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Poliovirus Hybrids Expressing Neutralization Epitopes from Variable Domains I and IV of the Major Outer Membrane Protein of *Chlamydia trachomatis* Elicit Broadly Cross-Reactive *C. trachomatis*-Neutralizing Antibodies

ANDREW D. MURDIN,[1]* HUA SU,[2] MICHEL H. KLEIN,[1] AND HARLAN D. CALDWELL[2]

Connaught Centre for Biotechnology Research, Willowdale, Ontario M2R 3T4, Canada,[1] and Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840[2]

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Trachoma and sexually transmitted diseases caused by *Chlamydia trachomatis* are major health problems worldwide. Epitopes from the variable domains of the major outer membrane protein are candidates for vaccine development. We have constructed hybrid polioviruses expressing sequences from major outer membrane protein variable domains I and IV. Antisera to the hybrids could, in combination, strongly neutralize 8 of the 12 *C. trachomatis* serovars most commonly associated with oculogenital infections and weakly neutralize the others.

Chlamydia trachomatis infects the epithelia of the conjunctivae and genital tract, causing blinding trachoma and a variety of sexually transmitted diseases (STDs) with sequelae that include pelvic inflammatory disease, ectopic pregnancies, and infertility (5, 20, 21, 28). The estimated cost of treating chlamydial STDs and their sequelae is over 4 billion dollars per year in the United States alone (28, 29). Trachoma, which is the leading cause of preventable blindness in developing nations, afflicts an estimated 600 million people worldwide (5). Development of an effective vaccine would be a valuable contribution to the control of chlamydial diseases. *C. trachomatis* isolates associated with trachoma and STDs can be immunotyped into 12 distinct serovars and two serogroups based on antigenic relatedness. Serovars A, C, H, I, J, and K are members of the C complex, whereas serovars B, Ba, D, E, F, and G are classified in the B and B-related complexes. Serovars A, B, Ba, and C are the causative agents of trachoma, while serovars D to K are primarily associated with STDs (5, 12, 20, 21).

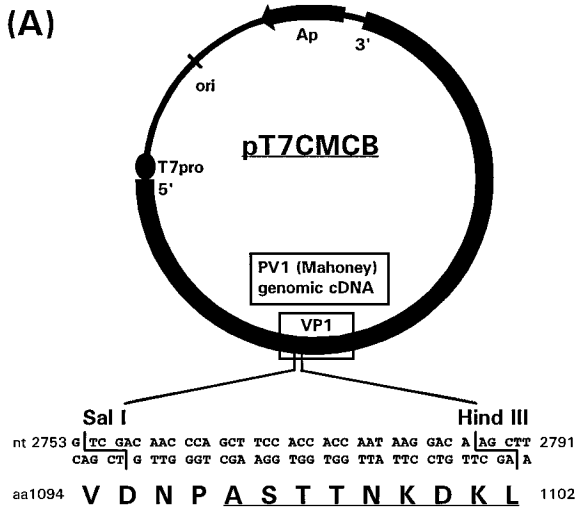
The pathophysiology of chlamydial oculogenital diseases is thought to involve repeated infections and the generation of a pathologic hypersensitivity response to chlamydial antigen(s) (8, 9, 25, 26). Past attempts to develop whole-cell chlamydial vaccines by using killed elementary bodies (EBs) frequently potentiated disease by sensitizing vaccinees, and any protection observed in these studies was limited to the immunizing serovar (7, 8). Consequently, an effective chlamydial vaccine will probably have to be based on a subunit immunogen capable of inducing a strong protective immune response without sensitizing the vaccinee. Moreover, it would be advantageous if such a subunit immunogen were capable of inducing protection against multiple serovars and could target broadly cross-protective immunity to mucosal surfaces, since these are the sites colonized by chlamydiae.

The most promising candidate antigen for the development of a subunit vaccine capable of stimulating local antichlamydial neutralizing antibodies is the chlamydial major outer membrane protein (MOMP) (3). The primary sequence of MOMP is highly conserved among serovars with the exception of four symmetrically spaced variable domains (VDI to VDIV) that exhibit considerable interserovar sequence variation (30). Neutralizing monoclonal antibodies (MAbs) that differentiate *C. trachomatis* isolates into serogroups and serovars recognize the MOMP, and the binding sites of MOMP-specific neutralizing MAbs have been mapped to VDI, VDII, or VDIV, depending on the serovar (2, 4, 10, 18, 22, 31, 32). These antigenic sites represent important targets for the development of a subunit or synthetic vaccine against *C. trachomatis*.

We are interested in using recombinant poliovirus-MOMP hybrids as models for the development of a live attenuated vector-based chlamydial vaccine since such vectors could theoretically elicit antichlamydial neutralizing antibodies at mucosal surfaces. Live attenuated polio vaccine is usually administered orally, with the object of inducing enteric mucosal immunity, but has also been reported to be capable of inducing detectable antibody in vaginal and uterine secretions following oral administration (17). We have previously described (14) a hybrid type 1 poliovirus, PV1-Ct7, which expresses MOMP sequences corresponding to VDI of serovar A, including the epitope VAGLEK recognized by the serovar A-specific neutralizing MAb A-20 (2, 31, 32). We showed that PV1-Ct7 is highly immunogenic and can induce neutralizing antisera cross-reactive with serovars A and C. To better assess the potential of this hybrid to evoke cross-reactive neutralizing antibody responses, we have extended our studies on the neutralizing activity of antisera generated against PV1-Ct7 and the related hybrid PV1-Ct8 to include other C-complex serovars. We have additionally extended our studies to include the construction of an additional family of hybrids expressing MOMP VDIV sequences that contain the highly conserved neutralizing epitope LNPTIAG found in all serovars except K (in which the equivalent sequence is LNPTITG) (2, 30–32).

Hybrid polioviruses were constructed by using the mutagenesis cartridge strategy of Murray et al. (16). The construction of PV1-Ct7 and PV1-Ct8, which express serovar A MOMP VDI sequences (Fig. 1), has been described previously (14). To

* Corresponding author. Mailing address: Connaught Centre for Biotechnology Research, 1755 Steeles Ave. West, Willowdale, Ontario M2R 3T4, Canada.



(B)

Clone	Sequence encoded (Chlamydial sequences underlined)	Viable virus recovered/ strain designation
pT7XLD	NPASTT NKD	Yes/PV1-XLD
pT7CtIVA1	NPASTTLNPTIAGAGDVKD	Yes/PV1-CtIVA
pT7CtIVB4	NPASTTLNPTIAGAGD	Yes/PV1-CtIVB
pT7CtIVC9	NPASTTLNPTIAGA KD	Yes/PV1-CtIVC
pT7Ct7	NPASTTSDVAGLEKDPVA	Yes/PV1-Ct7
pT7Ct8	NPASTTSDVAGLEKDP KD	Yes/PV1-Ct8

FIG. 1. Construction of poliovirus-chlamydia hybrids. By using a *Sal*I-*Hin*dIII mutagenesis cartridge, the PV1-Mahoney cDNA clone pT7CMCB was modified to encode amino acid sequences, including the LNPTIAG epitope, from B-complex *C. trachomatis* MOMP VDIV. The sequence TTLNPTIAGAGDVK is conserved among serovars B, Ba, D, and E. (A) The mutagenesis cartridge spans poliovirus nucleotides (nt) 2759 to 2785, which encode poliovirus amino acids (aa) 1094 to 1102. The BC loop of VP1 comprises amino acids ASTTNKDKL, which are underlined. (B) The poliovirus-specific nucleotide sequence within the cartridge was replaced with synthetic oligonucleotides encoding three amino acid sequences from B-complex *C. trachomatis* MOMP VDIV, as underlined in the lower part of the figure. Viable virus was recovered, as described in the text, from all three clones. The serovar A MOMP VDI sequences expressed by PV1-Ct7 and PV1-Ct8 are shown for comparison; their construction has been described previously (14).

construct the VDIV hybrids, synthetic oligonucleotides coding for conserved amino acid sequences from MOMP VDIV of the *C. trachomatis* B-complex serovars B, Ba, D, and E were cloned into a full-length cDNA of PV1-Mahoney in place of sequences coding for amino acids within the BC loop of poliovirus capsid protein VP1 (Fig. 1). Hybrid viruses were prepared from these cDNAs as described previously (14, 16, 27) and were designated PV1-CtIVA, PV1-CtIVB, and PV1-CtIVC (Fig. 1). In a single-step growth cycle, all were moderately impaired in comparison with PV1-XLD (data not shown), but all three hybrids grew sufficiently well to permit the easy preparation of virus stocks for further study.

The antigenic characteristics of PV1-CtIVA, PV1-CtIVB, and PV1-CtIVC are shown in Table 1. Susceptibility of these viruses to neutralization by MAb DIII-A3 was examined. DIII-A3 is specific to *C. trachomatis* MOMP and recognizes the LNPTIAG epitope located in VDIV. It binds to intact EBs of serovars B, Ba, D, E, L1, L2, F, G, and L3 in a dot immunoblot and neutralizes their infectivity in vitro (2, 31, 32). PV1-CtIVA was extremely sensitive to DIII-A3, whereas PV1-CtIVB required approximately 100-times-greater concentrations of the MAb to achieve the same degree of neutralization. Neutralization of PV1-CtIVC by DIII-A3 was not detectable at the highest concentration tested. In contrast, all three hybrids were sensitive to neutralization by convalescent sera obtained 120 days postinfection from cynomolgus monkeys that had recovered from a primary cervical infection with *C. trachomatis* serovar D, although titers against PV1-CtIVC were about fourfold lower than titers against the other two VDIV hybrids. The VDIV hybrids clearly express at least one *C. trachomatis*-specific epitope in a conformation which is presumably similar to the native conformation. Interestingly, the epitope (or epitopes) is not expressed equivalently on each hybrid, as evidenced by the neutralization titers of MAb DIII-A3 and the convalescent sera. The DIII-A3 (LNPTIAG) epitope is highly accessible on PV1-CtIVA and is progressively less accessible on the surfaces of PV1-CtIVB and PV1-CtIVC. This may be due to the progressive C-terminal truncation of the VDIV sequences expressed or to changes in the conformation of the VDIV sequence that affect the surface accessibility of the epitope.

Rabbits were immunized with the VDIV hybrid viruses as described previously (14). Pools of antisera were tested by Western blotting (immunoblotting) against solubilized chlamydial EB proteins to determine the specificity of the anti-MOMP response (14). Chlamydial EBs were purified by density gradient centrifugation (3) from HeLa 229 cells infected with *C. trachomatis* serovar A (strain Har-13), B (strain TW-5), Ba (strain AP-2), C (strain TW-3), D (strain UW-3/Cx), E (strain Bour), F (strain IC-Cal-13), G (strain UW-57/Cx), H (strain UW-4/Cx), I (strain UW-12/Ur), J (strain UW-36/Cx), or K (strain UW/31/Cx). These 12 strains represent the chlamydial serovars commonly associated with trachoma or chlamydial STDs. As shown in Fig. 2, rabbit antisera raised against PV1-Ct7 reacted very strongly with the homotypic serovar A MOMP and less strongly with the MOMPs of serovars C, I, and J. Antisera raised against PV1-Ct8 showed a slightly different profile, reacting approximately equally with the MOMPs of serovars A, C, I, J, and K. In contrast, antisera raised against the VDIV hybrids reacted strongly with the MOMPs of all 15 serovars.

These results confirm species-wide conservation of at least some VDIV epitopes, and more limited conservation of some VDI epitopes, in the context of the denatured MOMP. Antigenic cross-reactivity occurs even in cases where the identified epitopes (VAGLEK or LNPTIAG) are not completely conserved. Thus, antisera to PV1-Ct7 and PV1-Ct8 recognize the serovar A VDI sequence TTSDVAGLEKDPVA and also the equivalent sequences from serovars C and J (TTSDVAGLQN DPTT; differences are underlined), and I (TTKDVAGLEND PVA). Similarly, antisera to PV1-CtIVA, PV1-CtIVB, and PV1-CtIVC recognize sequences from VDIV of serovars B, Ba, D, E, F, G, C, A, H, I, and J (TTLNPTIAG) and of serovar K (TTLNPTITG). This finding indicates that the VDI and VDIV sequences expressed on the hybrids (Fig. 1) contain chlamydial epitopes in addition to VAGLEK and LNPTIAG. These additional epitopes are conserved between at least some serovars, and they can be effectively expressed by the poliovirus hybrids.

The specificities of the antisera for native MOMP were tested by dot blotting using viable EBs as the antigen (32). The cross-reactivity of the antichlamydial immune response elicited by the hybrids was not as great in the context of the native antigen. Antisera from rabbits immunized with PV1-Ct7 (rabbits 13 to 16) or PV1-Ct8 (rabbits 17 to 20) showed some

TABLE 1. Neutralization of poliovirus-MOMP hybrids by antichlamydial MAbs and sera

MAb or serum[a]	Neutralization titer[b] against:			
	PV1-CtIVA	PV1-CtIVB	PV1-CtIVC	PV1-XLD
DIII-A3	10,240	135	<20	<20
705				
Prebleed	<10	<10	<10	<10
Convalescent phase	299	423	92	<10
Anti-PV1	17,782	40,960	13,388	28,973

[a] MAb DIII-A3 was raised against *C. trachomatis* serovar D and is specific for the MOMP VDIV epitope LNPTIAG (2, 31, 32). Serum 705 was obtained from a monkey infected with *C. trachomatis* serovar D. Anti-PV1 is a PV1-specific rabbit pool.

[b] Reciprocal dilution of the serum or MAb giving a 50% endpoint in a neutralization assay versus 100 tissue culture 50% infective doses of virus conducted with Vero cells as described by Golding et al. (6). Assays were performed in microtiter plates, with eight replicates per sample per assay, and positive results were confirmed by repetition. Dilutions of DIII-A3 refer to a starting concentration of 1 mg/ml.

variation in specificity (Fig. 3A). Sera from rabbits 13, 14, 15, 17, 18, 19, and 20 reacted with EBs from serovars A, C, I, and J. Serum from rabbit 18 additionally reacted with serovar K EBs, but serum from rabbit 16 reacted only with serovar A EBs. Except for rabbit 19, the intensity of the reaction with the homologous serovar A was relatively constant between rabbits, whereas the intensity of the reaction with heterologous serovars was more varied. In contrast, sera from rabbits immunized with PV1-CtIVA (rabbits 24 to 26), PV1-CtIVB (rabbits 27 to 29), and PV1-CtIVC (rabbits 30 to 32) were more uniformly cross-reactive with B-complex serovars (Fig. 3B). All sera reacted very strongly with serovars D, E, and K. Most sera were immunoreactive with serovars B, Ba, F, and G, though this was usually weaker than the response to serovars D, E, and K and was more variable among the sera. Rabbits 27 and 31 reacted weakly with C-complex serovars (A, C, H, I, and J), and several other rabbits showed evidence of weak reactions with serovar A. In general, reactions were strongest with, but not limited to, B-complex serovars. Sera raised against PV1-XLD did not react with EBs of any serovar (not shown).

The rabbit antisera were tested for the ability to neutralize chlamydial infectivity for HaK cells (14). Antisera to PV1-CtIVA, PV1-CtIVB, and PV1-CtIVC were tested as pools (IVA, IVB, and IVC, respectively), and all showed essentially the same pattern of neutralizing activity (Table 2). All pools neutralized serovars Ba, D, E, and K strongly. Serovar G was neutralized strongly by pools IVB and IVC and to a lesser extent by pool IVA. Serovar A was neutralized weakly by pool IVC but not by pool IVA or IVB. Weak neutralizing activity against serovars B, F, and H was also observed. Interestingly, the sera in pool IVC, from rabbits immunized with PV1-CtIVC, was the most broadly cross-reactive although PV1-CtIVC expresses the shortest VDIV sequence. This finding suggests either that the VDIV sequence is expressed in a more favorable conformation on this hybrid or that a broader cross-reactivity was obtained by focusing the anti-VDIV response to epitopes, possibly immunorecessive, within the highly conserved sequence TTLNPTIAGA.

We also examined the neutralizing activity of antisera raised against the previously described VDI hybrids in more detail. Pooled anti-PV1-Ct7 and anti-PV1-Ct8 sera exhibited high-titer neutralizing activity against the homotypic serovar A (Table 2). In addition, they showed strong neutralizing activity

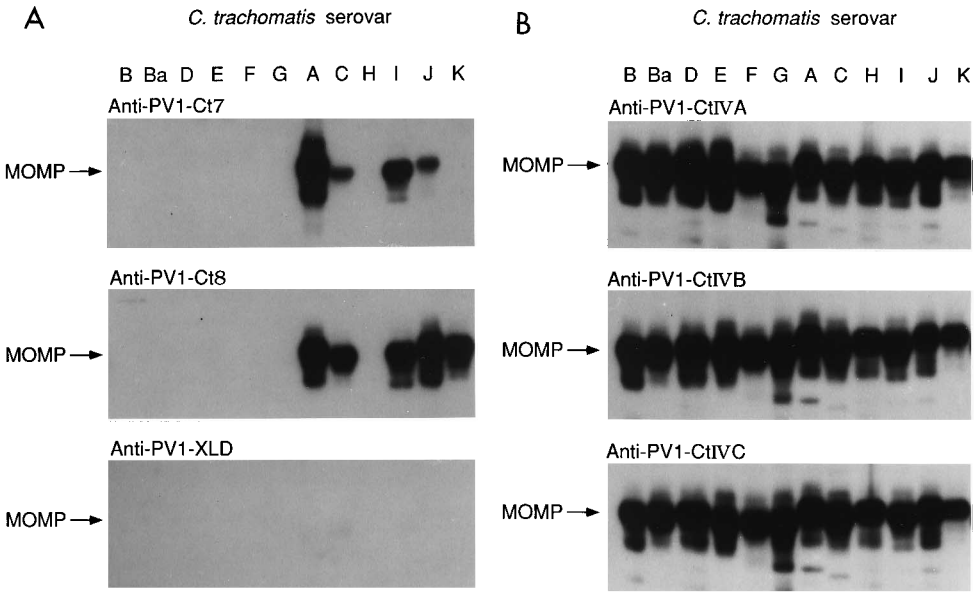


FIG. 2. (A) Specificity of pooled rabbit anti-PV1-Ct7 and anti-PV1-Ct8 antisera for MOMPs from 12 *C. trachomatis* serovars determined by Western blot analysis. Blots made with pooled anti-PV1-XLD antisera are shown as a control. (B) Specificity of pooled rabbit anti-PV1-CtIVA, anti-PV1-CtIVB, and anti-PV1-CtIVC antisera for MOMPs from 12 *C. trachomatis* serovars determined by Western blot analysis. Western blotting was conducted as described previously (14).

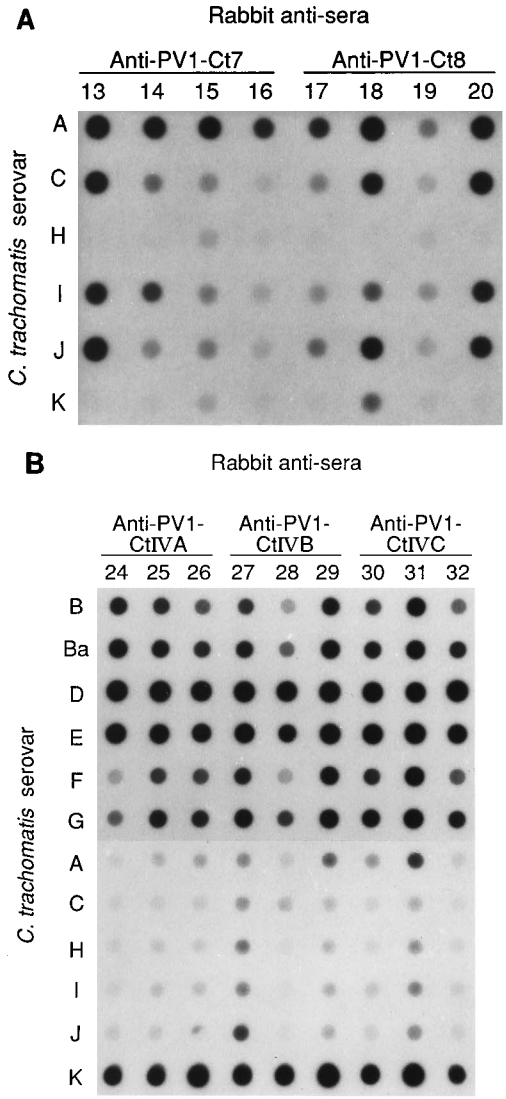


FIG. 3. (A) Specificity by dot immunoblots of rabbit anti-PV1-Ct7 (rabbits 13 to 16) and anti-PV1-Ct8 (rabbits 17 to 20) antisera for EBs of the *C. trachomatis* C-complex serovars (A, C, H, I, and J) and serovar K. (B) Specificity of rabbit anti-PV1-CtIVA (rabbits 24 to 26), anti-PV1-CtIVB (rabbits 27 to 29), and anti-PV1-CtIVC (rabbits 30 to 32) antisera for EBs of 12 *C. trachomatis* serovars by dot immunoblotting. Dot immunoblotting was conducted as described by Zhang et al. (32).

against serovar C, lower activity against serovar I, and weak activity against serovar J.

The immunogenic characteristics of the VDIV hybrids generally conformed to the known properties of the sequence expressed and of MAb DIII-A3, but there were a number of notable exceptions. Anti-VDIV hybrid antisera were species specific in a Western blot against the denatured MOMP, demonstrating that some linear epitopes within the VDIV sequences expressed are conserved species-wide, whereas DIII-A3 recognizes the denatured MOMP of all serovars except K (31). However, these epitopes are clearly masked in the context of the native EB of at least some serovars, as shown by dot immunoblot (in which native MOMP epitopes are retained [32]) or neutralization. The anti-VDIV MAb DIII-A3 recognizes serovars B, Ba, L1, L2, L3, D, E, F, and G by dot immunoblotting (2, 31, 32), whereas anti-VDIV hybrid antisera had relatively weak activity against B, Ba, F, and G but recognized D, E, and K strongly. This was true for all anti-VDIV hybrid antisera, regardless of how well DIII-A3 neutralized the hybrid. Weak activity against serovar A or J in the immunoblot was observed for one or two of the antisera. A similar specificity was evident at the level of neutralization. In particular, serovar K was neutralized strongly, even though it does not retain the VDIV LNPTIAG epitope and is not recognized by DIII-A3. That the antibodies elicited by the VDIV hybrids have a specificity distinct from that of DIII-A3 suggests that the DIII-A3 epitope is relatively immunorecessive in the context of the VDIV hybrids. We interpret the neutralization results to mean that there are stronger neutralization epitopes than those recognized by DIII-A3 within the VDIV sequence and that these epitopes may overlap with but are distinct from LNPTIAG.

It has been reported that synthetic peptides containing the LNPTIAG epitope are also capable of eliciting cross-reactive neutralizing antibodies. Su and Caldwell (24) have described a peptide expressing a VDIV sequence similar to that used here together with a chlamydial T-help epitope. The peptide induced neutralizing antibodies against serovars D (B complex) and G (intermediate complex) but not H (C complex), comparable to the results obtained with the VDIV hybrids; other serovars were not examined. Qu et al. (19) studied the immunogenicity of a peptide expressing the VDI sequence of serovar C and the VDIV sequence of serovar E. The B-complex VDIV sequence expressed on the VDIV hybrids is conserved in serovar E, whereas the serovar A VDI sequence expressed on the VDI hybrids is distinct from the equivalent serovar C sequence. Qu et al. (19) observed that their peptide induced neutralizing antibodies against serovars B, D, E, J, C, L3, and, weakly, F but not against serovars H, I, and A, whereas antisera to the VDI and VDIV hybrids could, in combination, strongly neutralize serovars A, C, I, Ba, D, E, G, and K and weakly neutralize serovars B, F, H, and J. Serovars L1, L2, and L3 were not tested in our study.

Thus, the specificity of the response elicited by the hybrids is distinct from but comparable to the specificity of the response elicited by synthetic peptides. Expressing the VDI and VDIV epitopes on poliovirus hybrids or as synthetic peptides does not substantially change their capacity to induce a cross-reactive immune response. In total, the two classes of hybrids could induce detectable neutralizing responses in rabbits to all 12 serovars tested, indicating that relatively few hybrids would be required for a candidate vaccine to be used against all chlamydial infections. However, the hybrids possess two potentially significant advantages over synthetic peptides. First, the hybrids are powerful immunogens; on a molar basis, the response to 0.5 pmol of hybrid (equivalent to 30 pmol of chlamydial epitope) is similar to that elicited by nanomolar amounts of peptide antigens (14, 19, 24). Second, as discussed previously (14), poliovirus hybrids should be able to induce a much stronger mucosal immune response than a synthetic peptide.

To use such hybrids as a vaccine, it would obviously be necessary to incorporate chlamydial T-cell help epitopes in addition to the B-cell epitopes. Potentially suitable T-cell epitopes are known (e.g., reference 24), and we have previously described expression sites on the poliovirus capsid which can be used to construct a hybrid simultaneously expressing two different epitopes (13, 15). Other sites in the poliovirus capsid proteins may also accommodate the expression of a foreign epitope. In addition, techniques for the expression of entire foreign proteins by poliovirus vectors have recently been described (1, 11), which may make it possible to express mul-

TABLE 2. Neutralization of *C. trachomatis* by rabbit anti-poliovirus-MOMP hybrid antisera[a]

Immunizing antigen	Neutralization titer[b] against *C. trachomatis* serovar:											
	B	Ba	D	E	F	G	A	C	H	I	J	K
PV1-XLD	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	16
PV1-CtIVA	16	256	256	256	<16	64	<16	<16	16	<16	<16	256
PV1-CtIVB	16	256	256	256	16	256	<16	<16	16	<16	<16	1,024
PV1-CtIVC	16	128	256	1,024	16	256	16	<16	16	<16	<16	2,048
PV1-Ct7	ND[c]	ND	ND	ND	ND	ND	1,024	512	<16	64	16	ND
PV1-Ct8	ND	ND	ND	ND	ND	ND	1,024	1,024	<16	64	16	ND

[a] Antisera were tested as pools prepared from all rabbits immunized with each antigen. Four rabbits were used for PV1-Ct7 and PV1-Ct8; three rabbits were used for the other antigens.

[b] Reciprocal of the highest dilution giving at least 50% reduction in chlamydial inclusion-forming units in a neutralization assay conducted with HaK cells as described previously (14).

[c] ND, not done.

tiple B- and T-cell epitopes from a single hybrid. Consequently, the expression by poliovirus vectors of sufficient epitopes to elicit both antichlamydial antibodies and appropriate cognate T-cell help would appear to be technically feasible.

It is clear from the dot blot results that while rabbits respond homogeneously with respect to some chlamydial serovars, the response with respect to several others is variable from rabbit to rabbit. This is particularly evident in the response to the VDI hybrids. Strains of inbred mice have been reported to vary in the response to synthetic peptides expressing the chlamydial epitopes used here, probably because of variations in T-help epitope restriction (19, 23). Nonresponsiveness could be overcome by the use of keyhole limpet hemocyanin as a carrier or the provision of a chlamydial T-help epitope. The rabbit-to-rabbit variation observed in this study is probably not due to lack of effective T-cell help, since this would be provided by the poliovirus vector. Indeed, all rabbits mounted a strong antipoliovirus response (data not shown). Rather, the variation is probably due to differences in the rabbit B-cell antigen receptor repertoire and ability to recognize chlamydial B-cell epitopes. We have observed that in rabbits immunized with the VDI hybrids, cross-reactive antisera preferentially recognize epitopes in the chlamydial sequence TTSDVAGLEK, whereas antisera with greater specificity for serovar A preferentially recognize epitopes in the chlamydial sequence VAGLEKD PVA (12a). It may be possible to elicit a more consistently cross-reactive response by modifying the expressed VDI sequence so that responses are preferentially directed toward the sequence TTSDVAGLEK, for instance by changing or deleting the VDI amino acids PVA. This is supported by the observation that antisera to PV1-Ct8, which expresses TTSD VAGLEKDP, tend to be more cross-reactive than antisera to PV1-Ct7, which expresses TTSDVAGLEKDPVA.

We have previously proposed that the use of poliovirus-chlamydia hybrids will allow us to address the role of mucosal immunity in chlamydial infections and have shown that VDI hybrids were viable and immunogenic. We have shown here that these hybrids can elicit neutralizing antibodies against several C-complex serovars (A, C, I, and J). It is also possible to express VDIV sequences on poliovirus hybrids that are viable and immunogenic. The VDIV hybrids elicit a broadly cross-reactive immune response which is able to neutralize many B-complex serovars. Thus, by using only two hybrids, it is possible to elicit neutralizing antibodies against most chlamydial serovars. This result, combined with the ability of poliovirus to induce a mucosal immune response, should greatly facilitate the study of the role of mucosal immunity in both trachoma and chlamydial STDs.

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