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Some characteristics of a secreted chlamydial antigen recognized by IgG from *C. trachomatis* patient sera

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SUMMARY

Chlamydia trachomatis serovars release a glycolipid antigen (GLXA) into the culture supernatant during the infective cycle. This antigen is recognized by IgG isolated from humans with a natural chlamydial infection; GLXA may be a major antigenic determinant of chlamydia. It can be immunopurified by molecular shift or affinity chromatography. Silver staining of SDS-PAGE gels demonstrates a pattern of bands that is essentially the same for preparations isolated by either method. GLXA can also be extracted from mature elementary bodies (EB). These preparations show the same pattern of silver staining bands, and the major bands are immunoreactive as shown by Western blot analysis. Isoelectric focusing studies demonstrate that purified GLXA has an acidic pI.

INTRODUCTION

Chlamydia trachomatis displays a variety of antigenic determinants, protein, glycolipid, lipopolysaccharide (LPS) (Newhall, 1988; MacDonald, 1985; Schacter & Caldwell, 1980; Levitt and Barol, 1987), which interact with host cell components. Earlier investigators had begun to define antigen interactions that permit parasite attachment and the subsequent invasion of cells (Hackstadt, 1986; Wenman & Mauser, 1986; Reddish *et al.*, 1986). Other studies have examined parasite antigen which may aid in eluding host defence mechanisms (MacDonald, 1985) and may play a significant role in the immunopathology associated with chlamydial disease (Sacks *et al.*, 1978; Watkins *et al.*, 1986; Taylor *et al.*, 1986). Both of these aspects are fundamental to successful pathogenesis. From a microbiological as well as clinical perspective, therefore, it is important to define the relevance and roles of various antigens during the course of the infective cycle.

Many of the studies cited above have investigated antigens isolated from purified chlamydial elementary bodies (EB). However, previous studies from this laboratory (Stuart, Tirrell & MacDonald, 1987; Stuart & MacDonald, 1986, 1984, 1982) indicate that chlamydia secrete a genus-specific antigen (GLXA) during the infection of McCoy cells *in vitro*. This glycolipid has been partially characterized. Mass spectrographic analyses indicate it contains polysaccharides, one of which may be gulose or a derivation of gulose (Stuart *et al.*, 1987; Stuart & MacDonald 1984). In addition, there is a lipid component that has fatty acids of chain length C_{17} and $C_{18:1}$, the latter indicating an unsaturated bond. This glycolipid antigen appears to be different from the characteristic LPS of gram-negative organisms (Caldwell & Hitchcock, 1984).

Current studies have been directed towards assessing the GLXA isolated from supernatants of cell cultures infected with various chlamydial serovars. Specifically, this work has examined the following: (i) the relevance of GLXA to naturally occurring chlamydial infections; (ii) its immunopurification from supernatants of infected cultures; and (iii) the characteristics of GLXA as defined by various biochemical techniques. The results from these analyses have determined the recognition of GLXA by antibodies raised during a natural infection, defined the apparent molecular weight of GLXA and its pI, and demonstrated the similarity of GLXA from different biovars. Cumulatively, these findings indicate GLXA may be a component important for the chlamydial life cycle and relevant to natural infections.

Abbreviations: ABTS, 2, 2′-azinobis-(3 ethylbenzthiazoline-6-sulphonic acid); BHK, baby hamster kidney cells; BSA, bovine serum albumin (Fraction V); BSB, borate-buffered saline; EB, elementary body; EIA, enzyme-linked immunoassay; ETOH, ethanol; FA, Fluorescent antibody; GLXA, glycolipid exoantigen; IgG, immunoglobulin G; IgM, immunoglobulin M; KSCN, potassium thiocyanate; LGV, lymphogranuloma venerium; MW, molecular weight, pI, isoelectric point, SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis, TBS, Tris-buffered saline (pH 8·0); TCA, trichloroacetic acid.

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MATERIALS AND METHODS

Cultivation of C. trachomatis

Previously described methods for organism cultivation using centrifuge-assisted infection (Stuart *et al.*, 1987) were followed, except for the changes defined below. McCoy cells rather than BHK were utilized and, instead of irradiation, cycloheximide (1 μg/ml; Whittacker overlay medium; Walkersville, MD) was incorporated to inhibit cell division.

Initial antigen isolation

Antigen was isolated from culture supernatants as described previously (Stuart *et al.*, 1987). Glycolipid antigen also was isolated from the water layer of a phenol/water extract of Renogafin (E.R. Squibb, Newbrunswick, NJ) -purified organisms grown in embryonated eggs (Westphal & Jann, 1965; Hourihan & MacDonald, 1980) by passing the fluid over an Octyl–Sepharose CL4B column (Pharmacia, Piscataway, NJ); the antigen was eluted with ETOH. For experiments, the remaining ETOH was pervaporated and the preparations resuspended in Tris-buffered saline (TBS).

External labelling of antibody preparations

IgG-containing preparations were labelled with ^{125}I using one of two methods. Ten to 25 μl of antibody-containing mouse ascites were labelled using Bio-Rad (Rockville Center, NY) enzymobeads and following manufacturer's specifications. Alternatively, rabbit IgG was purified from a 14% sodium sulphate fraction by gel filtration over a Sepharose CL 6B column (79·0 × 1·5 cm, 0·1 M Tris, 0·15 M NaCl, pH 8·0). The 155,000 MW material was labelled using chloramine T (Sigma, St Louis, MO) (Hunter, 1973). After either labelling procedure, samples were filtered over a 3 Sephadex G-25 column (Pharmacia) (0·5 × 6·0 cm) and TCA-precipitable counts determined to verify the incorporation of label into protein.

Molecular shift chromatography

Antigen–antibody complexes were prepared by combining antigen in TBS with either isolated IgG (Stuart *et al.*, 1987; Hourihan & MacDonald, 1980) or with IgG-containing fractions of ^{125}I-labelled ascites fluid. Complexes were allowed to form for 2–4 hr at 30° as described previously (Stuart *et al.*, 1987). One-millilitre fractions were collected; ^{125}I isotope emissions were detected with a Beckman Gamma 4000 counter. For molecular shifts involving IgG from humans, equal volumes of three individual patient sera, each with a fluorescent antibody (FA) titre $\geq$ 2560, were pooled prior to IgG isolation. The presence of human IgG in the peak of shifted material was verified by EIA using the general protocol described previously (Stuart *et al.*, 1987). The appearance of colour in the ABTS substrate was quantified by absorbance at 405 nm with a Dynatech Micro Elisa 580 automated reader.

Antigen purification

Antigen and monospecific antibody were retrieved from molecular shift fractions by dissociation of the immune complex with 5 M KSCN. The KSCN solution was passed over an Octyl–Sepharose CL4B column (3·0 × 1·5 cm) equilibrated in 5 M KSCN. The dissociated IgG eluted with the KSCN while the antigen was bound to the hydrophobic gel. After rinsing with three to four column volumes of distilled water, the antigen was eluted with 95% ETOH. Antigen was concentrated to 2–4 ml using heat and reduced pressure. It then was stored in ETOH at −20°. Alternatively, antigen was purified using an affinity column. Briefly, an IgM monoclonal antibody, raised by *in vitro* stimulation of mouse spleen cells with a GLXA preparation (Dr W. Jones, Hygeia Sciences, Newton, MA) was coupled to Affi-Gel according to manufacturer's specifications. Concentrates of Octyl–Sepharose eluants from control or infected cultures were resuspended in phosphate-buffered saline (PBS), then passed through the column. After rinsing with five column volumes of 0·1 M phosphate buffer (pH 7·2), specifically bound material was eluted with 5 M KSCN. The sample was desalted by passage over Octyl–Sepharose and elution with 95% ETOH.

Electrophoretic analysis

For isoelectric focusing, the Pharmacia PhastSystem™ and a pH gradient of 3–9 or 4–6·5 were used to determine the pI of isolated material. The pH gradient was generated by applying 2000 V, 2·5 ma and 3·5 watts for 75 Vh. One microlitre of sample, suspended in 0·1 M glycine, pH 8·0, was loaded at 200 V, 2·5 ma, 3·5 watts for 15 Vh. Electrofocusing was carried out at 2000 V, 2·5 ma and 3·5 watts for 410–460 Vh. Gels containing focused samples and standards (Bio-Rad) were silver stained according to Pharmacia specifications for native gels. Isolated antigen also was examined by SDS–PAGE using the Pharmacia PhastSystem™. Continuous 10–15% gradient gels were utilized and stained as previously described (Stuart *et al.*, 1987). All electrophoresis was carried out at 15°.

Western blot analysis

Ten to 15 per cent SDS–PAGE gels, containing electrophoresed samples, were removed from their backing and rinsed in transfer buffer (0·025 M Tris, 0·2 M glycine, pH 8·4, in 20% methanol) for 10–30 min. With a Bio-Rad electrotransfer cell, material was transferred either to a hydrophobic nylon membrane (Nylon 66) or to Immobilon (Millipore, Bedford, MA) at 50–70 volts, 14–20 amps for 60–90 min at 4°. Membranes were blocked overnight with 3% bovine serum albumin (BSA; Sigma)/TBS or 5% skimmed milk, then incubated 24 hr with normal serum, rabbit anti-chlamydia serum or IgG, or mouse ascites fluid diluted in 1% BSA/TBS. Subsequently, membranes were rinsed and incubated with 4-chloro-1-napthol peroxidase substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MN).

RESULTS

Potential biological functions, as well as biochemical characteristics, are fundamental to an understanding of the role of GLXA during *C. trachomatis in vivo* infections. In initial experiments, the biological relevance of GLXA to clinical infections was assessed using molecular shift chromatography. Immunopurified antigen was reacted with IgG isolated from sera of patients with clinically defined LGV. If this antigen were naturally occurring during infection and stimulated antibody production, then there should be reactive antibodies present in patient sera. Antigen–antibody complexes should form and be detectable as a molecular shift. As Fig. 1 shows, a shift did occur and therefore the antigen was recognized by the IgG derived from a pool of patient sera. As a further demonstration that the human IgG had been shifted to the void volume, fraction aliquots were tested by EIA for the presence of human IgG. These results also are shown in Fig. 1 and indicate that the protein peaks correspond to peaks of human IgG.

The recognition by patient antibody of GLXA from tissue culture supernatants indicated a biological relevance for this material. Other experiments therefore were carried out to compare GLXA purified from different sources by different methods. Antigen was immunopurified by molecular shift or by monoclonal antibody affinity column procedures, then com-

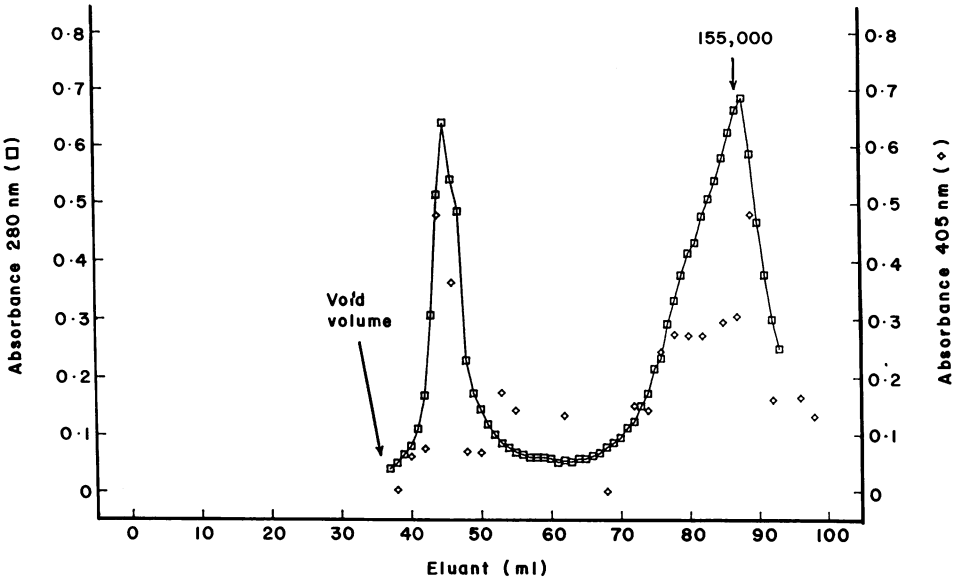

Figure 1. Absorbance profile at 280 nm of eluant from a Sepharose CL6B column (80 × 1·5 cm) equilibrated in 0·01 M BSB, pH 8·0. IgG isolated from LGV-positive human serum was reacted with GLXA affinity purified from serovar B-infected cell culture supernatants to permit antigen–antibody complex formation. The sample then was subjected to gel filtration chromatography (□). The protein was demonstrated to be human IgG by EIA testing. The absorbance at 405 nm (◇) of test aliquots is plotted relative to the protein profile. 155,000 indicates position of the 155,000 molecular weight standard (rabbit IgG).

pared using SDS—PAGE and silver staining. Fig 2 shows this comparison of the various preparations. Antigen purified by either method characteristically contains bands with apparent molecular weights (MW) of 62,000, 58,000, and 30,000. Serovar B organisms were isolated and purified from infected yolk sac material utilizing Renogratin isopicnic centrifugation. Antigen was prepared by hot phenol–water extraction as described in the Materials and Methods. SDS-PAGE analysis indicates a banding pattern very similar to that of tissue culture-derived glycolipid, as shown in Fig. 3 a, b. In a Western blot, the major bands of each preparation react with immune sera to produce an apparently identical band pattern (Fig. 4 a, b), while non-immune sera does not bind.

Isolated glycolipid antigen was further analysed by isoelectric focusing techniques. As Fig. 5 demonstrates, the antigen prepared from culture supernatants produced a band at an acidic pI (Fig. 5a). When loaded onto gels with a pI range of 4–6·5, the sample was resolved into two bands whose positions bracketed the pI 4·75 standard. Control preparations failed to show these bands (Fig. 5a, and b lane iii); samples prepared from yolk sac grown material appeared identical to the culture supernatant samples (not shown).

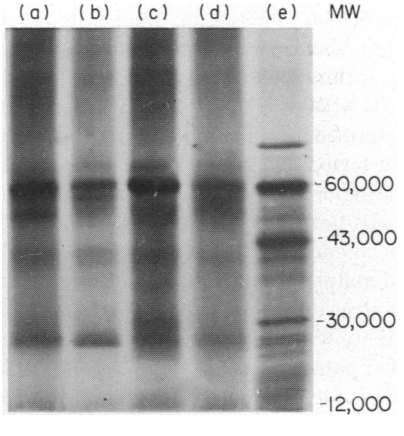

Figure 2. PhastSystem™ SDS–PAGE analysis of affinity or shift purified GLXA samples: 10–15% precast gels were electrophoresed for 65 Vh then silver stained. Lane (a–c): GLXA affinity purified from L_2, B and J; lane (d): shift purified GLXA from serovar J; lane (e): molecular weight standards. Note the band patterns appear very similar despite differing serovar sources or methods of immunopurification.

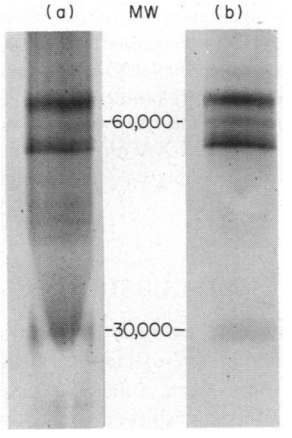

Figure 3. PhastSystem™ 10–15% SDS gel: samples of GLXA isolated from tissue culture or by hot phenol extraction of renografin-purified elementary bodies were subjected to SDS treatment and electrophoresed. Silver staining of the gel indicates that the banding pattern of tissue culture supernatant (a) or purified elementary body preparations (b) is very similar.

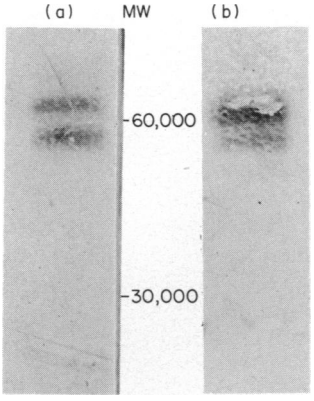

Figure 4. Western blot: immunoidentification of antigen bands transferred from SDS PhastSystem™ gels. Samples like those of Fig. 3 were electrotransferred to Nylon 66 membrane and probed with anti-chlamydial 3 rabbit IgG preparations followed by detection of bound IgG using an anti-rabbit IgG peroxidase conjugate. Similar, if not identical, major bands just above and below the 60,000 MW position are evident in material transferred from lanes with GLXA isolated from either culture media (a) or elementary bodies (b).

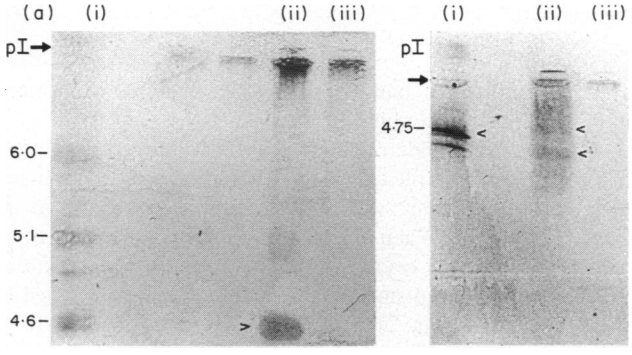

Figure 5. PhastSystem™ Isoelectric focusing analysis: samples were loaded onto the centre of precast 5% gel-containing ampholytes to generate gradients with pI of 3-9 gradient (a) or 4–6·5 (b). GLXA was affinity purified from supernatant of cultures infected with serovar B organism; control samples were similarly prepared from uninfected cultures. Samples were focused for 460 Vh, then silver nitrate stained. (a) Lane (i) pI standards; (ii) affinity-purified GLXA; (iii) sample purified from control culture supernatants. (b) Lane (i) protein standard with a pI of 4·75; (ii) affinity-purified GLXA; (iii), sample purified from control cultures. Arrows indicate loading position of samples; arrow heads indicate position of GLXA.

DISCUSSION

A genus-specific antigen, previously characterized as an exoglycolipid, can be obtained from infected cell culture supernatants. Studies presented here were aimed at assessing biological relevance utilizing molecular shift chromatography. An important finding was derived from the data obtained using IgG from sera of patients with lymphogranuloma venereum (FA titre against chlamydia ≥ 2560). The results clearly indicate that a significant fraction of this IgG population can recognize epitopes of the exoglycolipid isolated from *in vitro* culture media. The purpose of this experiment was to assess whether this GLXA was an artifact of *in vitro* cultivation or a component relevant to natural infections. The antigen used in this study was purified via affinity chromatography by binding to a monoclonal IgM antibody. The molecular shift data obtained using this purified component are significant since they demonstrate considerable epitope binding by IgG synthesized in response to a natural infection. The high proportion of antibodies in this IgG directed toward the GLXA antigen indicates the exoglycolipid may be an immunodominant group of chlamydia.

Other experiments were aimed at further defining GLXA. The genus-specific exoglycolipid was immunopurified from various serovars, including J, B, and L_2 either by an affinity column prepared with anti-GLXA monoclonal antibody or by dissociation of immune complexes isolated from molecular shift chromatography. The preparations were evaluated by SDS–PAGE. Although one cannot determine the specific molecular weight of glycolipid by this technique, two facts are evident from the data. First, a specific and reproducible pattern is generated by samples from the different biovars prepared by either method of immunopurification. Second, the pattern indicates a predominant fraction which resolves into two bands bracketing the 60,000 MW standard. This observation is of interest since SDS–PAGE profiles shown by others (Newhall, 1987) seem to indicate a major protein determinant with an approximate MW of 60,000; if silver staining were utilized as the indicator in such studies, there is a possibility that the exoglycolipid, and not the 60,000 MW protein, is being detected.

The SDS–PAGE analyses also indicate that a variety of molecular weight species exist and are detectable, with bands also occurring at 43,000 MW and 30,000 MW. This overall pattern is consistent with different serovars and appears to be identical whether the GLXA is isolated by monoclonal antibody affinity chromatography or dissociation of polyclonal antibody–antigen complexes isolated by molecular shift chromatography.

In other studies, isolated organisms propagated in yolk sac were examined for the presence of GLXA. These preparations showed that the exoglycolipid can be extracted from elementary bodies as well as being secreted by infected cell cultures. In SDS–PAGE preparations, the major silver-stained bands appear as a doublet in the 60,000 MW region, bracketing the region where protein of this molecular weight would migrate. For this reason it is again important that reagents specific for protein in the range of 30,000 MW or 60,000 MW not be cross-reactive with the GLXA described here. In addition, the SDS–PAGE banding patterns, transferred and probed with IgG in Western blots, reveal that the major components obtained from supernatant or from EB are identical. The GLXA thus appears to be a normal component of chlamydial EB and, although it must be extracted when purified mature organisms are used as a source, it is shed during the course of the infective cycle as demonstrated by immunoreactivity with sera from lymphogranuloma venerium (LGV)-infected patients.

The isolectric focusing data further characterize the GLXA and demonstrate bands with pI of 4·6 and 4·7. This isoelectric point indicates acid groups are associated with this antigen. Two aspects of these isoelectric focusing data are relevant. First, the existence of two bands of very similar, acidic pI, supports the contention that molecular weight heterogeneity may be a function of antigen breakdown. Certainly a sequential cleavage into epitope-containing blocks would result in the observed

heterogeneity and could explain the dual acidic bands which are found. Second, the acidic pI of purified GLXA is consistent with our earlier observations concerning the elution of antigen from DEAE-Sepharose columns (Stuart *et al.*, 1987; Stuart & MacDonald, 1982).

Chlamydia elaborate a number of antigens on their surface or secrete them into the surrounding microenvironment (MacDonald, 1985; Schacter & Caldwell, 1980). The studies presented in this paper indicate that an exoglycolipid is not only present on elementary bodies but is also secreted into infected cell culture media. Since circulating antibody of infected individuals is strongly reactive with GLXA, this antigen may constitute a major antigenic determinant perceived during a natural infection. It is therefore of interest to locate the determinants on infected cells or elementary bodies and these studies are now in progress.

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