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A Poliovirus Hybrid Expressing a Neutralization Epitope from the Major Outer Membrane Protein of *Chlamydia trachomatis* Is Highly Immunogenic

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Trachoma and sexually transmitted diseases caused by *Chlamydia trachomatis* are major health problems worldwide. Epitopes on the major outer membrane protein (MOMP) of *C. trachomatis* have been identified as important targets for the development of vaccines. In order to examine the immunogenicity of a recombinant vector expressing a chlamydial epitope, a poliovirus hybrid was constructed in which part of neutralization antigenic site I of poliovirus type 1 Mahoney (PV1-M) was replaced by a sequence from variable domain I of the MOMP of *C. trachomatis* serovar A. The chlamydial sequence included the neutralization epitope VAGLEK. This hybrid was viable, grew very well compared with PV1-M, and expressed both poliovirus and chlamydial antigenic determinants. When inoculated into rabbits, this hybrid was highly immunogenic, inducing a strong response against both PV1-M and *C. trachomatis* serovar A. Antichlamydia titers were 10- to 100-fold higher than the titers induced by equimolar amounts of either purified MOMP or a synthetic peptide expressing the VAGLEK epitope. Furthermore, rabbit antisera raised against this hybrid neutralized chlamydial infectivity both in vitro, for hamster kidney cells, and passively in vivo, for conjunctival epithelia of cynomolgus monkeys. Because poliovirus infection induces a strong mucosal immune response in primates and humans, these results indicate that poliovirus-chlamydia hybrids could become powerful tools for the study of mucosal immunity to chlamydial infection and for the development of recombinant chlamydial vaccines.

Chlamydia trachomatis infects the epithelia of the conjunctivae and of the genital tract, causing trachoma and a variety of sexually transmitted diseases which can lead to blindness and infertility, respectively (36, 37). There are 15 serovars of *C. trachomatis*; serovars A, B, and C are the causative agents of trachoma (10), while serovars D, E, F, G, H, I, J, and K are the most common causative agents of chlamydial sexually transmitted diseases (36, 37). Trachoma is the leading cause of preventable blindness in developing nations, and it is estimated that 500 million people suffer from trachoma, with as many as 7 million blinded by the disease (10).

The pathogenesis of trachoma involves repeated ocular infections and the generation of a deleterious hypersensitivity response to chlamydial antigen(s) (19, 20, 41, 42). The available evidence supports the hypothesis that secretory immunoglobulin A (IgA) is an important component of protection. In a primate model, ocular infection induces relatively rapid and persistent production of IgA in tears, whereas the presence of IgG in tears is transient, corresponding to the period of peak conjunctival inflammation and follicle formation (7). Protective immunity against chlamydial ocular infection in a subhuman primate model is homotypic, and resistance to ocular challenge is correlated with the presence of serovar-specific antibodies in tears (18, 46). Tears from infected humans neutralized the infectivity of homologous but not heterologous *C. trachomatis* serovars in owl monkey eyes (30), whereas passive humoral immunization with antitrachoma antibodies was not protective (32). Past attempts to develop whole-cell vaccines against trachoma have actually potentiated disease by sensitizing vaccinees (17, 19). Thus, an effective trachoma vaccine will probably have to be based on a subunit immunogen capable of inducing a strong and enduring mucosal neutralizing antibody response without sensitizing the vaccinee.

The most promising candidate antigen for the development of a subunit vaccine is the chlamydial major outer membrane protein (MOMP) (6). Other outer membrane proteins and the surface-exposed lipopolysaccharide are also highly immunogenic, but unlike the MOMP, antibodies to these antigens have not been shown to neutralize infectivity (51). The MOMP, which is the predominant surface protein, is an integral membrane protein with a mass of ca. 40 kDa and, with the exception of four variable domains (VDs), is highly conserved among serovars. The sequences of the four VDs have been determined for all 15 serovars (49). Antibodies capable of neutralizing chlamydial infectivity recognize the MOMP (24, 33, 40, 50, 51). The binding sites of MOMP-specific neutralizing monoclonal antibodies have been mapped for serovars A, B, and C (2, 8, 25, 38, 50, 51) and represent important targets for the development of synthetic or subunit vaccines. These sites are contiguous sequences of six to eight amino acids located within VDI or VDII (depending on the serovar) and VDIV.

Synthetic peptides containing epitopes recognized by neutralizing antibodies can induce neutralizing antibodies of the desired specificity (39), but immunization with these peptides by conventional means failed to induce mucosal immunity. Similarly, conventional immunization with MOMP would not be expected to induce significant mucosal immunity. It may, however, be possible to induce protective

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mucosal neutralizing antibodies against these epitopes by expressing them on poliovirus, which is known to efficiently induce a long-lasting mucosal immune response following infection of the gut (12, 13, 31). A number of heterologous epitopes have been successfully expressed on poliovirus (5, 28 [and references therein], 29). These epitopes are generally short, well-defined, contiguous epitopes, and most have been expressed within poliovirus neutralization antigenic site I (NAgI). This site, of which there are 60 copies per virion, includes the loop linking beta strands B and C (the BC loop) of poliovirus capsid protein VP1 (21). The BC loop is a surface-exposed loop of 9 amino acids (21) which can be replaced by at least 25 heterologous amino acids (27). The MOMP epitopes therefore seem to be ideal for expression on poliovirus, and hybrid polioviruses expressing chlamydial neutralization epitopes appear particularly attractive for use as experimental tools and even as potential vaccines.

In order to determine the feasibility of constructing useful poliovirus-chlamydia hybrids and to assess their immunogenicity, we have constructed a poliovirus hybrid expressing the well-characterized VAGLEK neutralization epitope (39, 50, 51) from VDI of *C. trachomatis* serovar A MOMP. This hybrid grows nearly as well as wild-type poliovirus and expresses a functional chlamydial epitope. Furthermore, in terms of the ability to induce a humoral immune response in rabbits, this hybrid is highly immunogenic compared with chlamydia-specific synthetic peptides or purified MOMP.

MATERIALS AND METHODS

Hybrid polioviruses. Hybrid polioviruses were constructed by the mutagenesis cartridge strategy developed by Murray et al. (29). Synthetic oligonucleotides coding for amino acids from VDI of the *C. trachomatis* serovar A MOMP were cloned into a full-length cDNA of poliovirus type 1 Mahoney strain (PV1-M) in place of sequences coding for some or all of PV1-M amino acids 1094 to 1102 (Fig. 1). Note that we use the four-digit code for poliovirus amino acids; thus 1095 is amino acid 95 of capsid protein VP1, for example. Various combinations of poliovirus- and chlamydia-specific amino acids were examined for their effects on viral growth and immunogenicity.

Transfection of Vero cells with RNA transcripts (45) of hybrid cDNAs yielded three hybrid viruses, designated PV1-Ct5, -Ct7, and -Ct8. Transcripts of cDNAs pT7Ct2 and pT7Ct4 were apparently noninfectious. Transfection with transcripts of the parental PV1-M cDNA (pT7XLD) (28) yielded a transfection-derived wild-type poliovirus designated PV1-XLD. The identities of PV1-Ct7, PV1-Ct8, and PV1-XLD were confirmed by sequencing the virion RNA through the region coding for the inserted amino acids.

Chlamydiae. *C. trachomatis* serovars A (strain Har-13), B (strain TW-5), and C (strain TW-3) were grown in stationary cultures of HeLa 229 cells, and chlamydial elementary bodies (EBs) were purified from infected cells by density gradient centrifugation as previously described (6).

MAbs. Monoclonal antibody (MAb) A-20 is specific for *C. trachomatis* serovar A. The hybridoma secreting MAb A-20 was generated from mouse splenocytes following intraperitoneal immunization with intact formalin-killed serovar A EBs. MAb A-20 binds to intact serovar A EBs in a dot immunoblot and neutralizes their infectivity for cultured eukaryotic cells. MAb A-20 is MOMP specific, and its binding site maps to the hexameric VAGLEK sequence located within VDI of serovar A MOMP (2, 50, 51). MAb B-B6 is a serovar B MOMP-specific antibody and was used as a negative control in virus neutralization assays; it was prepared in the same way as A-20 and is of the same isotype, IgG3 (51). For neutralization assays, dilutions were prepared from MAb stock solutions which had a concentration of 1 mg/ml.

Convalescent antichlamydia sera. Convalescent sera were obtained from cynomolgus monkeys that had recovered from a primary conjunctival infection with serovar A. Preimmune sera were taken prior to experimental infection, and convalescent sera were taken 45 days postinfection, approximately 2 weeks after the monkeys became culture negative and were free of any clinical signs (conjunctival hyperemia and follicles) of disease. The sera were heat inactivated at 56°C for 30 min prior to assay.

Antigens and inoculations. Rabbits were inoculated either with CsCl-purified PV1-Ct7 or PV1-XLD or with serovar A MOMP or synthetic peptide A8-VDI. Note that although the viruses used were live, poliovirus does not replicate in rabbits (22), and any response observed is effectively the response to an inactivated antigen. The synthetic peptide A8-VDI, a 40-mer, contains VDI of serovar A MOMP, including the B-cell epitope VAGLEK, and a MOMP helper T-cell epitope (39). MOMP was gel purified as described previously (6). The viruses were purified by banding on a CsCl density gradient to separate 155S virions from other viral antigens such as 80S empty capsids. By using the extinction coefficient for purified virus of $A_{260}^{1\%} = 74$ (44), the dose of virus per inoculation was determined from A_{260} values to be approximately 3.0×10^{11} virions, equivalent to 2.5×10^8 PFU as determined by plaque assay. This dose is equivalent to 0.5 pmol of virus or 30 pmol of the VAGLEK epitope, since each virion expresses 60 copies of the epitope. Similarly, the dose of the VAGLEK epitope per inoculation of serovar A MOMP or A8-VDI was 30 pmol, equal to 1.2 μg of MOMP and 0.16 μg of peptide.

On day 0, rabbits (four per antigen) were each inoculated simultaneously with one dose of antigen in Freund's complete adjuvant subcutaneously on the back and with one dose in saline intramuscularly. On days 14 and 28, the rabbits were each given one booster dose in Freund's incomplete adjuvant subcutaneously on the back. Blood samples were taken prior to the first inoculation and after 42 days. This protocol was based on that described previously for similar studies (1, 28, 29).

Poliovirus plaque and neutralization assays. Plaque assays were performed as described by Emini et al. (11). Sera were titrated for their ability to neutralize 100 50% tissue culture infectious doses of virus, using the method of Golding et al. (16). Vero cells were used as the substrate for all plaque and neutralization assays.

Western blots (immunoblots). Purified serovar A, B, and C EBs were solubilized in sample buffer and electrophoresed on a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel as previously described (23). Chlamydial polypeptides separated by SDS-polyacrylamide gel electrophoresis were transferred to nitrocellulose paper and incubated with rabbit antisera diluted 1:200 in phosphate-buffered saline (PBS) containing 1% dry milk and 0.5% Tween 20. Following overnight incubation with the antisera, the nitrocellulose paper was washed thoroughly in PBS-Tween 20, probed with ^{125}I-labelled protein A (Amersham, Inc.), washed again with PBS-Tween 20, and then dried. Radiolabelled chlamydial proteins were visualized by autoradiography.

Enzyme-linked immunosorbent assay (ELISA). Immulon 2 microtiter plates (Dynatech Laboratories Inc., Alexandria, Va.) were coated overnight at 4°C with 100 μl of peptide A

VDI (MOMP residues 61 to 85) or formalin-killed serovar A, B, or C EBs at 10 μg/ml in 0.05 M Tris buffer (pH 7.5) containing 0.15 M NaCl. Serial twofold dilutions of rabbit sera were tested in duplicate. Rabbit IgG was detected with alkaline phosphatase-conjugated anti-rabbit IgG (heavy and light chain specific; Zymed Laboratories Inc., San Francisco, Calif.) and then with substrate (5 mg of *p*-nitrophenyl phosphate in 10 ml of 0.1 M 2,2-amino-2-methyl-1,3-propanediol, pH 10.3). A_{405} was measured with an ELISA reader (Bio-Rad Laboratories, Richmond, Calif.).

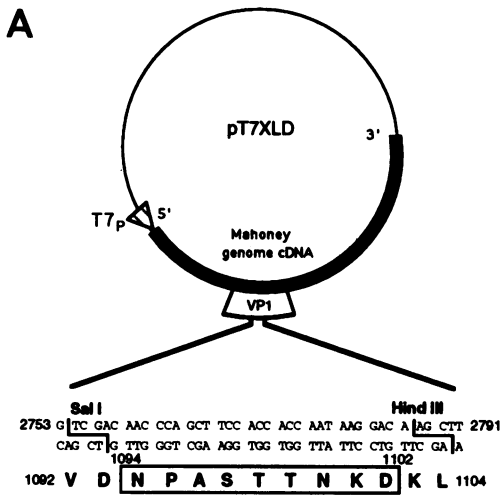
Pepscan-ELISA. The peptide-pin-based assay described by Geysen et al. (14, 15) was used to determine the fine specificity of the rabbit anti-PV1-Ct7 antisera. Sequential and overlapping octapeptides corresponding to the serovar A MOMP VDI sequence (residues 61 to 85) were synthesized on prederivatized polyethylene pins with a commercially available kit (Epitope Scanning Kit; Cambridge Research Biochemicals Inc., Wilmington, Del.) using f-moc chemistry as described by the manufacturer. Rabbit antisera diluted 1:500 were incubated with the individual peptide pins. Rabbit IgG bound to the peptides was detected by using alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies, as described for ELISA.

In vitro neutralization of chlamydiae. Hamster kidney (HaK) cells (10^5 cells per well) were grown overnight in flat-bottomed 96-well microtiter plates (Linbro; Flow Laboratories Inc., McLean, Va.). Serovar A and C EBs were diluted in SPG (0.25 M sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid [pH 7.2]). To determine serum neutralization titers, twofold dilutions of heat-inactivated (56°C for 30 min) sera were made in SPG. One hundred microliters of diluted sera was mixed with 100 μl of EBs in SPG at 6.6×10^5 inclusion-forming units (IFU) per ml and then incubated at 37°C for 30 min. After incubation, 50-μl aliquots from each mixture were inoculated in triplicate onto HaK monolayers. The monolayers were incubated at 37°C for 2 h, washed once with 100 μl of Hanks balanced salt solution (HBSS) and then fed with 200 μl of minimal essential medium containing 10% fetal calf serum and 1 μg of cycloheximide per ml. Cells were incubated at 37°C for 48 to 70 h, washed with HBSS, and fixed with absolute methanol.

To visualize chlamydial inclusions, methanol-fixed cells were incubated with a MAb to chlamydial lipopolysaccharide followed by a fluorescein-conjugated rabbit anti-mouse antibody (34). Fluorescent chlamydial inclusions were counted in 10 microscopic fields of view at ×100 magnification from triplicate wells. In neutralization assays, the percent reduction of IFUs was calculated by comparison with preimmune serum control values.

In vivo neutralization of chlamydiae. The ability of rabbit antisera to PV1-Ct7 to passively protect in vivo was assayed by using a monkey model of chlamydial ocular infection. A 300-μl volume of serovar A EBs in SPG (1.0×10^6 IFU) was mixed with 300 μl of a 1:100 dilution of pooled, heat-inactivated (56°C, 30 min) anti-PV1-Ct7 or preimmune sera and incubated at 37°C for 30 min. Immediately after the incubation, 20-μl portions of the serum-chlamydia mixtures were inoculated onto the upper and lower conjunctivae of both eyes of three cynomolgus monkeys.

Clinical disease developed by the monkeys was scored by evaluating hyperemia and follicle formation of the upper and lower palpebral conjunctivae of both eyes. The clinical grading scheme described by Taylor et al. (43) was used, and the data are expressed as aggregate inflammatory and follicle scores of both eyes for individual monkeys. All inoculations and clinical evaluations of monkeys were conducted with ketamine hydrochloride sedation.



B

Clone	Sequence encoded	Viable virus recovered/strain designation
pT7XLD	NPASTT NKD	Yes PV1-XLD
pT7Ct2	**VAGLEKDPVA**	No
pT7Ct4	N **PTTSDVAGLEKDPVA**	No
pT7Ct5	N **PTTSDVAGLEKDP**KD	Yes PV1-Ct5
pT7Ct7	NPAS**TTSDVAGLEKDPVA**	Yes PV1-Ct7
pT7Ct8	NPAS**TTSDVAGLEKDP**KD	Yes PV1-Ct8

FIG. 1. Construction of poliovirus-chlamydia hybrids. (A) By using a *SalI-Hin*dIII mutagenesis cartridge, the PV1-M cDNA clone pT7XLD was modified to encode amino acid sequences, including the VAGLEK epitope, from *C. trachomatis* serovar A MOMP VDI. The mutagenesis cartridge spans poliovirus nucleotides 2759 to 2785, which encode poliovirus amino acids 1094 to 1102, as shown in the lower part of panel A. (B) The poliovirus-specific nucleotide sequence within the cartridge was replaced with synthetic oligonucleotides encoding various amino acid sequences from *C. trachomatis* serovar A MOMP VDI (shown in bold print). Viable virus was recovered, as described in the text, from three of five clones encoding chlamydial sequences.

RESULTS

Growth characteristics of the hybrids. Using the cartridge mutagenesis method of Murray et al. (29), we constructed five hybrid cDNAs in which a portion of the sequence encoding NAgI of PV1-M was replaced with various sequences encoding regions of *C. trachomatis* serovar A MOMP VDI, including the VAGLEK neutralization epitope. RNA transcripts prepared from three of five hybrid cDNAs (pT7Ct5, pT7Ct7, and pT7Ct8) were infectious in Vero cells, producing viable hybrid viruses, whereas RNA transcripts prepared from the other two hybrid cDNAs did not produce virus after two independent transfections (Fig. 1). The three hybrids recovered were designated PV1-Ct5, PV1-Ct7, and PV1-Ct8. PV1-Ct5 grew very slowly and produced small, almost pinpoint, plaques in Vero cells. Because of its poor growth properties, it was not studied further. PV1-Ct7 and -Ct8 grew very well, producing plaques similar in size to or slightly smaller than those of PV1-XLD, respectively (data not shown). In a single-step growth cycle, both hybrids were slightly impaired compared with PV1-XLD; PV1-XLD grew faster than PV1-Ct8, which grew faster than PV1-Ct7 (Fig.

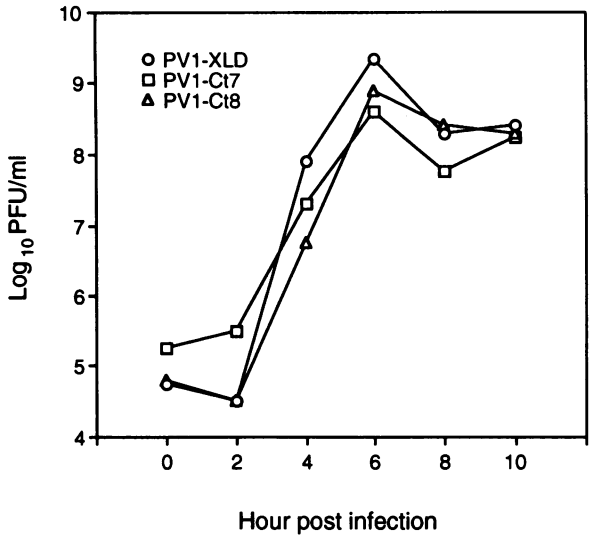


FIG. 2. Single-step growth curves of PV1-XLD, PV1-Ct7, and PV1-Ct8. Virus was grown and recovered as described previously (29) and then titrated by plaque assay. Points represent the mean values of titrations in duplicate wells.

2). The peak titers for PV1-Ct7 and PV1-Ct8 were only 0.6 and 0.75 $\log_{10}$, respectively, below the peak titer for PV1-XLD (Fig. 2). Consequently, preparing adequate quantities of the hybrids for further study was not a problem.

Antigenic characteristics of hybrid polioviruses. The antigenic characteristics of PV1-Ct7 and -Ct8 are shown in Table 1. Both were neutralized by MAb A-20, which is specific for the VAGLEK neutralization epitope from VDI of serovar A MOMP, indicating that this epitope is expressed in a recognizable and antigenic form on both hybrids. The higher titer against PV1-Ct7 suggests that this hybrid expresses the VAGLEK epitope in an antigenic configuration closer to that of the native epitope on intact chlamydiae. Significantly, the hybrids were also neutralized by convalescent sera from infected primates, indicating that the chlamydial sequence was expressed in a form recognizable during a natural infection. Although the titers in this case were low, it should be noted that the chlamydial infection generated only a weak humoral immune response and that the chlamydia-neutralizing titers of these sera were also low.

The hybrids induced a strong antipoliovirus response and were neutralizable by antipoliovirus (PV1-XLD) antisera, indicating that the changes to poliovirus NAgI had not significantly affected other antigenic sites on the virus. Since PV1-Ct7 was more readily neutralizable by MAb A-20, which suggested that it may express the VAGLEK epitope in a more appropriate conformation, this hybrid was selected for further study.

Immunogenicity of hybrid poliovirus. (i) Immunoblotting. The sera from rabbits immunized with PV1-Ct7 and PV1-XLD were tested by Western blotting against *C. trachomatis* serovars A, B, and C to determine the specificity of the anti-MOMP response. These analyses showed that all four rabbit antiserum samples raised against PV1-Ct7 reacted very strongly with the homotypic serovar A MOMP (Fig. 3). Moderate cross-reactivity against the heterologous serovar C MOMP was observed with one serum sample (rabbit 13), but the other three serum samples were not immunoreactive with serovar C MOMP. None of the anti-PV1-Ct7 antisera recognized serovar B MOMP. Sera raised against PV1-XLD did not react with the MOMPs of any of the three *C. trachomatis* serovars. These results show that PV1-Ct7 is capable of eliciting a consistent antibody response to serovar A MOMP and that the response is specific for the VDI sequence encoded by this hybrid.

TABLE 1. Neutralization of *C. trachomatis* and of poliovirus-chlamydia hybrids by antichlamydia and antihybrid sera and MAbs

Serum sample[a] or antibody	Neutralizing titer[b] against:			
	PV1-XLD	PV1-Ct7	PV1-Ct8	*C. trachomatis* serovar A
MAbs				
B-B6	<4	<4	<4	<16
A-20	<4	>2,048	601	2,048
Monkey convalescent sera				
831	<2	6	10	128
840	<2	23	16	128
939	<2	23	10	64
Rabbit sera[c]				
Anti-PV1-XLD	20,000	14,125	12,589	NT[d]
Anti-PV1-Ct7	28,183	ND[e]	ND	NT
Anti-PV1-Ct8	56,234	ND	ND	NT

[a] Results are shown for immune sera. Preimmune sera were also tested. All monkey preimmune sera had titers of <2 and all rabbit preimmune sera had titers of <4 against PV1-XLD, PV1-Ct7, and PV1-Ct8. Monkey preimmune sera had titers of <16 against *C. trachomatis*.

[b] Antivirus titers are the reciprocal dilution of the serum sample or MAb giving a 50% endpoint in a neutralization assay versus 100 50% tissue culture infective doses of virus. Antichlamydia titers are the reciprocal of the highest dilution of the serum sample or MAb giving at least 50% reduction in IFU assayed on HaK cells. Dilutions of MAbs refer to a starting concentration of 1 mg/ml.

[c] Pooled sera from blood samples taken 42 days after inoculation.

[d] The pooled sera were not tested (NT); see Fig. 5 for data on sera from individual rabbits.

[e] ND, not done.

(ii) Fine specificity of anti-PV1-Ct7 antisera. The sera from rabbits immunized with PV1-Ct7 were analyzed by pepscan-ELISA against overlapping octapeptides corresponding to the serovar A MOMP VDI sequence to ascertain the fine specificity of the anti-PV1-Ct7 response. The sera from all four rabbits showed a marked immunoreactivity with VDI octapeptides containing the VAGLEK sequence (Fig. 4). This result shows that PV1-Ct7 is capable of eliciting a consistent antibody response specific for the serovar A MOMP VDI sequence and, in particular, for the targeted VAGLEK epitope.

(iii) ELISA. The specificities and antibody titers of the anti-PV1-Ct7 sera were further tested by ELISA and compared with rabbit antisera raised against either purified serovar A MOMP or peptide A8-VDI (Table 2). The sera were tested against peptide A-VDI, which corresponds to serovar A residues 61 to 85 and contains the VAGLEK epitope, and formalin-fixed serovar A, B, and C EBs. Rabbit antisera raised against the control virus, PV1-XLD, did not react with any of the chlamydial antigens at the highest concentration of sera tested (1:32). All four rabbits immunized with PV1-Ct7 produced high-titer antisera (8,192 to 16,384) against the peptide A-VDI and intact serovar A EBs. The immunoreactivity with the EBs shows that the antibodies raised against PV1-Ct7 recognized the targeted chlamydial epitope(s) in the context of the native configuration of the

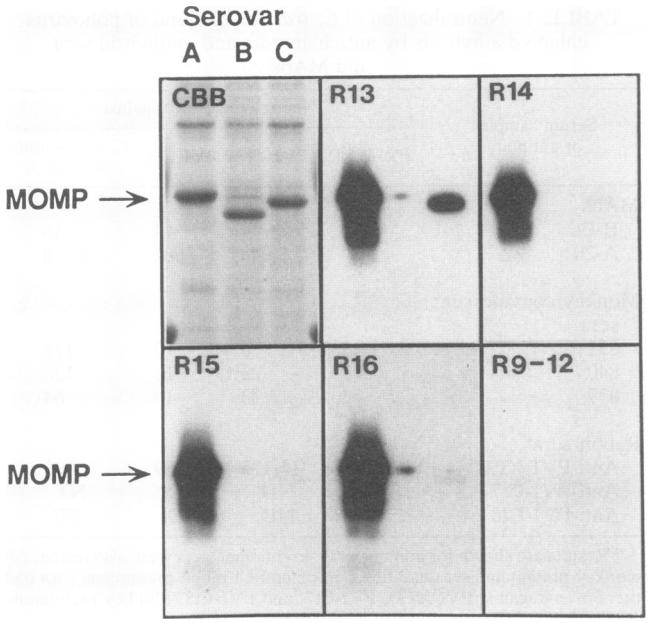


FIG. 3. Specificities of rabbit anti-PV1-Ct7 antisera for the MOMPs from *C. trachomatis* serovars A, B, and C by Western blot analysis. The panels show a Coomassie brilliant blue (CBB)-stained gel showing the MOMPs from serovars A, B, and C and the reactions with the three MOMPs of anti-PV1-Ct7 antisera from rabbits 13, 14, 15, and 16 (R13 to R16, respectively) (1:200 dilution). Pooled anti-PV1-XLD antisera from rabbits 9 to 12 (R9-12) did not react with the MOMPs. Western blotting was conducted as described in Materials and Methods.

MOMP on the chlamydial surface. Anti-PV1-Ct7 sera also exhibited considerable cross-reactivity against serovar C by ELISA. However, unlike the responses against serovar A, the responses against serovar C were clearly more variable among immunized rabbits, with titers ranging from 256 to 8,192. None of the anti PV1-Ct7 sera reacted in ELISAs with serovar B at a dilution of 1:32. The observed cross-reactivity between serovars A and C is not particularly surprising, since the primary sequences of their VDIs are very similar. Antiserum from rabbit 13 had an equivalent ELISA endpoint titer (8,192) against both serovar A and C EBs, which is inconsistent with the Western blot results shown in Fig. 3. The reason for this inconsistency is not understood, but it may reflect differences in antibody avidity or specificity for denatured and native forms of the MOMP.

In contrast to these results, rabbits inoculated with doses of purified serovar A MOMP or A8-VDI that contained the same molar amount of the VAGLEK epitope as in the dosage of PV1-Ct7 produced markedly lower and more variable antibody responses against both peptide A-VDI and serovar A EBs. These doses of purified MOMP and A8-VDI were significantly less (100- to 200-fold) than those we have previously found to induce antibody responses comparable in titer and specificity to those induced here by PV1-Ct7 (39). Nevertheless, these findings clearly demonstrate the superior immunogenicity of PV1-Ct7 in its ability to induce serovar A-specific antibodies.

In vitro neutralization of chlamydial infectivity by anti-PV1-Ct7 antisera. The rabbit anti-PV1-Ct7 antisera were tested for their ability to neutralize chlamydial infectivity for HaK cells. The results were consistent with those obtained by ELISA. All four serum samples exhibited consistent high-titer neutralizing activity against the homotypic serovar A (Fig. 5), in that for each serum sample, the dilution which caused a 50% reduction in serovar A infectivity was approximately 1:2,048. These sera also showed neutralizing activity against serovar C. However, except for sera from rabbit 13 (which caused a 50% reduction in serovar C infectivity at a dilution of approximately 1:2,048), the anti-serovar C neutralizing titers were lower and more variable than the anti-serovar A titers.

Passive in vivo neutralization of chlamydial infectivity for the monkey eye by anti-PV1-Ct7 antisera. The ability of rabbit anti-PV1-Ct7 antisera to neutralize chlamydial infectivity for HaK cells was a significant finding. However, it was important to determine whether the anti-PV1-Ct7 sera were capable of neutralizing chlamydial infectivity for cells which are more typically colonized by chlamydiae in the context of a naturally occurring ocular infection. We therefore tested the ability of rabbit anti-PV1-Ct7 sera to passively neutralize the infectivity of serovar A EBs for the conjunctivae of cynomolgus monkeys, an animal model that closely resembles human chlamydial ocular infection. As shown in Table 3, anti-PV1-Ct7 antisera were passively neutralizing in this context. All three monkeys inoculated with chlamydiae incubated with preimmune sera shed chlamydiae from their conjunctivae at 7, 14, and 21 days postchallenge. These monkeys also developed a mild follicular conjunctivitis characterized by conjunctival hyperemia and follicle formation. In contrast, those monkeys that received chlamydiae incubated with anti-PV1-Ct7 antisera were culture negative through day 21 postchallenge, and none developed clinical signs of conjunctivitis.

DISCUSSION

We have constructed and characterized two viable hybrid polioviruses (PV1-Ct7 and -Ct8) in which part of NAgI, the BC loop of VP1, has been replaced by sequences specific to VDI of the MOMP of *C. trachomatis* serovar A, including the well-characterized VAGLEK neutralization epitope. These hybrids were found to be only slightly growth impaired compared with their wild-type parent and grew better than many other previously described hybrids (see, for example, references 26 and 27). A third poliovirus-chlamydia hybrid (PV1-Ct5) grew poorly, and two other hybrids were apparently not viable. While we cannot rule out the effect of second-site mutations, it appears that the hybrids which grew best were those which retained some of the poliovirus VP1 BC loop amino acids on either side of the inserted sequence, particularly on the amino-terminal side. Conversely, those hybrids which grew poorly were those in which these amino acids had been deleted to keep the BC loop closer to its natural length. This result suggests that the sequence of the VP1 BC loop is more important than its length in determining the viability of poliovirus hybrids, which is consistent with our earlier observations (26, 27).

PV1-Ct7 and -Ct8 clearly express at least one *C. trachomatis*-specific epitope in a recognizable conformation which is presumably similar to that of the native epitope(s). The hybrids were neutralized both by MAb A-20, specific for the VAGLEK epitope of serovar A MOMP VDI, and by monkey convalescent sera. Rabbit antisera raised against PV1-Ct7 were strongly immunoreactive with octapeptides containing the sequence VAGLEK. The same antisera neutralized the infectivity of serovar A EBs both in vitro and, more significantly, in vivo. Thus, the expressed

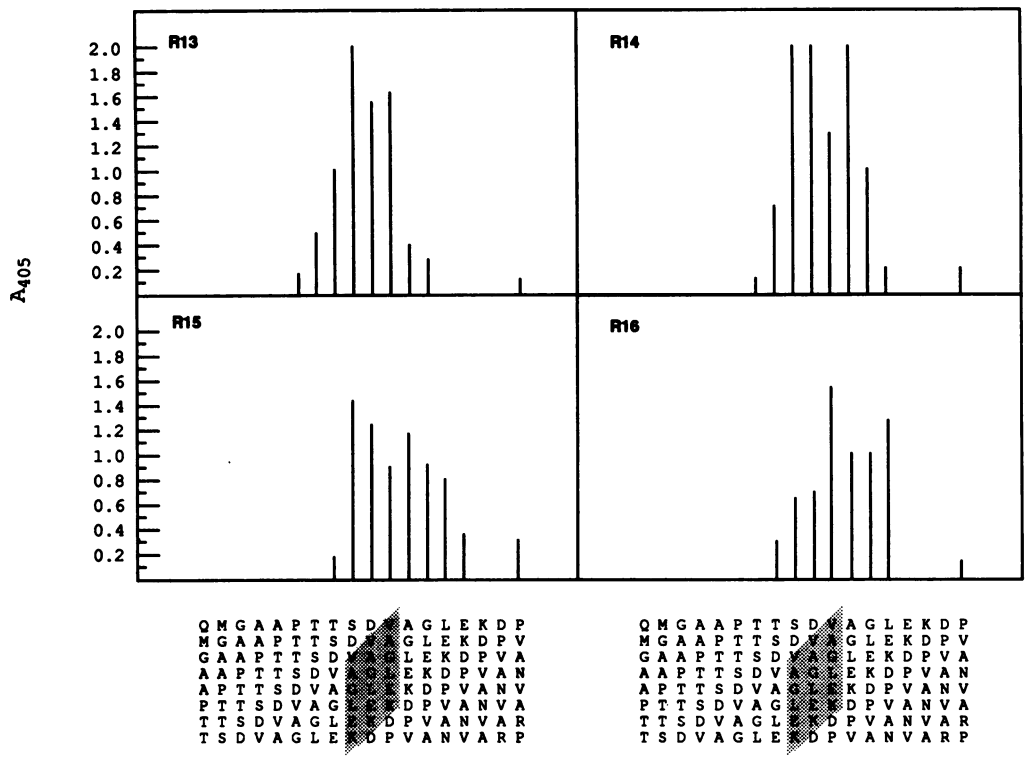


FIG. 4. Pepscan-ELISA of rabbit anti-PV1-Ct7 antisera. Sera from rabbits 13 to 16 (R13 to R16, respectively) were reacted with overlapping octapeptides corresponding to the serovar A MOMP VDI sequence. IgG antibodies reactive with the VDI octapeptides were detected with goat anti-rabbit IgG antiserum. The VDI amino acids are displayed in vertical columns across the bottom of the figure. The reactivities of the rabbit sera with the individual peptides are expressed as the A_{405} values and are represented by the solid vertical bars above the peptide sequences. The peptides displayed in the vertical columns represent sequential octameric sequences from VDI (residues 61 to 85), overlapping by a single amino acid. The Q at the top of the first column corresponds to residue 61, and the P at the bottom of the last column corresponds to residue 85.

epitope(s) were both recognized by and induced antibodies which were functionally relevant, including antibodies which were neutralizing in an appropriate animal model. Furthermore, PV1-Ct7 is a potent immunogen, consistently able to induce high titers of antichlamydia neutralizing antibodies. PV1-Ct7 was substantially more immunogenic, on an equimolar basis, than either purified A MOMP or synthetic peptide A8-VDI.

Purified MOMP and peptide A8-VDI have been proposed as potential antigenic components of a chlamydial vaccine (6, 9, 39, 47), and thus it is of particular interest that a poliovirus-chlamydia hybrid is so powerfully immunogenic in comparison. There are several possible explanations for the superior immunogenicity of the hybrid. One possibility is that poliovirus Th epitopes provide a better carrier effect by supplying cognate help to prime for a strong anamnestic response to successive doses of the hybrid. Another possibility is that the chlamydial epitopes expressed in the BC loop of VP1 are presented in a conformation that structurally mimics that of the epitopes on native MOMP, resulting in greater immunogenicity and the induction of antibodies with enhanced affinity for native MOMP. The second possibility would be consistent with earlier suggestions that the immunogenicity of isolated MOMP might be enhanced under conditions which maintain its native conformation (3).

We did not raise antisera to intact chlamydial EBs in this study, since they could probably not be included in any potential chlamydial vaccine. However, in other similar studies (5a), we have observed that doses of serovar A EBs containing approximately 50 nmol of the VAGLEK epitope induced serovar A-specific antibody titers comparable to those induced by the hybrids (30 pmol of the epitope) in this study. This result also suggests that the VAGLEK epitope and other VDI epitopes which may be present are presented at least as well on the poliovirus hybrids as they are on intact EBs.

Rabbits inoculated with PV1-Ct7 produced considerable cross-reactivity against serovar C EBs (Table 2). This result is not unexpected, considering that the VDIs of serovar A and C MOMP have very similar primary sequences (49). The region of serovar A VDI encoded by PV1-Ct7 is TTSDVA GLEKDPVA (Fig. 1); the equivalent sequence from serovar C, TTSDVAGLQNDPTT, differs by only four amino acids (underlined). Thus, a polyclonal antibody response generated against the serovar A sequence should elicit not only antibodies against the VAGLEK epitope but also antibodies against shared sequences that would correspond to antigenic determinants common to both these two closely related serovars. This cross-reactivity is very attractive in terms of providing a broader neutralizing response against multiple serovars, but the anti-serovar C reactivity generated by PV1-Ct7 was much more variable among rabbits than the anti-serovar A reactivity (Table 2 and Fig. 5). Although PV1-Ct7 is apparently unable to generate a consistent anti-serovar C response, it may be possible to improve the consistency and frequency of the cross-reactive response by

TABLE 2. Immunogenicity of poliovirus-chlamydiae hybrid PV1-Ct7

Immunogen[a]	Rabbit no.	ELISA antibody titer[b]			
		Peptide A-VDI[c]	Intact *C. trachomatis* EBs		
			Serovar A	Serovar B	Serovar C
PV1-XLD	9	<32	<32	<32	<32
	10	<32	<32	<32	<32
	11	<32	<32	<32	<32
	12	<32	<32	<32	<32
PV1-CT7	13	16,384	8,192	<32	8192
	14	8,192	8,192	<32	1024
	15	16,384	8,192	<32	512
	16	16,384	16,384	<32	256
Serovar A MOMP	5	256	<32	<32	<32
	6	512	256	<32	<32
	7	1,024	512	32	128
	8	128	128	32	32
Peptide A8-VDI	1	<32	<32	<32	<32
	2	256	64	32	32
	3	1,024	<32	<32	<32
	4	<32	<32	<32	<32

[a] Rabbit antisera were prepared against the immunogens which were standardized to contain equivalent amounts of the serovar A MOMP VAGLEK epitope (30 pmol per immunization per rabbit).

[b] ELISA titers are expressed as the reciprocal of rabbit serum dilutions giving an absorbance reading of 0.3. Absorbance values of preimmune sera were 0.1 or less.

[c] Peptide A-VDI corresponds to serovar A MOMP residues (61 to 85) and contains the VAGLEK epitope.

optimizing the expressed chlamydial sequence. For instance, we have found that sera from rabbits inoculated with PV1-Ct7 or PV1-Ct8 cross-react with several chlamydial serovars of the C complex in Western blot assays against MOMP and dot blot assays against intact chlamydiae and that some rabbits mount a response of approximately equal strength against at least four C-complex serovars (A, C, I, and J) (data not shown). It would be interesting to determine the epitope(s) recognized by these animals, since this might allow us to define a sequence which could reproducibly generate a strong C-complex cross-reactive response. Furthermore, the approach that we have used to construct a poliovirus-serovar A hybrid should be directly applicable to other chlamydial serovars. The sequence of serovar C VDI is very similar to that of serovar A VDI and should be readily expressible on a poliovirus-serovar C hybrid. On the basis of our findings with PV1-Ct7, a poliovirus-serovar C hybrid would be expected to be highly immunogenic, and importantly, it should induce a consistent anti-serovar C neutralizing antibody response.

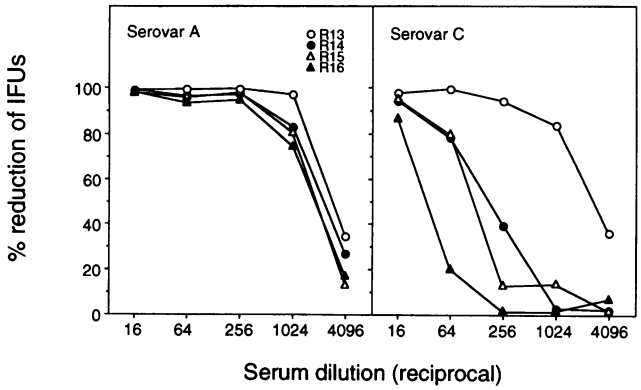


FIG. 5. Neutralization of *C. trachomatis* serovars A and C by anti-PV1-Ct7 antisera from rabbits 13 to 16 (R13 to R16, respectively). Pooled anti-PV1-XLD antisera were nonneutralizing (not shown). Neutralization assays were conducted as described in Materials and Methods.

It has been proposed that poliovirus hybrids expressing heterologous epitopes could be used as vaccines (5, 29), although, to our knowledge, no such vaccines have yet been developed. The hybrids that we have described were constructed in a virulent strain of poliovirus and are therefore not vaccine candidates. However, we have demonstrated that it is possible to construct poliovirus-chlamydia hybrids with excellent growth characteristics and that such hybrids are highly immunogenic compared with possible alternative chlamydial vaccine antigens. In a primate model that closely parallels human chlamydial ocular infection, antisera to PV1-Ct7 can neutralize chlamydial infectivity for cells of the conjunctival epithelia, the natural target cells and site of chlamydial infection. This result shows that antibodies reactive with one or more epitopes in the chlamydial sequence TTSDVAGLEKDPVA are capable of preventing chlamydial colonization of these cells. On the basis of these results, it becomes very attractive to construct poliovirus-chlamydia hybrids, using attenuated vaccine strains of poliovirus. It should be noted that, consistent with earlier observations (e.g., 1, 29), the hybrids are able to induce a strong antipoliovirus response even though poliovirus NAgI has been disrupted. Consequently, vaccine strain poliovirus-chlamydia hybrids could possibly be considered as supplements to, or even replacements for, vaccine strain polioviruses in an

TABLE 3. Passive neutralization of chlamydial infectivity for the monkey conjunctivae by anti-PV1-CT7 sera

Sera[a] and monkey no.	Chlamydial culture[b] on day postchallenge:			Clinical disease score[c] of challenged monkeys on day postchallenge:		
	7	14	21	7	14	21
Preimmune						
842	+++	+	++	+/−	++/−	++/++
880	+	+++	++	+/−	++/++	++/+++
686	+++	++	+++	+/−	++/−	++/++
Immune						
918	−	−	−	−/−	−/−	−/−
939	−	−	−	−/−	−/−	−/−
969	−	−	−	−/−	−/−	−/−

[a] Preimmune and immune sera from the PV1-CT7-immunized rabbits (rabbits 13, 14, 15, and 16) were pooled according to experimental groups and tested for their ability to neutralize *C. trachomatis* serovar A infectivity for the monkey conjunctivae as described in Materials and Methods.

[b] Culture results are expressed as the average number of chlamydial IFU per 3×10^5 HeLa 229 cells isolated from the conjunctivae of both eyes; −, 0 IFU; +, 1 to 10 IFU; ++, 10 to 100 IFU; +++, >100 IFU.

[c] The clinical disease results represent aggregate scores from both eyes of each monkey. Clinical disease scores for hyperemia are shown before the slash, and those for follicle formation are shown after the slash. The scores were determined as follows. For hyperemia: −, no hyperemia; +, mild hyperemia, ++, moderate hyperemia, +++, severe hyperemia. Follicle formation: −, no follicles, +, just present (1 to 3), ++, obviously present (4 to 10), +++, grossly present (>10).

immunization schedule that simultaneously immunizes the vaccinee against poliovirus and *C. trachomatis*.

The studies described here were undertaken to demonstrate the feasibility of constructing immunogenic poliovirus-chlamydia hybrids, and immunogenicity was assessed in terms of a humoral immune response in rabbits. Obviously it is important to examine the role of mucosal immunity in chlamydial infection, and poliovirus-chlamydia hybrids have the potential to be a powerful tool. Poliovirus infection is known to induce a strong mucosal antibody response following gut infection (12, 13, 31), and the common nature of the mucosal immune system means that this response should extend to the mucosal surfaces at risk of chlamydial infection, those of the conjunctivae and genital tract. Exposure of gut-associated lymphoid tissue to antigen ultimately results in the dispersal of antigen-sensitized immunoblasts to mucosal surfaces throughout the body, where they can initiate synthesis of antigen-specific secretory IgA (4, 31). Since cynomolgus monkeys have a long history of use as models for both poliovirus (35, 48) and chlamydial (41, 43) infections, it should be feasible to evaluate the immunogenicity and protective efficacy of orally administered attenuated poliovirus-chlamydia hybrids in a primate model of chlamydial disease. Such studies should allow us to demonstrate conclusively whether a strong mucosal immune response is in fact protective against chlamydial infection, which would be a valuable contribution to the development of a chlamydial vaccine.

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