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In Vitro and *in Vivo* Changes in Human Complement Caused by Silage

by Stephen A. Olenchock,* John J. May,† David S. Pratt,† Daniel M. Lewis,* Judith C. Mull,* and Lorann Stallones†

Aqueous extracts of silage samples from four farms in up-state New York were reacted *in vitro* with normal human serum. Hemolytic levels of complement component C3 were consumed in a dose-dependent fashion, and the four extracts differed in their relative activity rankings. Studies with chelated serum indicate that the alternative complement pathway is involved in the activation, and the active fragment C3b was demonstrated. Serum levels of hemolytic C3 and C4 *in vivo* were quantified before and after farmers performed their normal silo unloading operations. Although the study groups were small, suggestive evidence of *in vivo* complement consumption was found. IgE-related allergy did not appear to be of significance to the study groups. Complement activation may be an initiator of or contributor to adverse reactions in farmers who are exposed to airborne silage dusts.

Introduction

Pulmonary signs and symptoms associated with respiratory exposures to airborne vegetable dusts during farming are well documented. Hypersensitivity pneumonitis is associated with exposure to moldy hay (*1*) or contaminated grain (*2*). Allergy (*3*), asthma (*4,5*), chronic bronchitis (*6*), and grain fever syndrome (*7*) are reported in individuals who work in various grain storage environments. Recently, an acute febrile illness of short duration was identified in farmers who were unloading the contents of silos (*8*). This new entity was termed "silo unloader's syndrome" (*8*), and the role of silage in eliciting this illness is the focus of this study.

Specific attention is drawn to the reactions of silage with the human complement system, because such interactions could initiate or exacerbate events in the lung which could lead to pulmonary dysfunction of systemic manifestations of disease. Activation of complement has been demonstrated with other farm-related products such as grain and grain dusts (*9–11*), moldy hay dust (*12*), rice (*13*), sugar cane (*14*), and microorganisms which are associated with farmer's lung (*15*). It would seem reasonable, therefore, to continue this line of investigation with silage and to examine the role of complement activation as a potential disease mechanism in silo unloader's syndrome.

It is the purpose of this paper to report the *in vitro* activation of human serum complement by silage extract and to report *in vivo* changes in serum levels of complement components C3 and C4 in farmers who were unloading silos.

Materials and Methods

Silage

Samples of silage were obtained from four different farms in up-state New York. The samples were from moldy top hay silages which were brought into the clinic by patients who experienced adverse reactions to the silage dusts.

Extracts of the different silages were made individually by adding 0.5 g of silage to 25 mL of sterile, nonpyrogenic water (Travenol Laboratories, Inc., Morton Grove, IL). The mixtures were rocked at room temperature for 60 min, after which they were centrifuged for 10 min at 1000*g*. The supernatant fluid was removed and centrifuged again. Following the second centrifugation, the supernatant fluid was filtered through a sterile 0.45 μm pore size filter. The filtrate was centrifuged for 10 min at 1000*g* and the final supernatant fluid was frozen at −80°C until assayed.

The gram-negative bacterial endotoxin contents of the extracts were quantified in duplicate by a spectrophotometric modification of Limulus amebocyte lysate gel

*Immunology Section, Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, WV 26505.
†Section of Pulmonary Medicine, Mary Imogene Bassett Hospital, Cooperstown, NY 13326.

test (Pyrostat; Millipore Corp., Bedford, MA). Sterile, nonpyrogenic plasticware was used throughout these analyses. The results were analyzed by linear regression, compared to a standard curve, and reported in terms of nanograms of United States reference endotoxin per milligram of silage.

Complement Reactivity *in Vitro*

The dose-dependent effect of the silage extracts on the hemolytic activity of complement component C3 was tested *in vitro*. Aliquots of each extract (5, 10, 50, and 100 μL) were added to 0.5 mL of pooled normal human serum. Sterile, nonpyrogenic saline (Travenol Laboratories, Inc.) was added to each tube in order to achieve a total volume of 100 μL/0.5 mL of serum. The mixtures were incubated for 60 min at 37°C in a shaking water bath. Aliquots were assayed for hemolytic activity on C3 by the procedure recommended by the manufacturer (Cordis Laboratories, Miami, FL; 10), and the results were reported in terms of C3H50 U/mL. The median dose levels for relative complement activity ranking were defined by probit analysis (*16*).

Aliquots of each dose of the reaction mixtures were analyzed by two-dimensional electrophoresis for the conversion of complement component C3 *in vitro* as described previously (*10*). Additional aliquots of pooled normal human serum were chelated with 20 μL of 100 mM ethylenediaminetetraacetate (EDTA; Fisher Scientific Co., Pittsburgh, PA) or with 20 μL 100 mM ethyleneglycol-bis(β-aminoethyl ether)*N*,*N'*-tetraacetic acid (EGTA; Sigma Chemical Co., St. Louis, MO) before reaction with 50 μL silage extract in order to test for conversion of the alternative pathway of complement (*10*).

Complement Reactivity *in Vivo*

We examined the hemolytic or functional levels of C3 and C4 in the sera of farmers before unloading their farm's silo. The unloading operation provided a 45-min exposure period, and the hemolytic levels of the same complement components were quantified again 6 hr after exposure. Nonexposed individuals from the same farms served as controls for this study, and their sera were collected and analyzed in the same manner as the sera from the exposed workers. The materials for the assays were available commercially (Cordis Laboratories), and the procedures were as described by the manufacturer (*10*). Statistical comparisons were analyzed by *t*-test.

Allergy Testing

Total serum levels of immunoglobulin E (IgE) were quantified with the paper immunosorbent test (PRIST: Pharmacia Diagnostics, Piscataway, NJ) according to the manufacturer's recommendations. The results were expressed as international units of IgE per milliliter of serum (IU/mL).

The presence of specific IgE antibodies in the sera against fungi were assayed by the radioallergosorbent test (RAST; Pharmacia Diagnostics) as described by the manufacturer. Four major fungi were picked for the analyses, *Aspergillus fumigatus*, *Pencillium notatum*, *Mucor spp.*, and *Cladosporium spp.*, because these common organisms were among those isolated from the area's silage samples (I. W. Deep, personal communication). Results were expressed as percent of total counts of ^{125}I-labeled anti-IgE bound. Samples which bound at least twice the amount bound by the control sera were considered positive.

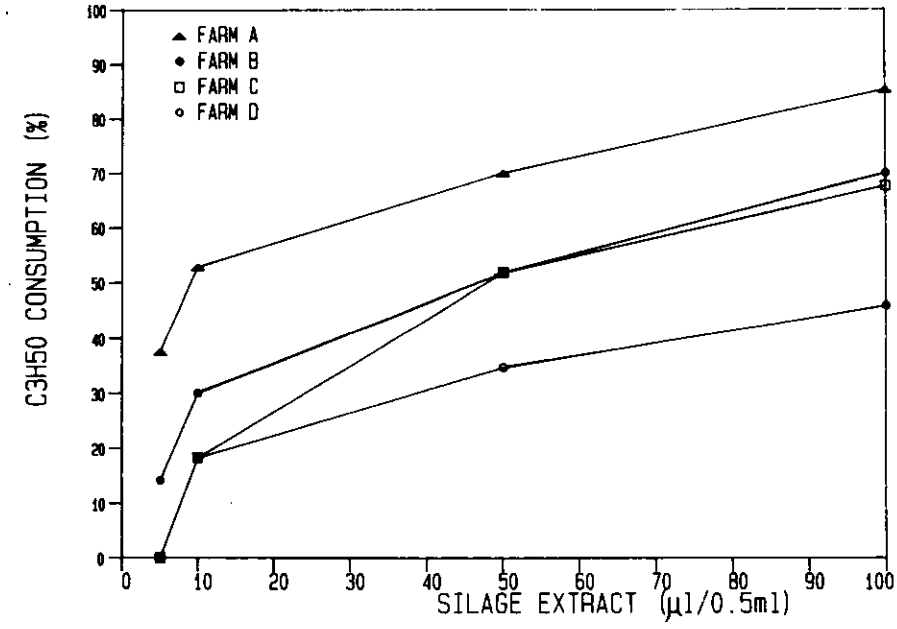

FIGURE 1. Percentage consumption (reduction) in hemolytic C3 in normal human serum which was reacted with increasing amounts of aqueous extracts of silage from four farms.

Results

Consumption of C3H50 *in Vitro*

Extracts of each of the four different silage samples were shown to consume normal human hemolytic complement component C3 in a dose-dependent fashion. Figure 1 illustrates the dose-related curves for the silage extracts. As the amount of silage extract increased, greater comsumption of hemolytic C3 was observed. There appeared to be a difference in the relative complement activity rankings of the silages in that the silage from farm A showed the greatest C3 consumption at all 4 data points (37.9–85.1% consumption). At the other extreme, the extract of silage from Farm D was the least active against C3, although still of considerable reactivity (0.0–46.0% consumption).

The relative activity rankings were calculated by probit analysis, and the results are shown on Table 1. The differences in relative anti-C3 activity of the extracts are reported in terms of the concentrations which consume 50% of the available serum C3H50 U/mL. Silage extract from Farm A was the most active, because only 9.97 μL was required to consume 50% of the C3H50 U/mL in 0.5 mL of the normal human serum. The extract from Farm D would require 144.52 μL, or greater than 14-fold more extract than silage from Farm A in order to achieve the same level of C3 consumption (50%).

Conversion of C3 *in Vitro*

Examination of aliquots of the extract-treated sera by two-dimensional electrophoresis revealed that C3 was converted to the active fragment C3b in a dose-dependent fashion. Figure 2 demonstrates immunochemically the conversion of C3 in the test mixtures. As the level of silage extract increased, the right arc in each panel increased which shows the activated C3b fragment. The left arc, native C3, consequently decreased as more was converted to the active form. The panels with only saline added show little nonspecific or background activation of C3.

When the normal human serum is pretreated with EDTA which blocks both classical and alternative complement pathway activation, no conversion of C3 to C3b is observed with any dose of extract (Fig. 3). Prior chelation of the sera with EGTA, which blocks the classical pathway activation but does not block the Ca-independent activation of C3 by the alternative pathway, resulted in the conversion of C3 to C3b as shown in Figure 3. Unchelated sera support conversion.

Table 1. Relative complement activity ranking and endotoxin levels for silage samples.

Silage extract, farm	50% $C3H_{50}$, μL/0.5 mL	Endotoxin, ng/mg
A	9.97	31.35[a]
B	37.50	31.97
C	46.33	6.32
D	144.52	0.06

[a] Average of duplicate values.

Endotoxin Levels

Table 1 shows also the concentrations of gram-negative bacterial endotoxins in the four silage extracts. The silage extracts from farms A and B had the highest and equivalent levels of endotoxins, 31.35 ng/mg and 31.97 ng/mg, respectively. The lowest concentration of endotoxins was found in the extract of silage from farm D, 0.06 ng/mg,

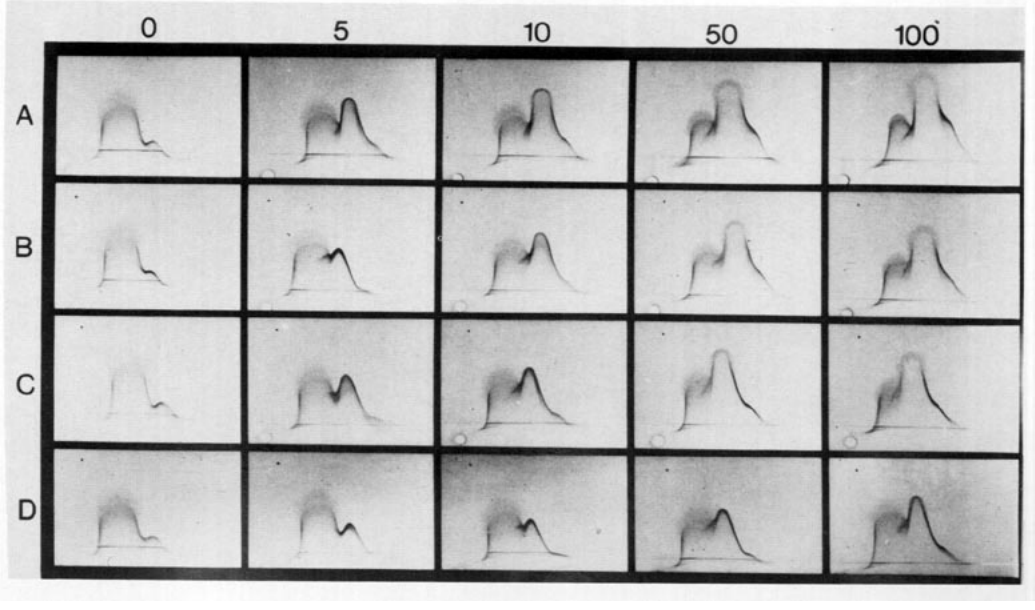

FIGURE 2. Two-dimensional immunoelectrophoresis of C3 from normal human serum which was reacted with increasing amounts (0–100 μL/0.5 mL of serum) of aqueous extracts of silage from four farms (A–D). In each frame the left arc is C3, and the right arc is C3b.

Table 2. Demography of study groups.

Characteristic	Exposed	Ill	Control
Number	9	3	4
Sex	Male	Male	Female
Mean age ± SE	33.4 ± 4.2	32.0 ± 4.4	48.5 ± 4.4
Age range	17–52	25–42	36–57

over 500-fold lower than in the extracts from farms A and B silages.

Hemolytic Complement Changes *in Vivo*

As a result of actual work-day practices, nine individuals were exposed to airborne silage dusts while unloading their silos. Of those nine, three men became ill and complained of headache, malaise, and low-grade fever (described elsewhere) (*8*). Table 2 shows the demographic characteristics of these individuals. Control individuals were the wives of the farmers, and therefore there were sex differences in the study populations. Additionally, the mean age of the controls was higher than the test group.

Table 3 shows the mean pre-exposure concentrations of hemolytic C3 in the sera of the study groups. Mean concentrations in the sera at 6 hr after exposure are shown also. No differences in the mean serum concentrations of the control group were noted between pre- and post-exposure times. However, the mean group concentration decreased approximately 500 C3H50 U/mL for both the whole exposed group and for those who became ill. These changes were not statistically significant at the $p < 0.05$ level.

Hemolytic or functional C4 concentrations dropped in the exposed and ill groups (Table 4), and the decrease in the ill group was significant statistically. The mean hemolytic C4 concentration in the sera of the control group increased during the same time period.

Table 3. Changes in serum hemolytic C3 levels ($C3H_{50}$) after silo unloading.

	Serum C3 levels		
Group (*N*)	Pre, U/mL	Post, U/mL	Significance
Control (4)	8500 ± 569[a]	8500 ± 436[a]	NS[b]
Exposed (9)	6933 ± 614	6422 ± 500	NS
Ill (3)	7267 ± 939	6733 ± 809	NS

[a] Mean ± standard error.
[b] NS = not statistically significant pre- to post-exposure as calculated by the paired *t*-test.

Allergy Status

No remarkable IgE patterns were observed in any of the study groups. The mean total IgE concentrations in the sera of the controls was 9.2 IU/mL (range, 1.0–23.0 IU/mL) and in the sera of the ill group, 16.3 IU/mL (range, 10.8–23.0 IU/mL). The whole exposed population, the active farmers, showed a mean total IgE level of 18.0 (range, 0.7–35.0 IU/mL). One exposed but not ill individual had markedly elevated total IgE (> 100 IU/mL) and was not included in the calculation of the group mean.

Only one individual had specific IgE to any of the fungi which were tested. Her serum was positive for IgE to *Aspergillus fumigatus*, *Penicillium notatum*, and *Cladosporium spp.* (borderline), and she was a member of the control group. No sera from members of the exposed or ill groups were positive for specific IgE to the antigens which were studied.

Discussion

Silage joins the list of other farm-related products such as grains (*9–11*) , moldy hay (*12*), rice (*13*), sugar cane (*14*), and associated microorganisms (*15*) that can activate human serum complement. Aqueous extracts of sil-

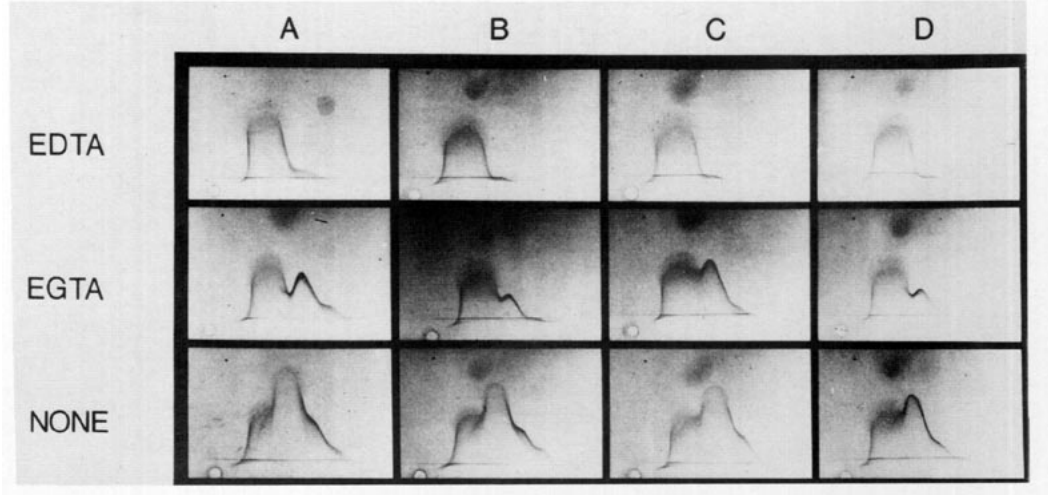

FIGURE 3. Two-dimensional immunoelectrophoresis of C3 from normal human serum which was chelated with EDTA or EGTA prior to treatment with 50 μL of aqueous silage extracts from four farms (A–D). In each frame the left arc is C3, and the right arc is C3b.

Table 4. Changes in serum hemolytic C4 levels ($C4H_{50}$) after silo unloading.

	Serum C_4 levels		
Group (*N*)	Pre, U/mL	Post, U/mL	Significance
Control (4)	49767 ± 12682[a]	53133 ± 12971[a]	$p<0.03$
Exposed (9)	33689 ± 5011	31244 ± 3937	NS[b]
Ill (3)	27167 ± 3815	24167 ± 3396	$p<0.04$

[a] Mean ± standard error.
[b] NS = not statistically significant pre- to post-exposure as calculated by the paired *t*-test.

age samples from four different farms in New York consume human hemolytic C3 in a dose-dependent fashion *in vitro*. Immunochemical studies show that native C3 is converted to active C3b, and that the alternative complement pathway is activated. Complement activation and the generation of active complement fragments can initiate or contribute to respiratory or systemic signs and symptoms of disease, either at the site of complement activation or as soluble mediators of inflammation (*17*). Because complement components are present in human airways (*18–20*), the potential exists for the direct interaction of inhaled silage dusts, represented in this study by aqueous extracts, and complement. Although the *in vitro* data suggest that the antibody-independent alternative complement pathway is activated by extracts of silage, the experimental design did not negate the potential role of classical pathway involvement as well.

Gram-negative bacterial endotoxins, which can activate both the classical and alternative complement pathways (*21*), were natural contaminants of the silage. Those samples which were the greatest activators of C3 (farms A and B) contained the highest concentrations of endotoxins. Likewise, the least complement reactive sample from farm D contained the least amount of endotoxins. The potential contribution of endotoxins in the observed consumption of human C3 must be considered, therefore. Additionally, other as yet unidentified complement activating components in the silage extracts are likely to be present as well (*22*). Beyond the activity on human complement, however, endotoxins may induce other inflammatory reactions as well by interacting directly with both humoral and cellular components in the lung, blood, and other tissues (*23,24*).

During the course of their regular job of unloading the farm silo, the farmers are exposed to airborne dusts which contain gram-negative bacterial endotoxins (unpublished data) as well as viable gram-negative bacteria, fungi, and thermophilic bacteria (W. G. Sorenson, personal communication). When one considers the relatively uncharacterized nature of airborne silage dusts, the full extent of the noxious and irritating respiratory insult is unknown. The individuals who were studied in this research were exposed to the unloading operations for approximately 45 min. Serum was collected both before and 6 hr after the work. Although the study and control groups are admittedly small, some interesting observations can be made from the data.

Changes in the hemolytic concentrations of serum C3 and C4 occurred in the test groups which did not occur in the control group. Serum C3 levels decreased as a result of exposure, but remained unchanged in the control group during the same time period. When considered in light of the *in vitro* data, one could suggest that C3 was activated *in vivo* by the alternative pathway. Control C4 levels increased during the 6 hr, but the C4 concentrations in the exposed and ill groups decreased. These data suggest that the classical complement pathway was activated as well. Additionally, the possibility exists that C4 is activated directly (*10,22*). Although statistical comparisons were made on the changes in C3 and C4 concentrations, it is obvious that the numbers which were studied are not of sufficient size to form the basis of a definitive statement concerning the *in vivo* changes in serum complement after exposure to silage dusts. In addition, the time-course of any change in complement levels should be followed by sequential analyses following exposure to silage dusts.

Direct immunologically mediated allergy appears to be of little consequence in the study groups. The exposed, and therefore the ill subgroup, was devoid of detectable specific IgE antibody to four major fungal organisms. Only one individual, a member of the control group, showed specific IgE to the allergens. It is conceivable, however, that the appropriate fungi were not tested although the organisms which were studied were found in silage from the general geographic area of the study groups. The three groups were also of similar total IgE levels. Only one member of the exposed (but not ill) group had a markedly elevated total IgE concentration. The serological studies, both of complement and of IgE, should be considered as preliminary because of the small group numbers.

In conclusion, the results of this study suggest that aqueous extracts of silage activate human complement *in vitro*. Preliminary evidence from serological analyses of complement levels in working farmers who are exposed to silage dusts suggests that *in vivo* changes in functional complement may occur also. Further studies of the *in vivo* changes are required before the actual contribution of this inflammatory mechanism of disease can be defined.

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Mention of company names or products does not constitute endorsement by the National Institute for Occupational Safety and Health.

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