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Stability and Immunogenicity of Empty Particles of Foot-and-Mouth Disease Virus

By

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With 5 Figures

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Summary

Three strains of foot-and-mouth disease virus were shown to contain significant amounts of naturally occurring 75S, empty particles as well as the infectious, 140S full particles. One of these strains — A Pando (1970) — was studied in detail.

The empty particles from this virus strain were shown to have an observed sedimentation coefficient of 67*S* in 0.04 M phosphate buffer; they were labile in SDS, non-infectious and probably RNA-free and, on heating, they broke down to 12S subunits as did the 140S particles. The empty particles differed from the full particles in their polypeptide composition since they contained VP_0, but there was no evidence for a diminished content of VP_4.

The 75S particles were shown to be present in significant amounts and to be stable to AEI inactivation. At 4° C they were stable for at least two years. In guinea pigs they were as immunogenic as the 140S particles. The antisera raised against the 75S particles had the same serological specificity in neutralization tests as sera prepared against the 140S particle. It was concluded that the 75S particles from the A Pando (1970) strain of FMD virus may provide as important a contribution as 140S particles to the immunogenicity of inactivated vaccines prepared from this virus strain.

Introduction

It is generally accepted that the immunogenicity of foot-and-mouth disease (FMD) virus is dependent on the full virus particle, which has a sedimentation coefficient of about 140S (7, 11, 26). The other two structural antigens; the empty particle and the protein subunits, respectively with sedimentation coefficients of 75S and 12S, are not considered to contribute substantially to the immunogenicity of FMD vaccines (11). Although Rowlands *et al.* (21) demonstrated that the empty particle possesses the D-antigenicity of the full particle, this antigen

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was nonetheless considered unimportant in terms of immunogenicity of vaccines since apparently it was unstable. The 12S protein subunits do not seem to induce significant amounts of neutralizing antibody (11, 20). Consequently, in recent years, sucrose density gradient and analytical centrifugation techniques have been incorporated in regular quality screening of inactivated antigen pools for FMD vaccine manufacture in order to assess their 140S antigen content (2, 12, 24, 25). As previously reported by TELLING (25), we have been surprised by the lack of regular correlation between 140S particle content of FMD antigen pools and their immunogenicity as formulated vaccines. This was most marked in the case of strain A Pando (1970) which regularly resulted in vaccines of high potency and yet only about 50 per cent of the vaccine antigen lots studied had detectable quantities of 140S particles. In contrast, strain O_1 BFS 1860 appeared to demonstrate a reasonable correlation between 140S particle content of antigen pools and potency of formulated vaccines.

The present study was undertaken to examine the stability and immunogenicity of the empty particles of foot-and-mouth disease virus in respect of vaccine production.

Materials and Methods

Virus Growth and Assay

Type A, strain Pando (1970), of foot-and-mouth disease virus has been in regular use for vaccine manufacture at Pirbright for seven years. Other strains were employed as specified under Results. The virus was grown in BHK 21 Clone 13 monolayer or suspension cells (8, 16). Virus infectivity was assayed by plaque titration in BHK 21 monolayer cells and the complement fixing antigen was titrated in microtitre plates using a modification of the technique described by CAPSTICK and others (9). Inactivation of infectivity was by two doses of 0.05 per cent v/v of acetylethyleneimine (AEI) at 24-hours interval with residual AEI being neutralized at 48 hours by 2 per cent sodium thiosulphate (6). The innocuity of inactivated antigens was tested *in vitro* in BHK 21 monolayer cells according to ANDERSON and others (1). In addition, those antigens which were formulated into vaccines were innocuity tested *in vivo* by the method of HENDERSON (14).

Virus Purification

The method of BROWN and CARTWRIGHT (5) was employed to purify the virus. When it was desired to obtain both 140S and 75S particles, the pelleted virus material was treated with 1 per cent Nonidet P_{40} (NP_{40}) instead of 1 per cent sodium dodecyl sulphate (SDS) prior to sucrose density gradient centrifugation since SDS disrupts the empty particles (21). The 15—45 per cent sucrose density gradients were centrifuged at 30,000 rpm for $2\frac{1}{2}$ hours in the MSE 3×23 ml rotor (Measuring and Scientific Equipment, Crawley, Sussex, England). Fractions of 1 ml were titrated for complement fixing antigen or infectivity.

Radioactively Labelled Virus

A Pando (1970) virus was grown in monolayer BHK 21 cells in the presence of 5 μCi/ml of ^{3}H-uridine or 2.5 μCi/ml ^{14}C-protein hydrolysate (Radiochemical Centre, Amersham, England) and purified as for unlabelled virus except that the ammonium sulphate precipitation step was omitted. The virus preparations were treated with ribonuclease at 100 μg/ml for 10 minutes at room temperature before the addition of NP_{40} in order to eliminate possible contamination of the 75S particles with ribosomes (21).

Polyacrylamide Gel Electrophoresis

SDS-disc polyacrylamide gel electrophoresis was carried out as described by MAIZEL (17) and the gels were fractionated using a Model AGDW-18 Autogeldivider (Savant Instruments, Inc. Flicksville, N.Y.). Samples were counted in a Beckman LS 3150

Liquid Scintillation counter using Beckman HP Readysolv scintillation fluid (Beckman Instruments International, Beckman RIIC Ltd., Eastfield Industrial Estate, Glenrothes, Fife, Scotland). Polypeptide molecular weights were estimated by comparison with the migration of ovalbumin and trypsin on parallel gels. The marker proteins were stained with Coomassie brilliant blue.

Analytical Centrifugation

The sedimentation rates (S-values) for the immunogenic fractions of A Pando (1970) virus were estimated by analytical centrifugation in an MSE Centriscan 75 using a 6-place rotor and 10 mm single sector cells. Centrifugation conditions were 20,000 rpm at 20° C in 0.04 M phosphate buffer pH 7.6, Schlieren optics with the knife edge at 80°.

Results

Figure 1 depicts the sedimentation profiles for six FMD vaccine virus strains. It is apparent that when virus pellets were treated with NP_{40} instead of SDS as in the method of BROWN and CARTWRIGHT (5), a number of strains demonstrated a significantly high proportion of 75S particles compared with 140S virions. Strain A Pando (1970) was selected as representing this group of viruses. For this strain the ratio of 75S to 140S complement fixing units varied from 1 : 1 to 3 : 1. In order to assess whether the 75S particles were disrupted by AEI treatment (21), an antigen pool from a vaccine production lot no. 273 was analysed before and after treatment. AEI treatment did not alter the sedimentation profile for the A Pando (1970) virus strain. Three complement fixing antigenic peaks, corresponding to 140S, 75S and 12S antigens, were demonstrated in AEI treated and untreated samples. In this batch of antigen, the 75S particles apparently were in greater quantity than the 140S particles (Table 1).

Table 1. *Stability of A Pando (1970) 140S and 75S antigens at 4° C*

			Nonidet P_{40} treated pellet SDG				SDS treated pellet—SDG			
			First (140S) peak		Second (75S) peak		First (140S) peak		Second (75S) peak	
Vaccine lot No.	Days of storage at 4° C	cfu per 0.025 ml concentrate	Peak cfu/ 0.025 ml	Total cfu in peak as % cfu of concentrate	Peak cfu/ 0.025 ml	Total cfu in peak as % cfu of concentrate	Peak cfu/ 0.025 ml	Total cfu in peak as % cfu of concentrate	Peak cfu/ 0.025 ml	Total cfu in peak as % cfu of concentrate
198	673	64	8	9.4	16	20.3	3	5.5	<2	Nil
199	671	45	11	25.6	11	25.6	6	10.0	<2	Nil
203	608	128	8	6.6	22	17.20	6	3.5	<2	Nil
204	612	16	2	6.3	3	15.6	2	6.3	<2	Nil
273 Expt I	10	512	22	5.8	64	14.6	Not done	Not done	Not done	Not done
273 Expt II	14	724	32	3.5	64	9.9	6	1.0	<2	Nil

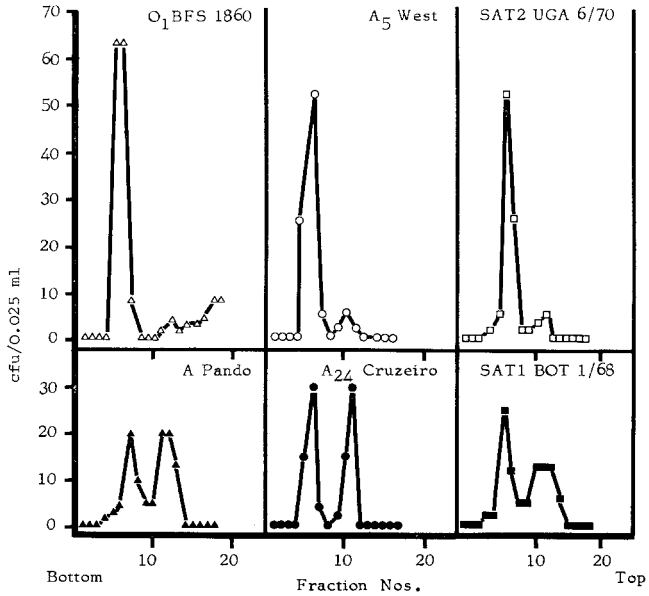

Fig. 1. Sucrose density gradient profiles of Six FMD vaccine strains

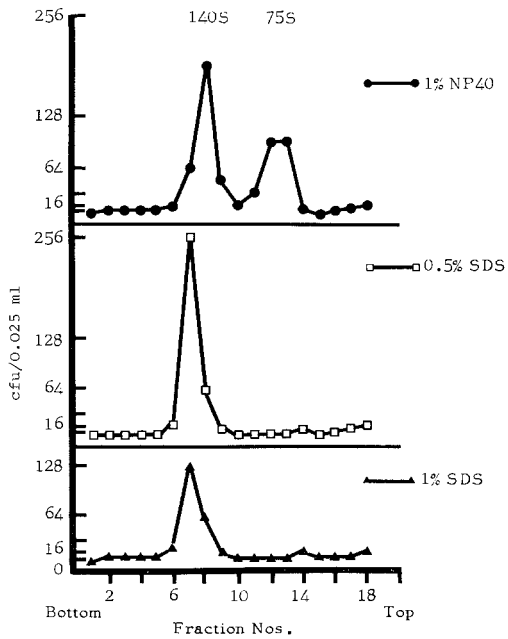

Fig. 2. Effect of SDS on A Pando (1970)—75S particles

Characterisation of the Two Main Antigenic Fractions of A Pando (1970) FMD Virus

Virus harvest from 2×10^9 BHK 21 monolayer cells was concentrated 100-fold by ammonium sulphate precipitation and ultracentrifugation and the resuspended pellet was treated with either 1 per cent NP_{40} or 0.5 per cent SDS or 1 per cent SDS prior to rate zonal centrifugation on sucrose density gradients. As demonstrated in Figure 2, the 75S particles were disrupted by SDS. Infectivity assays on the fractions from each gradient demonstrated only one peak corresponding to the 140S peak. Pooled fractions containing either the 140S or 75S antigens obtained from NP_{40} treated samples were dialysed overnight against 0.04 M buffer pH 7.6 and recycled on fresh gradients. The isolated fractions retained their sedimentation characteristics, each demonstrating a single peak corresponding to the parent peak.

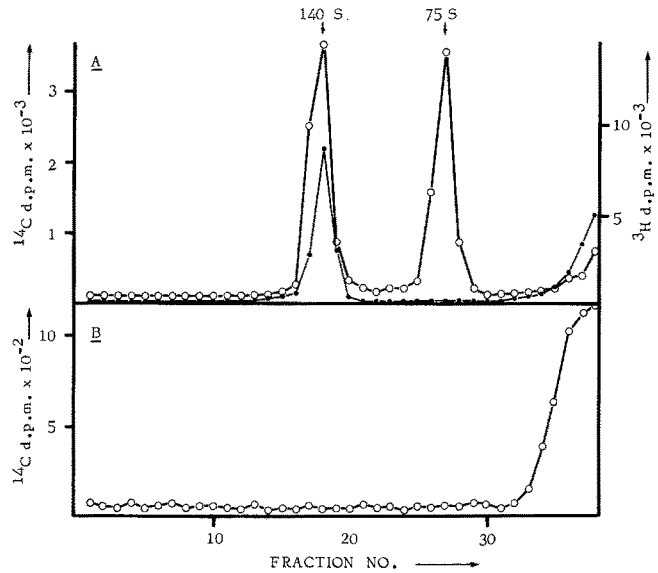

Fig. 3. *A* Co-sedimentation of ^{14}C-protein hydrolysate (o———o) and ^{3}H-uridine (•———•) labelled A Pando virus, *B* Sedimentation of A Pando 75S component after treatment with 1 per cent SDS

The use of radioactively labelled virus also demonstrated the presence of 140S and 75S particles in the virus preparation in approximately equal proportions and that only the 140S particles contained ribonuclease resistant RNA (Fig. 3A). The possibility that 75S particles of A Pando (1970) contain some RNAse-sensitive RNA (23) could not be excluded. When the fractions containing 75S particles were pooled and treated with 1 per cent SDS and re-run on a fresh gradient, all the radioactivity remained at the top of the gradient confirming the instability of 75S particles in SDS (Fig. 3B).

By analytical centrifugation of the A Pando (1970) virus pellet at 20,000 rpm, 2 peaks separated from the sample meniscus. The mean observed *S*-values in 0.04 M phosphate buffer at 20° C in 14 experiments were $128.00S \pm 14.39$ and

66.78$S \pm 0.08$ respectively. Occasionally a faster moving peak with an observed S-value ranging between 157.79S and 196.25S (mean 177.74$S \pm 15.76$) was detected. This was considered to represent aggregates. The two main peaks were resistant both to ribonuclease treatment (25 μg/ml for 15 minutes at room temperature) and to 1 per cent NP_{40}. Only the faster (128S) peak was resistant to 1 per cent SDS; this treatment obliterated the slower peak once again confirming the instability of empty particles to SDS. The observed S-rate of 128S for the full particles is similar to the values obtained by Strobbe and others (24).

The relative heat stability of the full and empty particles was tested in the following experiment. Strains A Pando (1970) and O_1 BFS 1860 were grown in BHK 21 monolayer cells and concentrated 100-fold. The resuspended pellets were each divided into 2 aliquots. One aliquot of each virus was heated at 54° C for 60 minutes and the second aliquot left at 4° C. Both aliquots were then subjected to rate zonal centrifugation on sucrose density gradients.

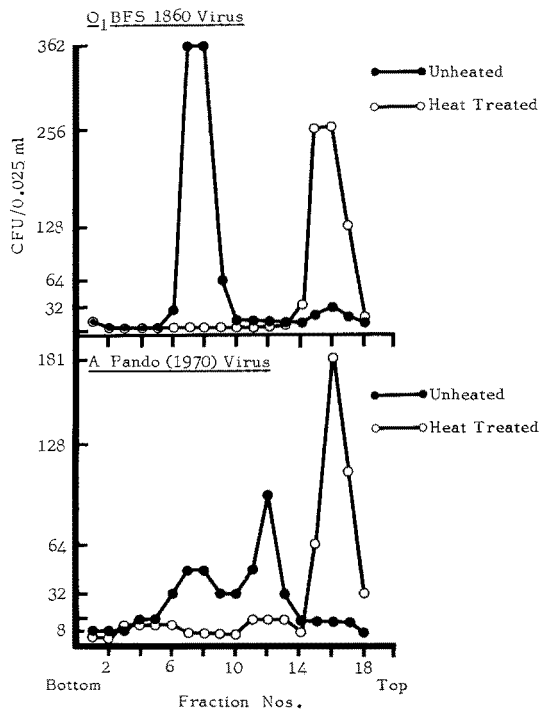

Fig. 4. Effect of heat on 140S and 75S particles

Figure 4 demonstrates that heating had resulted in the breakdown of the 140S antigens of O_1 BFS 1860 and A Pando (1970) viruses, and the 75S antigen of A Pando (1970) virus into 12S subunits indicating that the 75S particles were probably not degradation products of 140S particles. Moreover, it is evident that the A Pando (1970) 75S antigen was probably not more heat labile than the 140S antigen since after 60 minutes at 54° C there was still some residual 75S antigen detectable in the A Pando (1970) virus pellet.

Polypeptide Composition of A Pando (1970) 140S and 75S Particles

Figure 5 shows the result of polyacrylamide gel electrophoresis of ^{14}C-protein hydrolysate labelled A Pando (1970) isolated 140S (Fig. 5A) and 75S (Fig. 5B) particles. VP_1 appears to be partially resolved from VP_2 and VP_3, but these are not resolved from each other. The only significant difference between the polypeptide composition of the particles was the presence of a polypeptide of molecular weight 44,000, corresponding to the precursor protein, VP_0 (3), in the 75S particles and its absence in the 140S particles. This polypeptide represented about 7 per cent of the total protein in the 75S particles.

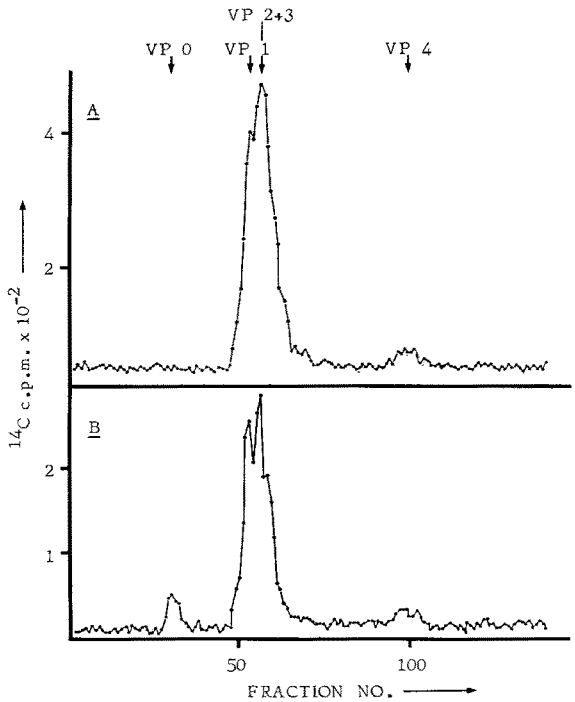

Fig. 5. Polyacrylamide gel electrophoresis of ^{14}C-protein hydrolysate labelled A Pando 140S particles (A) and 75S particles (B)

Stability of A Pando (1970) 140S and 75S Antigens in AEI Inactivated Antigens Stored at 4° C

SDG analyses were carried out on five vaccine production lots of AEI-inactivated unadsorbed A Pando (1970) antigens stored at 4° C for varying periods up to nearly two years. All the samples revealed both 140S and 75S antigen peaks in NP_{40} treated pellets, but SDS treated antigen pellets revealed only the 140S antigen peak (Table 1).

Comparative Immunogenicity of 140S and 75S A Pando (1970) Antigens

140S and 75S antigens were isolated from two vaccine production lots 273 and 198, representing freshly produced AEI-inactivated antigen and antigen which had been stored at 4° C for 673 days after AEI-inactivation. Each antigenic fraction was emulsified in Freund's incomplete adjuvant and 1 per cent Tween 80, and inoculated intramuscularly into four guinea pigs. The guinea pigs were bled at 28 days post inoculation and the sera from each group bulked. Table 2 summarises the neutralizing antibody response as measured in the microneutralization test (22). It is evident that both 140S and 75S particles of strain A Pando (1970) were immunogenic even after storage at 4° C for nearly two years as AEI-inactivated, unadsorbed antigens.

The specificity of antisera to the 140S and 75S antigens was tested in a plaque reduction test by titrating the sera against homologous (A Pando) and heterologous viruses. Table 3 summarises the heterologous relationships expressed as r values (i.e. heterologous titre: homologus titre; 18). Antisera to 140S and 75S antigens demonstrated similar specificities. They showed a close relationship between A Pando (1970) and A_{30} Uruguay viruses but a wide divergence with A_5 France 1/68 and A Philippine 10/75 strains.

Table 2. *Comparative immunogenicity of 140S and 75S A Pando (1970) virus particles*

Vaccine lot	Inoculum	Neutralizing antibody response 28 days p.i. (Log SN_{50}). n = 3
Lot 273 (10 days at 4° C)	140S Antigen (SDS treated)	1.49±0.37
	140S Antigen (NP_{40} treated)	1.84±0.16
	75S Antigen	2.14±0.16
Lot 198 (673 days at 4° C)	140S Antigen (NP_{40} treated)	1.42±0.12
	75S Antigen	2.20±0.04

Table 3. *Specificity of antisera to full (140S) and naturally occurring empty (75S) FMD virus particles of strain A Pando (1970) virus*

Antiserum	r—values[a] for following viruses			
	A Pando	A_{30} Uruguay	A_5 France 1/68	A Phil 10/75
A Pando (1970) 140S	1.00	0.56	0.003	<0.016
A Pando (1970) 75S	1.00	0.83	0.009	<0.006

[a] $r = \frac{\text{Heterologous serum neutralizing titre}}{\text{Homologous serum neutralizing titre}}$

Discussion

The presence of RNA-free virus-like particles in foot-and-mouth disease virus suspensions was demonstrated by the early work of Planterose and Ryan (19) and Graves *et al.* (13). These particles have been reported to have a sedimentation coefficient of 65 to 75S and they are often referred to as 75S or empty particles.

Their biochemical characteristics have been described recently by ROWLANDS and others (21), CAVANAGH (10) and LIEBERMANN and others (15). In our studies we have demonstrated that these particles have an observed sedimentation rate in 0.04 M phosphate buffer of 67S, are labile in SDS, are probably RNA-free and non-infectious, and on heating break down to 12S particles. The main difference in the polypeptide composition of the full and empty particles was the presence of significant amounts of VP_0 in the empty particle and its absence in full particles.

Empty particles of similar sedimentation rate can also be produced artifically by treatment of the 140S virions with EDTA (21) but it has not been shown that 75S particles represent a natural degradation product of the complete virion. The 75S particles of A Pando (1970) characterised in our studies contain at least a proportion of true procapsids, i.e. precursors of complete virion capsids (4), since (a) they contain both VP_0 and VP_4 whereas artificially produced 75S particles contain neither (21) and (b) they are capable of stimulating the production of neutralizing antibody. ROWLANDS and others (21) were unable to provoke a neutralizing antibody response using artificially produced empty particles even after their stabilization with formalin.

As demonstrated in Figure 1, different virus strains appear to contribute varying proportions of 75S particles to the total antigen pool. In our studies, strains A Pando (1970) and A_{24} Cruzeiro appear to be particularly rich in empty particles. The analysis of A Pando (1970) AEI-inactivated antigen lots which had been stored at 4° C for varying periods up to two years revealed that in several cases A Pando (1970) antigen pools contained more empty (75S) than full (140S) virus particles. In contrast to results described for two other strains of FMD virus (11, 21), the A Pando (1970) 75S particles appear to be stable both to AEI treatment and to prolonged storage at 4° C.

The antibody raised in guinea pigs to the 75S particles was demonstrated to possess both a high neutralizing capacity and the same serological specificity as antibody prepared against the 140S particle. It is unlikely that the demonstrated level of neutralizing antibody to the 75S antigen can be accounted for by the presence of contaminating 140S particles since the 75S antisera had significantly higher neutralizing antibody titres than those to the corresponding 140S antisera. This supposition is supported by the higher level of 75S compared to 140S particles in the antigen pools used in the experiment.

The requirements in vaccine manufacture for the FMD immunizing antigen are that it must be produced in abundance in the cell system used; it must be stable during vaccine manufacture and storage; it must be capable of eliciting specific neutralizing antibody. Until now it has been considered that these requirements are fulfilled only by the 140S particles. Our study has demonstrated that for strain A Pando (1970) FMD virus, at least, the 75S particles also meet these conditions. It is concluded that the 75S particles contribute significantly to the potency of vaccines prepared from this strain of foot-and-mouth disease virus.

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