Examination of chlamydial glycolipid with monoclonal antibodies: cellular distribution and epitope binding

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SUMMARY

A chlamydial glycolipid antigen (GLXA) is shed into the medium of *C. trachomatis*-infected cell cultures. This study screened monoclonal antibodies (mAb), prepared in different laboratories by immunization with embryonated eggs propagated elementary bodies (EB), for their ability to bind with infected cells and to react with purified GLXA isolated from supernatants of infected McCoy cells. The fluorescent antibody (FA) staining pattern exhibited by a number of mAb indicated that they bound antigen present within the inclusion and at the inner membrane surface of infected cells; the observed pattern differs significantly from the distribution seen when anti-lipopolysaccharide (LPS) (mAb) were used. The staining pattern observed by immunofluorescence was confirmed and extended by ultrastructure studies of immunogold-labelled, infected human endometrial gland epithelial cells (HEGEC) and a human endometrial carcinoma-derived cell line (RL95-2). Additionally, the immunoelectron microscopic studies revealed binding within the inclusion and on reticulate bodies, within the cell cytoplasm and at the surface of infected cells. The specificity of the reactive mAb, examined by molecular shift chromatography and isolated, affinity-purified GLXA, indicated that two mAb of the IgG isotype recognized an antigen which had been purified from tissue culture supernatants by affinity chromatography using an IgM mAb. The results suggest that GLXA is an important determinant whose role and function during *in vitro* and *in vivo* infections deserves further analyses.

INTRODUCTION

Previous studies have indicated that *Chlamydia trachomatis* produce and release a glycolipid antigen during the course of cultivation *in vitro* and that human sera from patients with clinically defined lymphogranuloma venereum (LGV) contain IgG antibodies which recognize glycolipid that has been affinity-purified from tissue culture supernatants. This finding suggested the glycolipid is produced during a natural infection *in vivo*. Obtaining specific monoclonal antibodies (mAb) and utilizing them to detect glycolipid distribution in infected cells would provide a key to assessing the possible role of this apparently dominant epitope during the natural infectious process.

A series of studies, therefore, was undertaken to examine, first, the distribution of glycolipid in infected cells, and then assess the reactivity of mAb presenting a strong staining pattern in fluorescent and ultrastructural analyses; the ultrastructural studies would provide more definitive evidence of the localization of chlamydial glycolipid exoantigen (GLXA) within infected cells. mAb presenting a common binding pattern were examined for their reactivity with the isolated glycolipid. Since glycolipid did not consistently bind to microtitre wells, the reactivity of mAb with GLXA could not be assessed by enzyme-linked immunoassay (EIA). Instead, mAb-GLXA binding, as well as competition between different antibodies for binding with GLXA, was examined by molecular shift chromato-
mAb binding to a chlamydial glycolipid epitope

Cultivation. Importantly, while assessment by this method is more cumbersome, it has the advantage of being carried out in liquid phase and so the natural configuration of GLXA is retained during interaction with antibodies.

The findings from these studies support four general conclusions. First, a number of mAb, generated by immunization with chlamydial elementary body (EB), harvested from embryonated eggs (EB-cs), react with glycolipid isolated from culture medium of infected cells. Second, the glycolipid is localized not within the chlamydial inclusion body but is also found in the inclusion membrane, the host cell cytoplasm, host cell surface and the surrounding micro-environment. Third, the molecular shift data indicate that at least some of the mAb which recognize the glycolipid antigen compete for binding to the same, or overlapping, epitopes. Finally, the evident competition demonstrated between different mAb strongly suggests that GLXA is a normal component of both EB and reticulate body (RB) forms and quite antigenic, routinely eliciting antibody production.

**MATERIALS AND METHODS**

*C. trachomatis*

Cultivation essentially followed the methods described previously, except as follows. McCoy cells were used and cell division was inhibited with cycloheximide (1 μg/ml; Whittacker overlay medium; Whittacker, Walkersville, MD). Serovars A, B, C and F also were grown in Corning roller bottles (Fisher Scientific, Pittsburg, PA) seeded with McCoy cells in 150 ml IMEMZO (Irvine Scientific, Santa Ana, CA) containing 3% foetal calf serum (FCS). At confluence, medium was replaced with 0–5–3 ml of inoculum in complete overlay medium. After rolling (3 r.p.m.) for 2 hr at 35°C, 200 ml of complete overlay medium were added to the bottles. Incubation continued at 35°C, 3 r.p.m., until termination. *C. trachomatis*, E/UW-5/Cx, a human urogenital isolate, and lymphogranuloma venerium (LGV) (L2/434/BU) were cultivated in McCoy cells as described by Moorman et al. Human endometrial gland epithelial cells (HEGEC) and the human carcinoma cell line (RL95-2) were cultivated following previously described protocols.

**Monoclonal production**

mAb were prepared by inoculating purified LGV-2 organisms from embryonated eggs (EB-cs) into BALB/c ByJ mice. Hybridoma cells were prepared by polyethylene glycol fusion of mouse myeloma cells (SP2/0 Ag 4-1) with spleen cells from the immunized mice following the method of Galfrie & Milstein. Antibody secretion was assessed by EIA screening for binding by hybridoma supernatants to microtitre wells coated with chlamydial EB. Bound immunoglobulin was detected with horseradish peroxidase (HRP) conjugated protein-A. Supernatants were tested against all 15 serovars of *C. trachomatis*, C. psittaci 6BC and meningogranulouis to demonstrate recognition of a genus antigen. mAb were isoyptyped with the Zymed MonoAB ID EIA kit (Zymed Lab Inc., San Francisco, CA). Alternatively, Renografin-purified, culture grown L2 serovar organisms were injected into BALB/c ByJ mice. An initial GLXA preparation (Ip), isolated from serovar B-infected McCoy cells as described previously, provided in vitro stimulation of spleen cells removed from primed animals. The resultant hybridomas were screened for binding to GLXA and EIA-positive clones were grown as ascites. An IgM mAb was isolated either by gel-filtration through Sepharose CL 6B (Pharmacia/LKB; Piscataway, NJ) or by low salt precipitation and used to prepare an affinity column.

**Affinity purification**

Initial preparations were isolated from supernatants of control or infected McCoy cell cultures as previously described.

Samples, in phosphate-buffered saline (PBS), were affinity purified by filtration over an Affigel-10 (Bio-Rad, Richmond, CA) column (0.7 × 5.0 cm) to which anti-GLXA IgM was coupled according to the manufacturer's specifications. Bound material was eluted in high salt, desalted by passage over Octyl-Sepharose CL 4B and stored at -20°C until used. Samples were assessed using the PhastSystem™ (Pharmacia) SDS-PAGE as described previously.

**External labelling of antibody preparations**

IgG was labelled with 125Iodine as described previously. Briefly, 10–25 μl of antibody containing mouse ascites were labelled using enzymobeads from Bio-Rad (Richmond, CA) and, subsequently, the 155,000 MW fraction was isolated by gel-filtration chromatography.

**Molecular shift chromatography**

Glycolipid does not consistently bind well to microtitre plates so GLXA/antibody interaction was assessed using molecular shift chromatography as described previously. Antigen preparations were combined either with isolated IgG or the IgG-containing fractions of 125I-labelled ascites fluid, then gel filtered. The larger molecular weight of GLXA/IgG complexes causes them to elute earlier from a gel filtration column than does IgG alone and they can be detected by absorbance at 280 nm and/or the c.p.m. of the 1-m1 fractions. 125I isotope emissions were detected with a Beckam 4000 Gamma counter. In competition experiments, EIA verified the presence of unlabelled, competing IgG in the eluant. Briefly, 100–μl aliquots were incubated in Dynatech Immunolon I Removawell strips (Dynatech, Chantilly, VA); after blocking, 1 hr with 1% BSA/PBS, bound antibody was detected using HRP-conjugated goat anti-rabbit or rabbit anti-mouse IgG (H&L specific, Jackson IMMUNOCHEMICAL Laboratary, West Grove, PA), diluted 1:1000 in PBS. Absorbance at 650 nm of the TMB substrate (Kirkegaard and Perry, Gaithersburg, MD) was quantified with a Vmax Kinetic Plate Analyzer (Molecular Devices, Palo Alto, CA).

**Fluorescent antibody staining**

Indirect fluorescent antibody (FA) staining of fixed cells followed the method of Nichols & McComb. Infected cells, grown on 12-mm coverslips in 24 multiwell plates (Fisher Scientific, Pittsburg, PA) were fixed with ice-cold MEOH and stored at -20°C until used. Subsequently, samples were incubated with anti-chlamydial or control antibody for 30 min at 37°C. Bound primary antibody was detected by incubation for 30 min at 37°C with an Fc-specific, rhodamine-conjugated rabbit anti-mouse IgG (Jackson IMMUNOCHEMICAL Laboratory, West Grove, PA). Photography utilized an Olympus 25, 35 mm camera (ASA 1600 Kodak film) mounted on a Zeiss A.7082 Oberkichen microscope, illuminated with a 12v/100z halogen lamp.
**Immunogold labelling**

A hormone responsive, human endometrial carcinoma-derived cell line RL-95-2\textsuperscript{5} or primary HEGEC were cultivated and infected with *C. trachomatis* as described previously.\textsuperscript{6} Cells, fixed with 2% paraformaldehyde:0.5% gluteraldehyde in 0.1 m Sorsenson buffer (pH 7.2), were embedded in Lowicryl K4M, using the procedure of Carlemalm *et al.*\textsuperscript{10} for low temperature preparation for immunoelectron microscopy. Thin sections, cut with glass knives, were collected on nickel grids and immunolabelled by a procedure adapted from Timms.\textsuperscript{11}

**RESULTS**

The cellular distribution of GLXA during *in vitro* infection was explored. Infected McCoy cells were examined at selected times post-infection by FA staining and the fluorescent pattern of mAb 89MS30, shown in Fig. 1, is typical of various mAb tested. The staining was characterized as follows: (i) inclusion bodies exhibited very bright fluorescence; (ii) fluorescence was apparent in the vacuole space surrounding this mass; (iii) in addition, fluorescence was localized apparently around the inner surface of the inclusion membrane as well as the infected cell surface (Fig. 1a, b, c). No fluorescence was demonstrated in controls, which consisted of identically infected cells, probed with normal mouse IgG and a Rhodamine-conjugated, affinity-purified second antibody (Fig. 1d).

To verify that the GLXA distribution observed for McCoy cells was not unique to this cell line, and to more specifically localize the *C. trachomatis* antigen binding, immunoelectron microscope studies of serovar E-infected RL95-2 cells were carried out. Figure 2 is representative of the immunogold labelling pattern on thin sections exposed to monoclonal 89MS30. Gold particles were evident within the inclusion, associated with developing RB and within 'space' of the inclusion. Additionally, the inclusion membrane, the rim of cytoplasm surrounding the inclusion, the cell-surface and the extracellular space were labelled (Fig. 2a, b). Uninfected cells processed identically showed limited background binding and no significant accumulations of gold particles (Fig. 2c).

In a third study, primary differentiated human endometrial epithelial cells were infected with the more virulent and invasive LGV serotype. In Fig. 3, TEM micrographs demonstrate that significant quantities of 89MS30 mAb binding material were present and have the same general distribution as was observed for serovar E-infected RL95-2 cells (cf. Fig. 2a, b). LGV-infected cells, however, appeared to contain more mAb binding material, both within the inclusion space and associated with the developing organisms. As a control, HEGEC cells infected with LGV were stained with the gold-conjugated second affinity antibody only, omitting the primary mAb, to show there was no significant non-specific binding (Fig. 3c). Thus, both ultrastructure series confirmed GLXA in the inclusion, and on RB. Additionally they demonstrated GLXA in inclusion-associated cytoplasm, and in the surrounding extracellular space.

To demonstrate the mAb binding constituted specific recognition of GLXA, a number of mAb which had presented similar binding patterns were tested. Since microtitre plates are variable in their binding capacity for GLXA, recognition of GLXA was assessed using the more cumbersome method of molecular shift chromatography rather than EIA. The molecular weight of the complex formed by antigen and its cognate antibody was greater...
mAb binding to a chlamydial glycolipid epitope

Figure 2. TEM immunogold staining of RL95-2 cells. Thin sections of RL95-2 cells infected with serovar E (a, b) or uninfected (c) probed with anti-GLXA mAb (89MS30; 1/100 in 1% ovalbumin/PBS) followed by colloidal gold-conjugated goat anti-mouse (1/50 in 1% ovalbumin/PBS) to detect the distribution of GLXA. Magnification: (a) × 27,000; (b) × 36,000; (c) × 42,000. Note minimal binding to cross-reactive epitopes on the eukaryotic cell (c).

Figure 3. TEM immunogold staining of HEGEC cells. Thin sections of HEGEC cells infected with LGV (a, b) HEGEC cells treated with 89MS30, followed by colloidal gold conjugate as described in Fig. 2. (c) Control, infected cells incubated only with the colloidal gold second antibody; note absence of non-specific labelling by the colloidal gold treatment. Magnification: (a) × 21,400. (b) × 28,000; (c) × 33,250.

The molecular shift technique was utilized next to verify that complex formation was antigen specific. Material, purified from control and infected culture supernatants utilizing an IgM affinity column, was reacted separately with aliquots of mAb 89MS30 IgG, gel filtered and the fraction aliquots tested by EIA to detect mouse IgG. Figure 5 shows a typical profile of absorbance at 650 nm. Two facts are evident: (i) mAb interacted with the affinity-purified GLXA, but not with control material, to form a multifraction shift peak; and (ii), as expected, mouse IgG molecules were present in this peak. Thus, the mAb recognized and specifically bound with the GLXA.

Subsequently, different mAb were screened using affinity purified, native GLXA, to assess and partially quantify their

than the weight of either component alone, therefore during gel filtration complexes eluted earlier than IgG alone. Initially, the dose dependency of the molecular shift peak size was verified by examining the absorbance profiles from interaction of different quantities of GLXA with a standard concentration of purified rabbit polyclonal anti-chlamydia IgG antibody. The observed absorbance at 280 nm (Fig. 4) demonstrated that larger quantities of antigen generated increasingly larger peaks eluting just after the column void volume. Thus, peak size was a quantitative reflection of antibody binding with antigen. Normal IgG did not produce a shift peak (data not shown).
suggested the presence of a GLXA-specific subpopulation of antibody molecules. Additional assays with this radiolabelled rabbit IgG. GLXA and a GLXA-specific monoclonal verified this subpopulation. As Fig. 7 illustrates, the level of radiolabelled rabbit antibody in the post-void volume shift peak fractions was significantly diminished in the presence of monoclonal 89MS30. Thus, among the rabbit B-cell clones, a significant portion produces antibody which interacts with the epitope recognized by the mouse monoclonal, and the mouse antibody can displace the rabbit antibody binding in the antigen/antibody complexes.

In each set of experiments, the same number of total c.p.m. and quantity of GLXA were used. Data from the different molecular shift assays therefore could be examined to determine, quantitatively, the decrease in c.p.m. associated with antibody competition. These comparisons are presented in Table I and illustrate three main points: (i) different mAb of the IgG isotype are reactive with the glycolipid purified with an IgM ligand affinity column; (ii) simultaneous interaction of two different mAb with affinity-purified GLXA demonstrated a quantitatively significant competition for binding with the antigen, resulting, for example, in a 69% decrease in the number of c.p.m. in the antigen/antibody complex-containing fractions; (iii) the rabbit polyclonal antibody also reacted with the GLXA purified by affinity chromatography, and in the presence of an anti-GLXA monoclonal the binding of radiolabelled rabbit antibody was diminished by 75%, suggesting it recognized the GLXA epitope; (iv) polyclonal interaction with non-affinity purified GLXA versus affinity-purified GLXA demonstrated an increase of 54% in the c.p.m. of the shift peak fractions. A broader spectrum of radiolabelled rabbit antibodies should recognize the broader spectrum of epitopes present in the non-affinity purified sample and therefore generate a larger peak containing antigen/antibody complexes.

**DISCUSSION**

GLXA is a genus-specific chlamydial antigen distinctly different from genus-specific LPS. The major differences include: (i) abundant secretion into the medium of infected cell cultures; (ii) facile isolation; (iii) a distinct banding pattern on SDS–PAGE; and (iv) a different isoelectric point.\(^2\) That the structures of GLXA and LPS\(^4\) are disparate has been confirmed by chemical analyses.\(^4\) No KDO, heptose, glucosamine, nor the fatty acids normally associated with lipid A, have been identified in GLXA preparations. Instead, galucose, mannose and possibly galactose constitute the major antigenic determinant and only two fatty acids appear to be associated with the purified antigen.\(^5\) It is notable that antibodies to GLXA are present in the antisera of individuals undergoing chlamydial infection and therefore GLXA may play a significant role in the process of infection, the replication of the organism, and the immune response of the host including, perhaps, the immunopathology associated with disease.\(^2\)

To begin delineating the function(s) in cellular biology for GLXA, observations of light and TEM samples were carried out and the results permit a number of conclusions. (i) The antigen is displayed at the surface of infected cells and within the inclusion body itself. (ii) Inside the inclusion, antigen is distributed on developing chlamydia, within the inclusion space, and on the inner surface of the inclusion membrane. (iii) Antigen

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**Figure 4.** Dose-dependency of complex peak. Profiles of absorbance at 280 nm generated by different quantities of Octyl-Sepharose-isolated (Ip) glycolipid antigen reacting with IgG from rabbits immunized with purified C. trachomatis EB-cs. The doses of antigen used, expressed as ml/eq, are equivalent, respectively, to volumes of 25 (Ο), 50 (△) or 75 (Ο) ml of supernatant from heavily infected cultures.
also is present in the cytoplasm of infected cells. Initial studies on the distribution of a chlamydia genus-specific antigen were carried out by Richmond & Stirling.\textsuperscript{12} Their results, utilizing polyclonal antibody, demonstrated membrane-bound antigen in infected cells treated with formalin, then carefully dried. The mosaic of membrane-bound antigen was developed by peroxidase-conjugated second antibody. Our results are similar to the immunofluorescent and membrane-bound TEM studies reported by these authors. In another study, in which a monoclonal to chlamydia-specific LPS was used, Karimi et al.\textsuperscript{13} demonstrated two different distributions: (i) cells whose entire surface was stained by immunofluorescence, a pattern never observed with anti-GLXA staining; and (ii) cells with a more punctate staining distribution similar to that reported here. An interesting aspect, unique to the present study, is the mosaic of antigen distribution within the inclusion, the infected cells and the microenvironment. The elaboration of the antigen in the infected cell is obvious by 36 hr post-infection and detectable on the surface of both RB and EB. In particular, significant quantities of GLXA appear to be associated with RB develop-
This investigation means of the exception of GLXA supernatants, the which remain in the infection assays of during infectionness and/or to appear complexes which during but the yet antigen-specific quantity of anti-GLXA its transport mechanism(s).

Table 1. Immunoglobulin interaction with glycolipid antigen

<table>
<thead>
<tr>
<th>Sample</th>
<th>C.p.m. shift peak</th>
<th>% change</th>
<th>C.p.m. IgG peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse monoclonal IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>87MS4*</td>
<td>0.51 x 10^6</td>
<td></td>
<td>0.650 x 10^6</td>
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<tr>
<td>87MS4* + GLXA</td>
<td>2.40 x 10^4</td>
<td></td>
<td>0.950 x 10^6</td>
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<tr>
<td>89MS30* + control</td>
<td>1.60 x 10^4</td>
<td></td>
<td>0.352 x 10^6</td>
</tr>
<tr>
<td>89MS30* + GLXA</td>
<td>3.90 x 10^4</td>
<td></td>
<td>0.630 x 10^6</td>
</tr>
<tr>
<td>89MS30* + GLXA</td>
<td>0.23 x 10^4</td>
<td>-69</td>
<td>0.604 x 10^6</td>
</tr>
<tr>
<td>+ 89MS35</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Rabbit polyclonal IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>88MS334* + GLXA</td>
<td>83.0 x 10^4</td>
<td>1.8 x 10^6</td>
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<td>0.7 x 10^6</td>
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<tr>
<td>+ 89MS30</td>
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</tr>
<tr>
<td>88MS334* + GLXA(1)</td>
<td>128.5 x 10^4</td>
<td>+54</td>
<td>1.4 x 10^6</td>
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</table>

* 125I-labelled IgG.

All control and GLXA preparations were affinity purified except (1). This was an initial GLXA preparation eluted from Octyl-Sepharose.

ment. The enzymatic mechanism(s) involved in synthesis, the initiation of this synthesis (whether by chlamydia or by the host cell as directed by the organism), the mechanism of GLXA transport through the cytoplasm to the host cell membrane and its secretion into the microenvironment all are significant considerations which remain to be clarified. In addition to distribution, the present TEM observations also indicate that the quantity of anti-GLXA mAb binding was increased when the infection was initiated with LGV rather than serovar E. This observation may be relevant to considerations of LGV invasiveness and/or virulence. During infection by either serovar, significant quantities of GLXA appear and a time-course study of GLXA production could help determine the nature of events. This investigation is in progress.

The data presented above also demonstrated features regarding GLXA/antibody interaction. (i) Complexes resulted from antigen-specific recognition by antibody and these complexes could be assessed by liquid phase quantification. (ii) Competition during liquid phase quantification provided a means to assess an antibody for its ability to recognize the antigen or to determine the relative abundance of anti-GLXA-specific antibodies amongst a polyclonal population. Thus, effective competition between different antibodies for specific epitope binding would diminish the c.p.m. in the antigen/antibody complexes (shift peak). However, the presence of the unlabelled, competing antibody would be detectable by EIA assays of complex peak fractions and these were the results obtained during the present studies. Thus, the shift analyses, as well as immunofluorescent staining, demonstrated that an array of mAb have been generated which recognize GLXA and they appear to compete for the same or an overlapping epitope.

The monoclonals tested in the present studies, with the exception of the IgM (utilized to make the affinity column for antigen isolation), were generated by immunization with EB-cs, yet they recognized an antigenic determinant purified from cell culture supernatants with an affinity chromatographic column in which an IgM monoclonal provided the specificity. Not only was the antigen recognized by a second monoclonal antibody, but the specific binding between antigen and antibody was inhibitable by yet a third IgG monoclonal also generated by immunization with EB-cs. The specific level of inhibition observed undoubtedly reflects differences in the concentration and the affinity of the two monoclonals. An IgG monoclonal antibody also was able to significantly inhibit the binding of polyclonal rabbit antibody to tissue culture-derived GLXA. These polyclonal antibodies are a subset of the total anti-GLXA population, generated by EB-cs immunization, since significantly increased binding (54%) occurred when Ip versus Ap GLXA was used.

Critical identification of a specific epitope, in terms of cellular distribution or biological interaction, demands a stringent analysis of the antibody utilized in defining it. That the mAb (89MS30) has a unique specific binding site to a GLXA epitope has been demonstrated by idiotypic analyses. Anti-idiotypic antibodies, raised in guinea-pigs, specifically inhibited the binding of GLXA by 89MS30. Additional data (L. L. An, manuscript in preparation) have confirmed that the anti-Id is the internal image of GLXA. It has been shown previously that human antibody generated during a natural infection with C. trachomatis recognizes and binds GLXA purified from tissue culture supernatants. When taken together, the present molecular shift data, along with the previous information strongly suggests that GLXA is an immunodominant group and its distribution among infected cells supports the suggestion that it may be significant to infections. Additionally, a high concentration of the antibody to chlamydia is directed against this antigen whether the host is inoculated with EB-cs (as shown here with the rabbit antiserum) or is undergoing a natural chlamydial infection. Seventy-five per cent of the rabbit anti-chlamydia IgG antibody was inhibited by anti-GLXA monoclonal antibody. In the case of human anti-LGV antiser (FA titre of > 2560) a considerable quantity of antibody was directed toward GLXA. Thus, synthesis and release of GLXA from LGV-infected cells (see above) coupled with high serum titres of anti-GLXA antibodies could lead to sequelae of pathology as a function of immune complexes. At present, similar quantitative data on the level of recognition for LPS or other genus-specific antigens is not available. However, the present results suggest that the B-cell repertoire has a number of anti-GLXA receptor bearing cells. This probably is not due to mitogenic expansion of the B-cell clones by GLXA since, unlike LPS, it is not mitogenic for lymphocytes in vitro (K. Troidle, manuscript in preparation).

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