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Isolated poliovirus capsid protein VP1 induces a neutralizing response in rats

(vaccine/immunoprecipitation)

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ABSTRACT **Antibodies were raised in rats against the poliovirus type 1 capsid proteins, VP1, VP2, and VP3. Antibodies directed against VP1 from type 1 poliovirus (Mahoney) neutralized type 1 but not type 2 poliovirus. Antibodies raised against VP2 and VP3 failed to neutralize type 1 virus. Thus, VP1 appears to be a neutralizing antigen for poliovirus and in its denatured form presents to the immune system its neutralizing determinants.**

The poliovirion has been shown to exist in two basic antigenic forms, N (or D) and C (or H), that are not crossreactive. The N antigenic form is associated with mature infectious virus (1). C antigenicity is observed in empty capsids isolated from infected cells, in mature virions that have been heated, modified, or degraded, and in virions that were bound to and eluted from membranes of suspectible cells (2–6). By using nonsusceptible animals for immunization, neutralizing sera have been found to recognize the N antigenic state and have been obtained only when native virions are injected. These neutralizing sera bind to native virions but do not recognize C antigenic structures. Similarly, C-antigen-specific antibodies recognize heated or disrupted virions and procapsid structures but do not bind to native virions (7–8). Recently, a hybridoma cell line producing neutralizing monoclonal antibodies has been generated from mice that were injected with native virions (9). In addition to binding to native virions, this antibody recognizes the 70S procapsid and the 14S assembly subunit, indicating that subunits of the virion previously thought to lack N antigenic sites do exhibit a neutralizing determinant. This raises the possibility that neutralizing antibodies could be induced by subunits of the virions and not only by the intact mature virion.

The determination of which of the poliovirus proteins can elicit a neutralizing response has proven elusive. The poliovirion contains four capsid proteins: VP1, VP2, VP3, and VP4. Neutralizing sera raised against mature virus and the monoclonal antibody do not recognize any of the capsid proteins selectively (9). The conversion of N-reactive virions to C-reactive particles is typically accompanied with the loss of VP4, suggesting initially that VP4 might be the neutralizing determinant (10, 11). Topological studies, however, indicate that VP4 is not located at the capsid surface (12). Several attempts to raise a neutralizing antiserum by using single isolated capsid proteins have yielded equivocal results. In one study, none of the antisera raised against VP1, VP2, VP3, or VP4 were neutralizing (13). In a more recent study, the antiserum from only one out of two rabbits injected with VP1 showed any neutralizing activity (14). The antigenic specificity of the antiserum was not characterized further.

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We present here data showing that all rats injected with isolated VP1 develop neutralizing antibodies to poliovirus. Rats injected with VP2 or VP3 generate antibodies that precipitate the cognate proteins and bind to virions but have no neutralizing ability.

METHODS

Production and Isolation of Poliovirus Capsid Proteins. Poliovirus type 1 (Mahoney) was isolated from infected HeLa cells. The capsid proteins VP1, VP2, and VP3 were isolated by $NaDodSO_4$/polyacrylamide gel electrophoresis (15). The virions were disrupted by boiling for 10 min in 1% $NaDodSO_4$/5 mM EDTA and then subjected to electrophoresis through a preparative 13% $NaDodSO_4$/polyacrylamide gel. The separated proteins were visualized as clear zones in the gel by precipitating the free $NaDodSO_4$ in the acrylamide gel with 2.5 M KCl. The protein bands were cut out and electroeluted overnight and then dialyzed against 10 mM Tris·HCl, pH 7.5/1 mM EDTA. The concentrations of the eluted proteins were estimated in polyacrylamide gels by staining intensity relative to those of protein standards of known concentrations.

Immunizations. Male Lewis rats, 8 wk old (Charles River Breeding Laboratories), were injected intraperitoneally and intradermally with emulsions containing 100 μg of the isolated proteins. To equalize variability among the individual rats, each protein was injected into three rats. The emulsions were made by mixing equal volumes of the protein solution and complete Freund's adjuvant. After 3 wk, the initial injection was followed by three booster injections 2 wk apart. Each booster injection contained 100 μg of protein in a 50% emulsion with incomplete Freund's adjuvant. Animals were bled from the ocular sinus 8–10 days after each booster injection. Sera were heated to 65°C for 30 min before storage at −20°C. They were checked for the presence of antibodies against the injected antigen using Ouchterlony double-diffusion plates.

Neutralizing Titers. The titer of neutralizing antibody present in antisera was measured by two methods. (*i*) Half neutralization value: Serial dilutions (1:2) of serum in phosphate-buffered saline were incubated with 200 plaque-forming units (pfu) of poliovirus in a final volume of 200 μl for 2 hr at 25°C and then overnight at 4°C. The entire sample was placed undiluted on HeLa cell monolayers in a standard plaque assay protocol. Neutralizing titer was defined as the serum dilution required to reduce the number of plaques by 50%. (*ii*) To quantitate the amount of virus that could be killed by a given amount of serum, poliovirus (10^7 pfu) was incubated with different dilutions of the rat serum in a final volume of 10 μl at 25°C for 2 hr and then overnight at 4°C. The number of surviving infectious particles was then measured in a standard plaque assay.

Abbreviations: pfu, plaque-forming unit(s); Staph A, *Staphylococcus aureus*; FMDV, foot-and-mouth disease virus.

Plaque Assays. The virus titers were determined using a cloned HeLa cell line S-3 that is carried in suspension but will also grow on plates. HeLa cells were plated at 2×10^6/60-mm plate in Dulbecco's modified medium/5% fetal calf serum. After 3 hr at 37°C, the medium along with any floating cells was removed by aspiration and 200 μl of the virus dilution (in phosphate-buffered saline) was placed on the monolayer. The plates were incubated for 30 min at 37°C. The plates were then overlayed with 5 ml of 1% agarose in Dulbecco's modified medium/5% fetal calf serum and incubated at 37°C for 36 hr (polio type 1, Mahoney) or for 54–72 hr (polio type 2, Lansing). All plaque assays were carried out in duplicate and plaques were visualized by staining with crystal violet.

[^{35}S]Methionine-Labeled Virions. HeLa cells in medium lacking methionine were infected with poliovirus at a multiplicity of 10 pfu per cell. Cells were labeled with [^{35}S]methionine (New England Nuclear; specific activity, 850 Ci/mmol; 1 mCi/liter; 1 Ci = 3.7×10^{10} becquerels) 3.5 hr after infection and harvested 6 hr after infection. Cells were lysed in 1% Nonidet P-40/10 mM Tris·HCl, pH 7.5/10 mM NaCl/1.5 mM $MgCl_2$ and nuclei were removed by centrifugation. The virus was collected by centrifugation in a 50 Ti rotor for 1 hr at 45,000 rpm. The virus pellet was suspended in 10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.1 M NaCl/0.5% $NaDodSO_4$ (TNE buffer/0.5% $NaDodSO_4$) and banded in CsCl. The virus band was isolated, dialyzed against TNE buffer, and stored at −20°C.

Immunoprecipitations. Immunoprecipitations were carried out in 10 mM Tris·HCl, pH 7.5/0.15 M NaCl/1% deoxycholate/1% Triton X-100/0.1% $NaDodSO_4$ (Ripa buffer) in the presence of ovalbumin at 0.5 mg/ml. In some cases, just prior to incubation with the antisera, samples were denatured by boiling for 10 min in 1% $NaDodSO_4$/5 mM EDTA and then centrifuged for 5 min in an Eppendorf centrifuge. [^{35}S]Methionine-labeled samples were incubated with a given amount of antiserum for 2 hr at room temperature and then for 30 min at 0°C with 10 times the serum volume of a 10% solution of formalin-fixed *Staphylococcus aureus* (Staph A) (New England Enzyme Center, Boston; Iggsorb). The Staph A was collected by centrifugation for 30 sec in an Eppendorf centrifuge, washed with three 1-ml portions of Ripa buffer/0.5 M NaCl and then with 1 ml of Ripa buffer/0.15 M NaCl. Staph A-containing samples were boiled in the $NaDodSO_4$ sample buffer for 10 min; the Staph A was removed by centrifugation and the supernatants were analyzed by electrophoresis through an 11% $NaDodSO_4$/polyacrylamide gel (15). The gels were fixed in 10% acetic acid/10% methanol, dried, and autoradiographed (Kodak, XAR film) at −70°C.

RESULTS

Antisera Reactive with Individual Polypeptides. To study the antigenicity of poliovirus proteins VP1, VP2, and VP3, they were isolated and individually injected into rats, and serum samples were collected after each boost. The sera formed precipitin lines with the injected antigen in Ouchterlony double-diffusion assays (data not shown).

To test the specificity and selectivity of these antisera against denatured poliovirus capsid proteins, [^{35}S]methionine-labeled poliovirions (Mahoney, type 1) were boiled in 1% $NaDodSO_4$ and then immunoprecipitated with the sera. The precipitated samples were displayed by electrophoresis through an 11% polyacrylamide gel and detected by autoradiography (Fig. 1*A*). Antisera raised against VP1 specifically precipitated the VP1 released from dissociated virions (lanes 3 and 4). Similarly, antisera raised against VP2 or VP3 precipitated only VP2 or VP3, respectively (lanes 6, 7, 9, and 10). None of the preimmune rat sera precipitated any specific virion proteins (lanes 2, 5, and 8).

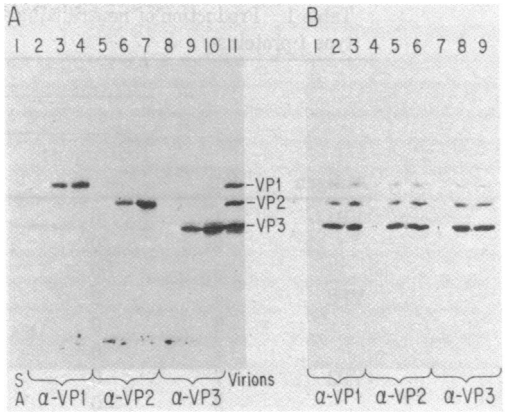

FIG. 1. Immunoprecipitation of $NaDodSO_4$-denatured and native virions with α-VP1, α-VP2, or α-VP3 serum. (*A*) [^{35}S]Methionine-labeled virions were boiled for 10 min in the presence of 1% $NaDodSO_4$/5 mM EDTA, and insoluble material was removed by centrifugation in an Eppendorf centrifuge for 10 min. The virions were precipitated with 5 μl of preimmune serum (lanes 2, 5, and 8) or with 2 μl (lanes 3, 6, and 9) or 5 μl (lanes 4, 7, and 10) of immune serum. Serum incubations were done in the presence of Ripa buffer for 2 hr at room temperature, formalin-fixed Staph A was added, and the mixture was incubated for 1 hr at 4°C. To monitor nonspecific background adsorption of the virion proteins to the Staph A, labeled virions were incubated with Staph A without addition of any serum (lane 1). [^{35}S]-Methionine-labeled virions were analyzed as markers (lane 11). (*B*) [^{35}S]Methionine-labeled virions were immunoprecipitated with 5 μl of preimmune serum (lanes 1, 4, and 7) or with 2 μl (lanes 2, 5, and 8) or 4 μl (lanes 3, 6, and 9) of α-VP1, α-VP2, or α-VP3 serum. The different intensities of VP1, VP2, and VP3 bands reflects the methionine content of each protein.

In every gel, several dilutions of [^{35}S]methionine-labeled virions at known protein concentrations and specific activity were displayed as protein standards. By comparing the intensities of the immunoprecipitated bands with those of the virion standards, an estimate was made of the number of antigen molecules precipitated by a known volume of serum. After the second boost, 2 μl of almost all of the rat sera precipitated a quantity of capsid protein equivalent to that found in 10^{10} particles. Because there are 60 molecules of each capsid protein in a virion, this represents almost 10^{12} molecules of antigen. Thus, each antiserum contains high titers of antibodies that are specific to only one capsid protein. The sera will be designated α-VP1, α-VP2, and α-VP3.

To test whether the sera recognized any antigenic determinants on native virions, immunoprecipitations were done using [^{35}S]methionine-labeled virions that had not been boiled in $NaDodSO_4$ (Fig. 1*B*). All of the antisera precipitated intact virions, although with various efficiencies. By comparing the intensities against virion standards, 2 μl of α-VP1 or α-VP2 precipitated approximately 10^9 virion particles and 2 μl of α-VP3 precipitated approximately 10^8 virions. Thus, only a small fraction of the antibodies produced against the denatured virion proteins recognized and bound to the proteins in their native state.

Neutralization Activity of Sera. Because the antisera recognized antigenic determinants on intact virions, they were tested for neutralizing activity. In one test, 200 pfu of poliovirus was incubated with 1:2 serial dilutions of serum and the number of surviving pfu was measured with a plaque assay. The titers for neutralization of one-half of the input virions showed that only α-VP1 contained any neutralizing activity (Table 1). Even after a third boost, α-VP2 and α-VP3 showed no neutralization activity. In contrast, after each boost with VP1, the neutralizing titers of α-VP1 increased such that, after the third boost with

Table 1. Production of neutralizing serum in rats inoculated with isolated poliovirus type 1 proteins

Antigen	Rat	50% neutralization titer of sera				
		Polio type 1				
		Preimmune serum	First bleeding	Second bleeding	Third bleeding	Polio type 2 (third bleeding)
VP1	1	0	1/2	1/5	1/30	0
	2	0	1/10	1/50	1/200	0
	3	0	1/4	1/6	1/60	0
VP2	4	0	0	0	0	0
	5	0	0	0	0	0
	6	0	0	0	0	0
VP3	7	0	0	0	0	0
	8	0	0	0	0	0
	9	0	0	0	0	0
Virions	10	0	$>10^4$			0.6×10^3*
	11	0	$>10^4$			1×10^3*
	12	0	$>10^4$			0.8×10^3*

* Determined on serum from the first bleed.

VP1, serum neutralizing titers had increased 10-fold over the titer obtained after the first boost. Although α-VP1, α-VP2, and α-VP3 immunoprecipitate VP1, VP2, and VP3 from poliovirus type 2 virions, respectively (data not shown), none of the rat antisera showed any neutralization activity against poliovirus type 2.

Because the serum titers were very low and neutralization activity was observed only with undiluted or very low dilutions of serum, a second neutralization protocol was used to test for the possibility of nonspecific inhibition. To this end, a high-titer stock of poliovirus was incubated with antiserum and the remaining virus was assayed after extensive dilution (Table 2). No significant neutralization was apparent when 5×10^7 pfu of poliovirus was incubated with undiluted preimmune sera, α-VP2, or α-VP3. In contrast, virus decreased by approximately 1.5 $\log_{10}$ when incubated with α-VP1 serum. Thus, rats injected with a single capsid protein, VP1, produce sera that are capable of neutralizing poliovirions. Also significant is the observation that all rats that were injected with VP1 generated neutralizing antibodies.

Anti-Virion Antibodies. Because the neutralizing titers of α-VP1 appeared low, the neutralizing titers of sera from rats injected with whole virions (α-Vir) were measured as a standard of comparison. As expected, the α-Vir titers after the first boost were several orders of magnitude higher than the α-VP1 titers achieved after the third boost (Tables 1 and 2).

Table 2. Effect of undiluted rat sera on 5×10^7 pfu of poliovirus type 1

Antigen	Rat	Virus titer after incubation with sera from each bleeding ($\times 10^{-7}$)			
		Preimmune serum	First	Second	Third
VP1	2	5.0	1.0	0.5	0.1
VP2	4	5.0		5.0	5.0
	5	5.0		4.0	
VP3	7	5.0		6.0	5.0
	8			4.0	
Virions	10	5.0	0.04*		
	11	4.0	0.1*		
	12	5.0	0.03*		

* A serum dilution of 10^{-3} was used for this test.

To test the ability of α-Vir to bind to capsid proteins in their natural or denatured conformations, native and $NaDodSO_4$-denatured virions were immunoprecipitated with α-Vir sera (Fig. 2). Consistent with the high neutralizing titers observed, the antisera quantitatively precipitated native virions (Fig. 2, lanes 1–6). Thus, after the first boost, 2 μl of serum could immunoprecipitate approximately 10^{10} virion particles. None of the α-Vir sera, however, recognized any of the denatured proteins (Fig. 2, lanes 7–12). Thus, antisera induced by virions appear to recognize different antigenic determinants than antisera raised against the individual proteins.

All antisera (α-VP1, α-VP2, α-VP3, and α-Vir) recognized and immunoprecipitated the capsid protein intracellular precursors from infected cell lysates in the absence of extensive denaturation (data not shown).

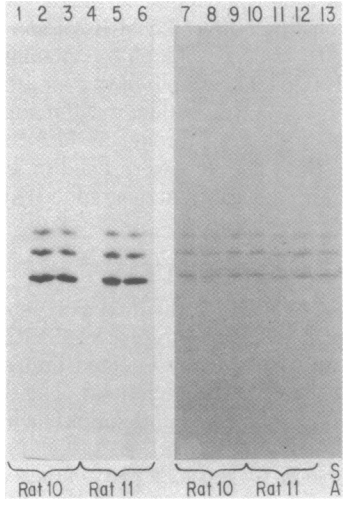

FIG. 2. Immunoprecipitation of $NaDodSO_4$-denatured and native virions with α-Vir serum. [^{35}S]Methionine-labeled virions, $NaDodSO_4$ denatured or intact, were precipitated with α-Vir sera from two rats (rat 10 and rat 11). Background absorption when virions were incubated with Staph A alone is shown in lane 13. Denatured virions were precipitated with 10 μl of preimmune serum (lanes 7 and 10) or with 5 or 10 μl of immune serum (lanes 8 and 11 and lanes 9 and 12, respectively). Intact virions were precipitated with 5 μl of preimmune serum (lanes 1 and 4) or with 2 or 5 μl of immune serum (lanes 2 and 5 and lanes 3 and 6, respectively).

DISCUSSION

Although most previous work on poliovirus neutralization has implied that only intact virions can elicit a neutralizing response, it is evident that rat sera prepared against a single polypeptide of the poliovirion, VP1, contain neutralizing antibody. Because comparable antisera against VP2 and VP3 had no neutralizing activity, it appears that only VP1 can stimulate a neutralizing response under our conditions of immunization. The neutralizing response to VP1 is much weaker than the response to virions but it is serotype specific and in this way mimics the response to virions.

The ability of poliovirus VP1 to elicit a neutralizing response might have been expected because the equivalent foot-and-mouth disease virus (FMDV) and mengovirus polypeptides have previously been shown to elicit such a response (16–21). Like poliovirus, these are picornaviruses although of the aphthovirus and cardiovirus subgroups rather than the enterovirus subgroup (22). Possibly all picornaviral VP1s would induce such a response.

Although α-VP2 and α-VP3 did not contain neutralizing antibodies, they did react with intact virions, implying that determinants of both VP2 and VP3 are on the viral surface. Previous radiolabeling and cross-linking studies had led to similar conclusions (12, 23).

Although VP1 clearly contains a neutralizing determinant, it is probably not the same neutralizing determinant to which the α-Vir antibodies bind because such antiserum did not immunoprecipitate the isolated proteins. In addition, hybridoma cell lines have been isolated in other laboratories from mice injected with poliovirions that produce neutralizing antibodies. These antibodies do not recognize VP1 from disrupted virions (9). Both lines of evidence suggest that the neutralizing determinants recognized by the α-Vir sera and α-VP1 are not identical.

The induction of neutralizing antibodies and the neutralization process are poorly understood phenomena, and the weakness of the neutralizing response elicited by isolated poliovirus VP1 could be due to many factors. Neutralizing antibodies must recognize the poliovirus proteins in their native conformation so that the $NaDodSO_4$-denatured VP1 protein may be less efficient than nondenatured VP1 protein for generating antibodies that recognize the native conformation. Also, the presence of the other capsid proteins (VP2 and VP3) in the virion may modify or alter the antibody response of the host to VP1. The interaction of VP1 with VP2 and VP3 may restrict the conformation of VP1 such that, in the presence of VP2 and VP3, the major VP1 immunogenic conformation of the neutralizing determinant presented to the animal is that recognized by the neutralizing antibodies. Alternatively, the association of VP2 and VP3 with VP1 may block or shield VP1 immunogenic sites that are predominant in the denatured VP1 protein. Thus, a higher proportion of the VP1-reactive antibodies generated in the α-Vir sera may be directed toward the VP1 neutralizing site. Finally, neutralizing antibodies raised from intact poliovirions may actually recognize VP1 in a complex with VP2 and VP3; hence these antibodies may be more efficient in neutralizing poliovirus than antibodies solely directed against VP1.

The existence of α-VP1 sera that bind to intact virions may allow the neutralization process to be studied in finer detail. Recent studies with FMDV have shown that the binding of FMDV virions to the cellular receptor is dependent on the corresponding VP1 protein from FMDV (24). Trypsin treatment of FMDV leaves the virion intact structurally, but the VP1 protein has been cleaved. The trypsinized FMDV is unable to bind to isolated plasma membranes containing the virus receptor or to intact cells. The observations that the poliovirus VP1 is capable of eliciting a neutralizing response suggests that this protein may be similarly responsible for mediating virus interactions with the cellular receptor. Thus, one possible mechanism of virus neutralization is that the neutralizing antibody to VP1 prevents the virus from binding to the cellular receptor.

The demonstration that VP1 can stimulate a neutralizing response suggests that it may be possible to produce a poliovirus vaccine using only VP1 determinants. The neutralizing response seen thus far in rats is probably too low to be protective but, by appropriate manipulations, a more immunogenic form of VP1 might be found. One promising route to producing such a subunit vaccine would be the use of synthetic peptides representing the VP1 sequence (20).

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