Analysis of the Humoral Immune Response to Chlamydial Genital Infection in Guinea Pigs

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Studies using the guinea pig model of chlamydial genital infection with the *Chlamydia psittaci* agent of guinea pig inclusion conjunctivitis (GPIC) have shown that serum and local antibodies play a role both in the resolution of infection and in protection against reinfection. Thus, this model is suited for further exploration of immune mechanisms and for vaccine studies with chlamydial macromolecules. We have further characterized the model by assessing the antigen-specific antibody response to experimental genital infection by using immunoblotting to assay both genital secretions and serum. The GPIC agent was characterized by analysis of outer membrane proteins, which indicated that the GPIC agent possessed a major outer membrane protein (MOMP), with a molecular mass of 39 kilodaltons (kDa), and a 61-kDa protein, analogous to cysteine-rich 60-kDa proteins or doublets of *Chlamydia trachomatis* strains. As indicated by immunoblotting, most infected animals produced serum immunoglobulin G antibodies to MOMP, the 61-kDa proteins, an 84-kDa outer membrane protein, and lipopolysaccharide. Such serum antibodies persisted for at least 813 days after primary genital infection. Immunoglobulin A antibodies against the 61-kDa proteins, lipopolysaccharide, and MOMP, but not the 84-kDa protein, were detected in secretions. Animals challenged with GPIC 825 days after primary infection became infected again despite the presence of serum antibodies, but the period of chlamydial shedding was significantly shorter and less intense than in primary infections. Although the specific mechanism is not known, these data suggest that a long-lasting immune effect is capable of altering the course of infection late after primary infection. Correlation of the antigen-specific antibody response and other immune parameters with the duration and degree of protective immunity induced by infection or vaccination may be helpful in further understanding the nature of such protective immunity.

The guinea pig model of chlamydial genital infection with the agent of guinea pig inclusion conjunctivitis (GPIC) closely resembles genital infections with *Chlamydia trachomatis* in humans (1). For example, the infection can be transmitted sexually (14) and perinatally to produce neonatal conjunctivitis (13). In the genital tract, the organism mainly infects squamous epithelial cells of the cervix and in normal animals is noninvasive (2). Although infection ascending to the endometrium and fallopian tubes does not usually occur naturally, it can be induced in the guinea pig by immunosuppressive (21) or hormonal (19) manipulation.

Although partial protection from reinfection may occur in human genital infections (9), the eliciting antigen(s) and immune mechanisms involved are poorly understood. However, studies of genital infections in the guinea pig GPIC system indicate that protective immunity occurs and that the humoral immune response plays a role both in the resolution of infections (18) and in protection against reinfection (17). Thus, the model is well suited for further exploration of immune mechanisms and the time frame within which they operate. It also can be used to evaluate whether protective immunity can be induced when purified chlamydial macromolecules, such as outer membrane proteins, are used as immunogens. To identify potential antigens for evaluation in such studies and to characterize the kinetics and antigen specificity of antibodies produced in response to genital tract infection with GPIC, we performed immunoblot analyses on serum samples or genital secretions or both obtained from 21 female guinea pigs during and after such infections.

MATERIALS AND METHODS

Chlamydiae and outer membrane isolation. The *Chlamydia psittaci* GPIC agent was originally provided by E. S. Murray, Boston, Mass. GPIC organisms used for electrophoretic analysis were grown in 150-cm² McCoy cell monolayers in the presence of 1 µg of cycloheximide per ml as previously described (4). Purified elementary bodies were obtained and stored according to established procedures (15). Chlamydial outer membranes were prepared by Sarkosyl extraction of whole purified elementary bodies (4). GPIC elementary bodies for intravaginal inoculation were prepared from infected yolk sacs as previously described (2).

Experimental animals and infection of guinea pigs. Hartley strain female guinea pigs weighing 400 to 500 g were obtained from Simonsen Laboratories, Inc., Gilroy, Calif. Although we have found this stock to be free of natural GPIC infection, all animals were determined before inclusion in experiments to be negative for antibodies to GPIC by the methods previously described (18).

Guinea pigs were infected with a suspension consisting primarily of elementary bodies. The suspension was prepared from infected yolk sacs in sucrose-potassium glutamate buffer (pH 7.2) containing 0.5 mg of gentamicin per ml and 0.5 mg of vancomycin per ml (2). All animals were infected by intravaginal inoculation with 0.05 ml of the particle suspension containing approximately 5×10^5 50% egg lethal doses (2). Isolation of chlamydiae was not done in this study. Rather, infection was assessed by obtaining vaginal wall scrapings and determining the percentage of inclusion-bearing cells on Giemsa-stained smears (2). We have found that in animals with detectable inclusions, prop-

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erly handled cultures are positive without exception (R. Rank, unpublished data).

Serum samples were routinely obtained by the collection of blood from the saphenous vein (11). Genital secretions were obtained from infected animals by a modification of the procedures of Lamont et al. (10). Surgical sponges (2 by 10 mm) were inserted into the vagina of an anesthetized animal and were retrieved 2 h later. The sponges were weighed before and after insertion, and the weight of the collected secretions was determined. Before antibody testing, the sponges were eluted in phosphate-buffered saline (pH 7.2) at a ratio of 0.2 ml of phosphate-buffered saline per 0.1 g of secretion. Specimens so eluted were held at -70°C until they were assayed.

SDS-PAGE and electrophoretic transfer. Chlamydial proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% polyacrylamide gels (15). Some gels were stained with Coomassie brilliant blue R-250, destained, and photographed (15). The apparent molecular masses of chlamydial proteins were estimated by comparison with a plot of migration distance versus the log of molecular mass with 68,000-molecular-weight (68K) bovine serum albumin, ovalbumin (45K), carbonic anhydrase (29K), and cytochrome *c* (11.7K) as standards. For gels used in immunoblotting, 280 μg of solubilized GPIC proteins were resolved on a 14-cm-wide slab gel consisting of a 2-cm stacking gel and an 8-cm separating gel. Resolved GPIC proteins were then electrophoretically transferred to nitrocellulose membranes, which were then sliced vertically into identical 0.4-cm strips (3).

Immunoblotting. Nitrocellulose strips bearing resolved GPIC elementary body proteins were used for immunoblotting. Blocking of unoccupied binding sites on nitrocellulose was accomplished by incubating the membrane at room temperature for 1 h in a solution containing 150 mM NaCl–10 mM Tris–0.5% (wt/vol) nonfat dry milk (Carnation) (8) (pH 7.4) as a blocking buffer or in the same buffer containing 10% (vol/vol) horse serum (20). Guinea pig serum samples were diluted 1:1,000 (vol/vol), and genital secretions were diluted 1:100 (vol/vol) in blocking buffer and used to probe 0.4-cm nitrocellulose strips bearing resolved GPIC proteins. Buffer containing unbound antibody was aspirated, and the nitrocellulose was washed five times over 30 min, each wash with 15 ml of 150 mM NaCl. To detect bound antibody, strips were incubated with a 1:10,000 (vol/vol) dilution of rabbit anti-guinea pig immunoglobulin G (IgG) (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.), followed by a repeat wash step and subsequent incubation with ^{125}I -labeled affinity-purified goat anti-rabbit IgG (Cooper Biomedical, Inc., West Chester, Pa.) at 150,000 cpm per strip. After a final wash cycle, the nitrocellulose strips were mounted and used to expose Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.). For experiments with vaginal secretions, antigen-specific binding of IgA antibodies was localized with α -chain-specific rabbit anti-guinea pig IgA (Miles Scientific) at a 1:1,000 (vol/vol) dilution followed by radioiodinated goat anti-rabbit IgG.

RESULTS

Characterization of GPIC proteins. The protein profile of purified elementary bodies of the GPIC strain is shown in Fig. 1, lane 1. The GPIC strain possessed a major outer membrane protein (MOMP) analogous to those of *C. trachomatis* strains (15). However, the apparent molecular mass of MOMP of the GPIC strain (39 kilodaltons [kDa]) was lower

than that of any *C. trachomatis* strain (the lowest was 42 kDa for serovar G) (15) when GPIC was directly compared with all 15 serovars of *C. trachomatis* by SDS-PAGE (data not shown). Many other GPIC proteins were resolved. Some of them included 72-, 33-, and 15-kDa proteins, as well as 61- and 84-kDa proteins. Lipopolysaccharide (LPS) was not seen on this Coomassie blue-stained gel. To identify those proteins that were present in the outer membrane, GPIC elementary bodies were extracted with Sarkosyl and analyzed by SDS-PAGE (Fig. 1, lane 3). MOMP was the predominant protein present. The second most prominent protein stained with Coomassie blue was one of approximately 61-kDa molecular mass, analogous to the 60-kDa outer membrane proteins observed in *C. trachomatis* strains (4). Several outer membrane proteins of higher molecular mass, including an 84-kDa protein, resolved in the GPIC strain (Fig. 1). These higher-molecular-mass outer membrane proteins appeared analogous to those outer membrane proteins in excess of 85 kDa reported by Hatch et al. (7). An

FIG. 1. Coomassie blue-stained 12.5% polyacrylamide gel showing resolved proteins of purified GPIC elementary bodies (lane 1), Sarkosyl supernatant (lane 2), and Sarkosyl pellet, representing GPIC outer membranes (lane 3). Positions of resolved chlamydial proteins are indicated on the right, with apparent molecular masses in kilodaltons. Positions of the molecular weight markers bovine serum albumin (68K), ovalbumin (45K), carbonic anhydrase (29K), and cytochrome *c* (11.7K) are on the left.

FIG. 2. Autoradiogram showing the immunoblot reactions of a control (no serum; lane A), a serum sample from preimmune guinea pig (animal 1, day -1), and 16 serum samples from female guinea pigs 21 to 41 days after intravaginal infection with GPIC (animals 1 to 16). The day on which each tested serum sample was obtained is given below each lane. Immunoreactive bands are identified by molecular mass in kilodaltons with lines on the right. The blocking reagent was 0.5% nonfat dry milk, as described in the text.

11-kDa outer membrane protein, analogous to the cysteine-rich proteins of similar molecular mass (7, 16), can also be seen on this gel (Fig. 1).

Characterization of the serum IgG response. Animals inoculated in the genital tract with GPIC develop an infection in which the largest numbers of organisms can be detected between 6 to 12 days postinoculation and which is resolved by 15 to 20 days postinoculation (18). Serum antibodies, measured by indirect immunofluorescence, first appear at 10 to 14 days and reach maximum titers by 21 to 28 days postinoculation (18). To determine the antigenic specificity of such antibodies, we performed immunoblotting on serum samples obtained from 16 animals at 21 to 41 days postinfection.

An autoradiogram showing the reactions of IgG antibodies for these sera (numbers 1 to 16) is depicted in Fig. 2. Each postinfection serum sample contained IgG antibodies which bound MOMP. The intensity of these reactions varied in the animals from weak (strip 2, day 30) to quite strong (strip 12, day 28). Intense reactions were observed with the 61-kDa protein for all serum samples, and all serum samples contained IgG antibodies against the 84-kDa protein. The immunological reactions located at the positions corresponding to MOMP, the 61-kDa protein, and the 84-kDa protein were also present when serum samples were used to probe nitrocellulose strips bearing GPIC outer membrane proteins (not shown). In addition, reactions were variable (Fig. 2) with several other proteins, including those of 15-, 19-, 27-, 33-, and 47-kDa molecular masses. Intense reactions were frequently observed with a doublet of 72-kDa molecular mass. Another common but variable reaction was with a diffuse band that comigrated with the tracking dye front of the SDS-PAGE gels and which is known to contain chlamydial lipopolysaccharide (5). This reaction was strong in several animals (strips 3, 4, and 6), weak in several others (strips 13, 14, and 16), and absent in others (strips 2, 7, and 15).

Thus, guinea pigs produced serum IgG antibodies against a number of polypeptides, including prominent outer membrane proteins, and LPS. The reactions observed by immunoblotting of sera from individual animals were variable in intensity for MOMP and LPS, but were more uniformly

intense for 61- and 84-kDa proteins. However, the courses of primary infections, as measured by inclusion scores, did not differ significantly among animals with weak versus strong responses to MOMP and LPS (data not shown).

Kinetics of the serum IgG response. To determine the kinetics of the serum IgG response, we analyzed sera obtained serially from infected animals. Figure 3 shows the reactions of sera obtained from four of the animals (Fig. 2, numbers 1, 2, 12, and 13) at various times in relation to experimental infection. Serum samples obtained from each animal before infection gave no significant antigen-specific binding. Within 6 to 7 days, faint reactions with minor components were detected. By days 12 to 14, reactions with the 61- and 84-kDa proteins were evident in serum samples from each animal and reactions with LPS could be seen in serum samples from two animals. In contrast, no samples showed detectable reactions with MOMP until days 20 to 21. Serum samples obtained from three animals at days 40 to 50 revealed weak reactions with MOMP, while a sample from one animal gave a strong reaction. In addition, strong reactions with LPS (animals 1 and 13) and the 61-kDa protein (animals 1, 2, and 13) persisted at least through days 40 to 50. These results suggested that serum IgG antibodies against some GPIC components may be less durable than others.

To extend these serological observations over longer periods and to observe the serum antigen-specific IgG response after late infectious challenge with GPIC, sera were analyzed from four animals (animals 17 through 20) which had a primary infection, repeated periodic bleedings during a prolonged follow-up period (813 days), and late infectious challenge with GPIC at day 825. Inclusion scores were done during the primary infection until they were negative on two consecutive occasions 3 days apart, but neither inclusion scores nor cultures were done during the prolonged follow-up until challenge at day 825. The courses of both primary and challenge infections (day 825) were assessed with inclusion scores determined on various days after inoculation (Fig. 4). The primary infections in these animals were typical of those seen in this model (18) in terms of duration and intensity of chlamydial shedding, as judged by inclusion scores. However, the courses of the challenge

FIG. 3. Autoradiogram showing reactions of sera obtained serially from four animals after intravaginal infection with GPIC. The animal designation is shown at the top and corresponds to numbers in Fig. 2. The days on which the individual serum samples were obtained after infection are shown beneath each lane. Days -1 or 0 indicate samples obtained just before experimental infection. Identifications of major immunoreactive bands (LPS, MOMP, 61K, and 84K) are on the right. The blocking reagent was 0.5% nonfat dry milk.

infections that developed were significantly attenuated ($P < 0.0001$) compared with the courses of primary infections in the same animals when inclusion scores were compared by a two-factor (primary versus challenge infection and inclusion scores each day) analysis of variance with repeated measures on one factor (inclusion scores each day).

An immunoblot analysis of serial serum samples obtained from one (Fig. 4, animal 18) of the four animals is shown in Fig. 5. MOMP reactions were seen at 21 days and remained through day 813, the last day the animals were tested before challenge. Antibodies to the 61-kDa protein showed a weak but definite reaction at day 14, strong reactions through day 513, and less-intense reactions through day 813. Reactions to MOMP, initially intense, also became less intense as time

FIG. 4. The course of GPIC genital infections resulting from primary and challenge inoculations in four animals (numbers 17 to 20). Inclusion scores of vaginal scrapings on each day are given as a percentage on the ordinate, and the number of days after primary infection is given on the abscissa. All animals had a primary infection induced on day 0, and all were challenged on day 825, as indicated by the arrows. Symbols: ●, animal 17; ○, animal 18; ▲, animal 19; and △, animal 20.

after the primary infection increased. In contrast, reactions to the 84-kDa protein were first seen at day 21 but became undetectable by day 209. LPS reactions also became detectable at day 21 and persisted through day 813. Serum samples obtained sequentially from the three other animals (animals 17, 19, and 20) have been analyzed in a similar fashion and also indicated that serum IgG antibodies persisted through at least day 813 (data not shown). However, the animal shown differed from the three other animals assayed in that two of the latter animals had intense reactions to the 84-kDa proteins that persisted through day 813 (Fig. 4, animals 19 and 20), while the third (Fig. 4, animal 17) gave less intense but persistent reactions to the 84-kDa protein.

After challenge with GPIC at day 825, intense reactions to MOMP, the 61-kDa doublet, and the 84-kDa protein developed by day 843, or 18 days after challenge (Fig. 5), similar to what was observed at the primary infection. In contrast, reactions to LPS became more intense by days 833 and 837 (8 to 12 days after challenge), earlier than seen with LPS at the primary infection. A complete set of vaginal-secretion samples from these four animals at both the primary and challenge infections was not available for assay, but representative specimens taken after the primary infection (animals 17, 18, and 19) are discussed below.

IgA antibodies in genital secretions. Figure 6 shows examples of the binding of IgA antibodies in vaginal secretions obtained from guinea pigs 13 to 50 days after intravaginal inoculation with GPIC. Results with secretions from animals 1 (Fig. 2 and 3), 18 (Fig. 4 and 5), 17 and 19 (Fig. 4), and 21 (not previously shown) are shown in Fig. 6. Secretions contained IgA antibodies that bound primarily the 61K protein and LPS, while reactions with MOMP were generally less intense. Reactions to a 15-kDa protein were observed when secretions from two of the five animals shown were assayed. In contrast, no antigen-specific binding of IgA was observed in sera (Fig. 6), although typical IgG reactions were seen with the same sera (not shown). The IgA antigen-specific reactivity of vaginal secretions was detectable by day 12 (animals 21 and 1) and decreased markedly by day 50 (animal 1). Serial vaginal secretions from five additional animals (data not shown) showed similar patterns of reactivity. The IgG reactions obtained with vaginal secretions were

FIG. 5. Autoradiogram showing the immunoblot reactions of a single animal (number 18) which was infected intravaginally with GPIC on day 0, had serial serum samples obtained through day 813, and was challenged with GPIC on day 825. Lane A is a control (no serum). The day after initial infection on which the serum samples were obtained is given below each lane. Postchallenge samples are shown as days after challenge infection for days 825 and beyond. The heavy bars indicate that the challenge infection was done on day 825. Major immunoreactive bands (LPS, MOMP, 61, and 84) are labeled. Blocking was done with 0.5% nonfat dry milk.

FIG. 6. Autoradiogram showing reactions of vaginal secretions obtained 13 to 50 days after intravaginal infection with GPIC. The immunoblots were done with rabbit anti-guinea pig IgA as described in the text. The left lane (CONTROL) is a buffer-only control. No preimmune secretions were available for assay. The animal numbers, corresponding to those in previous figures, are at the top of the lanes. Animal 21 had secretions from days 12, 18, and 25 assayed, as well as serum samples on days 18 and 25. Animals 17, 18, and 19 had secretions tested at one time, while animal 1 had secretions tested at two times. Immunoreactive bands (LPS, 15 Kd, MOMP, 61 Kd, and 84 Kd) are identified on the right. Blocking was done with 10% horse serum.

very similar to those seen with sera and were observed in secretions obtained 143 days after the primary infection in the six animals analyzed (data not shown).

DISCUSSION

Previous data indicate that humoral immunity plays a role in resolution of infection (18) and in protection against reinfection (17) in the guinea pig GPIC model. In those studies, anti-GPIC antibodies in sera and secretions were consistently detected in infected animals, and their appearance was correlated with the resolution of infection (18). The presence of such antibodies at high titers shortly after resolution of primary infections with GPIC was also correlated with solid protection against reinfection (17). However, serological data in these studies were obtained by methods that used whole GPIC elementary bodies as antigen and thus could not distinguish whether or not antibodies were produced against specific macromolecules. In this study, we used an immunoblotting method to determine whether specific chlamydial antigens induce production of IgG antibodies in sera and IgA antibodies in vaginal secretions during and after genital infection of female guinea pigs with the GPIC agent. In pilot experiments with guinea pig sera (data not shown), antigen-specific binding of IgM could not be demonstrated; therefore, IgM antibodies were not sought in the larger study.

The major reactions seen when serum samples from a group of 16 animals were subjected to immunoblot assay were those against MOMP, the 61-kDa protein, the 84-kDa outer membrane proteins, and LPS. Specific responses were first seen at days 12 to 14 and persisted through days 40 to 50

after infection in short-term kinetic studies. However, the intensity of some reactions seemed to decrease when serum samples obtained at 40 to 50 days were assayed. IgA antibodies in vaginal secretions were detected against MOMP, the 61-kDa doublet, and LPS in genital secretions. IgA antibodies were also detectable at days 12 to 13, but in contrast to IgG in sera, IgA antibodies in secretions became virtually undetectable in immunoblotting by day 50. In the small number of secretions analyzed in this study, reactions against MOMP were weak compared with reactions against the 61-kDa proteins and LPS, and reactions to the 84-kDa proteins were not observed. When paired specimens of guinea pig sera and secretions were probed for IgA binding (Fig. 6), no reactions were observed in the sera. Therefore, although we did not test specifically for the presence of secretory components, we concluded that the IgA detected in secretions was produced locally and was not derived from transudation of IgA into genital sites from serum.

Longer-term kinetic studies of serum IgG antibodies to chlamydial antigens in four animals monitored for 813 days after a primary infection indicated that such antibodies persisted for more than 2 years after the primary infection had resolved. However, since neither inclusion scores nor cultures were obtained during the prolonged follow-up period after the primary infection had resolved, we cannot rule out the possibility that persistence or intermittent shedding of chlamydiae occurred during this period, thus constituting a continuing antigenic stimulation. Three of these four animals developed detectable reinfections, as judged by inclusion scores of vaginal scrapings, when challenged at day 825, despite the presence immediately before challenge of detectable serum IgG antibodies to MOMP, the 61-kDa doublet, and LPS. However, in all four animals, the course of the challenge infection was significantly attenuated compared with that of the primary infection. In addition, the single animal which did not develop an infection as judged by inclusion scores and another animal with the lowest inclusion scores after challenge (Fig. 4) both had persistent intense reactions to the 84-kDa protein compared with reactions in the other two animals. These data parallel previous results of challenge experiments that were done with the GPIC guinea pig model of eye infection (12), except that the current experiments indicate that a presumed immune effect can modify the course of infection over a period of more than 2 years.

We currently do not know whether the serum antibodies detected at the time of challenge were responsible for the attenuation of the challenge infection. Earlier studies of genital infections of guinea pigs have indicated that solid immunity, as defined by negative inclusion scores of vaginal scrapings obtained after challenge, exists immediately (within 3 to 5 days) after resolution of a primary infection (17). More extensive studies are under way to determine the duration of such solid protection and when such protection wanes to become the limited protection (attenuated chlamydial shedding) observed in this study. Correlations of antigen-specific responses in sera and genital secretions may shed light on whether antibodies of certain specificities and at certain sites seem important in such protection.

Our results must be interpreted in light of the fact that the antigens used in this study in assays of both sera and secretions were treated by incubation in SDS and 2-mercaptoethanol at 100°C. It is likely that epitopes exist that are sensitive to one or more of these treatments and that, in the absence of renaturation, they will not be detected in immunoblotting. Nevertheless, immunoblotting of sera and

secretions in this model has identified a subset of proteins that may be worthy of further evaluation in vaccine studies in the model. In addition, correlation of immunoblotting data, serological data with nondenatured antigens, and measures of cell-mediated immunity may help to further define the nature of immunity in this model.

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