Protein Subunit Vaccines of Parainfluenza Type 3 Virus: Immunogenic Effect in Lambs and Mice

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(Accepted 17 February 1983)

SUMMARY

Protein subunit vaccines were prepared from a mixture of the haemagglutinin (HN) and fusion (F) glycoproteins of parainfluenza type 3 virus (PI-3). The glycoproteins were isolated in three different forms and characterized by their sedimentation coefficients: 30S protein micelles (a complex of several HN and F glycoproteins devoid of detergent and lipid), 18S protein–TX complexes (a complex of several glycoproteins containing the detergent Triton X-100), and 4S protein–TX complexes (probably monomers of the glycoproteins complexed to Triton X-100). These preparations were tested as vaccines in mice and lambs. The immune response in the mice was assayed both in the serum and in extracts from the lungs using an ELISA technique. Both of the multimeric complexes were highly immunogenic. The 30S protein micelles induced a high antibody response after two injections with either 10 or 1 μg protein. The serum IgG titres reached levels of about 90 μg/ml and 40 μg/ml respectively. Similar titres were reached with the 18S protein–TX complexes. After two injections of either the 30S or the 18S complexes IgA antibody responses were detected in the lung extracts. The 4S protein–TX complexes were poor immunogens and induced low antibody responses in mice. The lambs were vaccinated with the 30S protein micelles, and the immune response was evaluated serologically and in challenge experiments. The 30S protein micelles in an oil adjuvant induced detectable serum antibody titres as well as protective immunity against the pneumonia caused by the PI-3 virus.

INTRODUCTION

The effects of vaccines against virus diseases may be hampered by adverse reactions caused by components in the vaccines not necessary for induction of immunity. The safety of virus vaccines can be considerably improved by purification of the essential viral antigens. For enveloped viruses the surface glycoproteins seem to be the main antigens required to induce a protective immune response (Bachmayer et al., 1976; Cox et al. 1977; Hilleman, 1976; Hunsmann et al., 1981; Merz et al., 1980), but so far a generally applicable method for preparing effective vaccines from isolated surface proteins is lacking. Previous methods using a combination of organic solvents and detergents (e.g. Tween–ether) have given variable results (Ginsberg, 1975; Gross et al., 1977). Methods based on detergent extraction alone have proved more efficient (Bachmayer et al., 1976; Brady & Furminger, 1976). Our own studies have shown that the physical form of a membrane antigen has a considerable influence on its immunogenicity. We isolated the surface glycoproteins of Semliki Forest virus in three different forms using mild non-denaturing conditions (Helenius & Simons, 1975): (i) as protein monomers (complexed to detergent), (ii) as protein micelles (soluble spike protein octamers virtually devoid of lipid and detergent), and (iii) as virosomes, in which the glycoproteins were reconstituted into liposomes of egg lecithin. When these preparations were tested as vaccines against the lethal encephalitis caused by the virus in mice, it was found that the multimeric forms of the protein,
the protein micelles and the virosomes gave exceptionally efficient protection whereas the protein monomers were ineffective (Morein et al., 1978).

We are now extending our studies on the correlation of immunogenicity with antigen presentation to parainfluenza type 3 (PI-3) virus, a paramyxovirus. This virus causes local infections in the respiratory tract of man, lambs and in other animals. We have prepared different forms of the surface glycoproteins, the haemagglutinin/neuraminidase (HN) and the fusion (F) proteins and tested their immunogenic properties in mice and lambs. Our results show that protein micelles (the multimeric forms) are efficient immunogens in mice. In lambs an adjuvant had to be added to induce a protective immune response. Some of the results have been reported previously in a preliminary form (Simons et al., 1980; Morein et al., 1981).

METHODS

Virus growth and purification. Two strains of PI-3 virus were used. The Umea 23 strain was propagated in secondary calf kidney cells using Hanks' balanced salt solution with 0.5% lactalbumin hydrolysate, 2% horse serum and antibiotics, and this strain was used for the experiments with mice. The G2 strain was propagated in secondary foetal lamb kidney (Flk) cells using Eagle's minimal essential medium with 10% lactalbumin hydrolysate, 0.5% foetal bovine serum and antibiotics, and this virus was used for the experiments with lambs.

The virus was concentrated by hollow-fibre filtration (Amicon DC-2; exclusion limit 100000 daltons), and of 200 μl 8% sucrose containing 1% Triton X-100 in TN on top of a 12 ml 10 to 30% sucrose gradient containing 200 μl was layered onto 300 μl 15% sucrose containing 1% Triton X-100 and TN over a 12 ml sucrose gradient in centrifugation for 40 min at 4 °C in a SW27 Beckman rotor. The pellet was resuspended in TN to a concentration of about 10 mg/ml. The amount of virus protein was determined by the Lowry method (Lowry et al., 1951) with 3-5 mm SDS in the reaction mixture. The surface glycoproteins of the virus were labelled with the galactose oxidase-[3H]borohydride procedure (Luukkonen et al., 1977). The labelled PI-3 virus was separated from non-bound [3H]borohydride by centrifugation through 30% (w/w) sucrose onto a cushion of 60% (w/w) sucrose.

Isolation of PI-3 virus surface glycoprotein preparations

Protein micelles. The PI-3 protein micelles containing the virus surface glycoproteins, HN and F proteins were prepared essentially as described by Helenius & von Bonsdorff (1976) and Simons et al. (1978). About 1 mg of PI-3 virus in TN was solubilized, with 2% Triton X-100 together with [3H]-labelled virus. A sample volume of about 200 μl was layered onto 300 μl 15% sucrose containing 1% Triton X-100 and TN over a 12 ml sucrose gradient in centrifugation ranging from 20 to 50% (w/w). The centrifugation was performed at 40000 rev/min for 22 h at 20 °C. Fractions of 500 μl were collected from the bottom and aliquots were measured for radioactivity. The pooled fractions were dialysed against 0.05 M-Tris–HCl, pH 7.4, for 20 h in the cold, and concentrated by lyophilization. This preparation had a sedimentation coefficient of about 30S (see later) and is referred to as the 30S protein micelles. In some of the vaccination experiments 30S protein micelles were mixed with an equal volume of mineral oil adjuvant (Bayol-Falba).

PI-3 virus surface glycoproteins complexed to Triton X-100. PI-3 virus was solubilized with Triton X-100 as described above, and centrifuged for 20 h at 39000 rev/min at 4 °C through a sucrose gradient composed of a layer of 200 μl 8% sucrose containing 1% Triton X-100 in TN on top of a 12 ml 10 to 30% sucrose gradient containing 0.05% Triton X-100. About 50% of the HN and F glycoproteins sedimented in a peak with a sedimentation coefficient of about 18S and the other 50% sedimented much slower, at about 4S. The 18S complex is referred to as the PI-3 protein–TX complex (PI-3 virus glycoprotein complexed to Triton X-100) and the 4S complex is designated the 4S protein–TX complex.

Other methods. Polyacrylamide gel electrophoresis was done in slab gels of 10% acrylamide, and 0.27% N,N'-methylenebisacrylamide in the presence of SDS in a discontinuous buffer system (Laemmli, 1970). The samples were prepared with 1% SDS and 1% 2-mercaptoethanol and heated to 100 °C for 1 min.

The PI-3 surface glycoproteins were isolated by lentil lectin chromatography. The HN and F polypeptides were solubilized by suspending the PI-3 virus (1 to 2 mg/ml) in 2% Triton X-100 in TN at room temperature for 60 min and then applied to a column of Lentil–Sepharose 4B (Pharmacia) equilibrated with TN buffer containing 0.5% Triton X-100. After the column had been washed with 5 bed vol. of equilibration buffer, the immobilized glycoproteins were eluted with 2.5% methyl-α-D-mannoside dissolved in TN buffer.

The surface glycoprotein preparations isolated by sucrose density gradient centrifugation were characterized by sedimentation velocity analysis as described by Martin & Ames (1961) using 29S Semliki Forest virus protein micelles, 19S thyroglobulin, and 7S IgG as standards. Electron microscopy of negatively stained preparations was done as described by Simons et al. (1978). The amount of actin was measured by the DNase inhibition assay (Blikstad et al., 1978).
Table 1. Vaccination of mice with parainfluenza 3 virus glycoproteins

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>1st vaccination</th>
<th>2nd vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td>Serum samples were collected regularly</td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>10 µg 30S protein micelles</td>
<td>10 µg 30S protein micelles</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>1 µg 30S protein micelles</td>
<td>1 µg 30S protein micelles</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>0.1 µg 30S protein micelles</td>
<td>0.1 µg 30S protein micelles</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>10 µg 18S protein-TX complexes</td>
<td>10 µg 18S protein-TX complexes</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td>2nd vaccination*</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td>Serum samples were collected regularly</td>
</tr>
<tr>
<td>F</td>
<td>10</td>
<td>5 µg 4S protein-TX complex</td>
<td>5 µg 4S protein-TX complex</td>
</tr>
<tr>
<td>G</td>
<td>10</td>
<td>5 µg 18S protein-TX complex</td>
<td>5 µg 18S protein-TX complex</td>
</tr>
<tr>
<td>H</td>
<td>10</td>
<td>Mixture of 4S and 18S protein-TX complexes; 2.5 µg of each</td>
<td>Mixture of 4S and 18S protein-TX complexes; 2.5 µg of each</td>
</tr>
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* Extracts were collected from lung and trachea following necropsy of the mice, 6 weeks after the 2nd vaccination.

Table 2. Vaccination of lambs with parainfluenza 3 virus

<table>
<thead>
<tr>
<th>Group</th>
<th>No. in group</th>
<th>Treatment at weeks after vaccination:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>4</td>
<td>Unvaccinated</td>
</tr>
<tr>
<td>B</td>
<td>7</td>
<td>15 µg micelles TN buffer, intramuscular</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>15 µg micelles Bayol-Falba, intramuscular</td>
</tr>
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Experimental animals

Lambs. Hysterectomy-derived, colostrum-deprived lambs were reared under specific pathogen-free (SPF) conditions and allocated to groups as required.

Mice. BALB/c male mice were used. They were 6 weeks old at the start of the experiment and were obtained from Bomholtgard Ltd, Ry, Denmark.

Experimental designs

Vaccination experiments in mice. The mice were kept in cages of five, allocated to groups of ten and treated according to the experimental protocol (Table 1a, b). The mice were injected twice with an interval of 3 weeks subcutaneously with different doses of the PI-3 glycoprotein preparations. Blood samples were collected at weekly intervals by bleeding the mice from the retrobulbar plexus. Serum was prepared and stored at -20 °C until used. The mice were exsanguinated and killed 4 or 6 weeks after the second vaccination. The lungs were removed and the immunoglobulins were extracted in TN at the proportion of 1 ml buffer to 1 g trachea or lung tissue (Waller et al., 1980).

Vaccination experiments in lambs. Seventeen SPF lambs were used in the first experiment (Table 2) and 25 in the second. The lambs were injected intramuscularly with 1 ml of the respective vaccines. Blood samples were taken before vaccination and at weekly intervals thereafter until the end of the experiment. Sera were stored at -20 °C until examined. Three weeks after the second vaccination, each lamb was injected intratracheally with 8 ml and intranasally with 2 ml of the G2 strain of PI-3 virus (10^{5.5} TCID_{50}/ml, experiment 1; 10^{7.7} TCID_{50}/ml, experiment 2). Nasopharyngeal swabs for virus isolation were obtained daily from each lamb, held in transport medium on ice and inoculated onto Flk cells within 2 h. Swabs were then stored at -70 °C and positive samples...
were titrated retrospectively, as described previously (Wells et al., 1978). The lambs were killed 7 days after challenge by intravenous pentobarbitone and exsanguinated by severing the axillary vessels. Lung lesions, as viewed from the dorsal aspect, were recorded on lung diagrams (Wells et al., 1978).

**Immunological tests.** The haemagglutination inhibition (HI) test was performed as described previously (Wells et al., 1976).

The enzyme-linked immunosorbent assays (ELISA) to detect antibodies in mouse and sheep sera differed. The ELISA for mouse antibodies employed Dynatech M129B plates coated for 1 h at 37 °C with 100 µl/well of the Umea 23 strain of PI-3 virus (0-2 µg/ml). After three washings with phosphate-buffered saline (PBS) containing 0.05% Tween 20, 100 µl of the test sera diluted 1/1000 or 1/5000 or the extracts from lungs or tracheae diluted 1/500 were added to each well and the plates were incubated for 1 h at 37 °C. After three washings the plates were incubated for 1 h at 37 °C with 100 µl/well of a rabbit anti-mouse IgG (2 µg/ml) (Litton Bionetics Laboratory Products, Kensington, Md., U.S.A.), or with rabbit anti-mouse IgM or IgA, both 2 µg/ml (Miles Laboratories). After three washings, 100 µl of swine anti-rabbit immunoglobulin (8 µg/ml) conjugated with horseradish peroxidase (National Veterinary Institute, Stockholm, Sweden) was added and the plates were incubated for 1 h at 37 °C. After three washings, 100 µl of the substrate 5-aminosalicylic acid was added to the wells and the colour reaction was read at 490 nm after incubation for 60 min at room temperature. The absorbance values shown were corrected by deducting the mean values of samples from non-vaccinated control mice.

The amount of antibody was quantified according to the procedure of McLean et al. (1980).

The ELISA for detecting antibodies from sheep was performed in Dynatech M129A plates coated overnight at 4 °C with 100 µl/well of the G2 strain of PI-3 virus (8 µg/ml). A single buffer was used as diluent and washing fluid throughout the remainder of the test (Hommers et al., 1982). After three washings, 100 µl of the 1/150 diluted test sera were added to the plates and incubated for 2 h at 37 °C. After a further three washings, 100 µl of a rabbit anti-sheep immunoglobulin (0.3 µg/ml) conjugated with alkaline phosphatase was added to each well and incubated at room temperature for 3 h. The plates were washed three times, 100 µl of substrate was added (p-nitrophenyl phosphate) and the colour reaction determined at 405 nm after incubation for 1 h at room temperature.

**RESULTS**

**Preparation and characterization of the PI-3 virus surface glycoprotein complexes**

The polypeptide pattern of the purified PI-3 virus preparation is shown in Fig. 1 (a). The pattern is similar to that described for other parainfluenza viruses and to that previously described for PI-3 virus by Shibuta et al. (1979). The protein band with an apparent mol. wt. of 44 000 is actin. The DNase inhibition assay indicated that 1 to 3% of the protein content was actin. The 73 000 and the 51 000 mol. wt. bands we assign (by analogy with other paramyxoviruses) as the HN and the F glycoproteins respectively (see Choppin & Compans, 1975). These could be isolated by lentil lectin chromatography (Fig. 1b) and were the only polypeptides labelled with the galactose oxidase–[3H]borohydride procedure (not shown).

Galactose oxidase–borohydride 3H-labelled PI-3 virus was solubilized with Triton X-100 and applied to a sucrose gradient designed for isolation of protein micelles. After centrifugation, the radioactivity was distributed in two peaks, one in the lower, detergent-free portion of the gradient (Fig. 2a). SDS–polyacrylamide gel electrophoresis showed that the HN and the F glycoproteins respectively (see Choppin & Compans, 1975). These could be isolated by lentil lectin chromatography (Fig. 1b) and were the only polypeptides labelled with the galactose oxidase–[3H]borohydride procedure (not shown).

The peak fractions 6 to 10 in Fig. 2(a) were pooled and concentrated by lyophilization. The sedimentation coefficient of the protein was determined by velocity sucrose gradient centrifugation. The average value was about 30S.

We tested whether the PI-3 glycoprotein complexes were influenced by the lyophilization procedure used to concentrate the protein. A preparation of glycoprotein complexes obtained from 1 mg of PI-3 virus was divided into two halves. One portion was concentrated by vacuum dialysis in a collodion bag (SM 13200 Sartorius membrane filter) and the other was dialysed against 5 mM-Tris–HCl, 0-015 M-NaCl, pH 7-4 to remove the sucrose. The dialysed material was lyophilized and redissolved in water. The lyophilized protein complexes had the same
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Fig. 1. Polyacrylamide gel electrophoresis of PI-3 virus (Umea 23 strain) proteins in the presence of SDS. (a) Purified whole virus. Their apparent molecular weights are indicated (K = mol. wt. × 10^3). (b) HN and F glycoproteins isolated from the virus by lentil lectin chromatography. Protein stain was Coomassie Brilliant Blue.

distribution when analysed in a sedimentation velocity gradient as the material subjected to ultrafiltration (not shown). Furthermore, the lyophilization procedure gave a much higher recovery of the protein than did ultrafiltration, during which variable amounts of the protein were adsorbed to the walls of the collodion bag. From 1 mg of total PI-3 virus protein 0.13 mg of pure HN and F polypeptides could be recovered after lyophilization as protein micelles. After ultrafiltration the yields were about 50% lower.

Electron micrographs of negatively stained preparations of the PI-3-virus protein complexes showed a radial arrangement of spikes protruding from a central core (Fig. 3), probably formed by the association of hydrophobic protein domains (see Simons et al., 1978). The overall diameter was 32 ± 3 nm. These complexes are similar in size and appearance to the glycoprotein micelles isolated from Sendai virus (Scheid et al., 1972; Simons et al., 1978).

When 3H-labelled PI-3 virus solubilized into Triton X-100 was centrifuged in a sucrose gradient containing Triton X-100 (the sucrose gradient in Fig. 2 did not contain detergent), two peaks containing the PI-3 virus surface glycoproteins were observed (Fig. 4a). The more rapidly sedimenting peak had a sedimentation coefficient of 18S, and was enriched in the HN glycoproteins (Fig. 4b, lane 3). The more slowly sedimenting peak had a sedimentation coefficient of about 4S, and was enriched in the F glycoprotein (Fig. 4b, lane 2). Recentrifugation of the 18S and 4S protein-TX complexes in sucrose gradients containing 0.05% Triton X-100 showed that they were stable and did not convert into each other (not shown).
Fig. 2. (a) Preparation of the 30S protein micelles from PI-3 virus labelled with [3H]borohydride. Sedimentation was towards the left. For details see Methods. (b) Polyacrylamide gel electrophoresis in the presence of SDS. Lane 1, PI-3 virus; lane 2, sample from the pooled fractions 6 to 10; lane 3, sample from the pellet. Stain was Coomassie Brilliant Blue.

Fig. 3. Electron micrograph of the membrane glycoproteins of PI-3 virus isolated as 30S protein micelles. Bar marker represents 100 nm.

Response of mice to vaccination

We tested the immune response to the different preparations of the PI-3 virus glycoproteins in mice as experimental animals. The experimental protocol is given in Table 1. The antibody responses were measured with an ELISA technique weekly during the experimental period, or by haemagglutination inhibition at the end of the experimental period.

Both of the multimeric forms, the 30S protein micelles and the 18S protein-TX complexes induced a high immune response in the mice. One μg of the 30S protein micelles gave a detectable IgG response amounting to about 40 μg of antibodies per ml of serum after the second vaccination with the same dose (Fig. 5). Lower doses (0.1 μg) gave a much lower response. When the effect of a dose of 10 μg 30S protein micelles was compared to that induced by the same dose of 18S protein-TX micelles, the response to each preparation was found to be similar both in the IgM and IgG class of antibodies. The secondary response reached levels of about 100 μg IgG antibodies per ml of serum. The immune response as measured by HI assay 6 weeks after the second immunization correlated with that measured by the ELISA test, the maximum mean titres being 128. The immune response was also measured in extracts of lungs and trachea at the
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Fig. 4. (a) Preparation of the 18S and 4S protein-TX complexes from PI-3 virus labelled with \(^{[3}\text{H}]\)borohydride. Sedimentation is towards the left. For details see Methods. (b) Polyacrylamide gel electrophoresis in the presence of SDS. Lane 1, PI-3 virus; lane 2, sample from the 4S peak; lane 3, sample from the 18S peak. Stain was Coomassie Brilliant Blue.

end of the experimental period. An IgA response was detected in lung extracts from mice vaccinated with 1 and 10 µg of the 30S protein micelles and with 10 µg of the 18S protein-TX complexes.

Mice vaccinated with 5 µg of the 4S protein-TX complex showed no antibody response after the first vaccination (Fig. 6). After the second vaccination they responded with low titres (mean 8 µg IgG per ml of serum). A mixture of 4S protein-TX complexes with 18S protein-TX complexes seemed to lower the response to the multimeric spike protein form as was shown by mixing 2.5 µg of each of the two preparations prior to injection (Fig. 6). No response was detected after the first immunization, and after the second the response was as low as with 4S protein-TX complexes alone and clearly lower than after vaccination with 1 µg 30S protein micelles (Fig. 5). This shows that not only is the slowly sedimenting form of the virus surface glycoproteins poorly immunogenic, it also might suppress the antibody response to the multimeric form. The detergent itself has no suppressive effect since the 18S protein-TX complexes alone are highly immunogenic.

The experiments thus showed that both the 30S protein micelles and the 18S protein-TX complex are highly immunogenic in mice whereas the 4S protein-TX complex is a poor immunogen.

Response of lambs to vaccination

To test the efficiency of the PI-3 spike proteins in producing protective immunity in lambs, we used the virulent G2 strain to prepare the vaccine. We chose to focus on the 30S protein micelles since our previous studies with Semliki Forest virus had shown that this form of the spike proteins was an excellent vaccine. The first series of experiments was performed in SPF lambs with 30S protein micelles either alone or mixed with an oil adjuvant (Table 2).

Following the first vaccination only those lambs receiving micelles in adjuvant developed
antibodies detectable by ELISA or HI (Fig. 7 and 8). After the second vaccination, there was a further rise in antibody titres, and a slight increase in titre by ELISA in those lambs receiving micelles alone. Haemagglutination-inhibiting antibody was not stimulated in these lambs by vaccination. Challenge with live virus elicited a further rise in serum antibody levels in all lambs, this increase being particularly marked, by ELISA, in the animals vaccinated with micelles without adjuvant. In unvaccinated lambs low titres of antibody were detected after challenge.

**Response of lambs to challenge with PI-3 virus**

In this experiment (Table 2), the control lambs and lambs vaccinated with micelles without adjuvant developed a mild respiratory illness and pyrexia as described previously (Wells et al., 1976). In contrast to this, the lambs vaccinated with micelles in adjuvant developed no signs of illness.

Virus was recovered from the unvaccinated control lambs for 6 days, with peak titres 3 to 5 days after inoculation (Fig. 9). Virus was excreted from most lambs that received micelles without adjuvant, for a similar period and achieved similar titres. However, of those lambs receiving micelles in adjuvant, only one lamb provided evidence of virus replication. This group
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![Graph](image)

Fig. 6. Antibody response (mean values) in serum from BALB/c mice, measured with an ELISA technique following immunization with 5 μg 4S protein–TX complexes (○), 5 μg of the 18S protein–TX micelles (●) or 2.5 μg of the latter mixed with 2.5 μg of the 4S protein–TX complexes (△) as described in Table 1(b); non-vaccinated controls are also shown (▲).

![Graph](image)

Fig. 7. Antibody response in the IgG class in serum from lambs, measured with an ELISA technique following immunization with the 30S protein micelles. The animals were immunized as described in Table 2. ○, Mean value from four animals, non-vaccinated controls; ●, mean value from seven animals vaccinated twice with 30S protein micelles without adjuvant; ▼, mean value from six animals vaccinated twice with 30S protein micelles. Adjuvant was included in the first immunization.

of lambs also showed reduced pneumonic lesions; two lambs had no lesions at all. The other vaccinated lambs had lesions as extensive as those in the control lambs.

One further experiment was done using 25 lambs to test whether higher doses of the 30S protein micelles alone induced a protective immune response. Two vaccinations of 25 and 50 μg protein respectively were given to two groups of lambs. The results were no better than with two doses of 15 μg 30S protein micelles alone. These experiments thus showed that a protective immune response could be induced in lambs by vaccination with the 30S protein micelles in oil adjuvant. Although lambs vaccinated with micelles without adjuvant developed only low titres of IgG antibodies and were not protected, the rapid rise in antibody titres following challenge with live virus indicated the priming effect of such vaccination.
Fig. 8. Antibody response measured by haemagglutination inhibition in serum from lambs following immunization with the 30S micelles. The animals were immunized as described in Table 2. ○, Mean value from four animals, non-vaccinated controls; ●, mean value from seven animals vaccinated twice with 30S protein micelles without adjuvant; ■, mean value from six animals vaccinated twice with 30S protein micelles. Adjuvant was included at the first immunization. The numbers in the figure indicate the number of lambs that became seropositive.

Fig. 9. Recovery of PI-3 virus from nasal swabs after challenge (see Table 2). Group A (■), non-vaccinated lambs; group B (□), lambs vaccinated twice with 30S protein micelles without adjuvant; group C (△), lambs vaccinated twice with 30S protein micelles. Adjuvant was included in the first immunization.

**DISCUSSION**

With the possibility of producing the virus antigens responsible for induction of a protective immunity either by recombinant DNA technology or as synthetic antigens (Emtage & Carey, 1980; Kleid et al., 1981; Lerner et al., 1981; Muller et al., 1982), the interest in protein subunit
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vaccines has evidently increased. However, even if it does prove possible to produce the required antigens in large quantities, the problem of efficient presentation of the antigen remains. Not only is it uneconomical to use large doses for vaccination, but if the dose is too high the preparation might contain enough toxic components to cause adverse reactions, one of the problems one wants to avoid by the use of subunit vaccines. We have in a series of studies tried to analyse which form of virus surface glycoprotein is the most efficient as an immunogen. Our results with the PI-3 virus are essentially similar to those we earlier obtained with Semliki Forest virus (Morein et al., 1978; Balcarova et al., 1981). The monomer forms of the spike proteins are poor immunogens, whereas the multimer forms are highly immunogenic. In mice 0.1 μg of 30S protein micelles from the PI-3 virus gave almost as high an immune response as 5 μg of the 4S protein–TX complex. Moreover, it appeared that the 4S protein–TX complexes from PI-3 virus could suppress both the IgM and IgG responses against the virus surface glycoproteins when the mice were immunized at the same time with the highly immunogenic multimer form, the 18S protein–TX complex. The suppression could be due to the stimulation of T suppressor cells (see Gershon, 1974; Tada & Okumura, 1979) but more work is clearly needed to analyse this effect. This aspect is of importance since previous subunit vaccines against enveloped viruses have usually contained mixtures of protein micelles, protein monomers and lipids.

In lambs we did not obtain protection against pneumonia without including adjuvant in the first vaccination with the protein micelles prepared from the G2 strain of PI-3 virus. This was somewhat disappointing. In our previous experiments in mice with Semliki Forest virus, excellent protection was achieved with protein micelles alone. However, it should be emphasized that earlier studies with formalin-inactivated whole virus vaccines of PI-3 virus have shown that adjuvant also had to be included with those vaccines to achieve a protective response (Wells et al., 1976). The reason for the need of adjuvant in lambs is not known, but it may be due to species differences or to different requirements for establishing mucosal immunity as compared to humoral immunity. We have not yet differentiated in detail the antibodies induced by the vaccinations to the two different virus glycoproteins. However, preliminary immunoblotting experiments show that sera from mice and lambs (with antibodies detectable by our ELISA assays) contained antibodies to both the HN and the F proteins (B. Morein & M. Sharp, unpublished results). Clearly, the qualitative nature of the immune response will influence the protection afforded by the vaccination (see Merz et al., 1980).

The simple one-step procedure used to prepare micelles of membrane glycoproteins of PI-3 virus was originally devised for the Semliki Forest virus spike glycoproteins (Helenius & von Bonsdorff, 1976). In contrast to previous procedures to prepare micelles of virus spike proteins (Laver & Valentine, 1969; Scheid et al., 1972), this method seems generally applicable to amphiphilic proteins, which have a fairly large hydrophilic domain outside the membrane and a small hydrophobic protein domain in the lipid bilayer. The procedure has been used by us to make micelles of the surface glycoproteins of Sendai virus, fowl plague virus and vesicular stomatitis virus (Simons et al., 1978; Matlin et al., 1981, 1982). The procedure has also been used by Heinz & Kunz (1980) to prepare protein micelles of the surface glycoproteins of tick-borne encephalitis virus, by Skelly et al. (1981) for the hepatitis B virus surface antigen, and, somewhat modified by Schneider et al. (1980) for the Friend leukaemia virus glycoproteins. One additional advantage of the procedure is that further purification of the relevant antigens is achieved during sucrose gradient centrifugation. Actin which was present in our PI-3 virus preparation was decreased to almost undetectable levels in the 30S protein micelles (Fig. 2). This might be important since host components like actin could induce an adverse autoimmune response (Allison, 1977).

In conclusion, we would like to emphasize the importance of antigen presentation in vaccination studies. Our studies suggest at least one simple method to analyse a surface glycoprotein preparation before its use in immunogenicity studies. This can be done by sedimentation velocity analysis in the preparative or the analytical ultracentrifuge. Preparations can in this way be standardized and the problem of multimers versus monomers properly addressed.
We wish to thank Ulla Hallen, Patricia Marinello and Hilika Virta for expert technical assistance and Karl Matlin for a critical reading of the manuscript.

REFERENCES


(Received 6 December 1982)