Disoxaril stabilization and immunogenicity of poliovirus procapsids

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Disoxaril (5-[7-[4(4,5-dihydro-2-oxazolyl)phenoxy]-heptyl]-3-methylisoxazole) protects poliovirus procapsids against alkaline dissociation and thermal denaturation up to 42 °C. When added during the purification of procapsids, it enhances their yield and antigenic quality. Disoxaril increases the immunogenicity of both purified virions and procapsids in mice. Whereas untreated procapsids mainly elicit H-specific antibodies, disoxaril-treated procapsids yield high titres of neutralizing antibodies. The prospect of using disoxaril-treated procapsids in vaccines is discussed.

Introduction

Procapsids, i.e. empty protein shells of composition (VP0 + VP1 + VP3)60, are found in most poliovirus-infected cells. Provided denaturation is prevented during their preparation, they exhibit the same antigenicity (called N for native; Icenogle et al., 1981; Rombaut et al., 1982) and neutralizing antigenic sites (Rombaut et al., 1990) as mature virions. As a consequence, it should be possible to use procapsids in vaccines, although their sensitivity to thermal denaturation (Rombaut et al., 1989) may preclude this use, unless they can be chemically stabilized.

In this work, the possible use of disoxaril (5-[7-[4(4,5-dihydro-2-oxazolyl)phenoxy]-heptyl]-3-methylisoxazole), also referred to as WIN 51711; McKinlay, 1985) was investigated. This compound stabilizes the viral capsid and prevents uncoating by filling a hydrophobic pocket in capsid protein VP1 (Smith et al., 1986). It will be shown that disoxaril indeed protects procapsids against alkaline dissociation and thermal denaturation. Moreover, whereas untreated procapsids are poor immunogens in mice, disoxaril-stabilized procapsids elicit high levels of neutralizing antibodies. Disoxaril also enhances the immunogenicity of mature virions; finally, when added during the purification of procapsids, it greatly improved their yield.

Methods

Purification of radiolabelled procapsids. HeLa cells (107/ml) were infected with Mahoney (type 1) poliovirus at an input multiplicity of 100 p.f.u. per cell, and 15 μCi/ml of [35S]methionine was added at 3 h post-infection. After 2.5 h, the cells were collected by centrifugation, resuspended to a density of 2 × 109 cells/ml in 0-02 M-phosphate buffer pH 7-2 supplemented with 1 μg/ml disoxaril, and frozen at −20 °C.

After three cycles of freezing and thawing, the extracts were clarified by a short centrifugation. An equal volume of Genetron (1,1,2-trichlorotrifluoroethane) was added. After blending in a Sorvall omni-mixer and removal of the Genetron by centrifugation, the aqueous phase was centrifuged for 2 h at 160000 gav. The pellet was resuspended in 300 μl RSB buffer (10 mM-NaCl, 10 mM-Tris, 1.5 mM-MgCl2, adjusted to pH 7-2 with HCl) supplemented with 1 μg/ml disoxaril, and submitted to sucrose gradient centrifugation (15 to 30% sucrose in RSB buffer, supplemented with 1 μg/ml disoxaril, run for 3 h at 4 °C and 94000 gav). The 65S and 160S particles were collected and kept at −80 °C.

Monoclonal antibodies and Protein A-aided immunoprecipitation. Four monoclonal antibodies were directed against the neutralizing sites of poliovirus type 1 (Page et al., 1988; Rombaut et al., 1990): site 1 (antibody 95), 2 (antibody 36-5h2), 3A (antibody 424) and 3B (antibody 35-2b6). The non-neutralizing antibody 39-5b4 (Brioen et al., 1982) was also used; this antibody recognizes H antigen (i.e. virions heated for 20 min at 56 °C), but neither native virions nor procapsids.

Protein A-aided immunoprecipitation using microtitration plates is fully described elsewhere (Vrijisen et al., 1983). Briefly, 80 μl of antigen is mixed with 10 μl of antibodies. The mixture is allowed to stand for 1 h at 4 °C, and 40 μl of a 10% suspension of formalin-fixed Staphylococcus aureus of strain Cowan I is added. After 30 min, the plate is centrifuged for 15 min at 1100 g and a 50 μl sample of the supernatant is removed for radioactivity assay.

Immunization of mice. BALB/c mice were immunized intraperitoneally with 1 μg of purified antigen in 0-02 m-phosphate buffer pH 7-2, without adjuvant. The procedure was repeated on days 14 and 28, and the animals were sacrificed on day 35.

Disoxaril. This compound was obtained from the Sterling-Winthrop Research Institute (Rensselaer, New York 12144, U.S.A.). Stock solutions (1 mg/ml) were made in dimethyl sulphoxide and diluted in RSB buffer immediately before use.

Results

Disoxaril stabilization against thermal denaturation

Radiolabelled procapsids were prepared by the procedure of Rombaut et al. (1989); in this method, the main
purification step is a sucrose gradient centrifugation, followed by collection of the 65S material. The native condition of the procapsids was ascertained as described (Rombaut et al., 1989) using as criteria their sedimentation coefficient of 65S, pI 7.2, alkaline dissociability, presence of all N-antigenic sites and absence of H-antigenicity (results not shown). To assess the effect of disoxaril, these procapsids were incubated for 1 h at temperatures ranging from 37 °C to 52 °C, in RSB buffer with or without 1 µg/ml disoxaril, then tested by immunoprecipitation against four monoclonal antibodies targeted against the neutralizing sites 1, 2, 3A and 3B, and against one H-specific antibody. The results are shown in Fig. 1. In the absence of disoxaril and at temperatures higher than 37 °C, all neutralizing antigenic sites disappeared, and the particles were quantitatively converted from N to H antigen. In the presence of disoxaril, the same antigenic conversion went on, but required a temperature of at least 48 °C for completion within 1 h.

The possibility existed that site 1, comprising amino acids 83 to 104 of VP1, would be either disrupted or selectively protected by disoxaril, which binds to the canyon floor of the protein (Smith et al., 1986), but neither possibility was supported by the results. Therefore site 1, which is of minor importance in poliovirus type 1, was not included in further studies focusing on the immunodominant sites 2, 3A and 3B (Page et al., 1988).

Even in the presence of disoxaril, a slow antigenic conversion occurred at 43 °C and higher. A detailed study with 1 degree intervals in the 40 to 43 °C range showed that disoxaril prevented denaturation up to 42 °C (results not shown). The complete stability of the N-antigenicity at 42 °C is illustrated in Fig. 2: without disoxaril, antigenic conversion was complete in 20 min (a), but in the presence of 1 µg/ml of the compound there was still no measurable conversion after 20 h (b). The concentration dependence of the disoxaril protection was investigated by incubating procapsids for 1 h at 42 °C with a half-log series of disoxaril concentrations. Full protection was achieved at 0.1 µg/ml (Fig. 3).

Whereas thermal denaturation of untreated procapsids shifted their sedimentation coefficient from 65S to 74S (Rombaut et al., 1989), disoxaril-treated procapsids retained their sedimentation coefficient of 65S at 42 °C. Above this temperature, they were converted to 74S empty capsids (results not shown).

Disoxaril stabilization against alkaline dissociation

Procapsids are susceptible to alkaline dissociation (Marongiu et al., 1981; Onodera et al., 1986), with an optimal pH of 8.2 (Rombaut et al., 1987). Procapsids, prepared as described in the preceding section, were treated for 1 h at pH 8.2 and 4 °C, in the presence or
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Fig. 2. Time course of the denaturation of procapsids at 42 °C: effect of disoxaril. Radiolabelled procapsids in RSB pH 7.2 buffer containing either no drug (a) or 1 μg/ml disoxaril (b) were incubated at 42 °C. Samples taken at the times stated were submitted to immunoprecipitation as described in Methods, using monoclonal antibodies directed against neutralizing sites 2 (O), 3A (∆) or 3B (O), or against H antigen (■).

Absence of disoxaril. The pH was then lowered to 7.2 and the mixture analysed by sucrose gradient centrifugation. The results are shown in Fig. 4. Without disoxaril, the procapsids (trace a) were dissociated to 14S subunits (trace b); in contrast, no dissociation occurred in the presence of disoxaril (trace c). Even after 24 h at pH 8.2, these procapsids were still undissociated (results not shown).

Disoxaril-aided purification of virions and procapsids

For meaningful immunogenicity experiments, procapsids must be free of cellular material, and our usual virus purification procedure was adapted for that purpose (see Methods). The absence of host proteins in the procapsid material was shown by Coomassie blue staining of electropherograms, which revealed only the structural proteins VP0, VP1 and VP3. The whole purification procedure was carried out in the presence of 1 μg/ml disoxaril, control particles being simultaneously prepared without disoxaril. In three separate experiments, the yield of procapsids was increased four- to sixfold by disoxaril, without concomitant loss of virions (in fact the yield of virion material was also enhanced up to twofold). Table 1 shows the results of a representative experiment. It will be noted that the procapsids prepared in the presence of disoxaril were 100% N-antigenic, as compared to less than 80% for those purified in the absence of the compound.
Table 1. Purification* of virions and procapsids: effect of disoxaril

<table>
<thead>
<tr>
<th>Disoxaril during purification*</th>
<th>Virions</th>
<th>Procapsids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (c.p.m.)†</td>
<td>Antigenicity‡</td>
<td>Yield (c.p.m.)†</td>
</tr>
<tr>
<td>None</td>
<td>1.3 × 10⁶</td>
<td>100% N, 0% H</td>
</tr>
<tr>
<td>1 μg/ml</td>
<td>1.8 × 10⁶</td>
<td>100% N, 0% H</td>
</tr>
</tbody>
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* See Methods.
† From 10⁸ HeLa cells.
‡ Fraction of input radioactivity bound by anti-N and anti-H monoclonal antibodies in S. aureus-aided immunoprecipitation.

Table 2. Effect of disoxaril on immunogenicity of purified virions and procapsids

<table>
<thead>
<tr>
<th>Immunogen*</th>
<th>Average antibody titre†</th>
<th>Immunoprecipitation‡</th>
<th>Neutralization§</th>
</tr>
</thead>
<tbody>
<tr>
<td>N antigen</td>
<td>H antigen</td>
<td>Neutralization§</td>
<td></td>
</tr>
<tr>
<td>Untreated virions</td>
<td>2.8 (2.5–3.4)</td>
<td>2.5 (2.1–3.1)</td>
<td>2.8 (2.5–3.5)</td>
</tr>
<tr>
<td>Disoxaril-treated virions</td>
<td>3.8 (3.4–4.1)</td>
<td>3.4 (2.9–3.6)</td>
<td>4.1 (3.8–4.4)</td>
</tr>
<tr>
<td>Untreated procapsids</td>
<td>1.0 (0.5–1.6)</td>
<td>3.3 (3.1–3.4)</td>
<td>1.6 (1.5–1.7)</td>
</tr>
<tr>
<td>Disoxaril-treated procapsids</td>
<td>3.6 (3.2–4.0)</td>
<td>3.4 (3.0–3.5)</td>
<td>4.0 (3.6–4.4)</td>
</tr>
</tbody>
</table>

* See Methods for immunization procedure.
† Average of antibody titres in sera of four mice. The range is in parentheses.
‡ Titre is the log₁₀ of serum dilution causing precipitation of 50% of input antigen.
§ Titre is the log₁₀ of serum dilution causing 50% plaque reduction, using a 100 p.f.u. inoculum.

**Immunogenicity of disoxaril-treated virions and procapsids**

Groups of mice were immunized (see Methods) with 1 μg of either virions or procapsids, purified with or without disoxaril. Table 2 shows the results. The sera were tested against N and H antigens by S. aureus-aided immunoprecipitation. The untreated virions elicited comparable titres of anti-N and anti-H antibodies; the same held true for disoxaril-treated virions, except that the antibody titres were roughly 1 log₁₀ higher. With disoxaril-treated procapsids as the immunogen, the anti-N antibodies had an average titre of only 10⁻¹, as against 10⁻³⁻³ for the anti-H antibodies. In contrast, disoxaril-treated procapsids elicited comparable anti-N and anti-H titres, and as high as those elicited by disoxaril-treated virions.

The titre of neutralizing antibodies was 1.3 log₁₀ higher when the immunogen consisted of disoxaril-treated rather than untreated virions. Untreated procapsids elicited only a low titre of neutralizing antibodies, whereas disoxaril-treated procapsids elicited neutralizing antibodies as efficiently as disoxaril-treated virions. It will be noted that the titres of neutralizing antibodies responded to disoxaril treatment of the immunogen in exactly the same way as that of the N-specific antibodies assayed by immunoprecipitation.

**Discussion**

The polio vaccines presently in use have remained essentially unchanged for 35 years. Immunization against poliomyelitis began with J. Salk's formalin-inactivated vaccine (IPV; Salk *et al.*, 1954), followed by A. Sabin's live, attenuated, oral vaccine (OPV; Sabin, 1955). In developed countries, these vaccines were so successful that poliomyelitis was practically eradicated. In the Third World, however, poliomyelitis remained a
problem, despite mass vaccination campaigns. The main drawbacks of OPV are its genetic instability, in particular that of the Sabin 3 strain, and the need to maintain infectivity by an unbroken cold chain. IPV, on the other hand, is expensive to produce and administer, as it requires a primary and two booster injections. In view of the shortcomings of the classical vaccines, research on possible alternatives remains worthwhile.

The use of procapsids in a safe vaccine, free of genetic material, is an attractive possibility, but one beset with uncertainties and difficulties. (i) Even though native procapsids are N-antigenic and express all the neutralizing sites of mature virions (Rombaut et al., 1990), their immunogenicity remained unknown. (ii) Native procapsids are highly sensitive to thermal denaturation. At temperatures in excess of 25 °C, they are rapidly converted to H-antigenic, empty capsids. This thermal denaturation is slowed, but not suppressed, by millimolar concentrations of Mg$^{2+}$ (Rombaut et al., 1985, 1989). However, as shown in this paper, thermal denaturation of procapsids up to 42 °C could be prevented by disoxaril. (iii) Procapsids are broken down to 14S subunits when the pH exceeds 7.4, even at 4 °C (Rombaut et al., 1987). However, this alkaline dissociation was totally prevented by disoxaril, even at the optimum pH of 8.2.

When procapsids were purified in the presence of disoxaril, both their yield and antigenic quality were improved, without concomitant loss of virions. Presumably, disoxaril prevented the denaturation of procapsids, and thereby reduced losses by keeping them from sticking to centrifuge tubes and pellet material, as heat-denatured particles tend to do (Boeyé & De Rees, 1989).

The most striking feature of disoxaril-treated procapsids was their immunogenicity in mice. Untreated procapsids mainly induced H-specific antibodies, but only low titres of N-specific or neutralizing antibodies. Disoxaril treatment increased the average titre of N-specific or neutralizing antibodies by 2.4 to 2.6 $\log_{10}$. A similar, though smaller (1.0 to 1.3 $\log_{10}$) increase was observed with disoxaril-treated compared to untreated virions. Thus, disoxaril enhances the immunogenicity of both virions and procapsids; as a matter of fact, disoxaril-treated procapsids and virions are equally potent immunogens in mice.

In conclusion, disoxaril treatment seriously improves the prospect of using procapsids in vaccines. One remaining difficulty is that procapsids, classically prepared from infected cells, may be too costly for use in vaccines. However, this drawback may be overcome by recombinant DNA methodology, possibly involving the use of either a baculovirus expression vector in insect cells (Urakawa et al., 1989) or a plasmid in bacteria (Jore et al., 1989).

The authors are grateful to Dr D. Pevear (Sterling-Winthrop Research Institute) for providing disoxaril, to Drs M. Ferguson and K. Wiegers for the gift of monoclonal antibodies, to Dr R. Vrijsen for critically reading the manuscript, and to Monique De Pelsmacker, Alfonso De Rees and Solange Peeters for their excellent technical assistance. One of us (B.R.) is a Senior Research Assistant of the National Fund of Scientific Research (Belgium).

References


(Received 2 November 1989; Accepted 5 February 1990)