
Attempted Oral Immunization With Chlamydial Lipopolysaccharide Subunit Vaccine
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The effects of oral immunization with a recombinant vaccine expressing chlamydial lipopolysaccharide (LPS) on subsequent ocular challenge with Chlamydia trachomatis were studied in cynomolgus monkeys. Groups of four or five monkeys were given an oral vaccine containing 5 × 10⁸ parent or recombinant Escherichia coli on days 0, 14, and 35 and were challenged with either 2 × 10⁵ or 5 × 10⁵ inclusion forming units of viable purified elementary bodies on day 42. On clinical and microbiologic grounds, oral immunization failed to protect monkeys against subsequent ocular challenge. Anti-chlamydial IgG or IgA antibodies were not induced by oral vaccination, and the antibody response following ocular challenge was similar in vaccinated and nonvaccinated animals. Paradoxically, however, while nonvaccinated control animals developed antibodies against chlamydial LPS detectable by immunoblotting after chlamydial challenge, the LPS vaccinated animals did not. This study demonstrates that the oral recombinant vaccine expressing chlamydial LPS was ineffective in protecting against chlamydial eye infection and strongly suggests that chlamydial LPS may not be an important antigen for protective immunity against chlamydia. Invest Ophthalmol Vis Sci 28:1722–1726, 1987

It is generally assumed that an effective vaccine against trachoma will stimulate protective immunity at the mucosal surface of the eye. Previous studies have shown that the oral administration of an antigen can prime the eye to subsequent challenge with that antigen, and preliminary vaccine trials in a monkey model of trachoma have shown that protection equivalent to that seen after ocular infection can be induced by oral immunization with viable whole Chlamydia trachomatis elementary body (EB) vaccines. However, some of these oral preparations as well as systemic vaccines can also induce a hypersensitivity response with more severe disease occurring after ocular challenge. It seems clear that the immune response to C. trachomatis can be both protective and deleterious and that different chlamydial antigens may be responsible for the different types of immune response. For this reason, subunit vaccines may offer the most appropriate mechanism of stimulating protective immunity without inducing harmful sensitization. The first subunit chlamydial antigen preparation to be available in sufficient quantities for study is chlamydial lipopolysaccharide (LPS) expressed by a recombinant Escherichia coli. This report presents the first studies on the efficacy of a recombinant chlamydial LPS oral vaccine tested in the monkey model of trachoma.

Materials and Methods. Animals: Groups of young adult cynomolgus monkeys were obtained from Charles River Primates (Boston, MA). There were five monkeys in each group unless otherwise specified. All procedures described herein conform to the ARVO Resolution on the Use of Animals in Research.

Oral vaccination: Recombinant E. coli harboring plasmid pFEN 207 which expresses chlamydial LPS group antigen on its surface and the parent E. coli harboring only the pUC8 plasmid vector were cultured in LB medium and adjusted to contain 1 × 10⁸ organisms per ml. Monkeys were fasted overnight; and immediately after neutralization of gastric contents with 5 ml of sodium bicarbonate solution, the enteric dose of 5 ml suspension (5 × 10⁸ organisms) was administered via a gastric tube.

Oral vaccine, or the parent E. coli, was given on days 0, 7, 14, and 35; and the ocular challenge was given on day 42.

Ocular challenge with C. trachomatis: C. trachomatis serovar B (TW-5) was grown in mass tissue culture. Purified EBs were prepared by centrifugation through renograffin and resuspended at various dilutions in phosphate-buffered saline. Ocular inoculations were adjusted to 1 × 10⁹, or 2.5 × 10⁷ infection
forming units (IFU) per ml. Twenty μl of suspension was placed into each conjunctival sac, giving an ocular inoculation of approximately $2 \times 10^3$, or $5 \times 10^3$ IFU per eye.

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, and superior fornix conjunctiva as well as 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. At each examination, serum was obtained and tears were collected using small cellulose sponges. Although both eyes were examined, specimens were taken only from the left eye to eliminate the possibility of artifactitious changes in the right eye.

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 (TW-5). IgG was assayed using goat anti-human IgG (Hyland Labs, Costa Mesa, CA) which cross-reacts with monkey immunoglobulin heavy chains, and IgA was assayed with rabbit anti-monkey IgA (Nordic Immunological Labs, El Torro, CA).

Immunoblotting: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were essentially done as previously described. Preparatory SDS-PAGE was performed using whole-cell lysates of the B serovar on a 12.5% gel. After SDS-PAGE, chlamydial polypeptides were electroblotted onto nitrocellulose paper (NCP) and the NCP was then incubated with "blotto" for 30 min at room temperature. NCP was then cut into 0.5 cm strips, and individual strips were incubated with samples of monkey tears followed by $^{125}$I Protein A. In following this technique, tear antibody was diluted approximately 1:5, and 1.0 ml of the diluted specimen was incubated overnight at room temperature with an NCP strip containing chlamydial antigens. NCP strips were washed extensively in PBS and then incubated with 5 ml of $^{125}$I Protein A ($5 \times 10^4$ cpm/ml) in PBS for 2 hr at room temperature. The NCP strips were washed thoroughly, dried, and subjected to autoradiography. Immunoblotting was performed with tears collected from animals given either parent or recombinant E. coli and then challenged with the highest titer ocular inoculation ($5 \times 10^3$ IFU).

Results. Overall there was no significant difference between the clinical or microbiologic response observed after ocular challenge with animals in any of the groups (Fig. 1). Monkeys in each group developed acute, self-limited chlamydial conjunctivitis. The two groups of monkeys given the recombinant E. coli expressing the chlamydial LPS ("vaccinated groups") did not show any evidence of protection nor was there evidence for a dose response to the varying strength of challenge inoculum. There was, however, no evidence of sensitization or worsening of disease in the vaccinated monkeys.

Serology: None of the monkeys had preexisting antichlamydial IgG or IgA antibodies in either serum or tears at the start of these studies. None of the animals in the vaccinated groups developed serum or tear IgG or IgA antibodies prior to ocular challenge. After challenge, there was a brisk IgG and IgA antibody response in both serum and tears in each of the vaccinated and nonvaccinated groups. There was no consistent or significant difference between the response of any one group of monkeys compared to the others. The kinetics of IgG and IgA responses in the immunized animals was similar to that seen in the nonimmunized animals (data not shown) and previously reported in naive animals.

Immunoblotting: The immunoreactivity of monkey tear IgA antibodies to individual antigens of the C. trachomatis B serovar is shown by the immunoblots in Figure 2. The tear IgA antibody response to the 57 Kd polypeptide and to major outer membrane protein (MOMP) were very similar for both groups. The most pronounced difference in responses between monkeys of the two groups was against chlamydial LPS. Monkeys vaccinated with E. coli expressing the LPS group antigen did not have detectable tear IgA antibodies specific for chlamydial LPS. In contrast, three of the four monkeys immunized with the parent strain of E. coli showed an intense tear IgA antibody response to LPS. One monkey in each study group (marked as Z-4 and T-7) had weak to nondetectable tear IgA antibodies as determined by immunoblotting.

Discussion. This study is the first attempt to use a recombinant subunit vaccine against Chlamydia. Oral vaccine with recombinant E. coli expressing chlamydial LPS antigen failed to show any protective effect against subsequent ocular chlamydial chal-
Inoculum Index

Ocular response expressed as the mean follicular index and the mean inflammatory index following challenge with serovar B (error bars denote standard error of the mean). The upper bar graph shows the frequency of positive chlamydia cultures. The smallest bar off the baseline indicates that all cultures on that date were negative. (A) Five naive monkeys challenged with $2 \times 10^3$ IFU (from ref. 1). (B) Four monkeys orally immunized with recombinant \textit{E. coli} and challenged with $2 \times 10^3$ IFU.

Fig. 1. Ocular response expressed as the mean follicular index and the mean inflammatory index following challenge with serovar B (error bars denote standard error of the mean). The upper bar graph shows the frequency of positive chlamydia cultures. The smallest bar off the baseline indicates that all cultures on that date were negative. (A) Five naive monkeys challenged with $2 \times 10^3$ IFU (from ref. 1). (B) Four monkeys orally immunized with recombinant \textit{E. coli} and challenged with $2 \times 10^3$ IFU.

The outcome of these prolonged and expensive studies was disappointing. The immunizing schedule and the timing of the ocular challenge followed those we have used in previous studies with the addition of an extra boost given on day 35. The animals were challenged 1 week later. In retrospect, the lack of protection could have been predicted since the immunizing regimes that were used did not induce anti-chlamydial IgG or IgA antibodies detectable by the microimmunofluorescent assay in either serum or tears. However, this same assay primarily detects antibodies directed against MOMP rather than the chlamydial LPS. Of even more relevance to that prediction was the absence of the anti-chlamydial LPS anti-

Fig. 1 (continued) (C) Five monkeys orally immunized with parent \textit{E. coli} and challenged with $5 \times 10^2$ IFU. (D) Five monkeys orally immunized with recombinant \textit{E. coli} and challenged with $5 \times 10^3$ IFU.
Fig. 2. Immunoreactivity by immunoblotting of tear IgA antibodies to individual antigens of C. trachomatis. (A) Four monkeys orally immunized with parent E. coli and challenged with 5 x 10^3 IFU. (B) Five monkeys orally immunized with recombinant E. coli and challenged with 5 x 10^3 IFU.

bodies at the time of challenge as demonstrated by the immunoblotting of tears from five vaccinated animals.

Even though oral vaccination with recombinant chlamydial LPS neither induced antichlamydial antibodies nor gave protection, it was not entirely without immunological effect, and its lack of protection cannot simply be dismissed as an ineffective preparation. It was shown, almost paradoxically, that orally vaccinated monkeys failed to develop anti-LPS antibodies after challenge. Whereas three of the four nonimmunized monkeys tested by immunoblotting developed tear IgA antibodies against chlamydial LPS by day 28, none of the five vaccinated monkeys so tested developed these antibodies. This suggests that the oral vaccination might have even induced some degree of immune tolerance to chlamydial LPS.

Since these studies were initiated, several reports have appeared which suggest that in chlamydial infection the immunological response to LPS may be less important than previously thought. Neutralization studies have shown that monoclonal antibodies against chlamydial LPS do not inactivate viable chlamydia, whereas monoclonal antibodies against surface exposed epitopes of MOMP do block infectivity of EB both in vitro and in vivo. Those findings, together with the results of the present studies, suggest that the immune response to LPS is probably not important in protection against chlamydial infection. It has also been suggested that LPS may be important in the deleterious hypersensitivity response seen in chlamydial infection, though it has been shown more recently that LPS does not induce an inflammatory or delayed hypersensitivity response in ocular immune animals. Although an antigen which is extractable in Triton-X 100 detergent does induce such a response, these findings together with the data presented in this report indicate that chlamydial LPS is probably not a key factor in the deleterious response to chlamydial infection.

Key words: Chlamydia trachomatis, lipopolysaccharide, mucosal immunity, vaccine, trachoma

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pled fluxes of Na+ and Cl~ through the secretory cell basal-lateral plasma membranes, but the precise icated. Since lacrimal gland fluid formation was par-
ionic influx mechanisms have not yet been delin-
characterized by plasma-like concentrations of Na +

Na+ /H+ Antiporter in Lacrimal Acinar Cell Basal-Lateral Membranes

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The first step in the formation of lacrimal gland fluid is believed to depend on transport systems which couple a flux of Cl− ions to the passive influx of Na+ ions across the acinar cell basal-lateral plasma membrane. The transport systems which mediate these fluxes have not yet been character-
tized, but a review of previous studies (Parod and Put-
ey, Am J Physiol 239:G106, 1980) raises the possibility that Na+/H+ antiporters might represent a major pathway for Na+ influx. This conclusion is of interest, because anti-
porter mediated Na+ influx can, potentially, drive net C1− fluxes. We have now examined a sample of basal-lateral membrane vesicles from rat exorbital lacrimal gland to ver-
ify the presence of a Na+/H+ antiporter activity. Imposi-
on of an outward H+ gradient caused a 4.4-fold increase in the 22Na influx rate, while imposition of an outward Na+ gradient accelerated H+ uptake as determined by changes in acidine orange absorbance. All transport experiments were done in the presence of valinomycin and symmetrical K+ concentrations, eliminating the possibility of conductive Na+ or H+ fluxes driven by diffusion potentials. The pH gradient dependent Na+ influx was completely inhibited by 1 mM amiloride, indicating that it was mediated by a Na+/H+ antiporter similar to those described in other tis-

Materials and Methods. Analyzed reagent grade d-sorbitol was from J. T. Baker (Phillipsburg, NJ), 22Na from Amersham (Arlington Heights, IL), and 3H-mannitol from ICN (Costa Mesa, CA). All other chemicals were reagent grade and were obtained from standard suppliers. Male Sprague-Dawley rats, 240–260 gm, were used in conformity with the ARVO Resolution on the Use of Animals in Research.

For each experiment, 16 exorbital lacrimal glands were cut into 5 mg fragments and placed in a modi-
ified Krebs improved Ringer I bicarbonate buffer (KRB) saturated with 95% O2-5% CO2. After a 55 min incubation at 37°C, the fragments were transferred to isolation buffer and subjected to homogenization, differential sedimentation, and equilibrium density gradient centrifugation. KRB, isolation buffer, and density gradient media all contained 0.2

* Y. Saito, manuscript submitted.