon after inhaling grain dust (doPico et al., 1982), and we reasoned that this pathophysiological process probably involves, in the lung, the rapid extraction of soluble active agents from the dust materials.

Table 1 confirms earlier work by Olenchock et al. (1980b) that an aqueous extract of spring wheat dust consumes human complement components C1, C4, C2, and C3, thus showing that the classical pathway is active. Olenchock and co-workers also showed, by immunoelectrophoresis, that factor B is activated by swd extract, thus implicating the alternative pathway (Olenchock et al., 1978). In the present work we have confirmed that conclusion by the hemolytic assay of C3 in serum that has been treated with swd extract with and without chelators (Table 1).

The serum C4 consumption we obtained using EA agrees with the finding of Ziccardi (1981): i.e., C4 consumption ceases after 3 min. These experiments (both Ziccardi's and ours) were carried out using undiluted serum. Ziccardi postulates that these concentrations are the most physiologic and that, in this circumstance, the C1-Inh acts promptly to terminate the reaction at 3 min. When such reactions take place in highly dilute serum, complement activation continues for hours (Borsos et al., 1964), presumably because the C1-Inh concentration is so low as to render it (C1-Inh) ineffective. Since C4 consumption by swd extract continued until 11 min, we reasoned that this material might be interfering with the action of C1-Inh. The data shown in Fig. 3 do not support this suggestion. The swd extract has no apparent effect on purified C1-Inh. In contrast to our result, the work of Giclas (1982) and of Chan-Yeung et al. (1980) is of some interest. These workers have been concerned with asthma occurring in people working in red cedar sawmills. The active agent in red cedar wood is plicatic acid. They showed that plicatic acid inactivates C1-Inh so that control of C1 is prevented or compromised. The end result is activation of C1 and consumption of components of the complement cascade. Thus, although both cedar wood dust and spring wheat dust produce pulmonary distress, they apparently activate complement by different mechanisms. The slower

C4 consumption kinetics of swd extract compared to EA (Fig. 2) are unaccounted for at the present time.

We are currently dealing with three complement-related activities in swd extract: classical pathway activator, alternative pathway activator, and C2 inhibitor. (The reduction of C4 consumption kinetics shown in Fig. 1 is not a readily defined parameter, but this might constitute still another complement-related activity in this dust.) The chemical nature of these soluble complement reactive substances is largely undefined, but some inferences are possible. The behavior of the three activities on G-200 Sephadex indicates that they are macromolecular. Table 2 shows that the classical and alternative pathway activators were not affected by proteolytic enzymes, DNAse, or RNAse, indicating these substances are not protein or nucleic acid. The vulnerability to periodic acid suggests they are carbohydrate: Fig. 2 then leads us to entertain the possibility that the classical and alternative pathway activators are polysaccharide, at least in part. In this connection, it is interesting that Schultz and Arnold (1981) have isolated a small polysaccharide from ant venom that activates C1.

The C2 inhibitor presents an interesting problem (Table 2). Treatment with proteolytic enzymes produced a consistant small enhancement of activity beyond what might result from experimental error. Furthermore, two RNAses had no affect on this activity, while a third RNAse produced a substantial decrease in the C2 inhibitor. Finally, if the swd extract was pretreated with chymotrypsin, even a previously inactive RNAse produced a substantial loss of this activity. This constellation of properties leads us to tentatively propose the following. The C2 inhibitor of spring wheat dust is an RNA moiety associated with protein, and this complex displays a molecular weight of about 36,000 (Fig. 3). The protein masks the RNA; if the protein is digested there is a small increase in C2 inhibitor activity (Table 2A), as well as an emergence of RNAse vulnerability [Table 2B, bovine pancreas RNAse sample (b)]. Bovine pancreas RNAse (a) (Table 2) presumably was active without the preliminary chymotrypsin treatment because of protease contamination; pancreas, which is the source of this RNAse, is a rich source of proteases, and such contamination could readily occur. The reaction of C2 with nucleic acid does have a precedent of sorts. Gardner et al. (1980) identified a major DNA binding protein of serum as factor B, and factor B is a structural and functional analog of C2 (Stroud et al., 1979).

The presence of endotoxin in swd extract raises the question of how the complement-reactive substances of this dust are related to the endotoxin; endotoxins have been shown capable of activating both the classical and alternative pathways (Gewurz et al., 1968; Morrison and Kline, 1977). The experiment shown in Fig. 4 indicates that a major portion of the classical pathway activator can be separated from the endotoxin by gel filtration on Sepharose 4B. The two gel filtration experiments (Figs. 3 and 4) ap-

parently indicate different size ranges for the classical pathway activators. Assuming this agent(s) is polysaccharide in nature, Fig. 3 indicates that the major portion of this activity has a molecular size of 200,000 or greater, while Fig. 4 suggests that peak II (lacking endotoxin) has a molecular weight of 30,000 or less. These disparate results are difficult to reconcile. It seems that forces other than gel filtration determine the distribution of the classical pathway activator on Sepharose 4B. For example, a charged polysaccharide might interact with the residual charge of the agarose of Sepharose 4B (Gel Filtration Theory and Practice, 1979), thus retarding its emergence from the column. Whatever the reason, it allowed us to demonstrate that a significant portion of the classical pathway activator is distinct from endotoxin.

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