

Activation of the alternative complement pathway by extracts of cotton dust

M. R. WILSON,* A. SEKUL,† R. ORY,† J. E. SALVAGGIO*
and S. B. LEHRER*

*Clinical Immunology Section of the Department of Medicine, Tulane Medical Center,
and the †Southern Regional Research Center, USDA, New Orleans, Louisiana, U.S.A.

(Received 21 August 1979; accepted for publication 26 September 1979)

Summary

Extracts of cotton dust were tested for their ability to activate the alternative complement pathway in fresh normal human serum (NHS). Alternative pathway activation was determined by a haemolytic assay utilizing glutathione-sensitized human erythrocytes, consumption of alternative pathway components in terms of alternative pathway CH50 units and an immunoelectrophoretic assay to detect split products of activation of factor B. All assays were performed under conditions that have been shown to block the initial steps of classical pathway activation but permit activation of the alternative complement pathway. Results demonstrate that the cotton dust extracts could consume alternative complement pathway proteins in a dose-response manner. The complement activating factor is probably endotoxin since a cotton dust extract obtained by an extraction method for endotoxin yielded the greatest activity.

Introduction

Workers exposed to cotton dust are affected by a symptom complex known as byssinosis. The initial symptoms are cough, chest tightness, and a feeling of breathlessness particularly on Mondays after a week-end away from the mill (Mareska & Heyman, 1845). Some workers may not experience symptoms after a lifetime of exposure to dust, but others may have symptoms after brief exposure. Coincident with symptoms, it has been demonstrated that a decrease in expiratory volumes (FEV₁) occurs over the course of a shift on Monday (McKerrow *et al.*, 1958). Thus, any hypotheses for pathogenic mechanisms must take into consideration the variability observed in symptoms during a workweek.

Several mechanisms have been suggested as an explanation of the symptoms of byssinosis. It has been postulated that the release of pharmacological agents such as

Correspondence: Dr S. B. Lehrer, Clinical Immunology Section of the Department of Medicine, Tulane Medical Center, 1700 Perdido Street, New Orleans, Louisiana 70112, U.S.A.

histamine or other mediators producing bronchoconstriction may be important (Bouhuys & Lindell, 1961), but the mechanism of release is unclear. Endotoxin which is present in cotton dust may be important in the elaboration of mediators (Cavagna, Foa & Vigliani, 1969) or in potentiating their actions on smooth muscle (Weiner & Zweifach, 1966). Endotoxin can effect release of histamine via the complement system and the complement activation can cause human basophils to release histamine via the anaphylatoxins, C3a and C5a (Grant *et al.*, 1976). Since endotoxin is known to directly activate the alternative complement pathway we studied the effects of cotton dust on the complement system. In this report, we demonstrate, with *in vitro* assays, that cotton dust and extracts of cotton dust can effectively activate the alternative complement pathway.

Materials and methods

Cotton dust samples and extractions

Cotton cardroom dust was collected from a commercial textile mill. This material was passed through a sieve to obtain respirable dust particles (RD) of about 20 μm . Two hundred grams of cardroom dust was extracted with 500 ml of deionized water for 15 min at 25°C $\times$ 3. The process was repeated twice with 250 ml of water. The extracts were pooled, centrifuged at 9,000 *g* and the supernate lyophilized. This was labelled soluble dust (SD) fraction. SD was extracted three times with 85% methanol (1:5 w/v) for 30 min at 25°C. Soluble material of the methanol extraction (SD-ME) was evaporated to dryness. Methanol-insoluble material was dissolved in ammonium sulphate (80% saturation) and soluble material (SD-1) was dialysed against deionized water and then lyophilized. Precipitated material (SD-2) was dissolved in water, dialysed against PBS, and lyophilized.

A trichloroacetic acid (TCA) extraction (commonly used to extract bacterial endotoxins) was performed on the cardroom dust according to the methods described by Nowotny (1969). In this method cotton dust was homogenized in 5% cold TCA, centrifuged and the supernate dialysed exhaustively against ion depleted water. Dialysed extracts were filtered (Whatman No. 1) and pH adjusted to 7.4 with NaOH, and concentrated in vacuum. Two volumes of cold methanol containing 0.2% MgCl_2 was added to the concentrate. The resulting precipitate was isolated by centrifugation (5,000 *g*), resuspended in, and dialysed exhaustively against, ion depleted water. In testing for alternative complement pathway activation, all lyophilized samples were resuspended in sodium phosphate (0.01 M), buffered (pH 7.4) sodium chloride (0.15 M) (PBS) at concentrations varying from 0.0001 mg/ml to 10 mg/ml.

Activation of the alternative complement pathway

Activation of the alternative pathway was tested by three different methods. The first was a hemolytic assay to determine the ability of RD, SD, SD-ME, SD-1, SD-2 or endotoxin extracts to produce complement-dependent lysis of glutathione-sensitized human erythrocytes (GSHE) under conditions in which the early steps of the classical pathway were blocked by the addition of ethylene glycol tetra-acetic acid (EGTA). GSHE cells are sensitive to lysis by the C5-C9 complexes in the presence of magnesium (Götze & Müller-Eberhard, 1972). Normal human serum (NHS) was obtained fresh from healthy donors. Sera were negative for precipitins to an aqueous extract of the

respirable dust. NHS treated with EGTA was the source of C5-C9 complexes generated during cotton dust activation of the alternative pathway.

Samples (0.01 to 10 mg) were incubated with 100 μ l of undiluted fresh NHS with EGTA (10 mM) and $MgCl_2$ (0.5 mM). These conditions have been shown to block classical pathway activation but permit activation of the alternative pathway (May, Rose & Frank, 1973). After mixing, GSHE (5×10^7 cells) were added and the mixture incubated in a shaker water bath at 37° for 60 min. Two ml of cold PBS were added, mixed and centrifuged. The amount of free haemoglobin in the supernate was determined spectrophotometrically at 412 nm. Maximum lysis by 100 μ g of inulin was considered as 100% activation for comparative purposes. Greater than 5% lysis of the GSHE cells was considered significant activation of the alternative pathway.

The second method for detecting alternative pathway activation was a haemolytic assay to determine the consumption of alternative complement pathway proteins in terms of alternative pathway CH50 units (APCH50). This assay was performed using a technique described by Platts-Mills & Ishizaka (1974) as modified by Fine & Harper (1977). NHS with EGTA (10 mM) and $MgCl_2$ (0.5 mM) were incubated with samples of RD, SD, SD-ME, SD-1 or SD-2 in a dose range of 0.01 mg to 10 mg, or with TCA extract of cotton dust in a dose range 0.001 to 1 mg/ml for 30 min at 37°C. NHS (diluted 1:10 in veronal buffered saline, 1% gelatin, 0.002 M $MgCl_2$, and 0.008 M EGTA [VBS-G-MgEGTA]) in volumes ranging from 0.4 to 1.2 ml was added to various amounts of VBS-G-MgEGTA to bring the final volume to 1.8 ml. Rabbit erythrocytes (0.2 ml) suspended in VBS-G-MgEGTA (1.0×10^9 cells) were added and the mixture incubated at 37°C for 60 min. Fresh guinea pig serum (2.5 ml) diluted 1:50 in EDTA (10 mM) buffer was added to supply C3-C9 and incubation continued for 30 min. After centrifugation, optical density of the supernatant fluid (541 nm) was determined and percent lysis (y) calculated. The volume of serum was plotted against $y/1-y$ on a log scale to determine 50% end point and values expressed in APCH50 units.

The third method for demonstrating alternative pathway activation was factor B conversion into split products. In this assay, 10 μ l of respirable cotton dust or extracts of cotton dust (10 mg/ml in PBS) and 20 μ l of fresh NHS were incubated in the presence of EGTA (10 mM) and $MgCl_2$ (0.5 mM). The mixture was electrophoresed on a microscopic slide coated with 1.5% agarose using veronal buffer (pH 8.6) (ionic strength, 0.05) and EDTA (0.02 M). The slide was developed with monospecific antisera to factor B (a gift from Doctor Carlos Arroyave, Scripps Clinic and Research Foundation, La Jolla, California). Conversion of factor B indicating alternative complement pathway activation was detected by the appearance of split products with gamma and alpha mobility (Arroyave, Wilson & Tan, 1976). Fresh NHS treated with EGTA and $MgCl_2$ incubated for the same length of time was used as the negative control. NHS treated with EGTA and $MgCl_2$ and activated with inulin was used as a positive control.

Results

Six samples of cotton dust extract were tested in the two assays. The results of the GSHE lysis assay and factor B conversion assay are shown in Table 1. All samples produced activation of the alternative complement pathway as demonstrated by significant lysis of GSHE cells when compared to a PBS control which produced no lysis of GSHE cells. The range of GSHE lysis produced by the various samples other

Table 1. Activation of the alternative complement pathway by cotton dust extracts*

Sample	GSHE lysis (%)†	Factor B Conversion‡
RD	33 ± 1.3§	+
SD	18 ± 1.0	+
SD-ME	22 ± 1.2	+
SD-1	19 ± 1.0	+
SD-2	23 ± 1.3	+
TCA Extract	88 ± 2.0	+
PBS Control	0	-

* Cotton dust samples were tested at a final concentration of 250 µg/ml, except for endotoxin which was tested at a final concentration of 25 µg/ml.

† Greater than 5% lysis was considered positive for alternative pathway activation. The percentage lysis was calculated by using an inulin-activated pathway GSHE cell lysis as the 100% standard.

‡ (+) indicates immunoelectrophoretic conversion of factor B into split products of activation with alpha and gamma mobility.

§ The value represent the mean and standard deviation of four experiments performed simultaneously.

than TCA extract was 18 to 33%. The TCA extract of cotton dust lysed 88% of GSHE cells in the assay system. It is important to point out that the concentrations of TCA extract assayed were 10 times less than that of the other cotton dust samples. Therefore, as expected, this extract of cotton dust was a potent activator of the alternative complement pathway.

Also shown in Table 1 are the results of the immunoelectrophoretic assay to detect conversion of factor B by various cotton dust samples. All samples produced conversion of factor B into split products with alpha and gamma mobility when compared to a PBS control which produced no split products.

In order to demonstrate a dose-response effect of the cotton dust samples on the alternative complement pathway, various doses of each sample were reacted with fresh serum under conditions in which the classical complement pathway was blocked, but the alternative pathway was functional. The percentage consumption of alternative pathway proteins was then determined in these samples. Results are displayed in Fig. 1. As illustrated, all cotton dust samples had approximately the same dose-response effect on the alternative complement pathway except for the TCA extract of cotton dust which was approximately 10 times more effective in activating the alternative complement pathway than the soluble cotton dust extracts or respirable dust.

Discussion

The results of this study demonstrate that soluble extracts of cardroom cotton dust effectively activate the alternative complement pathway. This was detected by three

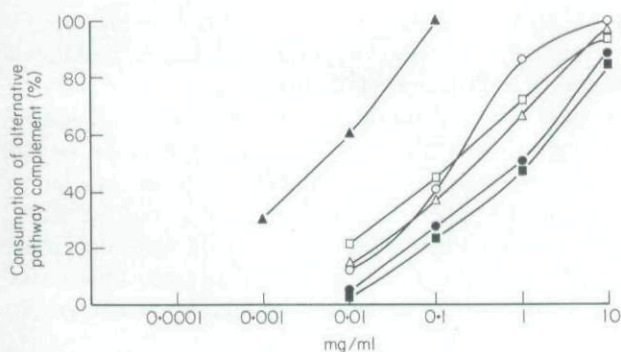


Fig. 1. The dose response curves of SD (closed circles), SD-1 (○), SD-2 (■), SD-ME (◻), R.D. (△) and endotoxin (▲). Endotoxin extracted from cotton dust shows ten-fold greater activity in consumption of alternative complement pathway proteins.

assays which measure alternative complement pathway activation: complement-dependent lysis of GSHE cells, immunoelectrophoretic conversion of factor B, and consumption of alternative pathway protein. It has been shown by other investigators that activation of the complement proteins, particularly C3 and C5, produce split products C3a and C5 respectively which are anaphylatoxins capable of releasing histamine from human basophils (Grant *et al.*, 1976). Furthermore, it has been demonstrated that alternative complement pathway proteins exist in a native state in human lung as shown by studies on broncho-alveolar wash fluid (Robertson *et al.*, 1976). Bouhuys, Lindell & Lundin (1960) and Bouhuys & Lindell (1961) have suggested that soluble histamine-releasing factors present in cotton dust may release histamine and other mediators from lung tissue and produce reversible narrowing of small airways. Our data suggest that in addition to the possible direct effect of histamine-releasing factors on lung tissue, there may be soluble factors in cotton dust which, after prolonged exposure, activate the complement system and subsequently produce release of histamine via anaphylatoxins, ultimately producing inflammation and tissue destruction.

Our data also indicate that endotoxin (presumably present in the TCA cotton dust) was a potent activator of the alternative pathway. At an equivalent weight to the soluble cotton dust extracts, it had approximately 10 times more activity in our assay system. Because of a febrile response in workers exposed to contaminated cotton dust, several investigators have suggested an association between 'mill fever' and inhalation of dust which contains endotoxin. However, Cavagna and associates (1969) using human subjects and an animal model of byssinosis demonstrated that aerosolized endotoxin could increase pulmonary resistance which led them to suggest that endotoxin may be important in producing some of the symptoms of byssinosis. It is likely that endotoxin has several biological effects in addition to producing the febrile response seen in 'mill fever'. In fact, these investigators demonstrated in certain subjects that when the FEV₁ decreased, there was change in body temperature or white blood cell counts. As our data suggest, endotoxin may be at least one important component of cotton dust which produces increased airway resistance via the complement system through elaboration of anaphylatoxins.

The hypothesis that endotoxin activating the complement system is responsible for release of histamine in the lungs of workers with byssinosis, must also explain the

unusual variation in symptoms which begin at the start of a working week, usually Monday, after 2 days away from the mill. It has been demonstrated that the half life of factor B of the alternative pathway is 75 h (Wilson *et al.*, 1976). Thus it is possible that a worker away from exposure to dust would synthesize, and replenish his levels of complement protein. And, arriving at the mill after a 2-day absence, he would experience his most severe symptoms. As these proteins are activated and depleted in pulmonary tissue, symptoms would be less pronounced toward the end of the week. Thus, this hypothesis may explain the phenomenon of diminution of symptoms after prolonged exposure. Further experiments in this area should measure alternative pathway proteins during work exposure in order to determine whether the alternative pathway is activated *in vivo*.

Acknowledgments

We wish to thank Ms Helen Bezbek for her excellent technical assistance and Mrs Marlene Szczepanski for help in preparing this manuscript. This work was supported by the USDA Research Agreement (12-14-7001-898) and the NIAID (AI-13401).

References

- ARROYAVE C.M., WILSON, M.R. & TAN, E.M. (1976) Serum factors activating the alternative complement pathway in autoimmune disease. Description of two different factors from patients with systemic *Lupus erythematosus*. *Journal of Immunology*, **116**, 821.
- BOUHUYS, A., LINDELL, S.E. & LUNDIN, G. (1960) Experimental studies on byssinosis. *British Medical Journal*, **1**, 324.
- BOUHUYS, A. & LINDELL, S.E. (1961) Release of histamine by cotton dust extracts from human lung tissue *in vitro*. *Experientia*, **17**, 211.
- CAVAGNA, G., FOA, V. & VIGLIANI, E.C. (1969) Effects in man and rabbit of inhalation of cotton dust or extracts and purified endotoxins. *British Journal of Industrial Medicine*, **26**, 314.
- FINE, D.P. & HARPER, B.L. (1977) Clinical adaption of a hemolytic alternative complement pathway assay. *Clinical Research*, **25**, 357A.
- GÖTZE, O. & MÜLLER-EBERHARD, H.J. (1972) Paroxysmal nocturnal hemoglobinuria. Hemolysis initiated by the C3 activator system. *New England Journal of Medicine*, **286**, 180.
- GRANT, J.A., SETTLE, L., WHORTON, E.B. & DUPREE, E. (1976) Complement-mediated release of histamine from human basophils. *Journal of Immunology*, **117**, 450.
- MARESKA, J. & HEYMAN, J. (1845) Enquête sur le travail et la condition physique et morale des ouvriers employés dans les manufactures de coton à Gand. *Annales de la Société Médicale de Gand*, **16** page 245.
- MAY, J.E., ROSE, W.F. & FRANK, M.M. (1973) Paroxysmal nocturnal hemoglobinuria. Alternate-complement-pathway mediated lysis induced by magnesium. *New England Journal of Medicine*, **289**, 705.
- McKERROW, C.B., McDERMOTT, M., GILSON, J.C. & SCHILLING, R.S.F. (1958) Respiratory function during the day in cotton workers: a study in byssinosis. *British Journal of Industrial Medicine*, **15**, 75.
- NOWOTNY, A. (1969) Extraction of Bacterial O-Antigens (Endotoxins). In: *Basic Exercises in Immunology*. Springer-Verlag, New York, p. 25.
- PLATTS-MILLS, T.A.E. & ISHIZAKA, K. (1974) Activation of the alternative pathway of human complement by rabbit cells. *Journal of Immunology*, **113**, 348.
- ROBERTSON, J., CALDWELL, J.R., CASTLE, J.R. & WALDMAN, R.H. (1976) Evidence for the presence of components of the alternative (properdin) pathway of complement activation in respiratory secretions. *Journal of Immunology*, **117**, 900.
- WEINER, R. & ZWEIFACH, B.W. (1966) Influence of *E. coli* endotoxin on serotonin contractions of the rabbit aortic strip. *Proceedings of the Society for Experimental Biology and Medicine*, **123**, 937-939.
- WILSON, M.R., ARROYAVE, C.M., NAKAMURA, R.M., VAUGHAN, J.H. & TAN, E.M. (1976) Activation of the alternative complement pathway in systemic lupus erythematosus. *Clinical and Experimental Immunology*, **26**, 11.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.