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Antigenic Heterogeneity of an Alkali-Soluble, Water-Soluble Cell Wall Extract of *Coccidioides immitis*

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The antigenic composition of an alkali-soluble, water-soluble cell wall extract of *Coccidioides immitis*, designated C-ASWS, was assessed by two-dimensional immunoelectrophoresis against goat antisera to C-ASWS and coccidioidin. The results established that C-ASWS from mycelia or spherule cell walls is heterogeneous in composition, containing two distinct antigenic components. One is present as a polymer that is antigenically identical to a polymeric antigen in coccidioidin, designated antigen 2. The other component detected in C-ASWS presented an unusual precipitin pattern in that a cathodal leg was demonstrable in the absence of an anodal leg. This incomplete precipitinogen was also detected in coccidioidin. In addition to the finding that C-ASWS is antigenically heterogeneous, the results provide evidence that the conformational and/or configurational structure of the C-ASWS antigen 2 (or antigen 2-like polymer) is altered during physicochemical extraction. This conclusion is based upon the finding that the immunoelectrophoretic profile of the C-ASWS polymer differs from that of coccidioidin antigen 2. The C-ASWS polymer is characterized by having a small cathodal precipitin peak connected to a large anodal peak, whereas coccidioidin antigen 2 is characterized by a predominant cathodal peak.

A series of reports have established that an alkali-soluble, water-soluble cell wall extract from mycelia and spherules of *Coccidioides immitis*, designated C-ASWS, is effective in eliciting delayed-type hypersensitivity responses (5, 7, 8, 14), detecting immunoglobulin E antibody to *C. immitis* (4), reacting with tube precipitin antibody (6), and protecting mice against challenge with viable arthroconidia (11). This broad spectrum of biological activity equates with those exhibited by coccidioidin (CDN) prepared as an autolysate or a toluene-induced lysate of *C. immitis* mycelia and spherulin prepared as an autolysate of the spherule-endospore phase of *C. immitis*. The notable exception is that C-ASWS is nonreactive with anti-*Coccidioides* complement-fixing antibody.

Antigenic analysis by two-dimensional immunoelectrophoresis (2D-IEP) of C-ASWS in tandem with CDN against a reference burro anti-CDN serum provided evidence that C-ASWS from mycelial or spherule cell walls was homogeneous in composition (6). That is, a single component was detected having antigenic determinants identical to those of a large polymer, designated antigen 2 (Ag_2), in CDN. In the present study, the antigenic composition of the C-ASWS extracts was evaluated in 2D-IEP with a goat antiserum to CDN and antisera to the mycelial- and spherule-derived C-ASWS. Two new findings emerged. (i) The C-ASWS extracts are not homogeneous in composition but contain, in addition to Ag_2, a second antigenic component detected as a partial precipitinogen; and (ii) the IEP profile of C-ASWS polymer differs from that of CDN Ag_2, suggesting that C-ASWS is denatured during physicochemical extraction.

MATERIALS AND METHODS

Antigens. C-ASWS was isolated from mycelial and spherule cell walls (designated C-ASWS-M and C-ASWS-S, respectively) as detailed in a previous report (6). In brief, Formalin-killed mycelia and spherules of *C. immitis* Silveira were mechanically disrupted in a Braun homogenizer for 4 min at 2,000 strokes per min. Cell walls were collected by centrifugation, treated with trypsin, and then extracted with 1 N NaOH for 3 h at 25°C. The alkali-soluble extracts were dialyzed against distilled water, filtered (1.2-μm pore size), and lyophilized. CDN (lot TS6772; obtained from Demosthenes Pappagianis, University of California at Davis) was prepared as a toluene-induced lysate of mycelia of *C. immitis* Silveira (12). Before use, CDN was dialyzed against distilled water and lyophilized.

Antisera. Antisera to the C-ASWS extracts and CDN (TS6772) were obtained by immunizing goats intramuscularly with 10 mg (dry weight) of antigen in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.), followed by booster injections of antigen (5 mg) at 1- to 3-month intervals. Goats were bled from the jugular vein 5 to 7 days after each immunization. The immunoglobulin fraction was obtained by precipitation of sera with an equal volume of saturated ammonium sulfate. Precipitated immunoglobulins were dialyzed against distilled water and then lyophilized and stored at −20°C.

2D-IEP. The various techniques of 2D-IEP were performed according to published procedures (1, 10, 15). Antigen (20 μl) was applied in a well (4 mm in diameter) cut 2 cm from the side and bottom of a gel bond film (FMC Corp., Marine Colloids Div., Rockland, Maine) coated with 1% agarose (type I; Sigma Chemical Co., St. Louis, Mo.). First-dimension electrophoresis was performed for 1 h at 10 V/cm. The gel was removed 5 mm parallel to the first-dimension run and replaced with 1% agarose containing immunoglobulin. Electrophoresis in the second dimension was performed at 3 V/cm for 17 h at 15°C. The gels were washed with saline followed by distilled water and then dried and stained with Coomassie brilliant blue R. For tandem 2D-IEP, antigens were admixed with an equal volume of 2% SeaPlaque agarose (FMC Corp.) maintained in a liquid state at 37°C. The antigen-agarose mixtures (20 μl) were applied to wells cut 7 mm apart, and after the agarose solidified. first- and second-dimension electrophoreses were performed as described above. For intermediate gel 2D-IEP, antigen was

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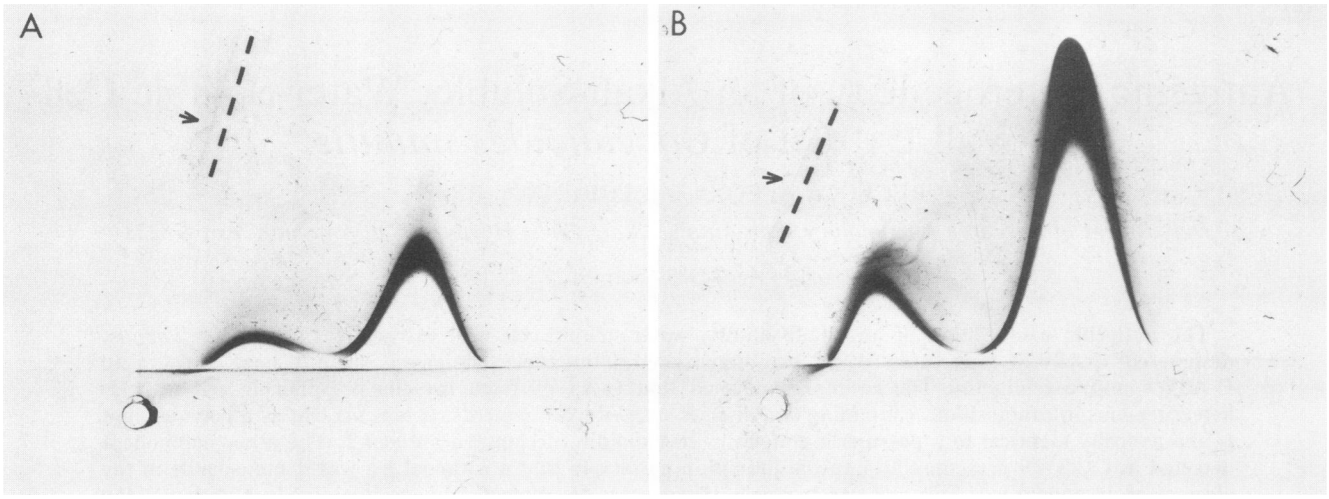

FIG. 1. Antigenic heterogeneity of the C-ASWS extracts as assessed by 2D-IEP against goat anti-CDN. C-ASWS-M (200 μg) (A) and C-ASWS-S (200 μg) (B) against goat anti-CDN (162 μg of immunoglobulin per cm^2 of gel). Dotted lines delineate the incomplete precipitinogen. In the first-dimension run the anode is to the right; in the second dimension the anode is to the top.

subjected to electrophoresis in the first dimension, and after this, an agarose strip (1.5 cm in width) containing immunoglobulin was poured parallel to the first-dimension gel. The intermediate gel was allowed to solidify, and agarose containing a second immunoglobulin was applied to the remaining second-dimension gel area. Electrophoresis was then performed across the two gels at 3 V/cm for 17 h.

RESULTS

Antigenic heterogeneity of C-ASWS. The antigen composition of C-ASWS-M and C-ASWS-S was evaluated in 2D-IEP against goat antiserum to CDN and homologous goat antisera. The results obtained with anti-CDN are depicted in Fig. 1. Two components were present in C-ASWS-M (Fig. 1A) and C-ASWS-S (Fig. 1B). The predominant antigen was a polymer having a cathodal precipitin peak connected to a larger anodal peak. The second component presented an unusual precipitin pattern in that a partial cathodal leg was demonstrable and an anodal leg was not, except at extremely high concentrations of antigen.

C-ASWS-M and C-ASWS-S differed quantitatively in that C-ASWS-M contained more of the incomplete precipitating antigen (designated IPA) and, conversely, C-ASWS-S contained more of the polymeric antigen. Qualitative differences were not demonstrable; i.e., coelectrophoresis of C-ASWS-M and C-ASWS-S (antigens placed in the same well) resulted in the fusion of the IPAs and the fusion of the two polymers, thereby establishing antigenic identity (data not shown).

The IEP patterns of the C-ASWS extracts against homologous antisera are shown in Fig. 2. C-ASWS-M (Fig. 2A) showed a profile similar to that observed in 2D-IEP against goat anti-CDN (Fig. 1A), except that the cathodal leg of the

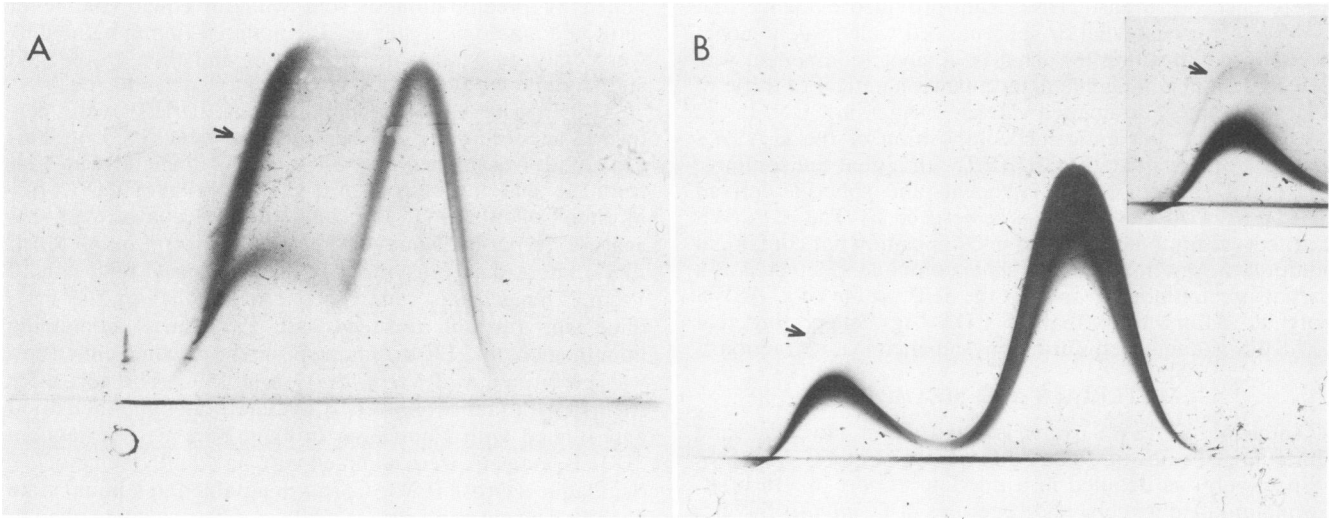

FIG. 2. Antigenic heterogeneity of the C-ASWS extracts as assessed by 2D-IEP against homologous antisera. (A) C-ASWS-M (100 μg) against goat anti-C-ASWS-M (162 μg/cm^2 of gel) and (B) C-ASWS-S (200 μg) against goat anti-C-ASWS-S (405 μg/cm^2 of gel). Arrows identify the incomplete precipitinogen. Inset in panel B is a double print to show the IPA in C-ASWS-S.

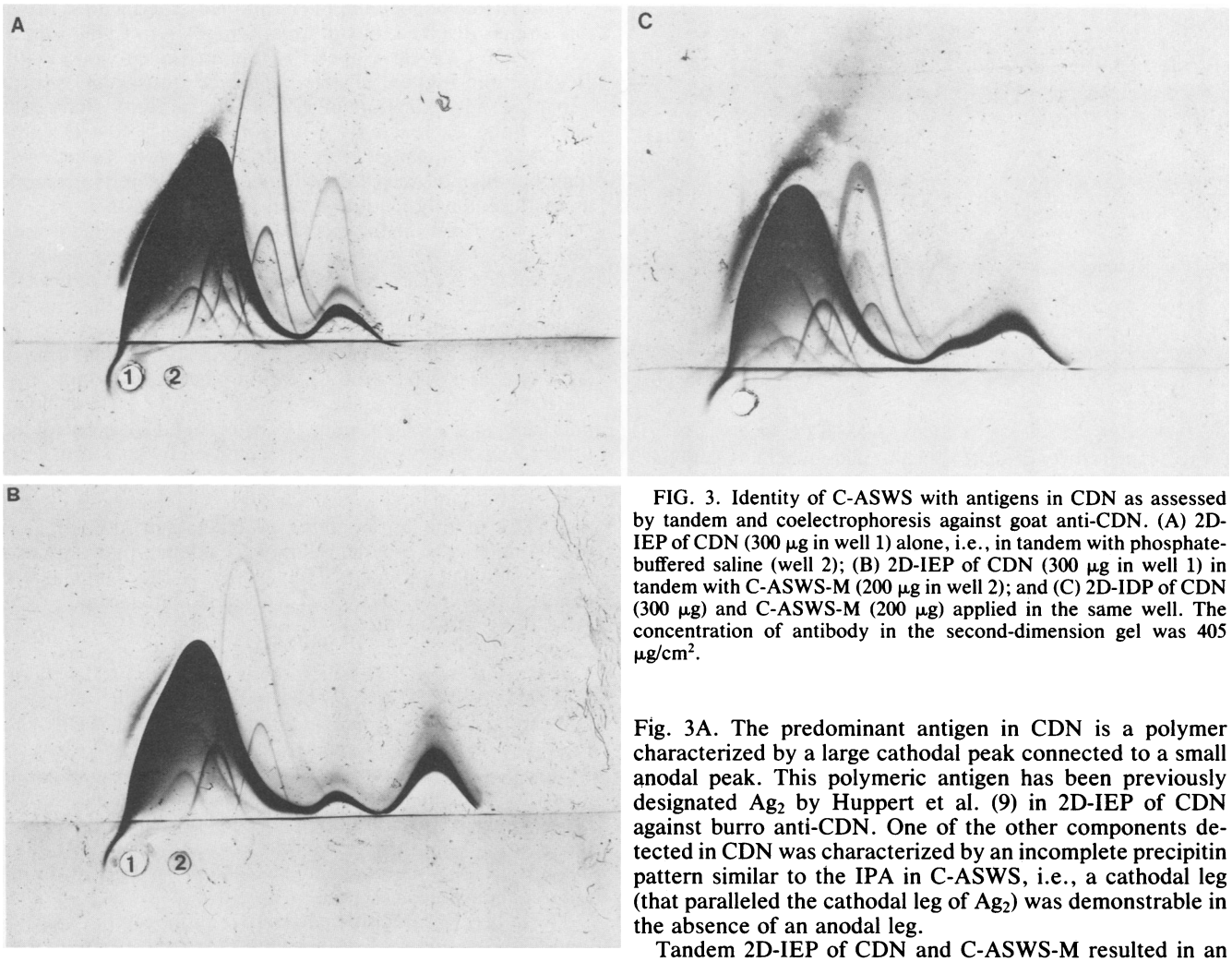

FIG. 3. Identity of C-ASWS with antigens in CDN as assessed by tandem and coelectrophoresis against goat anti-CDN. (A) 2D-IEP of CDN (300 μg in well 1) alone, i.e., in tandem with phosphate-buffered saline (well 2); (B) 2D-IEP of CDN (300 μg in well 1) in tandem with C-ASWS-M (200 μg in well 2); and (C) 2D-IDP of CDN (300 μg) and C-ASWS-M (200 μg) applied in the same well. The concentration of antibody in the second-dimension gel was 405 μg/cm^2.

IPA was extended and appeared to fuse with the cathodal leg of the polymer. This precipitin profile suggested (and was originally interpreted as) a partial or total identity of antigenic determinants between the two components. This fusion was not demonstrable, however, when extremely high concentrations of antigen were employed, a result that argues against antigenic analogy. The latter interpretation is further supported by the results obtained in 2D-IEP of C-ASWS-S against homologous antiserum. The IPA in C-ASWS-S paralleled but did not fuse with the cathodal peak of the polymer (Fig. 2B). Moreover, at high concentrations of C-ASWS-S, the precipitin line of the IPA was extended and observed to cross the anodal peak of the polymer, ergo a reaction of nonidentity (not shown).

The IEP patterns obtained in 2D-IEP of C-ASWS-M against goat antiserum to C-ASWS-S and in 2D-IEP of C-ASWS-S against goat anti-C-ASWS-M were identical to those obtained with homologous antisera. These results further establish the antigenic identity of C-ASWS-M and C-ASWS-S.

Antigenic comparison of C-ASWS and CDN. The identity of the two components in the C-ASWS extracts with antigens in CDN was evaluated by using the technique of tandem 2D-IEP. The antigenic composition of CDN alone, i.e., in tandem with phosphate-buffered saline, is depicted in Fig. 3A. The predominant antigen in CDN is a polymer characterized by a large cathodal peak connected to a small anodal peak. This polymeric antigen has been previously designated Ag_2 by Huppert et al. (9) in 2D-IEP of CDN against burro anti-CDN. One of the other components detected in CDN was characterized by an incomplete precipitin pattern similar to the IPA in C-ASWS, i.e., a cathodal leg (that paralleled the cathodal leg of Ag_2) was demonstrable in the absence of an anodal leg.

Tandem 2D-IEP of CDN and C-ASWS-M resulted in an altered precipitin pattern of Ag_2 in CDN (Fig. 3B). This altered pattern is evidenced by the addition of a connecting anodal peak that corresponds to the anodal peak of the C-ASWS polymer (Fig. 1A), a finding that establishes the antigenic identity of the C-ASWS polymer with CDN Ag_2.

No other CDN precipitinogens were affected by tandem 2D-IEP including the IPA. Fusion of the IPA in CDN with C-ASWS during tandem 2D-IEP would be difficult to demonstrate, however, in the absence of an anodal leg. To circumvent this problem, CDN and C-ASWS-M were coelectrophoresed in the first- and second-dimensional runs. The results (Fig. 3C) clearly show an increase in the precipitin pattern of the IPA in CDN when compared with Fig. 3A. This additive effect establishes the antigenic identity of the IPA in CDN and C-ASWS. Coelectrophoresis of CDN and C-ASWS-M also caused a predictable change in the precipitin pattern of CDN Ag_2.

Further evidence that the IPA in C-ASWS is analogous to the CDN IPA is provided by the results obtained in 2D-IEP of CDN against goat antiserum to C-ASWS-M (Fig. 4). Namely, high concentrations of goat anti-C-ASWS detected, in addition to Ag_2, the IPA in CDN.

Evidence for altered conformational structure of C-ASWS Ag_2. Although the preceding results establish the antigenic identity of the C-ASWS polymer with Ag_2 in CDN, the anomalous finding exists that the confirmation and/or configuration of the C-ASWS and CDN Ag_2 polymers differ,

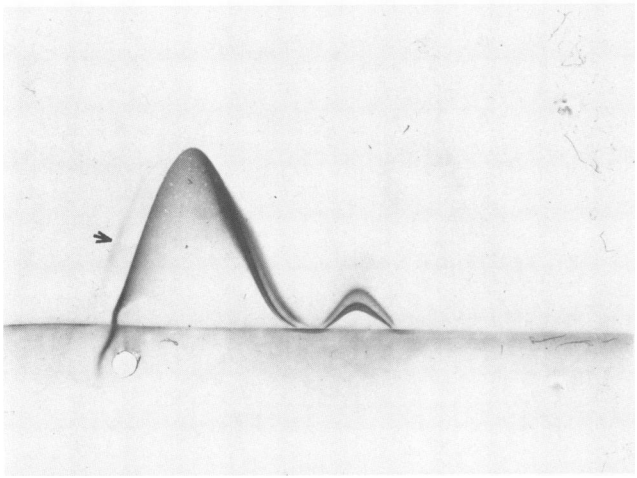

FIG. 4. Reactivity of CDN with goat antiserum to C-ASWS-M. CDN was assayed at a concentration of 300 μg. The second-dimension gel contained goat anti-C-ASWS-M at a concentration of 1.62 mg/cm^2. Arrow identifies the incomplete precipitinogen.

i.e., the relative heights of the cathodal and anodal peaks are reversed. This pattern occurs regardless of whether 2D-IEP is performed with homologous or heterologous antiserum. Hence, the differences in polymeric configuration or conformation or both are not attributed to differences in antibody specificities.

Assuming that Ag_2 in CDN represents the native cell wall polymer, these results indicate that the C-ASWS polymer is altered during physicochemical extraction. However, since the CDN used in this study was prepared as a toluene-induced lysate of mycelial cells, the possibility exists that CDN Ag_2 does not represent the native configuration or conformation of the cell wall polymer. This argument is refuted by the finding that 2D-IEP of Smith's CDN lot 64 D4, prepared as an autolysate of mycelia in the absence of toluene (13), yielded an Ag_2 polymer having a profile identical to that of the toluene-induced lysate (data not shown).

Comparison of goat and burro antibody specificities. Previous studies directed toward identifying Ag_2 in CDN utilized a burro anti-CDN developed by Huppert and co-workers (9). Because the supply of this reference antiserum is near depletion, identification of Ag_2 in the present study was based upon its reactivity with goat antisera to CDN and C-ASWS. A requisite of this study is, therefore, to establish that the polymer detected with the goat antisera is the same as that previously identified with burro anti-CDN.

A comparison of the specificities of burro and goat antisera for CDN Ag_2 was accomplished by the technique of intermediate gel 2D-IEP. The reactivity of burro anti-CDN with CDN, i.e., across an intermediate gel containing buffer only, is shown in Fig. 5A. Inclusion of goat anti-C-ASWS-M in the intermediate gel resulted in the in situ precipitation of Ag_2, as evidenced by the precipitin pattern in the intermediate gel and the absence of reactivity of Ag_2 with burro anti-CDN in the second-dimension gel (Fig. 5B). None of the other CDN components were precipitated by goat antiserum to C-ASWS-M. In situ adsorption of CDN Ag_2 (as well as other CDN antigens) was also effected by inclusion of goat anti-CDN in the intermediate gel (data not shown). The preceding results provide definitive evidence that the polymer designated as Ag_2 in this study is the same as the polymer previously described with burro antiserum.

DISCUSSION

The results of the present study confirm our earlier report (6) that C-ASWS from mycelia or spherule cell walls contains antigenic determinants identical to those of CDN Ag_2. However, in contrast to that report, the C-ASWS extracts were shown to be heterogeneous in composition, containing a second antigen characterized as a partial or incomplete precipitinogen. In addition, evidence is presented to support the possibility that the configuration and/or conformation of the Ag_2 (or Ag_2-like polymer) in C-ASWS is altered during physicochemical extraction.

The failure to detect the partial precipitinogen in C-ASWS (and CDN) in previous studies with burro anti-CDN (6, 9) is not attributed to the lack of burro immunoglobulin to this antigen but rather to the extremely low titer of antibody

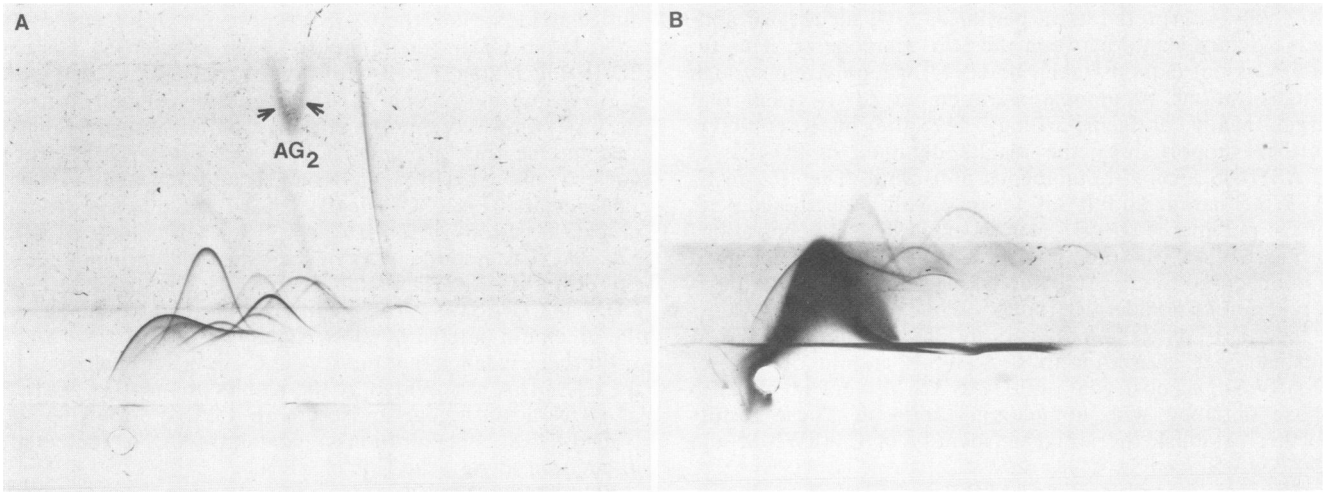

FIG. 5. Antibody specificities of burro anti-CDN and goat anti-C-ASWS for CDN Ag_2 as assessed by 2D-IEP with an intermediate gel. (A) Intermediate gel contained phosphate-buffered saline. (B) Intermediate gel contained goat anti-C-ASWS-M at a concentration of 670 μg/cm^2. Remainder of second-dimension gel contained burro anti-CDN at a concentration of 177 μg/cm^2. CDN was applied at a concentration of 100 μg in gels A and B.

response. That is, concentration of burro immunoglobulin established the presence, albeit weak, of antibody to the partial precipitinogen. It is also noteworthy that the antibody response of the burro to Ag_2 was considerably less relative to that obtained with goat anti-CDN. These differences in antibody responses are probably attributed to differences in the composition of the CDN preparations used as immunogens. In other words, the antigen preparation used to immunize the burro was an admixture of a toluene-induced lysate and broth culture filtrate (9), whereas the goat antiserum was directed against the toluene-induced lysate only.

Detection of only Ag_2 and the incomplete precipitinogen in C-ASWS is in disagreement with a recent study by Cole et al. (3). These investigators reported that the C-ASWS-S contains, in addition to Ag_2, a component designated AgCS, demonstrable by fused rocket IEP of C-ASWS-S against burro anti-CDN. The presence of AgCS in the C-ASWS extract prepared by Cole et al. (3) cannot be argued. However, the method that they used to isolate C-ASWS was a modification of the protocol used by us. Namely, the cell walls were not treated with trypsin before alkaline extraction nor was the alkali-soluble, water-insoluble fraction that precipitates during dialysis removed before analyses. We have found these two procedures to be essential in the purification of C-ASWS. On this basis, the C-ASWS extracts prepared in our laboratory were analyzed by the same fused rocket IEP system with burro anti-CDN and reference CDN. These analyses, performed by one of the collaborators (P. Starr) in the study by Cole et al. (3), failed to reveal the presence of AgCS. Hence, the differences in the two studies are most likely attributed to differences in the techniques used to isolate C-ASWS.

The finding that the C-ASWS polymer (from mycelia or spherules) contains antigenic determinants identical to those on CDN Ag_2 but differs from CDN Ag_2 in its IEP profile suggests a denaturation effect(s) of physicochemical extraction. Whether this denaturation occurs during mechanical disruption of the mycelia cells or in subsequent steps is not known. It is not attributed to the treatment of cell walls with trypsin before alkaline hydrolysis; i.e., the alkaline extract of non-trypsin-treated cell walls yielded an identical polymer. The most probable cause of polymeric alteration is the exposure of the walls to 1 N NaOH, a procedure that is known to have deleterious effects on cell wall polymers, particularly heteroglycans (2, 16).

An important issue raised by the results of this study is whether the previously reported biological activities of C-ASWS-M and C-ASWS-S (4–8, 11, 14) are attributed to the Ag_2 (or Ag_2-like) polymer, the incomplete precipitinogen, or both. The increased biological activity of C-ASWS-S as compared with C-ASWS-M together with the higher content of the Ag_2 in the spherule-phase extract implies the importance of the polymer. However, a definitive answer to this question awaits purification of Ag_2 and the incomplete precipitinogen. We have previously reported the isolation of Ag_2 by solid-phase immunoadsorption of CDN with goat antisera to C-ASWS (R. A. Cox, M. Huppert, and L. A. Britt, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, F43, p. 299). Because the affinity of the goat antisera for the incomplete precipitinogen in CDN is low, only trace amounts of the latter antigen are present in the column eluate (desorbed) fraction. Separation of Ag_2 and the IPA is in progress, using a combination of ion-exchange and solid-phase immunosorption.

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