In vitro activation of the alternative pathway of complement by settled grain dust*

Stephen A. Olenchock, Ph.D., Judith C. Mull, B.S., Pervis C. Major, Ph.D., Michael J. Peach, III, M.S., M.P.H., Mary E. Gladish, and Geoffrey Taylor, M.D. *Morgantown, W. Va.*

Settled grain dust was collected from several active grain elevators in the Superior-Duluth areas of the United States. Particle size distribution (47% <5 μm) and endotoxin contamination (429 ng/gm) of the dust were similar to those reported for the airborne parent dust. Human complement was activated in vitro in a dose-response manner which could be quantified. This hemolytic consumption was via the alternative pathway as defined by ethylenediaminetetraacetic acid/ethylene-glycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid (EDTA/EGTA) differential serum chelation, factor B conversion, and complement reductions in serum from guinea pigs deficient in C4. It is proposed that continuous low-dose exposure to aerosolized, biologically active rafter dust could contribute to the respiratory insult of grain workers.

Agricultural dusts are recognized as representing a serious safety hazard to grain elevators because of their index of explosibility and strong to severe explosion severity.[1] Once ignited, grain and settled grain dusts will burn, thereby providing fuel to subsequent fires.[2] Recent epidemiologic investigations of grain handlers have suggested that grain dust is a potential health hazard to workers as well.[3–5] Our laboratories have recently demonstrated a potential biological mechanism which could occur in response to the inhalation of grain dust.[6] Respirable airborne grain dusts activate the alternative complement pathway in vitro and, it then may be argued, have the potential to elicit respiratory pathophysiology by the inflammatory sequelae produced as a result of complement activation.[7] During the average working day, airborne dust from grain transport within the elevator exposes grain workers to multiple acute challenges of high dust levels. The airborne dust eventually settles on most exposed horizontal surfaces, and continuous mechanical agitation provides a source of long-term low-dose exposure to the respiratory systems of workers. It is the purpose of this paper to examine the effects of settled grain dust on the human complement cascade.

MATERIALS AND METHODS

Rafter dust samples

Settled dust was collected from rafters and ledges in active terminal grain elevators in the Superior-Duluth areas of the United States. In the memories of the workers queried, none of these areas had been cleaned, thereby indicating that no cleaning had taken place for at least 20 to 30 yr prior to dust collection for our experiments.

The collected material was pooled, stored in a vacuum dessicator, and used throughout the experiments. Measurement of particle size was made with the use of light microscopy and a Porton graticule (BGI, Inc., Waltham, Mass.).

Factor B activation by rafter dust

Normal human serum (NHS) was pooled from members of our laboratory and reacted with increasing amounts (0.1 to 20.0 mg) of settled rafter dust. This serum was negative by counterimmunoelectrophoresis for precipitins to aqueous extracts of settled rafter dust and 8 individual airborne grain dusts. Pooled serum was used fresh or it was frozen at −88 C until used. The 0.5-ml aliquots were incubated in a 37 C

From the Division of Respiratory Disease Studies, Appalachian Laboratory for Occupational Safety and Health, National Institute for Occupational Safety and Health.

Received for publication May 23, 1978.

Accepted for publication July 24, 1978.

Reprint requests to: Stephen A. Olenchock, Ph.D., Immunology Section, Department of Health, Education and Welfare, United States Public Health Service, CDC, ALOSH, DRDS, 944 Chestnut Ridge Road, Morgantown, WV 26505.

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

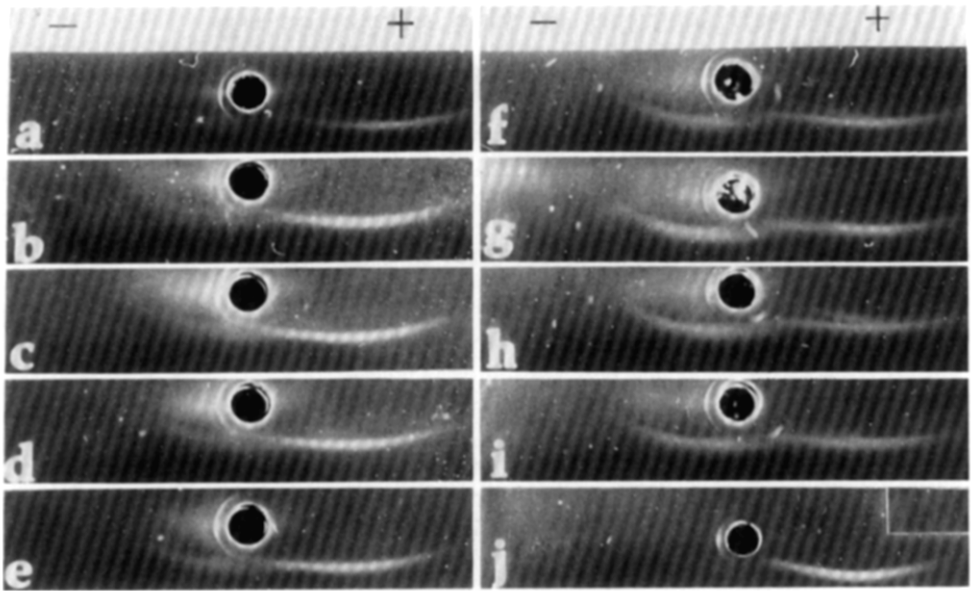

FIG. 1. Immunoelectrophoresis of factor B in pooled normal human serum incubated with increasing amounts of settled rafter dust. *a* and *j*, Control serum without dust. *b*, Serum with 0.1 mg dust. *c*, 0.25 mg. *d*, 0.5 mg. *e*, 1 mg. *f*, 5 mg. *g*, 10 mg. *h*, 15 mg. *i*, 20 mg. *Bb* arc is to the left of the well.

TABLE I. Particle size distribution of settled rafter dust

Particle size (μm)	Rafter dust (%)
<5	46.9
5-10	21.3
10-20	15.9
20-40	15.9
Total	100.0

water bath with constant shaking at 125 strokes/min for 1 hr. The samples were then centrifuged at 900 g for 7 min and the supernatant fluids removed.

Aliquots (8 μl) of each treated serum were subjected to electrophoresis at 4 C in 1% agarose with pH 8.4 buffer for 45 min at 200 v. Conversion of factor B of the alternative pathway of complement was demonstrated by developing the electrophoresis slides with rabbit antiserum to human C3 activator (β_2-glycoprotein II; Behring Diagnostics, Sommerville, N. J.) for 24 hr in the cold. After examination for the characteristic double arc of factor B conversion, photographs were taken. As a negative control, NHS without prior reaction with settled dust was treated in a similar manner.

Hemolytic complement consumption

The hemolytic activity of the complement in the identical supernatant fluids from treated and untreated NHS was quantified in terms of CH_{100} U/ml by radial diffusion and subsequent lysis of sensitized erythrocytes in gel (Quantiplate; Kallestad, Chaska, Minn.) and in terms of CH_{50} U/ml by the tube method of Mayer.[8]

The median dose level for relative dust anticomplementary activity ranking was computed by probit analysis.[9]

Factor B conversion in chelated NHS

Aliquots (0.5 ml) of NHS were treated with chelators by a modification[6] of the method of Fine and associates[10] before reaction with 5 mg of settled rafter dust. Disodium ethylenediaminetetraacetate (EDTA; Fisher Scientific Co., Fair Lawn, N. J.) or ethylene-glycol-bis-(β-amino-ethyl ether) N,N′-tetraacetic acid (EGTA; Sigma Chemical Co., St. Louis, Mo.) was added (25 μl/0.5 ml) at a concentration of 100 mM and the sera examined for factor B conversion. NHS without dust was treated with chelators as a negative control.

Complement consumption in C4-deficient guinea pig serum

Serum aliquots (0.5 ml) negative to precipitins to settled grain dust and pooled from 25 guinea pigs genetically deficient in the fourth component of complement (C4D) and pooled precipitin-negative serum from 25 inbred guinea pigs with an intact complement cascade (S13; NIH strain 13) were reacted with 1.0 mg of settled rafter dust by a previously defined protocol.[11] In brief, the procedure, which takes into consideration the consistently lower C2 levels in C4D than in normal guinea pigs,[12] involved adding a standard amount (100 CH_{50} Units) of purified guinea pig C2 to each serum after incubation and centrifugation. Standard hemolytic CH_{50} levels were then quantified in duplicate by the method of Mayer[8] at a serum dilution of 1:250 with

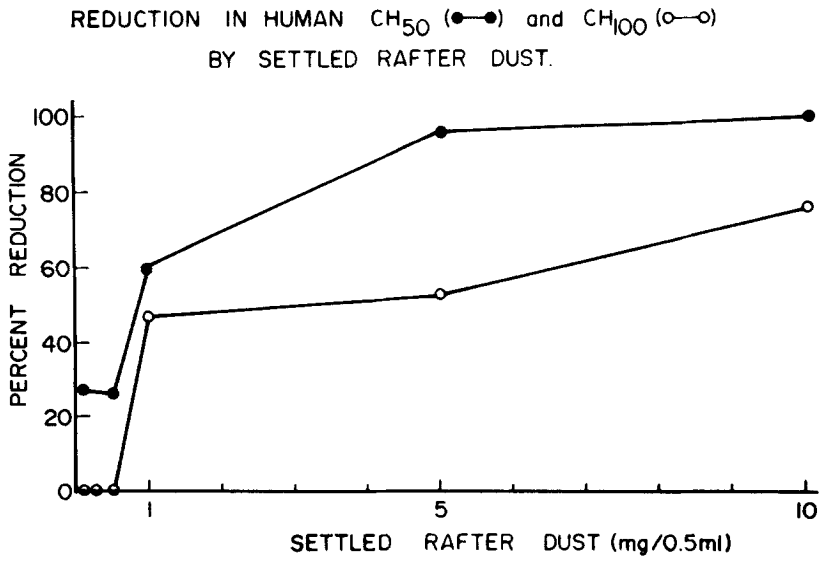

FIG. 2. Percent reduction in CH_{100} U/ml (○——○) and CH_{50} U/ml (●——●) from pooled normal human serum incubated with increasing amounts of settled rafter dust.

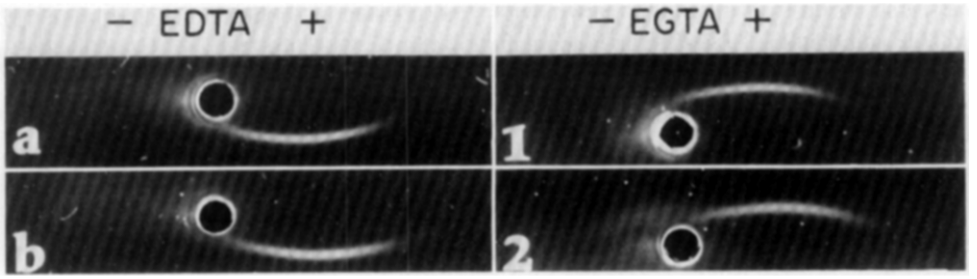

FIG. 3. Immunoelectrophoresis of factor B in pooled normal human serum (NHS) which was first chelated with either EDTA or EGTA. *a,* NHS + EDTA. *b,* NHS + EDTA + rafter dust. *1,* NHS + EGTA. *2,* NHS + EGTA + rafter dust. *Bb* arc is to the left of the well in No. 2.

the use of sensitized erythrocytes complexed with stable guinea pig complement components C1 and C4 (EAClgp4gp; Cordis Laboratories, Miami, Fla.). C4D and S13 sera without dust were similarly assayed in octuplicate as CH_{50} controls. C4D sera were also evaluated at a dilution of 1:250 and undiluted with the use of sensitized erythrocytes (EA) not complexed with complement components.

Limulus amebocyte lysate assay

Settled rafter dust (1 gm) was extracted with 10 ml of sterile nonpyrogenic water (Travenol Laboratories, Inc., Morton Grove, Ill.) by rocking for 5 min at room temperature, centrifuged at 900 g for 5 min, and filtered (0.45 μm). Sterile nonpyrogenic plastic ware was used throughout the assay. The extracted sample was assayed at a dilution of 1:30 with the use of a spectrophotometric modification of the *Limulus* amebocyte lysate gel test (Pyrostat; Worthington Biochemical Corp., Freehold, N. J.) which was capable of detecting 0.1 ng *Klebsiella* endotoxin equivalents per ml. Sample results were compared with a standard curve and analyzed by linear regression.

RESULTS

Settled rafter dust

Settled dust more than 8-cm deep could be found on the rafters, light fixtures, and most of the elevated exposed horizontal surfaces in 7 of the 8 grain elevators visited. The amounts of dust varied with the location and operations in the elevators. For example, dust was often more than 30-cm deep on floors and corners of beams. The particle size distribution of the settled rafter dust is shown in Table I. All the dust examined was less than 40 μm in size with approximately 47% in the range of 5 μm or less.

Factor B conversion and CH_{50} consumption

Factor B was readily converted to Bb in a dose-dependent fashion when incubated with specific precipitin-free NHS (Fig. 1). Greater amounts of dust caused increasingly intense Bb arcs with increased gamma mobility upon electrophoresis. Quantification

TABLE II. CH_{50} reduction in C4-deficient and normal guinea pig serum by settled rafter dust

Guinea pig strain	Control serum CH_{50} (U/ml)	Rafter dust serum* (U/ml)	CH_{50} reduction (%)	
C4D†	383.9‡	359.3	6.4	$p < 0.01$
S13§	458.7‖	391.0	14.8	$p < 0.01$

*1.0 mg/0.5 ml.
†NIH strain genetically deficient in C4. Guinea pig C2 added just prior to CH_{50} using EAC1gp4gp cells.
‡Mean of 8 controls (SD, ±10.9).
§NIH strain 13 guinea pigs. Guinea pig C2 added just prior to CH_{50} using EAC1gp4gp cells.
‖Mean of 8 controls (SD, ±19.1).

of the hemolytic activity of the same supernatant fluids used for electrophoresis verified the dose-response relationship. Fig. 2 shows the percent reduction of the total hemolytic activity (CH_{100} U/ml) as well as the percent reduction in CH_{50} U/ml for each concentration of rafter dust. In both hemolytic assays, increasing amounts of dust resulted in greater reductions in available complement. It should be noted that there is a significant difference ($p < 0.02$) between the hemolytic complement consumption by the CH_{100} and the CH_{50} methods.

To test the hypothesis that settled rafter dust differed from the various airborne grain dusts in its ability to activate the complement cascade in vitro, relative anticomplementary activity ranking was defined by probit analysis. The rafter dust concentration which consumed 50% of the available serum CH_{50} U/ml was 530 μg/0.5 ml. The mean concentration of 8 airborne grain dusts which consumed 50% of the available CH_{50} U/ml was 170 μg/0.5 ml with a range of 30 to 330 μg/0.5 ml.[11]

Activity in chelated human serum and C4D guinea pig serum

To examine the hypothesis that the observed factor B conversion and complement consumption was by the alternative pathway, settled rafter dust was reacted with NHS which was first chelated with either EDTA or EGTA (Fig. 3). EDTA completely blocked factor B conversion by 5 mg of settled dust, whereas the same amount of dust caused the conversion of factor B in serum chelated with EGTA, which strongly implies that complement consumption was by the alternative pathway. Neither EDTA nor EGTA converted factor B in the absence of dust.

Settled rafter dust consumed hemolytic complement in serum from guinea pigs genetically deficient in the fourth component of complement thereby providing further evidence that complement was activated by the alternative pathway. In order to provide toxicity comparisons of settled rafter dust with the previously reported 8 airborne grain dusts (range, 2% to 37%), percent CH_{50} reduction by 1.0 mg of settled rafter dust is presented in Table II. This reduction, although small, is significantly different from the control serum ($p < 0.01$). Settled rafter dust elicited a greater CH_{50} reduction in S13 guinea pig serum than in C4D serum.

Untreated sera from C4D and S13 guinea pigs were also tested in duplicate at a dilution of 1:250 for CH_{50} activity with the use of standard sensitized sheep erythrocytes (EA) without C4 or C2 added. No hemolysis could be detected with the C4D serum, but the S13 serum contained 435.5 CH_{50} U/ml. Only with undiluted C4D serum could even a nominal value of 1.6 CH_{50} U/ml be obtained, which indicates a nonfunctional hemolytic system in the C4D guinea pigs when the classical complement pathway was evaluated with EA. On the other hand, the classical complement pathway of the S13 guinea pigs was functional.

TABLE III. Comparison of endotoxin levels in settled rafter dust and airborne grain dust extracts

Dust	Endotoxin* (ng/gm dust)
Settled rafter dust	429.0
Range of 8 airborne dusts	331.2-439.5

*Assayed as nanograms FDA *Klebsiella* endotoxin equivalents per 10-ml extract of 1.0 gm dust.

Endotoxin level in rafter dust extract

Gram-negative endotoxin contamination of the settled rafter dust was assayed by the *Limulus* amebocyte lysate (LAL) technique. Table III illustrates the endotoxin levels of the rafter dust extract as well as the range of endotoxin levels in 8 airborne grain dusts reported previously.[11] A purified lipopolysaccharide extracted from a *Klebsiella sp.* is used by the Bureau of Biologics of the Food and Drug Administration (FDA) as an endotoxin standard.[13] Therefore, the results of the LAL assay are reported as FDA *Klebsiella* endotoxin equivalents.

DISCUSSION

Settled grain dust or "rafter dust" originates from the airborne dust generated by transporting grain in an elevator. Like the parent dust, the largest fraction of particle size distribution is in the 5 μm or less range which indicates that as the dust ages (approximately 20 to 30 yr) the particles are not coalescing into larger

masses. This dust is still very active in converting factor B to the active Bb. Our previous studies[6] have suggested that the complement activation was not a surface area phenomenon. Likewise, others have shown that glass wool and silica do not convert factor B and consume human complement in vitro.[14] The apparent dose-response relationship observed by increased intensity and gamma migration of the Bb arcs was quantified by both CH_{100} immunodiffusion and CH_{50} tube techniques. The CH_{50} tube assay was more sensitive than the CH_{100} assay in detecting complement consumption by the lower concentrations of dust. In fact, this difference in sensitivity was significant at the level of $p < 0.02$. Gewurz and Suyehira[15] have described the radial diffusion hemolytic technique as a screening procedure for use before the CH_{50} tube assay is used. However, for laboratory studies, the greater sensitivity of the tube assay is required to observe small changes in complement consumption.

Statistical analysis and relative anticomplementary activity ranking of the settled grain dust indicated that the rafter dust was less "toxic" to the hemolytic complement system than 8 airborne parent grain dusts. This decreased reactivity was approximately 18-fold less than the most active parent dust, rye, but separated by less than a 2-fold difference from the least active airborne dust, sunflower seed. When the reported age of the settled dust is considered, however, these results indicate a considerable retention of biologic activity.

In vitro experiments with the use of electrophoretic analysis of the conversion of human factor B by rafter dust differentiated the roles of the classical and alternative complement pathways by serum chelation with EDTA or EGTA.[10] EDTA completely blocked the conversion while EGTA permitted the calcium-independent activation of the alternative pathway as demonstrated by suboptimal factor B arc conversion.[16] The ability of settled rafter dust to activate the alternative complement pathway was verified by observed complement consumption in serum from guinea pigs genetically deficient in C4. These animals have been shown by others to maintain a functional alternative complement pathway in the absence of a functional classical pathway.[12, 17] Although we did not attempt to quantify C4 in the C4D serum, we did verify the absence of any functional classical pathway by use of standard sensitized erythrocytes (EA) in the CH_{50} tube assay with C4D serum. Only nominal hemolysis occurred with undiluted serum. Since greater CH_{50} consumption occurred in S13 guinea pig serum than in the C4D serum, it might be suggested that differences in strain reactivity to 1.0 mg of settled rafter dust were observed. Alternatively, it may reflect an enhancement of the alternative pathway lysis by the functional classical pathway in S13 serum. Perhaps the lipid A moiety of contaminating bacterial lipopolysaccharides (LPS) activated the classical complement pathway independent of antibody as described by Morrison and Kline.[18] Table III illustrates the endotoxin level in an extract of the rafter dust which verifies the contamination of the dust with LPS. The level observed in the settled dust is within the range of endotoxin contamination of 8 airborne grain dusts indicating that increased contamination does not occur with age. It should be noted, however, that positive results in the LAL assay may be due not only to gram-negative bacterial endotoxin but also many other materials[19–21] which might be expected to contaminate grain dust and thereby elevate the LAL values. Furthermore, since the endotoxin level of the settled dust is within the range of those of the 8 airborne grain dusts and the CH_{50} reactivity is considerably less than that of the parent dusts, it can be argued that endotoxin may play a minor role in the biological mechanism we report in these studies. We have previously shown[11] that airborne rye dusts from two different sources varied 4-fold in CH_{50} "toxicity" yet had similar endotoxin contamination, therefore extending the argument that the role of endotoxin in these observations may be minor. In any case, gram-negative bacterial endotoxin is capable of activating the alternative complement cascade,[18, 22–24] recruiting leukocytes in airways,[25] and acting as a mitogen.[20] Therefore, it has the undemonstrated potential to contribute to the respiratory insult of grain workers. Another recently reported potential contributor which is probably also a contaminant of settled rafter dust is gram-positive cell wall teichoic acid.[26] However, its specific in vivo contribution has not been determined.

Settled grain dust is continually aerosolized during the day-to-day operations of a grain elevator. The elevator itself has an inherent natural vibration resulting from moving belts, trippers, belt-driven pulleys, and personnel carriers such as manlifts and elevators. A major source of ventilation is obtained from open doors and windows which allow wind to disrupt the dust. Finally, cleaning operations such as sweeping and vacuuming generate aerosols of settled grain dust. All of these operations result in a constant low-dose exposure to dust which we have demonstrated in vitro to be active against the human alternative complement pathway. This long-term exposure may not in itself cause disease but could be one of many respiratory insults resulting in multifaceted biological events contributing to respiratory pathophysiology in grain handlers.

We thank Mr. Martin Petersen for his statistical analyses, Ms. Alice Greife for sizing the dust, Mr. Michael Moore and Mr. Robert Irwin for their photographic assistance, and Mrs. Debra Spurgeon for her help in preparing this manuscript.

REFERENCES

1. Jacobson, M., Nagy, J., Cooper, A. R., and Ball, F. J.: Explosibility of agricultural dusts, United States Department of the Interior, Bureau of Mines Washington, 1961.
2. Factory Mutual Engineering Corp.: Combustible dusts, *in* Handbook of industrial loss prevention, ed. 2, New York, 1967, McGraw-Hill Book Co., Inc., pp. 66-1–66-16.
3. DoPico, G. A., Reddan, W., Flaherty, D., Tsiatis, A., Peters, M. E., Rao, P., and Rankin, J.: Respiratory abnormalities among grain handlers, Am. Rev. Respir. Dis. **115:**915, 1977.
4. Tse, K. S., Warren, P., Janusz, M., McCarthy, D. S., and Cherniack, R. M.: Respiratory abnormalities in workers exposed to grain dust, Arch. Environ. Health **27:**74, 1973.
5. Warren, P., Cherniack, R. M., and Tse, K. S.: Hypersensitivity reactions to grain dust, J. ALLERGY CLIN. IMMUNOL. **53:**139, 1974.
6. Olenchock, S. A., Mull, J. C., Major, P. C., Gladish, M. E., Peach, M. J., III, Pearson, D. J., Elliott, J. A., and Mentnech, M. S.: Activation of the alternative pathway of complement by grain. I. C3PA conversion and quantification of complement consumption by rye, Clin. Allergy **8:**125, 1978.
7. Osler, A. G., and Sandberg, A. L.: Alternate complement pathways, Prog. Allergy **17:**51, 1973.
8. Mayer, M. M.: Complement and complement fixation, *in* Kabat, E. A., and Mayer, M. D., editors: Experimental immunochemistry, ed. 2, Springfield, Ill., 1961, Charles C Thomas, pp. 133-240.
9. Finney, D. J.: Probit analysis, ed. 3, Cambridge, 1971, Cambridge University Press, pp. 1-37.
10. Fine, D. P., Marney, S. R., Jr., Colley, D. G., Sergent, J. S., and Des Prez, R. M.: C3 shunt activation in human serum chelated with EGTA, J. Immunol. **109:**807, 1972.
11. Olenchock, S. A., Mull, J. C., Gladish, M. E., Peach, M. J., III, and Major, P. C.: Airborne grain dust activation of complement, Proceedings of the International Symposium on the Effects of Grain Dust on Human Health, New York, Academic Press, Inc. (In press.)
12. Frank, M. M., May, J., Gaither, T., and Ellman, L.: In vitro studies of complement function in sera of C4-deficient guinea pigs, J. Exp. Med. **134:**176, 1971.
13. Selzer, G. B.: Preparation of a purified lipopolysaccharide for pyrogen testing, Bull. Parenter. Drug Assoc. **24:**153, 1970.
14. Wilson, M. R., Gaumer, H. R., and Salvaggio, J. E.: Activation of the alternative complement pathway and generation of chemotactic factors by asbestos, J. ALLERGY CLIN. IMMUNOL. **60:**218, 1977.
15. Gewurz, H., and Suyehira, L. A.: Complement, *in* Rose, N. R., and Friedman, H., editors: Manual of clinical immunology, Washington, D. C., 1976, American Society for Microbiology, pp. 36-47.
16. Des Prez, R. M., Bryan, C. S., Hawiger, J., and Colley, D. G.: Function of the classical and alternate pathways of human complement in serum treated with ethylene glycol tetracetic acid and $MgCl_2$-ethylene glycol tetraacetic acid. Infect. Immun. **11:**1235, 1975.
17. Ellman, L., Green, I., Judge, F., and Frank, M. M.: In vivo studies of C4-deficient guinea pigs, J. Exp. Med. **134:**162, 1971.
18. Morrison, D. C., and Kline, L. F.: Activation of the classical and properdin pathways of complement by bacterial lipopolysaccharides (LPS), J. Immunol. **118:**362, 1977.
19. Brunson, K. W., and Watson, D. W.: Limulus amebocyte lysate reaction with streptococcal pyrogenic exotoxin, Infect. Immun. **14:**1256, 1976.
20. Elin, R. J., Sandberg, A. L., and Rosenstreich, D. L.: Comparison of the pyrogenicity, limulus activity, mitogenicity, and complement reactivity of several bacterial endotoxins and related compounds, J. Immunol. **117:**1238, 1976.
21. Kotani, S., Watanabe, Y., Kinoshita, F., Kato, K., Harada, K., Shiba, T., Kusumoto, S., Tarumi, Y., Ikenaka, K., Okada, S., Kawata, S., and Yokogawa, K.: Gelation of the amoebocyte lysate of *Tachypleus tridentatus* by cell wall digest of several gram-positive bacteria and synthetic peptidoglycan subunits of natural and unnatural configurations, Biken J. **20:**5, 1977.
22. Dierich, M. P., Bitter-Suermann, D., Konig, W., Hadding, U., Galanos, C., and Rietschel, E. T.: Analysis of bypass activation of C3 by endotoxic LPS and loss of this potency, Immunology **24:**721, 1973.
23. Gewurz, H., Shin, H. S., and Mergenhagen, S. E.: Interactions of the complement system with endotoxic lipopolysaccharide: Consumption of each of the six terminal complement components, J. Exp. Med. **128:**1049, 1968.
24. Marcus, R. L., Shin, H. S., and Mayer, M. M.: An alternate complement pathway: C-3 clearing activity, not due to $\overline{C4}$,$\overline{2A}$, on endotoxic lipopolysaccharide after treatment with guinea pig serum; relation to properdin, Proc. Natl. Acad. Sci. **68:**1351, 1971.
25. Hudson, A. R., Kilburn, K. H., Halprin, G. M., and McKenzie, W. N.: Granulocyte recruitment to airways exposed to endotoxin aerosols, Am. Rev. Respir. Dis. **115:**89, 1977.
26. Winkelstein, J. A., and Tomasz, A.: Activation of the alternative complement pathway by pneumococcal cell wall teichoic acid, J. Immunol. **120:**174, 1978.