Oral Immunization Against Chlamydial Eye Infection

Hugh R. Taylor, Elaine Young, A. Bruce MacDonald,* Julius Schachter,† and Robert A. Prendergast

The effects of enteric administration of different preparations of *Chlamydia trachomatis* prior to ocular challenge with live chlamydia were compared to the immunity that develops after recovery from ocular infection. Oral immunization with either live homologous serovar B or with formalin-killed heterologous serovar L2 did not influence the response to subsequent ocular challenge. Although oral immunization with live serovar led to protection against heterologous ocular challenge with serovar B, oral immunization with noninfectious UV-irradiated serovar L2 led to more severe and prolonged disease. An immunizing regimen designed for maximal mucosal and systemic immunity also resulted in protection against homologous ocular challenge. Although protection was correlated with the presence of serum IgA antibodies, no clear mechanism for the protective ocular immunity to chlamydial infection has emerged. These studies show that it is possible to stimulate mucosal immunity to induce protection against subsequent ocular challenge with *C. trachomatis* that is equal to that which follows prior ocular infection. Invest Ophthalmol Vis Sci 28:249-258, 1987

In an animal model for trachoma, monkeys develop resistance to chlamydial eye infection after recovering from a primary ocular infection. However, this resistance is only partial, since animals still develop infection following challenge, although it is milder and of shorter duration. Although there is evidence for protective immunity in chlamydial infection, much of the tissue damage associated with trachoma also appears to be immunologically mediated. It has been suggested that the antigens responsible for protection may be different from those causing the deleterious reactions; if it were possible to specifically stimulate only the protective immune response, then this might result in a safe and effective trachoma vaccine. Such a vaccine obviously would have a tremendous impact on the many millions of children who live within endemic areas.

The successful cultivation of *Chlamydia trachomatis* in the early 1950s opened the way for attempts to develop vaccines against trachoma. During the 1960s, a number of groups carried out studies on trachoma vaccines in animals and in humans with vaccines designed to stimulate systemic immunity. These preparations contained, almost without exception, killed whole elementary bodies (EB) and were given systemically either by intramuscular or subcutaneous injection. The overall experience of these studies was that some mild, limited protection often did occur, but it was, at best, of short duration, lasting less than 2 yr in humans. Furthermore, several of these studies also showed that a worsening of disease, or hypersensitivity to chlamydia, could be induced by an “inadequate” vaccination with either low potency vaccines or challenge with heterologous serovars. Because of both the lack of efficacy and concerns about safety, interest in trachoma vaccines waned.

In humans, infection with *C. trachomatis*, with the exception of the invasive L serovars, appears to be almost always confined to the mucosal surface. Many of the complexities of the mucosal immune system have been elucidated in the last few years. For example, successful regimens for specifically stimulating mucosal immunity have been successfully exploited for other infections such as cholera, where preceding systemic vaccines were of only limited efficacy. Previous studies in monkeys and rats have shown that enteric presentation of antigen leads to antigen-specific priming of the conjunctiva, and preliminary studies in guinea pigs suggest partial protection to ocular challenge with *C. psittaci* may follow distant mucosal infection. In addition, there have been tremendous advances in the methods for identifying and producing purified antigens in large amounts, should an appropriate antigen be identified.

For these reasons, it seemed appropriate to reexamine the possibility of developing a vaccine for trachoma that specifically stimulates mucosal immunity, is easy to administer, and does not induce hypersen-
sitivity. This report presents a series of studies of the effect of distant mucosal immunization with purified chlamydial EB on subsequent ocular infection with C. trachomatis in a monkey model.

Materials and Methods

Animals

Groups of young adult cynomolgus monkeys were obtained from Hazelton Laboratories (Alice, TX). There were five monkeys in each group, all procedures described herein conform to the ARVO Resolution on the Use of Animals in Research.

Preparation and Administration of C. trachomatis

Serovar B (HAR-36) and L2 were mass-cultured in tissue culture. Purified EB were prepared by centrifugation through renograffin and resuspended at the appropriate dilutions in phosphate-buffered saline. C. trachomatis, serovar E (Bour strain), was grown in the yolk sac of embryonated hen eggs, and EB were purified by centrifugation through renograffin.

Ocular inoculations were adjusted to 1 x 10^5 infection-forming units (IFU) per ml, which is equivalent to 10^3.2 egg lethal dose 50 per ml. Twenty microliters of suspension was placed into each conjunctival sac, giving an ocular inoculation of approximately 2 x 10^3 IFU per eye.

Enteric doses consisted of 5 x 10^8 EB. Monkeys were fasted overnight; immediately after neutralizing gastric contents with 5 ml of sodium bicarbonate solution, the enteric dose was administered via a gastric tube. Rectal inocula contained 1 x 10^5 IFU and was administered directly in the rectum during proctoscopy. Preparations for intramuscular injection contained 5 x 10^10 EB per ml. This had been emulsified 1:1 with incomplete Freund’s adjuvant for subsequent injections. At each time, animals were given a total of 1 ml at two sites.

Where indicated, viable agent was inactivated either by ultraviolet irradiation (UV) or by the addition of formalin. For UV inactivation, a suspension of EB was placed in a plastic dish to a depth of 2 mm and the open dish placed 10 cm from a Westinghouse 40 W germicidal UV lamp (George W. Gates and Co., Inc., Franklin Square, NY) for 120 min. The suspension was continuously agitated by magnetic stirrer. Alternatively, EB were inactivated by the addition of formalin to a final concentration of 0.01%. Inactivation was confirmed by three serial culture passages using multiple dilutions of the inactivated agent. The detailed immunizing protocol and ocular challenge for each group of monkeys are given in the results section and in the respective figures.

Examination and Specimen Collection

The clinical response of each eye was graded for a number of individual signs that were then combined to express the clinical response as two simplified indices as previously described. Briefly, the “follicular index” quantitates the follicular response in the bulbar, limbal, superior tarsal, and superior fornix conjunctiva. The “inflammatory index” summarizes the nonspecific signs of inflammation: specifically, hyperemia or injection of the bulbar, superior tarsal, superior fornix conjunctiva, and ocular discharge. Examinations were performed in random order without informing the examiner of the monkey identification. Recovery from disease was defined as a follicular index of less than one and an inflammatory index of zero.

Conjunctival swabs were collected at each examination for chlamydial reisolation cultures in a cycloheximide-McCoy cell tissue culture system. Although both eyes were examined, specimens were taken only from the left eye to eliminate the possibility of artifactitious changes in the right eye. Rectal swabs were also taken for culture from monkeys in one group.

Serology

Tears and serum were collected for microimmunofluorescent serologic tests against whole chlamydial EB. Each specimen was separately titered against purified preparations of serovar B and serovar L2 EB except for specimens from the maximally immunized group, which were assayed against serovar E EB. Commercially available reagents cross-reacting with monkey immunoglobulin heavy chains were used: goat antihuman IgG (Hyland Labs; Costa Mesa, CA) and goat antihuman IgM (Kallestad; Austin, TX). IgA was assayed using rabbit antimonkey IgA (Nordic Immunological Labs; El Torro, CA).

Lymphocyte Proliferation Assay

Peripheral blood mononuclear leukocytes (PBML) were isolated from animals as previously described. PBML were suspended at a concentration of 1.5 x 10^5/ml in Minimum Essential Medium (MEM) supplemented with 25 mM Hepes buffer, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (all from Gibco; Grand Island, NY). Triplicate wells containing 1.5 x 10^3 PBML and 100 μl of antigen (10^7 serovar L2 EB) or medium in a total volume of 200 μl were cultured in round bottom plates (Linbro; McLean, VA) at 37°C in 5% CO2. One μCi 3H-thymidine was added to each well on day 3. Cultures were harvested 18 hr later, and radioactivity was measured in a liquid scintillation counter. The Stimulation Index (SI) was determined as the ratio of
counts per min (cpm) in test wells over cpm in control wells.

**Results**

**Primary and Secondary Responses in Nonimmunized Monkeys**

Five naive monkeys receiving a single ocular inoculation with live *C. trachomatis*, serovar B, rapidly developed an acute, self-limited inclusion conjunctivitis (Fig. 1). Nonspecific signs of inflammation (inflammatory index) resolved within 1 month, whereas the follicular response (follicular index) waned more slowly. All animals had clinically recovered by 12 wk. After recovery, these animals demonstrated ocular immunity to chlamydial infection and were relatively resistant to ocular challenge with the same organism (Fig. 1). Clinical disease resolved within 6 wk of challenge. Positive chlamydial cultures isolated from conjunctival swabs were obtained for 6 wk after the initial infection but only for 2 wk after ocular challenge.

These monkeys had a strong, serovar-specific antibody response with IgM, IgG, and IgA antibodies appearing in both serum and tears following primary infection (Fig. 2). Serum serovar-specific IgG titers increased with secondary challenge with little change in the titers of the other antibody classes.

**The Effect of Oral Immunization With Live Agent**

Two groups of five monkeys each were immunized orally at weeks 0, 1, and 2 with live chlamydia of either serovar B or serovar L2 and given an ocular challenge with serovar B at week 3. The clinical and microbiological response of monkeys orally immunized with live serovar B EB was essentially the same as nonimmunized animals (Fig. 3A). They had recovered clinically within 12 wk, and positive chlamydial cultures persisted for 4 wk. However, monkeys immunized with live serovar L2 had milder disease than nonimmunized monkeys (Fig. 3B) and responded in a similar way to ocular immune animals (vide supra). Clinical disease resolved in 7 wk, although one animal had mild persistent clinical disease and gave weakly positive conjunctival cultures for 8 wk. One monkey fed live serovar L2 died 5 wk after ocular challenge. No evidence for disseminated chlamydial infection was found on autopsy.

Monkeys immunized with live serovar B developed moderate serum serovar B-specific IgM and IgG titers within 2 wk of immunization, but neither serum IgA antibodies nor tear antibodies of any class were detected until after ocular challenge (Fig. 4A). Two weeks after ocular challenge, there was a rapid appearance of antibodies of all three isotypes in the tears. There was a
Fig. 2. Mean serovar B-specific antibody titers in tears (top) and serum (bottom) of five naive monkeys given a single ocular inoculation of live serovar B *C. trachomatis* on week 0 and challenged with the same serovar on week 18.

Fig. 3. Clinical ocular response following challenge with serovar B of (A) five monkeys orally immunized with viable serovar B *C. trachomatis*; (B) five monkeys orally immunized with viable serovar L2 *C. trachomatis*. The mean follicular and inflammatory indices and the frequency of positive chlamydial cultures are shown. The response of naive animals is shown in broken lines for comparison. Error bars represent SEM.
corresponding increase in serum IgM and IgG, and IgA also appeared in serum at this time. The monkeys immunized with live serovar L2 developed comparable serum serovar B-specific IgM and IgG titers but also developed serum IgA antibodies after immunization (Fig. 4B). Antibodies were not detected in tears before challenge, although a rapid tear antibody response followed ocular challenge. In this group, IgA and IgM could be detected within 1 wk of challenge compared with 2 wk in the animals orally immunized with serovar B and 3 wk in nonimmunized animals. High titers of IgM persisted in the serum and tears of both immunized groups of monkeys. For both immunized groups, the mean serovar-specific antibody titers for serovar B or serovar L2 for each antibody class did not differ by more than one dilution (data for serovar L2 not shown).

The Effect of Oral Immunization With Inactivated Agent

Because oral immunization with viable serovar L2 produced some protection to ocular challenge, we next examined the effect of oral immunization using inactivated serovar L2. Two groups of five monkeys each were immunized orally following the previous schedule. At weeks 0, 1, and 2, they were given serovar L2 EB inactivated by either formalin or UV irradiation. They were challenged with ocular serovar B at week 3. The clinical and microbiologic response in the monkeys immunized with formalin-inactivated serovar L2 EB was similar to that of nonimmunized animals (Fig. 5A). Clinical recovery was seen by 10 wk, although positive conjunctival cultures were obtained for up to 10 wk. In contrast, oral immunization with UV-inactivated serovar L2 resulted in the marked prolongation of clinical disease and positive culture isolates (Fig. 5B). In this group, disease resolution was delayed for over 5 months, and positive cultures were obtained for up to 10 wk after challenge in 80% (4 of 5) of the animals.

Two monkeys orally immunized with formalin-inactivated serovar L2 EB developed serovar B-specific IgM antibodies before ocular challenge. These two animals also had low serum titers of antiserovar L2 IgM and IgG antibodies (titers of 1:16 for each) 3 wk after the initial feeding. After challenge, the serum antibody responses in this group to serovar B (Fig. 6A) and serovar L2 (data not shown) were essentially the same as nonimmunized monkeys, although lower titers of each
isotype were found in tears. Oral immunization with UV-inactivated serovar L2 EB induced a serum IgM response to both serovar B and L2 in one monkey (titers of 1:128 for each serovar). Following ocular challenge, the serum antibody response to serovar B (Fig. 6B) was similar to that observed in nonimmunized animals (as shown in Fig. 2), although the IgM response was much greater and more prolonged than in normal monkeys. The tear IgM and IgG response was identical to non-immunized animals following primary ocular infection; however, the tear IgA response was both delayed and depressed.

The Effect of Maximal Immunization
In an attempt to induce maximal mucosal immunity, six monkeys were given an initial rectal infection on weeks 0 and 5 followed by distant mucosal (oral) and systemic (intramuscular) boosting on weeks 8, 12, and 14. In this experiment, serovar E was used for both immunization and ocular challenge. Mild proctitis developed in all monkeys after the first rectal inoculation with chlamydia, and more marked but still self-limiting proctitis followed the second. Positive chlamydia cultures from the rectum confirmed the establishment of infection (Fig. 7). There was no spread of infection to the eyes following rectal inoculation or oral immunization. After ocular challenge, these animals developed a short-lived but vigorous clinical response (Fig. 7) similar to the ocular immune animals (Fig. 1). Microbiological evidence of infection cleared within 2 wk.

After the first rectal inoculation, serovar E-specific IgM or IgG was not found in serum or tears, but one animal developed low serum titers of IgA (1:32). Following the second rectal inoculation, low titers of serovar E-specific IgG and IgM were found in serum, and moderate titers of IgA were found in all animals (Fig. 8). After oral and systemic boosting, extremely high titers of IgA and IgG and moderate titers of IgM appeared in the serum of all animals. Low titers of IgA and IgG, but not IgM, also appeared in tears at this time. Moderate titers of IgG antibodies (mean titer 1:320) were also detected in rectal secretions, but IgM was not found. Rectal secretions were not tested for IgA. Following ocular challenge, serum IgG and IgM titers did not change, but serum IgA antibodies reached even higher titers (range 28 days post-challenge was 1:16,384 to 1:32,768). Tear serovar E-specific IgA and IgG rose rapidly to very high titers, and low titers of IgM also appeared. The titers of IgG in tears exceeded those in serum, suggesting that local production rather than transudation was responsible for much of the increase in tear antibody.

**Fig. 5.** (A) Clinical ocular response following challenge with serovar B of five monkeys orally immunized with formalin-inactivated serovar L2 C. trachomatis and challenged with serovar B at week 3; (B) five monkeys orally immunized with UV-irradiated serovar L2 C. trachomatis and challenged with serovar B at week 3. The mean follicular and inflammatory indices and the frequency of positive chlamydial cultures are shown. The response of naive animals is shown in broken lines for comparison. Error bars represent SEM.
Cell-Mediated Immunity

Cell-mediated immunity assayed by in vitro lymphoproliferative assays of circulating lymphocytes did not reflect the development of ocular immunity to chlamydial infection. Neither the ocular immune monkeys nor the animals successfully immunized with live serovar L2 developed a significant cell-mediated immune response. Cellular immunity to whole EB was observed only in animals immunized by intramuscular injection. In this group, the mean SI (± standard deviation) at week 0 was 1.5 ± 0.4 and at week 16 (ocular challenge), 11.2 ± 4.8. Parallel in vitro lymphocyte proliferation assays using purified chlamydial lipopolysaccharide antigen also failed to demonstrate statistically significant sensitization of peripheral blood lymphocytes.

Discussion

The present series of experiments were designed to compare the effect of different immunizing regimens with the ocular immunity that develops following recovery from chlamydial ocular infection. Specifically, we wished to determine whether prior distant mucosal exposure to whole chlamydial EB would protect against subsequent ocular challenge. The oral immunizing

Fig. 6. (A) Mean serovar B-specific antibody titers in tears and serum of five monkeys orally immunized with formalin-inactivated serovar L2 C. trachomatis and challenged with serovar B at week 3; (B) five monkeys orally immunized with UV-irradiated serovar L2 C. trachomatis and challenged with serovar B at week 3.

Fig. 7. Clinical ocular response of six monkeys given rectal inoculations of serovar E C. trachomatis on weeks 0 and 5; combined oral and intramuscular boosting on weeks 8, 12, and 14; and an ocular challenge with the same serovar at week 16. The mean follicular and inflammatory indices and the frequency of positive chlamydial cultures are shown. For the first 20 wk, both rectal and ocular cultures were taken [(R) indicates positive rectal cultures]. Error bars denote the SEM.
methods were similar to those used in experiments in monkeys with cholera toxoid. Those studies showed that oral immunization would prime the conjunctiva for subsequent ocular challenge. Four chlamydial antigen preparations were investigated for immunization potential: viable homologous EB (serovar B), viable heterologous EB (serovar L2), and nonviable heterologous EB (serovar L2) inactivated by either the addition of formalin or UV irradiation. Following oral immunization, resistance to ocular challenge was induced only in animals given viable serovar L2 EB.

Each of the oral antigen preparations induced detectable serum antibody levels in at least some animals, but serovar-specific antibodies were not found in the tears of any orally immunized animal before ocular challenge. The highest serum antibody titers were found in the groups orally immunized with viable organisms. Only the group given viable serovar L2 had detectable levels of IgA antibodies in serum prior to challenge, and these animals had a brisk tear antibody response after ocular challenge. Both of these features are indicative of priming of mucosal immunity. There was no evidence for gastrointestinal colonization by viable serovar L2 in the one animal autopsied 5 wk after ocular challenge. Although rectal cultures were not performed routinely in these monkeys, positive rectal cultures were not obtained in this group or following enteric boosting in the maximally immunized group when rectal cultures were performed routinely. It is of interest that to be effective oral shigella vaccines must consist of living, although not necessarily invasive, organisms. Nonviable organisms were ineffective, and this was not related to the total antigenic mass. It is believed that the nonviable bacteria are not adequately presented by the antigen-presenting cells in Peyer's patch. Whether similar mechanisms operate for chlamydia is unknown. With viable serovar L2 organisms, there is the potential for invasion and replication in lymphoid tissue that does not exist for the other serovars, and this could markedly alter the immunizing potential of viable serovar L2.

The animals orally immunized with viable serovar L2, however, were not better protected than monkeys that had recovered from ocular infection. For this reason, the almost heroic combined mucosal and systemic immunizing schedule was used to induce maximum immunity without resorting to ocular infection. These monkeys were partially resistant to challenge, but they were still not better protected than the ocular immune animals, despite extraordinary titers of IgA and IgG in both serum and tears and demonstrable cellular immunity.

From these studies, it is not clear whether components of humoral immunity are important in resistance to ocular infection. Tear antibodies were not induced without ocular infection except in the maximally immunized group, but the brisk tear antibody response that followed ocular challenge did suggest some form of immunologic priming of the conjunctiva in some groups. An analogous situation has been found with oral immunizing studies using cholera toxoid where oral immunization primed the conjunctiva but specific antibody-containing cells were not seen prior to ocular challenge. It is always difficult to tell whether antibodies in tears from inflamed eyes are locally produced in the conjunctiva or are part of the inflammatory transudate from serum. A huge increase in the numbers of B lymphocytes in the conjunctiva has been found following chlamydial infection; moreover, as tear antibody titers may exceed serum titers, it seems likely that, in large part, antibodies found in the tears are locally produced. The presence of antichlamydial IgA antibodies in serum did correlate with resistance to ocular infection, but it is unclear whether this is a direct effect of the serum antibodies themselves or whether
the presence of serum IgA is merely an indicator of some other priming of the immune system against subsequent chlamydial infection. None of the oral immunizing schedules induced tear antibodies prior to ocular challenge, although both ocular immune animals and maximally immunized animals had tear IgA antibodies and were resistant to challenge. In several animal models, it has been the presence of local antibodies rather than serum antibodies that has correlated with resistance to chlamydial infection. Whether IgA antibodies in either serum or tears act by preventing new infection or by limiting existing infection is unknown.

A marked worsening of disease or hypersensitivity was induced in animals orally immunized with UV-inactivated serovar L2 EB. Both formalin treatment and UV irradiation block chlamydial replication, but UV inactivation does not prevent specific attachment and phagocytosis of the EB by cells. Monkeys fed UV-inactivated EB showed no characteristics in their serologic or cellular immune response that could explain their prolonged infection and exaggerated response or that would suggest what mechanisms might be involved. Grayston and Wong have emphasized that “inadequate” trachoma vaccines can induce hypersensitivity, especially if they are of a heterologous serovar as was the case in these monkeys. Heterologous immunization, however, did not lead to sensitization with the ineffective formalin-inactivated EB, and viable serovar L2 gave good protection even against heterologous challenge with serovar B. It should be noted that serovars B and L2 are antigenically related, both belonging to the “B group.” The comparability of the serovar-specific antibody response in our studies reflects this. It may be that heterologous challenge using organisms from the “C group” would induce more severe disease than heterologous challenge within the same group. However, it seems unlikely that ocular disease could be more severe than the prolonged disease seen in the group orally immunized with UV-inactivated serovar L2 EB and challenged with B. The mechanisms involved in the hypersensitive response are not clear and require further examination, as they may shed light on the deleterious features of the host immune response to chlamydial infection that must be avoided by any prospective vaccine.

In summary, these studies do show that it is possible by oral immunization to induce some degree of protective immunity to subsequent chlamydial ocular infection. Although this supports the notion that mucosal immunity is important in protecting against chlamydial infection, the overall lack of efficacy of the various oral immunization regimens, except for the pathogenic live serovar L2, and the failure to obtain better protection than that induced by recovery from previous ocular infection were disappointing. One explanation for this may lie in the use of whole EB as the immunizing antigen. It is now clear that chlamydial EB are antigenically complex and express a number of distinct antigens. If the immune response to chlamydia is both protective and destructive, immunization with whole EB may well induce both types of response. A purified antigen preparation, therefore, might be more successful in eliciting protection than vaccines using the antigenically complex whole organism. We are currently exploring the efficacy of oral immunization with recombinant bacteria that express a single specific chlamydial antigen. Finally, it may be that enteric immunization alone may not be able to prime the conjunctiva sufficiently to completely resist subsequent ocular challenge, and studies assessing topical ocular and combined oral and ocular immunization are planned.

Key words: chlamydia, trachoma, mucosal immunity, vaccination, monkeys

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References