CHLAMYDIAL DISEASE PATHOGENESIS
Ocular Hypersensitivity Elicited by a Genus-specific 57-kD Protein

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The pathogenic mechanisms leading to the development of blinding trachoma are not well understood. Primary infection with Chlamydia trachomatis usually results in a self-limiting, mucopurulent, follicular conjunctivitis that resolves without adverse sequelae. However, upon repeated ocular exposure to C. trachomatis, chronic inflammation and progressive trachoma may develop (1-5). Thus, it has been suggested that much of the tissue damage observed during the early stages of trachoma is immunologically mediated (1, 6, 7). Repeated exposure to chlamydial antigens either by reinfection or by the establishment of persistent or recurring infections could function as the antigenic stimulus for chronic inflammation.

Data from previous studies suggest that delayed hypersensitivity (DH) to chlamydial antigens may be involved in the pathogenesis of trachoma. For example, Monnickendam et al. (8) demonstrated, using a guinea pig model of chlamydial conjunctivitis, that repeated ocular challenges led to chronic conjunctivitis, followed by pannus and scarring of the conjunctiva. This chronic inflammation lasted for several months and was characterized by a predominantly mononuclear cellular infiltrate. Furthermore, the chronic conjunctivitis was not associated with the presence of chlamydial inclusions, a finding similar to that observed in trachoma. Data obtained from early trachoma vaccine trials also support the hypothesis that ocular DH may be a deleterious immune response that leads to blinding trachoma. In those studies, prior vaccination resulted in more severe clinical disease upon challenge than observed in nonvaccinated groups (9-13). Moreover, challenge with a heterologous serovar of C. trachomatis often induces a much more intense ocular inflammatory response (9, 11, 14). Because ocular hypersensitivity to chlamydial antigens is believed to be associated with the development of blinding trachoma, it is of interest to identify chlamydial antigens that elicit such a response and determine their role in the pathogenesis of chlamydial disease.

Recently, we demonstrated that a soluble Triton X-100 extract of chlamydial elementary bodies (EBs) (TX-100 extract) elicited an ocular DH reaction in both immune
guinea pigs and monkeys (15, 16). TX-100 extracts from strains of both C. trachomatis and C. psittaci elicited an ocular DH response, indicating that the response was to a common antigenic determinant (genus specific) (15). LPS, a chlamydial group antigen, is the major component of the extract, but purified LPS did not elicit an ocular DH response in immune animals (15, 16). In addition, purified chlamydial major outer membrane protein (MOMP), which is present in only trace amounts in the extract, is similarly negative when tested in immune animals (16). Two other genus-specific antigens present in the TX-100 extract are proteins having molecular masses of 45 and 57 kD. In this study, we purified these proteins by immunoaffinity chromatography and demonstrated that the 57-kD protein, but not the 45-kD protein, elicited an ocular hypersensitivity response characterized by a predominantly mononuclear macrophage and lymphocyte cellular infiltrate in immune guinea pigs.

Materials and Methods

Organisms. The C. trachomatis serovars A/Har-13, B/TW-5, Ba/Apa-2, C/TW-3, D/UW-31, E/Bour, F/IC-Cal-13, G/UW-57, H/UW-4, I/UW-12, J/UW-36, K/UW-31, L1/LGV-440, L2/LGV-434, and L3/LGV-404, C. psittaci strains guinea pig inclusion conjunctivitis (GPIC), and meningopneumonitis (Mn) were grown in HeLa 229 cells, and EBs were purified by discontinuous density centrifugation in Renografin (E. R. Squibb and Sons, Princeton, NJ) (17). Inclusion-forming units (IFU) were determined by methods described previously (18).

Animals, Chlamydial Infection, and Ocular Hypersensitivity. Male and female Hartley guinea pigs, 8–12 wk old from a chlamydial-free colony, were used throughout these studies. Animals were bred and maintained at the Rocky Mountain Laboratories, Hamilton, MT. Animals were infected by placing 10 μl containing 10 ID₅₀ (10 × 10⁵ IFU) of GPIC onto the lower conjunctiva as described previously (15). Conjunctiva of infected guinea pigs were culture negative by 4 wk after infection. These guinea pigs are referred to as ocular immune and were used to test for ocular hypersensitivity 6–8 wk after primary infection. Ocular hypersensitivity was assessed by placing 25 μl of the appropriate antigen solution onto the lower conjunctival sac. The hypersensitivity response was assessed clinically at 2, 12, 18, 24, 48, and 72 h and was scored using a scale of 0 to 4 (15) (0, negative; 1, slight hyperemia and edema of the lower palpebral conjunctiva; 2, hyperemia and edema of the lower palpebral conjunctiva with slight hyperemia of the bulbar conjunctiva; 3, overt hyperemia and edema of the lower palpebral and bulbar conjunctiva; and 4, same as 3 with the addition of mucopurulent exudate). Peak inflammation was observed at 24 h after instillation of antigen. The time course of the inflammatory response and the nature of the cellular infiltrate (see Fig. 3) has led us to refer to this response as an ocular DH.

Affinity Chromatography and Antigen Purification. The protein A binding fraction of a polyclonal monospecific rabbit antisera against the genus-specific 57-kD chlamydial protein and an mAb (purified IgG), reactive against the 45-kD genus-specific chlamydial protein (GPIC-IV BI, IgG, a kind gift from Dr. You-Xun Zhang, Rocky Mountain Laboratories), were used to prepare the affinity columns. The polyclonal anti-57-kD antisera was prepared by immunizing rabbits with isolated immunoprecipitin bands excised from two-dimensional immunoelectropherograms as previously described (19). The immunoprecipitates used as immunogen in the preparation of the anti-57-kD antisera correspond to the single common crossreacting antigen observed by crossed immunoelectrophoresis (20). mAb GPIC IV-BI was prepared by immunizing BALB/c mice with GPIC EBs and following previously described procedures (21, 22). The purified antibodies were covalently crosslinked to the support matrix as described previously (23). 1-ml packed volume of swollen protein A-Sepharose CL-4B (Sigma Chemical Co., St. Louis, MO) beads was gently mixed with 10 mg (at 1 mg/ml in 50 mM PBS, pH 7.2) of either anti-45-kD or anti-57-kD antibody at 22°C for 45 min. The immunomatrix (protein A-Sepharose antibody) was washed three times with 100 mM borate buffer, pH 8.2, followed by a single 20-ml wash with 200 mM triethanolamine, pH 8.2. The antibody was covalently crosslinked to the protein A-Sepharose by
resuspending the immunomatrix in 20 ml of freshly prepared 20 mM dimethylpimelimidate-
dihydrochloride in 200 mM triethanolamine, pH 8.2, and gently mixed for 45 min at 22°C. The
immunomatrix was pelleted by light centrifugation and resuspended in 1.0 ml of 20 mM ethanolamine, pH 8.2. After 5 min at 22°C, the immunomatrix was washed once with 10 ml of 100 mM borate buffer, pH 8.2, poured into a column, washed with 20 ml of PBS, and stored at 4°C until used.

The 45 and 57-kD chlamydial proteins were purified from a Triton X-100 soluble extract of GPIC EBs (15). 10 ml of the soluble GPIC extract was preabsorbed with 0.1 g of protein A-Sepharose for 45 min at 22°C to remove nonspecifically binding components of the extract. The preabsorbed antigen extract was sequentially passed through the anti-45-kD and anti-57-kD columns, respectively. Each column was washed with 20 ml of 50 mM phosphate buffer, pH 7.2, containing 500 mM NaCl and 0.5% Triton X-100. Bound antigen was eluted with 3.0 M potassium thiocyanate (KSCN) in PBS. 1-ml fractions were collected, dialyzed overnight against PBS at 4°C, and analyzed for purity by SDS-PAGE and immunoblotting. Approximately 500 and 300 μg of protein were eluted from the anti-45-kD and anti-57-kD columns, respectively. Fractions containing purified protein were assayed for their ability to elicit ocular hypersensitivity as described above.

**SDS-PAGE, Electrophoretic Transfer, and Immunoblotting.** SDS-PAGE was performed using 12.5% polyacrylamide gels as described by Dreyfuss et al. (24), except chlamydial whole-cell lysates and samples were prepared with 2X Laemmli sample buffer (25). The apparent molecular masses of chlamydial proteins recognized by the anti-57-kD and anti-45-kD antibodies were determined by comparing the migration distances of these immuno-reactive proteins with a plot of migration distance vs. the log molecular mass of several protein standards (Bio-Rad Laboratories, Richmond, CA). Electrophoretic transfer and processing were done as described previously (22).

**Histology.** Guinea pigs were killed with T-61 euthanasia solution (Hoechst Corp., Somerville, NJ). The upper and lower eyelids were removed, fixed in neutral-buffered 10% formalin, and stained with hematoxylin and eosin as described previously (15).

### Results

**Characteristics of Chlamydial Proteins.** The SDS-PAGE polypeptide profile of the 15 serovars of *C. trachomatis* and two strains of *C. psittaci* are shown in Fig. 1 A. The 57- and 45-kD proteins are indicated by arrows and the MOMPs are the major staining protein bands that appear in the bracketed area of the gel. The genus specificity of the anti-57-kD and anti-45-kD antibodies are demonstrated in Fig. 1 B and C, respectively. The anti-57-kD antiserum reacted with a similar molecular mass protein in all chlamydial strains tested. Similarly, the anti-45-kD antibody reacted with all strains, although more strongly with the 45-kD protein of the *C. psittaci* strains GPIC and Mn. The weaker reactivity to the *C. trachomatis* strains might be explained by quantitative differences in this protein among strains or may simply reflect stronger reactivity to the immunizing species. Slight variability in Mr of this protein among strains was also observed. These data demonstrate the genus specificity of the anti-57-kD and anti-45-kD antibodies, and the prominence of these proteins in chlamydial EBs.

**Immunoblot Analysis of Purified Chlamydial Antigens.** The 45- and 57-kD chlamydial proteins and LPS are genus-specific constituents and major components found in the soluble fraction of the TX-100 extract of GPIC EBs. This extract causes an ocular hypersensitivity response in ocular immune guinea pigs (15). Since the major genus-specific constituent of this extract (LPS) failed to induce ocular hypersensitivity, we purified the genus-specific 45- and 57-kD proteins to determine whether they were constituent of the extract that could elicit such a response. These proteins were
FIGURE 1. Chlamydial specificity of the polyclonal rabbit anti-57-kD antiserum and the anti-45-kD mAb. (A) Coomassie brilliant blue-stained gel of all 15 serovars of C. trachomatis and two strains of C. psittaci, Mn, and GPIC. The MOMP of each vary in Mr and are the major staining polypeptides indicated by the bracket. (B) Immunoblot probed with polyclonal monospecific anti-57-kD antiserum. (C) Immunoblot probed with mAb GPIC IV-B1. The polyclonal anti-57-kD and mAb anti-45-kD antibodies were monospecific and reacted with proteins found on all 15 C. trachomatis serovars and two C. psittaci strains. In some serovars the 45-kD protein comigrated with the MOMP (serovars A, C, H, I, and J) and was thus difficult to distinguish on the Coomassie-stained gel. However, it could be distinguished when probed with the anti-45-kD antibody. The 57- and 45-kD proteins are major genus-specific proteins found on EBs.
purified using immunoadfinity chromatography (Fig. 2). The soluble TX-100 extract of GPIC EBs contains a number of immunoreactive proteins recognized by antiserum raised to GPIC EBs (Fig. 2, lane 2). Passage of this extract through the anti-45-kD column followed by passage through the anti-57-kD column efficiently removed the 45- and 57-kD proteins (Fig. 2, lanes 3 and 5, respectively). They were then eluted from the columns as antigenically homogeneous proteins (Fig. 2, lanes 4 and 6). Homogeneity of the protein preparations was also demonstrated by Coomassie brilliant blue and silver staining of SDS-PAGE gels (data not shown). Noteworthy is the finding that both the 45- and 57-kD proteins migrate as single bands in EB preparations, but were observed as doublets in the extract and purified fractions.

**Ocular Hypersensitivity Elicited by Chlamydial Antigen Preparations.** Chlamydial an-
tigen preparations and affinity-purified proteins were tested for their ability to elicit an ocular inflammatory response in immune and naive guinea pigs (Table I). The purified 57-kD, but not the 45-kD chlamydial protein, elicited an inflammatory response when administered topically to the conjunctival surface of ocular immune guinea pigs. The intensity of the inflammatory response elicited by the purified 57-kD protein (3.1) was similar to that elicited by the soluble TX-100 extract (3.4). Depleting the extract of the 45- and 57-kD proteins did not render the extract noninflammatory. However, the intensity of the ocular inflammation was marginal (2.3) and waned more quickly than the response elicited by the extract containing these proteins.

**Histological Profile of Ocular Hypersensitivity Responses.** To determine the cellular characteristics of the inflammation elicited by the various antigen preparations, hematoxylin- and eosin-stained sections of the palpebral conjunctiva were examined at the time of peak inflammation (24 h post-challenge) (Fig. 3). The inflammatory response elicited by the soluble TX-100 extract and the purified genus-specific 57-kD protein were indistinguishable (Fig. 3, B and C). Both these preparations elicited a subacute inflammatory response characterized by lymphoid hyperplasia and a submucosal infiltrate consisting primarily of mononuclear macrophages and lymphocytes. Occasional polymorphonuclear neutrophils (PMN) were observed at the mucosal surface. In contrast, the inflammatory response elicited by the extract

| Challenge antigen preparation | Immune | Naive |
|-----------------------------|--------|-------|
| *C. psittaci* (GPIC) TX-100 extract (lane 2) | 15/15 (3.4)<sup>4</sup> | 0/6 (<1) |
| GPIC TX-100 extract V<sub>a</sub> anti-45 kD (lane 3) | 10/10 (3.3) | 0/6 (<1) |
| Purified 45-kD protein (lane 4)<sup>6</sup> | 1/10 (<1) | 0/6 (<1) |
| GPIC TX-100 extract V<sub>a</sub> anti-57 kD (lane 5) | 10/10 (2.2) | 0/6 (<1) |
| Purified 57-kD protein (lane 6)<sup>6</sup> | 9/10 (3.1) | 0/6 (<1) |
| TX-100 buffer<sup>3</sup> | 0/6 (<1) | 0/6 (<1) |

<sup>3</sup> Purified GPIC EBs (10<sup>10</sup> IFU) were extracted with 10 ml of 25 mM PBS containing 0.5% Triton X-100 for 30 min at 37°C and centrifuged at 100,000 g for 1 h. The resulting soluble extract was used as hypersensitivity antigen. Fractions of this antigen preparation, corresponding to those described in Fig. 2, were tested for hypersensitivity. Guinea pigs were challenged by placing 25 μl of antigen preparation onto the lower conjunctival sac. Animals challenged with the GPIC TX-100 extract or column passed extracts received ~6-8 μg of protein. Those challenged with the purified 45- or 57-kD proteins received ~1-2 μg of protein.

<sup>4</sup> Ocular hypersensitivity was assessed 24 h after challenge. Inflammation was scored on a scale of 0 (negative) to 4 (overt hyperemia and edema of the conjunctiva with mucopurulent exudate). A clinical score of 2 was considered positive. Data are presented as the number of guinea pigs eliciting a positive ocular hypersensitivity response (clinical score >2.0) over the total number tested.

<sup>5</sup> Mean clinical score.

<sup>6</sup> Purified 45- and 57-kD proteins were mixed 1:1 with 2X Triton X-100 buffer before use.

<sup>7</sup> PBS + 0.5% Triton X-100.
Figure 3. Hematoxylin- and eosin-stained sections of the palpebral conjunctiva from ocular immune guinea pigs 24 h after challenged with (A) TX-100 buffer; (B) soluble TX-100 extract of GPIC EBs; (C) immunopurified 57-kDa protein; and (D) TX-100 extract of GPIC EBs depleted of the 57- and 45-kDa proteins. See footnotes to Table 1 for dosages of antigen administered. M, mucosal epithelium; SM, submucosa; P, polymorphonuclear neutrophil; L, lymphocyte. MΦ, macrophage.
depleted of the 45- and 57-kD proteins was more acute and characterized by a marked PMN infiltrate (Fig. 3 D).

In some instances, DH responses in the guinea pig have been shown to be examples of cutaneous basophil hypersensitivity. In fact, certain antigens elicit conjunctival cutaneous basophil hypersensitivity (26). Therefore, Giemsa-stained sections of the palpebral conjunctiva from chlamydial-antigen-challenged guinea pigs were examined for the presence of basophils. Only very few basophils (1%) were observed in the infiltrates. Thus, because the inflammatory response elicited by the TX-100 extract and the purified 57-kD protein was primarily mononuclear (macrophage and lymphocyte) and delayed in appearance (24 h), we have characterized it as an ocular DH.

Discussion

Hypersensitivity to chlamydial antigens has long been thought to play an important role in the pathogenesis of trachoma. Early studies by Collier (7) demonstrated that the inflammation in the conjunctiva of persons with trachoma was not associated with the presence of chlamydial inclusion bodies, and led him to hypothesize that the inflammation was the result of DH to chlamydial antigens present in the conjunctival epithelium. The literature is replete with observations that support this hypothesis. For example, several attempts to vaccinate against trachoma have resulted in more severe disease upon reinfection with a heterologous C. trachomatis serovar (9, 11, 14). This deleterious response is thought to result from re-exposure to a genus-specific chlamydial hypersensitivity antigen. In addition, marked conjunctival inflammation is observed in patients with severe trachoma, even though chlamydiae cannot be demonstrated by culture or cytological assays (7, 27, 28). The deleterious nature of the ocular hypersensitivity has also been demonstrated using the guinea pig model of chlamydial ocular infection (8, 15, 29). Collectively, the studies concerning human and animal models of chlamydial ocular infection strongly implicate a role for DH in the pathogenesis of trachoma. A better understanding of the antigens that elicit such responses will likely be important to the understanding of the adverse sequelae of many chlamydial infections.

Several studies have attempted to characterize chlamydial antigens capable of eliciting DH responses. In most instances the hypersensitivity is directed toward chlamydial group antigens (10, 15, 30-32). We previously demonstrated that the soluble fraction of a Triton X-100 extract of Chlamydia induced delayed conjunctival hypersensitivity in ocular immune guinea pigs and subhuman primates (15, 16). The antigen(s) eliciting this response was a chlamydial group antigen since extracts prepared from strains of both chlamydial species, but not unrelated Gram-negative organisms, were capable of causing ocular inflammation. In the present study, we purified, from the soluble fraction of TX-100-treated GPIC EBs, two genus-specific chlamydial antigens having approximate molecular masses of 45 and 57 kD. The purified 57-kD antigen elicited an ocular DH response when applied to the conjunctiva of immune guinea pigs, whereas the 45-kD antigen failed to elicit such a response. The extract depleted of the 45- and 57-kD proteins elicited an ocular hypersensitivity response, though clearly distinguishable clinically and histologically from that elicited by the crude extract or purified 57-kD protein. This response was not surprising since a
number of chlamydial antigens, including LPS, remain in the Triton X-100 extract after affinity purification of the 45- and 57-kD proteins, which could separately or cooperatively stimulate such a response.

The 57-kD chlamydial hypersensitivity antigen is common to all 15 serovars of *C. trachomatis* and *C. psittaci* strains GPIC and Mn (Fig. 1). This antigen is associated with both EBs and reticulate bodies (15), and appears not to be the 60-kD cysteine-rich protein found in the Sarkosyl insoluble outer membrane fraction of *Chlamydia* (33) (our unpublished data). Although we previously stated that the hypersensitivity antigen was heat labile, our present observations indicate differently. The Triton X-100 soluble extract of GPIC EBs and the purified 57-kD protein elicited an ocular DH response after heating at 60°C for 30 min (data not shown). In addition, we found that the 57-kD protein was not surface exposed on EBs, as determined by indirect immunofluorescence and dot immunoblot analysis of intact, whole EBs.

The relationship between the 57-kD DH antigen that we have described and the 57- and 60–62-kD antigens that are immunoreactive with sera from patients with tubal obstruction (34) and active chlamydial infection (35), respectively, is not known. Antibody reactivity to these proteins as well as other chlamydial antigens is observed in patients and experimental animals experiencing an acute infection (35–38). In one study, women with tubal infertility had a distinctive antigen-specific antibody response to an immunodominant 57-kD protein (34). Whether tubal infertility is the result of persistent chlamydial infection or results from postinfection inflammatory damage is not known. Regardless of the cause, the resulting scarring of the fallopian tubes is very reminiscent of the persistent inflammation and tissue damage associated with trachoma. Because of the immunogenicity of this protein and because we can demonstrate the release of this protein from persistently infected cells (our unpublished observations), we believe it to be a major immunogen involved in the development of the sequelae associated with chronic chlamydial infections.

Recently, there has been considerable interest in a family of conserved and highly cross-reactive bacterial proteins referred to as Common antigens (39–45). These proteins are highly immunogenic, have similar electrophoretic mobilities in SDS-PAGE electrophoresis, and several have been shown to be heat-shock proteins (43, 45, 46). The precise cellular function of many Common antigens is unclear. However, the *Escherichia coli* Common antigen, groEL, is an ATPase and is believed to be involved in the post-translational assembly of oligomeric proteins (47–49). We do not know the relationship between the chlamydial 57-kD protein and other Common antigens, however our rabbit anti-57-kD antiserum reacts with similar molecular mass proteins from a number of different Gram-negative bacteria (Dr. F. Nano, personal communication). The 65-kD “common” mycobacterial antigen has been shown to play a dominant role in the immune response to mycobacteria (40). Moreover, a role for this antigen in the etiology of experimental autoimmune arthritis has been suggested (40, 50, 51). Interestingly, chlamydiae have been implicated in the development of a reactive arthritic condition known as Reiter’s disease (52, 53). Although most of the evidence for implicating chlamydiae in this disease is circumstantial, patients do demonstrate antibody reactivity to a chlamydial protein having a similar molecular weight, and chlamydial antigen has been used to induce arthritis in experimental animal models (54, 55). Therefore, it is conceivable that the highly im-
munogenic 57-kD chlamydial protein that stimulates the production of cross-reactive antibodies and DH responses may play a role in the development of such arthritic diseases.

Our studies have demonstrated the ability of the genus-specific, 57-kD chlamydial protein to elicit ocular DH in immune guinea pigs. We believe that this highly immunogenic protein may also contribute to the acute and more chronic sequelae observed in human chlamydial infections. Indeed, it will also be of interest to determine the relationship of the chlamydial 57-kD antigen to common bacterial antigens possessing similar properties. Further understanding of this protein and the immunological sequelae it induces may be key to understanding the pathogenesis of many chlamydial diseases.

Summary

Recurrent or persistent infections with Chlamydia trachomatis are thought to provide the antigenic stimulus for the chronic inflammation associated with blinding trachoma. We used the guinea pig model of inclusion conjunctivitis to identify chlamydial antigens that may be involved in this deleterious immune response. We purified from chlamydial elementary bodies a genus-specific 57-kD protein that elicited an ocular hypersensitivity response when placed topically onto the conjunctiva of ocular immune guinea pigs. This response was characterized by a predominantly mononuclear macrophage and lymphocyte cellular infiltrate of the submucosal epithelium. The clinical and histological findings were consistent with those of a delayed hypersensitivity response. These data demonstrated that the 57-kD chlamydial protein was a potent stimulator of ocular delayed hypersensitivity. Our findings may be critical to understanding the pathogenesis of the debilitating chlamydial diseases associated with chronic inflammation, such as trachoma and many urogenital syndromes.

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