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Immunogenic, non-infectious polio subviral particles synthesized in *Saccharomyces cerevisiae*

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Polioviral genes coding for P1, the precursor for the structural proteins, and 3CD, the viral protease, were cloned in a *Saccharomyces cerevisiae* inducible expression system. N-antigenic empty capsids could be isolated from the yeast cell extract provided that pirodavir, a capsid-binding compound and capsid stabilizer, was added during the induction period and during purification. Purification was by immunoaffinity chromatography. The purified empty capsids had the same immunogenicity as poliovirus virions. The techniques described might be useful for the production of new non-infectious vaccines.

Growing insight into the morphogenesis of poliovirus and detailed knowledge of the antigenicity of the various (sub)viral particles involved have made poliovirus an attractive model system to study the feasibility of a vaccine based on subviral particles. Prospects for the development of such a vaccine are very good.

(1) Procapsids, which are empty protein shells of composition $(VP0 + VP1 + VP3)_{60}$, directly isolated from infected cells, have the same antigenicity as virions (N antigen). Like virions, they possess all four neutralizing antigenic sites of poliovirus (Rombaut *et al.*, 1990*a*).

(2) These subviral particles can be stabilized against thermal denaturation by capsid-binding compounds such as disoxaril, R 78206 or pirodavir. The stabilized procapsids are excellent immunogens (Rombaut *et al.*, 1990*b*, 1991).

(3) Procapsids can be made with a minimum of genetic material (coding sequence for P1; the capsid precursor and 3CD; the virus-encoded protease) *in vitro* (Jore *et al.*, 1991).

Different systems have been explored for the synthesis of polio subviral particles on a preparative scale. In two of these expression systems, baculovirus recombinants in insect cells (Urakawa *et al.*, 1989) and vaccinia virus recombinants in mammalian cells (Ansardi *et al.*, 1991), empty capsids were synthesized. The empty capsids isolated from the baculovirus expression system induced only a very weak neutralizing response in mice (Urakawa *et al.*, 1989). No data on the immunological response of the empty capsids isolated from the vaccinia virus expression system seem to be available (Ansardi *et al.*, 1991).

We decided to use a third expression system: autonomously replicating expression vectors in the yeast *Saccharomyces cerevisiae*. P1- and 3CD-coding sequences (derived from serotype 1, Mahoney), provided with translational start and stop codons, were cloned in yeast vectors using inducible (GAL) promotors. It could be shown that during the induction period, capsid proteins VP0, VP1 and VP3 were synthesized and that these proteins were assembled into empty capsids, with a sedimentation value of 74S. About 0·1–1 mg of particles was obtained from 1 l of yeast culture (Jore *et al.*, 1994). However, using a competition immunoprecipitation assay, it was shown that the empty capsids were H antigenic (Rombaut *et al.*, 1994), meaning that these particles are denatured. They do not possess any neutralizing determinant. The particles therefore cannot induce neutralizing and protective antibodies. The surface of these particles is identical to that of H antigen obtained by heating virions for 20 min at 56 °C (Hummeler & Hamparian, 1958).

The aim of this paper is to show that N-antigenic empty capsids can be synthesized in this same yeast expression system, provided pirodavir (a capsid-binding compound) is added to the yeast cells. The N-antigenic empty capsids were purified using immunoaffinity chromatography with monoclonal antibodies (MAb). The purified N-antigenic empty capsids were able to induce N-specific and neutralizing antibodies in mice.

The construction of the *S. cerevisiae* expression system is fully described elsewhere (Jore *et al.*, 1994). Briefly, pLOP254, a plasmid carrying an inducible (GAL7 promoter driven) transcription unit for P1 (the capsid proteins precursor), and plasmid pLOP380, carrying an inducible (GAL1 promotor) transcription unit for 3CD (the viral protease), were jointly introduced and stably maintained in *S. cerevisiae* strain 334. Extracts were prepared after induction by 1% galactose and incubation for 16 h at 30 °C. Glass beads were used for lysis of

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Table 1. Antigenicity of partly purified empty capsids of yeast

Concentrations obtained by competition assay (nM)*	Pirodavir concentration (μg/ml)†			
	0	0·1	1	10
N-antigenic	0	0	1·2	3·3
H-antigenic	3·2	3·0	2·4	0

* Competition immunoprecipitation assay (Rombaut *et al.*, 1994).
† Pirodavir, also called R 77975, ethyl-4-[3-[1-(6-methyl-3-pyridazinyl)-4-piperidinyl]ethoxy]benzoate, was synthesized by the Janssen Research Foundation. Stock solutions (10 mg/ml) of pirodavir were made in dimethyl sulphoxide and diluted in PBS (137 mM NaCl, 2·7 mM KCl, 9·5 mM sodium phosphate, pH 7·3).

yeast cells. Lysis buffer was PBS buffer supplemented with 1% NP40. Supernatant was clarified by centrifugation at 10000 ***g*** for 5 min at 4 °C.

Analysis of the supernatant by a competition immunoprecipitation assay (Rombaut *et al.*, 1994) revealed that only H-antigenic empty capsids were formed (see Table 1), confirming previous results (Jore *et al.*, 1994). In contrast, during *in vivo* assembly in infected cells, N-antigenic empty capsids or procapsids are formed (Rombaut *et al.*, 1990*a*). This discrepancy might be due to the lack of the putative 'natural stabilizer' (or natural ligand) in yeast cells. This natural stabilizer is only abundantly present in infected HeLa cells. Uninfected cells are deficient in this stabilizer (HeLa cells) or possess only limited quantities (reticulocytes) (Rombaut *et al.*, 1991; Jore *et al.*, 1991, and unpublished results). The natural stabilizer protects the procapsids against denaturation during assembly (Rombaut *et al.*, 1991). The natural stabilizer consists, probably, of one or more (sphingosine-like) lipid substituents and can be found in the pocket underneath the canyon of the polio virion (Filman *et al.*, 1989). The natural stabilizer can be mimicked by capsid-binding compounds, such as pirodavir, disoxaril or R 78206 (Rombaut & Boeyé, 1991). These compounds bind to the same hydrophobic pocket and can stabilize the capsid more than the natural stabilizer does (Grant *et al.*, 1994). It might be worth noting that denaturation of N-antigenic empty capsids occurs at a temperature as low as 25 °C (Rombaut *et al.*, 1989) and this thermal denaturation can be blocked by the addition of capsid-binding compounds (Rombaut *et al.*, 1990*b*).

Because yeast cells might be deficient in the natural stabilizer different concentrations of pirodavir, a capsid-binding compound, were added to the medium at the start of induction. During the extraction and further purification of the extract, the concentration of pirodavir was kept constant. Results are shown in Table 1 for partly purified empty capsids from yeast extracts. Partial purification by a combination of high-performance size-exclusion chromatography (HPSEC) and sucrose gradient ultracentrifugation (SGU) was as described (Jore *et al.*, 1994). Concentrations of the empty capsids were determined using a competition immunoprecipitation assay using MAbs against N and H antigen, respectively. The assay method has been designed for unlabelled, unpurified antigens of poliovirus. The method is based on competition between the unlabelled antigen and a standard quantity of radiolabelled antigen, in the presence of a limiting amount of an N- or H-specific MAb. The immune complexes are removed by protein A-bearing, fixed staphylococci. The method is free from cross-reaction between N and H antigen, and has a detection limit of approximately 1 nM (Rombaut *et al.*, 1994). At a pirodavir concentration of 1 μg/ml, a mixture of H- and N-antigenic empty capsids was obtained, whereas at 10 μg/ml of pirodavir, only N-antigenic empty capsids were found (Table 1). Note that the total concentration of empty capsids (sum of H- and N-antigenic empty capsids) is independent of the pirodavir concentration indicating that the addition of pirodavir (10 μg/ml) to the yeast cells only results in a conformational change of the assembled empty capsids.

To determine the immunogenicity of the N-antigenic empty capsids, a proper purification scheme for the empty capsids had to be developed. The HPSEC and SGU purified N-antigenic empty capsids (see above) were still contaminated with yeast proteins (data not shown). To avoid tolerance problems in an immunization protocol, and in order to compare the immunogenicity of different particles, N-antigenic empty capsids should be free of any contamination. It was therefore decided to purify the empty capsids from yeast extracts using immunoaffinity purification.

MAb 33-5b5 is N-specific and directed against neutralizing site 3B (Page *et al.*, 1988). As site 3B is formed upon assembly of 14S subunits into N-antigenic empty capsids, monoclonal antibody 33-5b5 recognizes only virions and N-antigenic empty capsids (Rombaut *et al.*, 1990*a*). The antibody is therefore an ideal tool in the immunoaffinity purification of the yeast extract. It would only recognize the N-antigenic empty capsids in the mixture of yeast components and viral proteins. MAb 33-5b5 was bound to CNBr-activated Sepharose 4B (Pharmacia). A small column of about 5–7 ml of gel was prepared according to the manufacturer's protocol (Pharmacia) with 2 g of CNBr-activated Sepharose 4B and 1 ml of mice ascites containing MAb 33-5b5.

Using radiolabelled virions or procapsids purified from infected cells, the capacity of the column was determined. At least 200 μg of radiolabelled virions or procapsids could be bound to the column without any significant loss during washing procedures (retention of particles $> 98\%$), hereby confirming the fact that 33-5b5 is a high-affinity antibody. The specificity of the column was tested by loading the column with radiolabelled H-antigen (virions heated for 20 min at 56 °C) or radiolabelled 14S subunits. No radioactivity was retained by the column and more than 99% of the input radioactivity was found in the eluate using binding buffer.

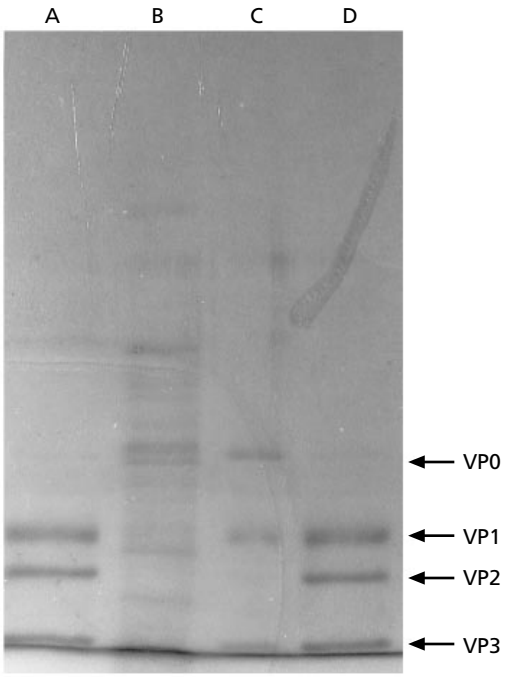

Fig. 1. Polypeptide pattern of N-antigenic empty capsids purified from yeast by immunoaffinity chromatography. Separation was by SDS–PAGE and staining was by Coomassie blue. Lanes A and D, poliovirus virions; lane B, yeast extract; lane C, empty capsids purified from the yeast extract by immunoaffinity chromatography (see text).

Table 2. Immunogenicity of empty capsids extracted from yeast in the presence of pirodavir

	Average antibody titre†		
	Immunoprecipitation‡		Neutralization§
Immunogen*	N-antigen	H-antigen	
N-antigen	4·3 (3·9–4·7)	3·8 (3·3–4·3)	4·3 (4·2–4·5)
H-antigen	1·5 (1·1–1·8)	4·2 (4·0–4·3)	1·5 (1·5–1·7)
Yeast empty capsids	4·0 (3·6–4·5)	3·5 (3·3–3·8)	4·3 (4·0–4·6)

* Mice each were immunized intraperitoneally with 1 μg of purified antigen (Serotype 1, Mahoney) in PBS buffer supplemented with incomplete Freund's adjuvant. The procedure was repeated on days 14 and 28, and the animals were sacrificed on day 35.
† Average of antibody titres in sera of five mice. The range is in parentheses.
‡ Protein A aided immunoprecipitation using microtitration plates is fully described elsewhere (Vrijsen *et al.*, 1983). Titre is the $\log_{10}$ of serum dilution causing precipitation of 50% of input antigen.
§ The plaque assay was used (see Rombaut *et al.*, 1994). Titre is the $\log_{10}$ of serum dilution causing 50% plaque reduction, using a 100 p.f.u. inoculum.

Elution of the bound antigen from the immunoaffinity column was with 100 mM triethylamine buffer, pH 11·5. Due to the high affinity of MAb 33-5b5 for virions or N-antigenic empty capsids, these relatively harsh conditions were necessary to break the antigen–antibody interactions. Several other less stringent elution conditions were tested, but all failed to elute the bound antigen completely (data not shown). To neutralize the high pH used to elute the antigen from the column, the collection tubes contained a buffer (50 mM phosphate, pH 6·8).

To prevent N-antigenic empty capsids from being dissociated into 14S subunits due to the high pH of the elution buffer (Rombaut *et al.*, 1982, 1989), pirodavir (10 μg/ml) was added. It is known that pirodavir and other capsid binding compounds protect N-antigenic empty capsids against alkaline dissociation (Rombaut *et al.*, 1990*a*).

In the final setup, a yeast cell extract was prepared from 0·5 l of culture and brought onto the immunoaffinity column. More than 99% of the cell extract components were eluted with the binding buffer. After the washing procedure, bound antigen was eluted with the elution buffer. About 50 μg of proteins was collected (results not shown) and further analysed. Using the competition immunoprecipitation assay, it could be shown that these 50 μg of proteins were N-antigenic and that roughly the same amount of N-antigenic particles was present in the unpurified yeast cell extract. Sucrose gradient analysis proved that they had the same sedimentation value as empty capsids (74S; results not shown) and SDS–polyacrylamide gel electrophoresis followed by Coomassie blue staining showed that the collected antigen contained almost only viral capsid proteins VP0–1–3 (Fig. 1). In conclusion, using immunoaffinity purification N-antigenic empty capsids could be purified almost completely from the yeast extract. About 100 μg of N-antigenic empty capsids could be isolated from 1 l of yeast culture.

As purified particles were now available, the immunogenicity of these yeast-purified N-antigenic empty capsids could be determined and compared with virions (N antigen) or H antigen. Groups of mice were immunized (see Table 2) with 1 μg of either N or H antigen or with yeast empty capsids. The sera were tested against N and H antigens by *S. aureus*-aided immunoprecipitation and the neutralization titre was determined using a plaque assay (see also Table 2). Results (Table 2) show that the yeast empty capsids and N antigen elicited comparable titres of anti-N antibodies. These titres are roughly 2·5 $\log_{10}$ higher than the anti-N antibody titre of H antigen. The titre of neutralizing antibodies is 2·8 $\log_{10}$ higher when the immunogen consisted of N antigen or yeast empty capsids rather than H antigen.

To summarize.

(1) Yeast strain 334 supports formation of poliovirus empty capsids which are N-antigenic provided pirodavir is added during the induction period and during purification.

(2) N-antigenic empty capsids can be purified completely

and directly from lysed, clarified yeast cells using immunoaffinity chromatography.

(3) These empty capsids have the same immunogenicity as poliovirus virions.

Although these yeast purified N-antigenic empty capsids might constitute a safe, new polio vaccine, the techniques described here might be useful for the formulation of other (picornavirus) vaccines (e.g. hepatitis A vaccine).

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