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Precipitation of Serum Proteins by Extracts of Cotton Dust and Stems

Identification of β-Lipoprotein and Production of Specific Antibodies[1]

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Sera from 59 cotton textile workers were examined by gel diffusion and counterimmunoelectrophoresis for the presence of precipitating antibodies to extracts of cotton bract, carpels, stems, leaves, immature cotton lint, and cardroom cotton dust. Precipitating antibodies to these extracts were not detected in any of the cotton workers' sera. However, the aqueous extracts of cardroom cotton dust and cotton stems were found to contain polyphenolic tannins that interacted with and precipitated beta-lipoprotein and gamma-globulins in a pseudoimmune fashion. Normal human and animal sera, as well as sera from active cotton textile workers, all exhibited identical patterns of reaction with these two extracts. The *in vivo* role this pseudoimmune interaction may play in the pathogenesis of byssinosis is unknown at this time.

INTRODUCTION

Serological studies of potential immune mechanisms in byssinosis have been encumbered by cotton plant constituents which precipitate serum proteins in a nonimmunological manner. Taylor *et al.* (1971) were the first to encounter this problem during the preparation of their "cotton antigen" from cotton bract material. They isolated this "antigen" to serologically titrate sera from byssinotic and nonbyssinotic cardroom workers and normal controls. Although they found significant differences in mean titers between cardroom workers and controls, and between byssinotic and nonbyssinotic cardroom workers, it is interesting that all of their control sera showed some reaction with this "antigen." Edwards and Jones (1973) determined that this same interaction was nonimmunological and applied the term "pseudoimmune" to it.

The aim of this present paper is to report that cardroom cotton dust and cotton stem material contain a similar pseudoimmune precipitating agent, and that elaborate chemical purification schemes are not necessary to demonstrate it. It is further our purpose to identify the serum proteins which react with this agent.

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

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MATERIALS AND METHODS

Specimen collection. Bract, carpels, stems, leaves, and immature cotton lint were collected manually from frost-killed field cotton near Lubbock, Texas. Cardroom cotton dust was collected at Khartoum North, Sudan.

Preshift Monday serum samples were collected from 59 cotton textile workers and stored at −20°C. Sera were also collected from 35 nonexposed normal volunteers and stored similarly.

Extract preparation. The individual plant parts and cardroom cotton dust were ground to 60 mesh. These particles were then extracted separately at 4°C in sterile nonpyrogenic water (20 g/liter). The supernatant fluids were filter sterilized and concentrated 40-fold by pervaporation (4°C), and stored at −20°C.

Animal immunizations. Antisera to cotton bract and leaves were raised in rabbits which received multiple intramuscular injections of the respective extracts over a 4-week period. Preimmunization sera served as negative controls.

Serologic analyses. Human and rabbit sera were examined for evidence of immune and pseudoimmune precipitation by the various methods of gel diffusion (Burrell, 1974), counterimmunoelectrophoresis (Gordon *et al.*, 1971), and immunoelectrophoresis (Burrell, 1974).

Precipitation of sera with extracts. Serum from normal human subjects, cotton textile workers, and a commercial pooled serum preparation which lacked beta-lipoprotein were each separately mixed in 1-ml quantities with 0.5 ml aliquots of either the cardroom cotton dust or stem extract. These mixtures were incubated for 30 min at 37°C and finally centrifuged for 20 min at 10,000*g* to remove the resultant precipitates. The supernatant fluids were removed and stored at −20°C.

Staining procedures for agarose slides. Permanent records of precipitin arc patterns were made by staining the agarose slides with 0.15% amido black 10 B (Biorad Laboratories, Richmond, Calif.) according to standard techniques (Hirschfeld, 1968). The lipoprotein pseudoimmune precipitates were detected using a Sudan black B staining technique (Polysciences, Inc., Warrington, Pa.) (Uriel, 1964).

Removal of plant polyphenolic compounds. Plant polyphenolic tannins were removed from the aqueous extracts prior to the concentration step by adsorption with insoluble polyvinylpolypyrrolidone (PVP; Calbiochem, San Diego, Calif.) (Selvendran and Isherwood, 1967; Loomis and Battalle, 1966).

RESULTS

Examination of Human Sera for True Precipitating Antibodies

All control and worker serum samples were negative for precipitating antibodies against extracts of plant parts and cardroom cotton dust as determined by gel diffusion and counterimmunoelectrophoresis.

Demonstration of Pseudoimmune Precipitins in Human and Rabbit Sera

During the course of the examination of human and rabbit sera for specific precipitating antibodies, it was noted that extracts of stems and cardroom cotton dust precipitated certain serum proteins in a nonimmunological reaction. We adopt the nonimmunological terminology of Edwards and Jones (1973) who

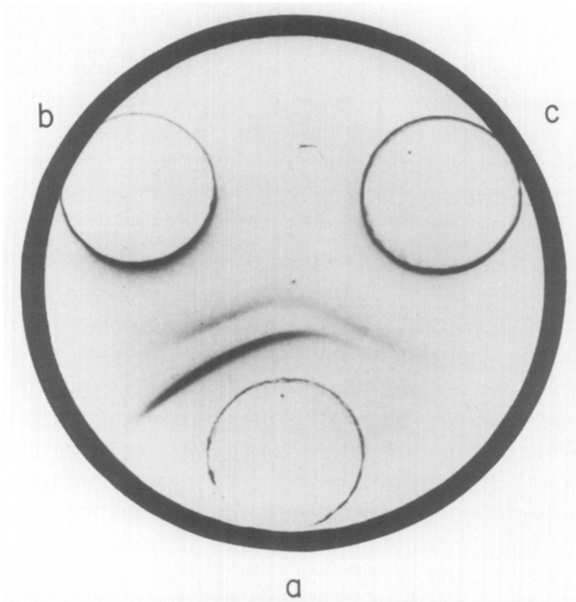

FIG. 1. Gel diffusion of cotton textile worker serum (a) with cotton stem extract (b) and cotton cardroom dust extract (c). Precipitation shows lines of identity between (b) and (c).

showed that the precipitation of serum proteins by cotton dust extract was not a classical antigen–antibody reactivity since the precipitation occurred with five separate myeloma IgG samples, isolated heavy and light chains, and Fab and Fc fragments of IgG. In our study every control serum, worker serum, and rabbit serum which we tested showed the same precipitation pattern which is illustrated in Fig. 1. One large, distinct band close to the serum well and one small, diffuse band close to the extract well were present. Further, extracts from geographically distinct plant strains reacted similarly with all sera. Extracts prepared from cotton stems which were grown in the United States or the Sudan caused the precipitation of the same serum proteins from rabbit sera, from normal human sera, and from the sera of active cotton textile workers. Finally, a concentrated extract of tea leaves precipitated the same serum proteins (data not shown) in all sera tested. Of interest, the control sera were obtained from a group of individuals who by religious preference abstained from coffee and tea.

Identification of Precipitation Bands as β-Lipoprotein and a Gamma Region Protein

Admixture of human sera with extracts of either cardroom cotton dust or stems resulted in the disappearance of the large, distinct band when the sera were examined by gel diffusion (Fig. 2). A commercial reference serum which lacked beta-lipoprotein did not demonstrate the large distinct band after gel diffusion either neat or after mixing with the extracts (Fig. 2).

Immunoelectrophoresis of the control and worker sera resulted in two bands of precipitation when developed with cotton dust or stem extracts. Prior mixing of

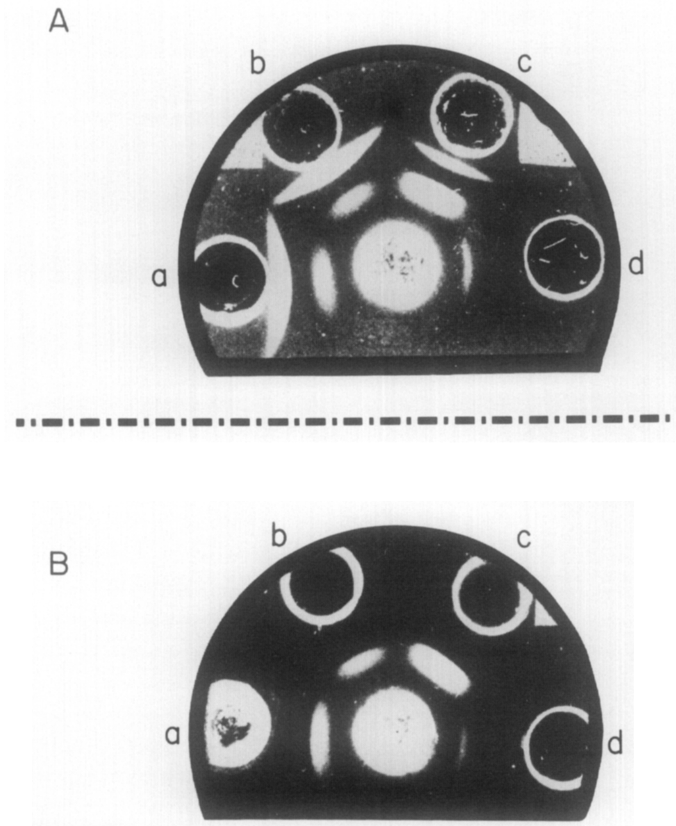

FIG. 2. Gel diffusion of cotton stem extract (center well) with normal human serum (a), cotton textile workers' serum (b, c), and a commercial reference serum which lacked beta-lipoprotein (d). (A) Precipitation pattern before admixture of sera with extract; (B) after admixture.

the sera and precipitation with either extract removed the beta-migrating band while the gamma-migrating band was unaffected (Fig. 3).

The precipitation bands which were observed in Fig. 1 were uniformly positive when stained with the general protein stain, Amido black. Likewise, the large distinct band of precipitation which was closest to the serum wells in the gel diffusion plates stained positively for lipoprotein. In a separate experiment, the beta-region band but not the gamma-region band observed after immunoelectrophoresis (Fig. 3) stained positively for lipoprotein when Sudan black B was used.

Gel diffusion of untreated human serum with antiserum to human beta-lipoprotein and with stem extract resulted in a line of identity which demonstrated that the large distinct band of precipitation contained human beta-lipoprotein (Fig. 4).

The immunoelectrophoretic band which was observed in the gamma-region was not identifiable when reacted in gel with specific antibody against human IgG, IgA, or IgM (data not shown).

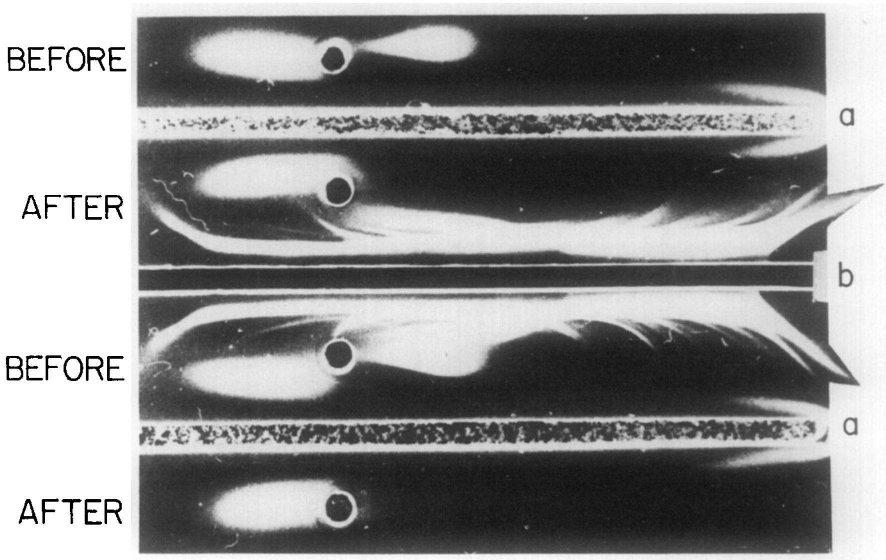

FIG. 3. Immunoelectrophoresis of cotton textile worker serum BEFORE and AFTER admixture with cotton stem extract. Slide developed with cotton stem extract (a) and anti-human serum (b). Anode is to the right.

Rabbit Immune Sera

The rabbit immunization experiments showed that cotton bract and cotton leaf material contained antigens which were similar as shown by a line of identity (Fig. 5). Anti-bract serum not only yielded a true reaction with cotton bract or leaf material but also reacted in a pseudoimmune fashion with the cotton stem extract. Neither reaction diminished the intensity of the other.

Identification of the Plant Agent Responsible for the Pseudoimmune Precipitation Reaction

The plant agent responsible for precipitation of the beta-lipoprotein and gamma-globulin was suspected of being a cotton plant polyphenolic tannin since a similar reaction was seen with a concentrated extract of tea leaves. Identical extracts of cotton stems and cardroom cotton dust were prepared, but were first mixed with polyvinylpolypyrrolidone (PVP) to remove plant tannins before the extract was concentrated (Selvendran and Isherwood, 1967; Loomis and Battalle, 1966). Comparison of the PVP-treated and non-PVP-treated extracts by gel diffusion with normal sera or sera from active cotton textile workers revealed that the PVP-treated extracts did not precipitate the two serum proteins in a pseudoimmune reaction (Fig. 6).

DISCUSSION

We were unable to demonstrate in the sera of 59 cotton textile workers true precipitating antibodies to extracts of cotton bract, carpels, stems, leaves, immature cotton lint, or cardroom cotton dust. The extracts were immunogenic as shown by animal immunizations from which we obtained precipitating antibodies

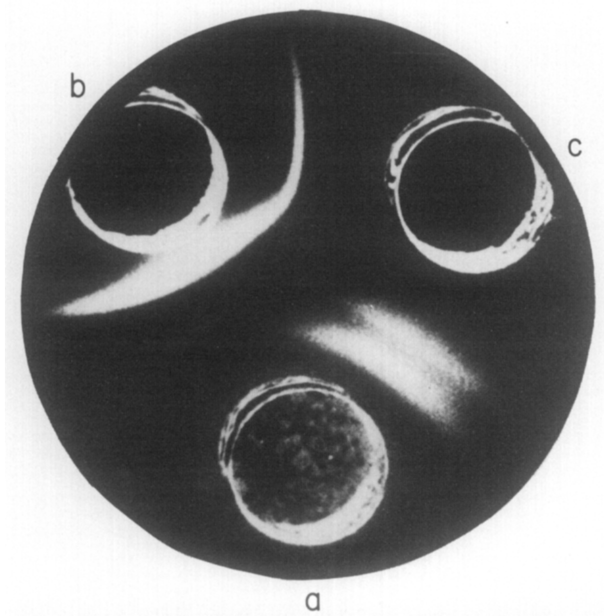

FIG. 4. Gel diffusion of cotton stem extract (a) with cotton textile worker serum (b) and antiserum to human beta-lipoprotein (c). Precipitation shows line of identity between (b) and (c).

to cotton bract and leaves. It is possible that in byssinosis the presence of serum antibodies is not indicative of dust exposure as it is with other vegetable dust diseases (doPico *et al.*, 1976). It is also possible that a disease mechanism, other than true antigen–antibody immune complex formation (if not a combination of mechanisms) is of at least equal importance in the development of byssinosis.

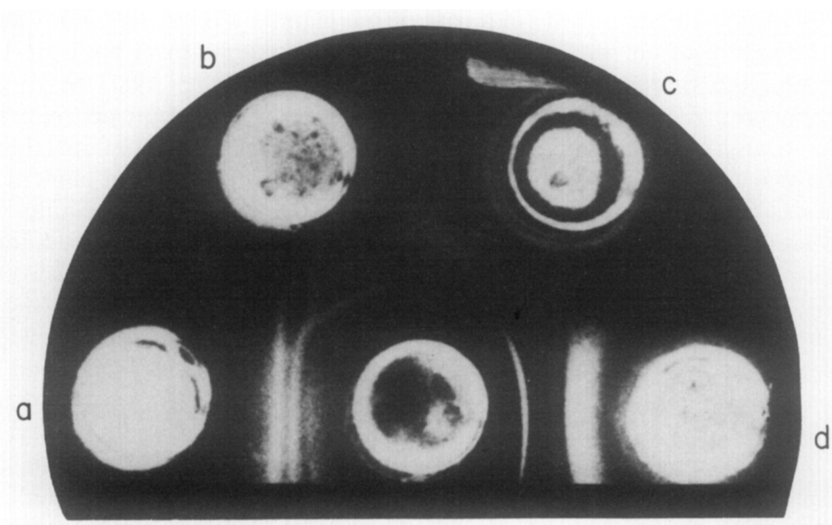

FIG. 5. Gel diffusion of serum from a rabbit (center well) which was immunized with cotton bract extract. Cotton bract extract (a); cotton leaf extract (b); cotton carpel extract (c); and cotton stem extract (d). Line of identity between (a) and (b) and pseudoimmune reaction between rabbit anti-bract serum (center well) and cotton stem extract (d).

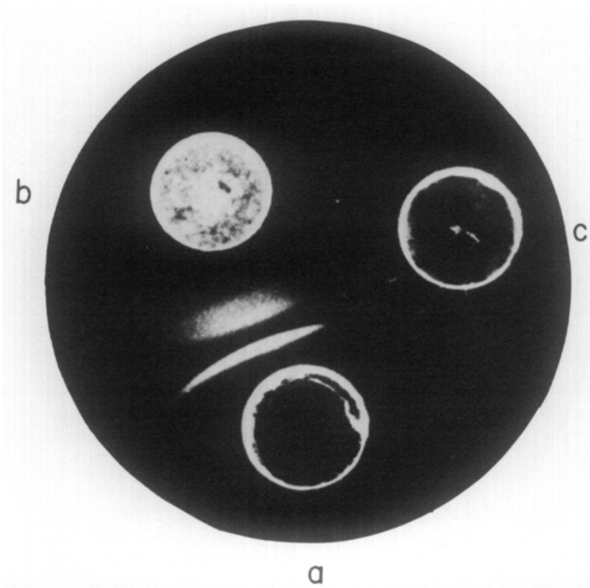

FIG. 6. Gel diffusion analysis of the pseudoimmune reaction between the cotton stem extract (a) and serum from an active cotton textile worker (b). Pretreatment of the stem extract with polyvinylpolypyrrolidone (c) eliminates this pseudoimmune reaction.

This study demonstrated that a plant tannin is responsible for the pseudoimmune precipitation reaction. While other investigators (Taylor *et al.*, 1971; Edwards and Jones, 1973) found a similar agent in cotton bract, our study found it only in extracts of cotton stems and cardroom cotton dust. This difference is unresolved at present, but may be due to the time of the year the plant parts or samples were collected, to the maturity of the plants used in the various studies, or to the nature of our methodologies. That cotton stems from both the United States and the Sudan contained the active agent implies that the observed pseudoimmune precipitation is neither an artifact of collection procedures nor unique to any one geographic area. Cardroom cotton dust contains the plant tannin since it probably contains stem material. Alternatively, other plant material which was contained in the dust may have been the source of the tannin.

The identification of the agent which was responsible for the pseudoimmune precipitation reaction was aided by the finding that an extract of tea leaves possessed the same characteristic. Tea and other plant leaves have been shown to contain large quantities of plant tannins, which are known to interact with and complex proteins of several biochemical types (Selvendran and Isherwood, 1967; Loomis and Battalle, 1966). Polyvinylpolypyrrolidone (PVP) was used in this study because it is a specific insoluble adsorbent for polyphenolic tannins (Selvendran and Isherwood, 1967). After adsorption of the stem, cardroom cotton dust, and tea extracts with PVP, these extracts no longer precipitated serum proteins. From these observations, it was concluded that a plant polyphenolic tannin was probably the agent that was responsible for the pseudoimmune precipitation reaction. Whether this is a single tannin or a heterogeneous group of tannins is unknown.

Only serum proteins which migrated electrophoretically in the beta- and gamma-regions were precipitated by the plant tannin, in a manner which is similar on electrophoresis to the precipitation of alpha-, beta-, and gamma-regions by phytohemagglutinin (Osunkoya and Williams, 1971). We identified the beta-region protein as human beta-lipoprotein. However, we were unable to identify the specific gamma-region proteins involved, even with the use of monospecific antisera. The most probable reason for this is that the plant tannin complexed with the immunoglobulins (perhaps with the Fc region) such that the immunoglobulin class-specific antigenic determinants were masked. In data not presented here, however, Cohn Fraction II (mostly IgG) was precipitated in agar gel by the plant tannin and yielded a reaction of identity with the pseudoimmune precipitate from the gamma-region. These observations imply that the gamma-region precipitate contained predominantly IgG.

It is interesting to note that mixing the human sera with either cardroom cotton dust extract or cotton stem extract removed all the beta-lipoprotein. Prior mixing did not, however, remove the gamma-globulin reaction. Attempts to quantify serum immunoglobulins in the sera after mixing with either extract yielded dubious results. Apparently enough unreacted tannin remained in the sera to precipitate the anti-human immunoglobulin sera in the radial immunodiffusion plates. Unlike the results of Edwards and Jones (1973), accurate measurements of our radial immunodiffusion plates were impossible to achieve and therefore interpret. However other authors have observed that phytohemagglutinin (a plant lectin) is also capable of precipitating serum proteins after gel diffusion or immunoelectrophoresis (Osunkoya and Williams, 1971). Therefore the exact chemical nature of the precipitating agent merits further investigation.

In conclusion, we identified the material in extracts of cotton stems and cardroom cotton dust which precipitate normal human beta- and gamma-globulins in a pseudoimmune fashion. We further identified the beta-globulin as human beta-lipoprotein. The possible role which the pseudoimmune precipitation reaction plays in the pathogenesis of byssinosis is not defined by the present study. Nor is it clear whether this reaction occurs *in vivo*. If it did occur, one could speculate that aggregation of gamma-globulins, specifically or in a pseudoimmune manner, could initiate several biological reactions via complement activation (Muller-Eberhard, 1975). Further examination of that reaction, as well as the role of beta-lipoprotein in the pathogenesis of byssinosis would be necessary to verify our suggestion. In any case, the *in vitro* observation of this pseudoimmune reaction emphasizes the problems which are associated with laboratory investigations of the immunological aspects of byssinosis.

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REFERENCES

Burrell, R. (1974). "Experimental Immunology." Burgess, Minneapolis.

doPico, G. A., Reddan, W. G., Chmelik, F., Peters, M. E., Reed, C. E., and Rankin, J. (1976). The value of precipitating antibodies in screening for hypersensitivity pneumonitis. *Amer. Rev. Resp. Dis.* **113**, 451–455.

Edwards, J. H., and Jones, B. M. (1973). Pseudo-immune precipitation by the isolated byssinosis "antigen." *J. Immunol.* **110**, 498–501.

Gordon, M. A., Almy, R. E., Greene, C. H., and Fenton, J. W. (1971). Diagnostic mycoserology by immunoelectroosmophoresis: a general, rapid, and sensitive microtechnique. *Amer. J. Clin. Pathol.* **56**, 471–474.

Hirschfeld, J. (1968). Immunoelectrophoresis—procedure and application to the study of group specific variations in sera. *In* "Handbook of Immunodiffusion and Immunoelectrophoresis" (O. Ouchterlony, Ed.), pp. 149–150. Ann Arbor Science Pub., Ann Arbor, Mich.

Loomis, W. E., and Battalle, J. (1966). Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry* **5**, 423–438.

Muller-Eberhard, H. J. (1975). Complement. *Annu. Rev. Biochem.* **44**, 697–724.

Osunkoya, B. O., and Williams, A. I. O. (1971). Precipitation of serum proteins by phytohaemagglutinin. *Clin. Exp. Immunol.* **8**, 205–212.

Selvendran, R. R., and Isherwood, F. A. (1967). Identification of guanosine diphosphate derivatives of D-xylose, D-mannose, D-glucose, and D-galactose in mature strawberry leaves. *Biochem. J.* **105**, 723–728.

Taylor, G., Massoud, A. A. E., and Lucas, F. (1971). Studies on the aetiology of byssinosis. *Brit. J. Ind. Med.* **28**, 143–151.

Uriel, J. (1964). The characterization reactions of the protein constituents following electrophoresis or immune-electrophoresis in agar. *In* "Immunoelectrophoresis Analysis: Applications to Human Biological Fluids" (P. Grabar and P. Burtin, Eds.), pp. 35–36. Elsevier, New York.