Measles Virus Fusion Protein Presented in an Immune-stimulating Complex (Iscom) Induces Haemolysis-inhibiting and Fusion-inhibiting Antibodies, Virus-specific T Cells and Protection in Mice

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SUMMARY

Immune-stimulating complexes (iscoms), which have recently been shown to be highly effective for the antigenic presentation of membrane proteins of viruses, were prepared with affinity-purified fusion (F) protein of measles virus (MV), using an adaptation of the standard method for iscom preparation. Immunization of monkeys with the F iscom preparation induced biologically active anti-F protein antibodies as was shown in haemolysis inhibition and cell–cell fusion inhibition tests. A whole MV iscom preparation, which also contained the haemagglutinin protein, induced not only haemolysis-inhibiting antibodies, but, in contrast to the F iscom preparation, also haemagglutination-inhibiting and virus-neutralizing antibodies. In addition the F iscom preparation was shown to activate measles virus-specific T cells in mice. This was demonstrated by the generation of an MV-specific delayed type hypersensitivity response in F iscom-immunized animals and by the isolation of T cell clones specific for MV F protein with the T helper phenotype. Vaccination of mice with MV iscom or F iscom protected them from MV-induced fatal encephalopathy. The data concerning the immunogenicity of MV proteins presented in iscoms are discussed in relation to their potential for the development of an inactivated measles vaccine.

INTRODUCTION

Measles is an acute disease of major biological importance to humans, which is caused by a member of the family Paramyxoviridae. The two membrane glycoproteins of measles virus (MV), the haemagglutinin (H) protein and the fusion (F) protein, play an important role in the induction of protective immunity. The H protein of paramyxoviruses in general is involved in the adsorption of the virus to cell receptors and in haemagglutination activity. The F protein of paramyxoviruses in its biologically active form (in the case of MV two covalently coupled subunits of 40000 and 20000 to 25000 M_r) is responsible for virus–cell fusion, the formation of syncytia by infected cells, and haemolysing activity of the virus (Scheid *et al.*, 1972; Scheid & Choppin, 1974, 1977). Although live measles vaccines have proven to be highly effective, the availability of an effective and safe inactivated vaccine would still have considerable advantages. Inactivated measles vaccines prepared by Tween–ether or formaldehyde treatment are able to induce virus-neutralizing (VN) and haemagglutination-inhibiting (HI) antibodies. However, individuals immunized with these types of vaccines proved insufficiently protected against infection and in some cases illness following exposure to live virus was more severe than in unvaccinated individuals (Fulginiti *et al.*, 1967). It has been suggested by Norrby and co-

workers that the lack of protection associated with these inactivated MV vaccines was due to an 'incomplete' antibody response to the F protein, as no haemolysis-inhibiting (HLI) antibodies were induced (Norrby et al., 1975). Consequently, for the construction of an effective inactivated measles vaccine the presentation of the F protein in a proper immunogenic form is considered to be of major importance (recommended by the Committee on Issues for New Vaccine Development, 1985).

A novel structure for the antigenic presentation of membrane proteins from enveloped viruses has been shown to be highly effective in inducing both humoral and cell-mediated immune responses: the immune-stimulating complex (iscom) (Morein et al., 1984; Morein & Simons, 1985; Osterhaus et al., 1985; Wahren et al., 1987; Howard et al., 1987). It was demonstrated that MV membrane proteins presented in iscom form elicited not only VN and HI antibodies, but also (non-HI) HLI antibodies in rats (Morein et al., 1984).

In order to examine further the role of the F protein presented in MV iscoms in the induction of the anti-MV response, we incorporated affinity-purified F protein into iscoms in the absence of other MV proteins. We showed that this preparation without further addition of adjuvants induced HLI antibodies but no VN or HI antibody in monkeys, rabbits and mice. The F iscom preparation also induced an MV-specific delayed type hypersensitivity (DTH) response in BALB/c mice, from which T cell clones specific for MV F protein could be isolated. Vaccination of mice with MV iscoms or F iscoms protected them from MV-induced fatal encephalopathy. These results, which emphasize the importance of the presentation of the F protein in a proper immunogenic configuration, are discussed in relation to the development of a safe and effective subunit vaccine against measles.

METHODS

Virus and cell culture. MV (Edmonston B strain) was twice plaque-purified and propagated in Vero cells grown in a microcarrier culture as described (Van Wezel et al., 1978a, b). Culture supernatant was concentrated 20-fold in a hollow fibre system with a mol.wt. cut-off of 10^6 (Amicon) and the virus was further purified by discontinuous sucrose gradient centrifugation according to methods described for mumps virus (Örvell, 1978).

Production, selection and characterization of mouse monoclonal antibodies. BALB/c mice were immunized three times subcutaneously at weekly intervals with 25 μg MV F_1 component, purified by a modification of Laemmli's (1970) SDS–PAGE as described previously (Rezelman et al., 1980). Nine days after the last inoculation, mice were boosted three times intravenously at daily intervals with purified MV and 2 days later spleen cells were fused with P3-X63-Ag8.653 BALB/c myeloma cells as previously described (Osterhaus et al., 1981). Culture fluids were screened for anti-MV antibodies by an indirect ELISA and thereafter in a radioimmunoprecipitation assay for their MV protein specificity. Positive hybridomas were cloned twice from single cells, expanded and injected into Pristane-treated mice for ascites production. Monoclonal antibodies were purified by affinity chromatography using Protein A–Sepharose CL-4B according to the procedure recommended by the manufacturer (Pharmacia).

Immunoaffinity chromatography (IAC). IAC purification of the F protein was carried out as previously described with minor modifications (Varsanyi et al., 1984; De Vries et al., 1987). Briefly, 2% Triton X-100 was used to solubilize the membrane proteins of the concentrated virus suspension. The insoluble debris was sedimented by ultracentrifugation. One mg of purified monoclonal antibody was coupled to 0·3 g CNBr-activated Sepharose 4B according to the procedure recommended by the manufacturer (Pharmacia). The solubilized antigens were circulated four times through a column of 0·7 cm diameter containing the immunosorbent (40 ml/h.cm²). After adsorption the column was washed with 10 column volumes of 2% Triton X-100 or 1% octylglucoside in 20 mM-Tris–HCl pH 7·8, 1 mM-EDTA, 150 mM-NaCl (TEN buffer). Desorption of proteins was achieved by elution with 5 M-NH₄SCN in TEN buffer containing 2% Triton X-100 or 1% octylglucoside. Fractions were dialysed and analysed by SDS–PAGE under reducing and non-reducing conditions.

Preparation of F iscoms, F–Quil A micelles and MV iscoms. Cholesterol (from porcine liver; Sigma) and phosphatidyl ethanolamine (from egg yolk; Sigma) dissolved in a molar ratio of 1 : 2 in chloroform were dried as a thin film in a stream of nitrogen. A solution of 1% octylglucoside in TN buffer (20 mM-Tris–HCl pH 7·8, 150 mM-NaCl) was added to prepare a lipid mixture of 1 mg/ml and subsequently agitated vigorously with a vortex mixer.

Six-hundred μg of the lipid mixture was added to 300 μg IAC-purified F protein in 2% Triton X-100. After addition of 0·1% (w/v) Quil A (Spikoside; Iscotec, Sweden) the mixture was layered over a 0·1% Quil A-containing 10 to 60% sucrose gradient in TN buffer and centrifuged in a SW27 rotor (Beckman) at 19000 r.p.m. for 18 h. Gradient fractions were dialysed against TN buffer and analysed by double-antibody ELISA for measurement of the F protein and by electron microscopy. Preparations were negatively contrasted on glow-discharged formvar-carbon-coated copper grids using 2% ammonium molybdate at pH 5·0. The grids were examined in a Philips

electron microscope. F iscom-containing fractions were pooled and layered over a 20 to 60% discontinuous sucrose gradient in TN buffer without Quil A, to separate the iscoms from free Quil A. Following a similar procedure, but without adding lipids to the F protein, F–Quil A micelles were formed during centrifugation. To avoid undefined aggregation of the F protein, F–Quil A micelles were not further purified from free Quil A. The MV iscom was prepared according to the method described by Morein *et al.* (1984).

Double-antibody ELISA for measurement of the F protein. Purified monoclonal antibody $F_1$4-21 was diluted in 50 mM-carbonate buffer pH 9·6 at a protein concentration of 5 µg/ml and adsorbed onto 96-well high-activated microELISA plates (Flow Laboratories) at 100 µl/well by overnight incubation at 20 °C. The plates were washed after all incubation steps with Tween 80 (0·5%) in water in an automated washing device. After incubation for 30 min at 37 °C with dilution buffer [0·5% bovine serum albumin, 0·05% Tween 20 in phosphate-buffered saline (PBS)] wells were incubated for 2 h at 20 °C with 100 µl F protein-containing fractions serially diluted in dilution buffer. Purified monoclonal antibody $F_1$7-21 was conjugated with horseradish peroxidase (Wilson & Nakane, 1978) and used as a second antibody (2 µg/ml). The substrate reaction was carried out with 100 µl/well of 0·1 mg/ml 3,3′,5,5′-tetramethylbenzidine solution in 0·11 M-sodium acetate–citric acid buffer pH 5·58 supplemented with 0·006% H_2O_2 and after 10 min was stopped with 100 µl 2 M-H_2SO_4. The absorbance at 450 nm was read in a Titertek Multiskan apparatus (Flow Laboratories). The amount of F protein present in each fraction was calculated from the linear part of the curves obtained.

Immunization experiments. For immunization experiments, six 18-month old cynomolgus monkeys (*Macaca fascicularis*) kept at the National Institute of Public Health and Environmental Hygiene (Bilthoven, The Netherlands) colony were used. All animals were tested for the absence of MV-specific antibodies in ELISA and VN, HI, HLI and cell–cell fusion inhibition (FI) tests. Two monkeys were immunized intramuscularly twice with the F iscom preparation at an interval of 4 weeks, using 10 or 50 µg doses per inoculation respectively; two were immunized similarly with 10 or 50 µg doses respectively of the F–Quil A micelle preparation, and the last two monkeys were immunized once with 10 µg of the MV iscom preparation. Essentially the same experiments were carried out in specific pathogen-free rabbits and mice. The F iscom and F–Quil A micelle preparations both contained 20 µg/ml protein, as determined by the Bradford (1976) protein assay. The amount of Quil A in the F iscom or F–Quil A micelle preparations was less than 10 µg/ml or 460 µg/ml respectively, as measured by an electrophoresis lysis method as previously described (Sundquist *et al.*, 1983). Blood samples were collected before starting the experiment and at weekly intervals for 10 weeks, and thereafter at intervals of 4 weeks. Sera were heat-inactivated and stored at −20 °C before use.

Serological tests. VN, HI and HLI tests were carried out according to the methods described by Norrby & Gollmar (1972, 1975). Anti-MV ELISA was performed by a method described by Sheshberadaran *et al.* (1983) with minor modifications. In short a 20-fold concentrated MV-infected culture supernatant was diluted in PBS at a protein concentration of 10 µg/ml and adsorbed onto 96-well microELISA plates (Flow Laboratories). Horseradish peroxidase-conjugated anti-human IgG (γ chain-specific) was used as the second antibody (Cappel Laboratories). The substrate reaction was carried out as described for the double-antibody ELISA. FI tests were carried out as previously described (De Vries *et al.*, 1987); FI titres were expressed as the highest serum dilutions causing a 50% reduction of the number of syncytia per well.

Induction and measurement of DTH. Four groups of three female 12-week-old BALB/c mice were each immunized once subcutaneously with 0·25 or 1·0 µg F iscom, with 0·25 or 1·0 µg F–Quil A micelles, with 50 µg u.v.-irradiated MV or with PBS. Purified MV was u.v.-irradiated with a dose sufficient to inactivate the virus, but preserving other biological properties like haemagglutination and haemolysing activities. Mice were challenged 7 days later (Smith & Ziola,1984) by a subcutaneous injection with 10 µg (15 µl) of the u.v.-inactivated MV or with uninfected Vero cell antigen into the left or right ear respectively.

At different times after challenge, ear swelling was measured using a micrometer (Mitutoyo, Tokyo, Japan). Ear swelling was expressed in µm, as the mean value ± S.D. measured in the three mice per group. Background swelling responses of non-immunized control mice were subtracted from the responses of the immunized animals. Clinical and histological examinations were carried out according to routine procedures.

Generation of murine T cell clones. BALB/c mice were immunized twice intramuscularly with 1·0 µg per dose F iscom at an interval of 4 weeks. Four months after the last immunization spleen cells were isolated by sedimentation on a density gradient (Lympholyte-M; Cedarlane Laboratories, Hornby, Canada). Cells were cultured in flat-bottomed 24-well plates at a density of 1·5 × 10^6 cells per well in Iscove's modified Dulbecco's medium, supplemented with 2 mM-L-glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml), 5 × 10^{-5} M-2-mercaptoethanol (IMDM-S), 10% foetal calf serum and 5% interleukin-2 (IL-2)-containing supernatant from rat splenocytes, which had been stimulated for 24 h with concanavalin A, and in the presence of 0·5 µg/well or 20 µg/well u.v.-inactivated MV. After four days the cells were collected and lymphoblastoid cells were isolated by gradient centrifugation on Percoll (Kurnick *et al.*, 1979). T cell clones were obtained from blastoid cells, culturing the cells by limiting dilution in IMDM-S supplemented with 10% foetal calf serum and 5% IL-2 supernatant and in the presence of 0·75 µg u.v.-inactivated MV and 3 × 10^5 irradiated (1500 rad) syngeneic spleen cells per well.

T cells were maintained in culture by weekly restimulation with fresh irradiated spleen cells and MV antigen. At various intervals, the cells were tested for MV reactivity in a proliferation assay.

The marker profile of the T cell clones was estimated by incubation of the cells with rat anti-Thy1.2, anti-Lyt-1, anti-Lyt-2 and anti-L3T4 monoclonal antibodies and a subsequent incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat immunoglobulin according to the procedure recommended by the manufacturer (Becton-Dickinson Immunocytometry Systems).

T cell proliferation assay. Viable T cells were separated from non-viable cells 7 days after antigen stimulation on Lympholyte-M and were resuspended in IMDM-S supplemented with 10 µg/ml insulin (Sigma, I 1882), 32 µg/ml bovine serum albumin (Boehringer), 1·2 ng $ZnCl_2$ (Sigma) and a mixture of soybean lipids (Boehringer). T cells (1 × 10^4) were cultured in 96-well, round-bottomed microtitre plates in the presence of 3 × 10^5 irradiated (1500 rad) syngeneic spleen cells, which had been incubated for 2 h with the appropriate antigen. Antigens used in this assay were 0·6 µg u.v.-inactivated MV, 0·3 µg IAC-purified F protein, 0·3 µg IAC-purified H protein (De Vries *et al.*, 1987), 0·3 µg of a mixture of NP and M proteins (Fig. 1, lane 2) and, as a control, Vero cell antigen. After 48 h incubation at 37 °C in a humidified atmosphere of 5% CO_2 in air, cultures were pulsed with 0·5 µCi of [^{3}H]thymidine and harvested onto glass fibre filters 18 h later. [^{3}H]Thymidine incorporation was measured by liquid scintillation spectroscopy. Results are expressed as mean c.p.m. ± S.D. of triplicate cultures.

Protection experiments. In three independent experiments, 12-day-old BALB/c mice were immunized subcutaneously twice at an interval of 6 days with F iscom or MV iscom preparations, each containing 0·1, 0·3 or 1·0 µg, or with PBS as a control. Three days after the last immunization all mice were challenged intracerebrally (i.c.) in one of the hemispheres with 30 µl of a 2·5% (w/v) brain suspension of the rat brain-adapted CAM R/40 strain of MV (CAM/RBH), which dilution was shown to kill about 50% of the suckling mice within 11 days with symptoms of acute encephalopathy. This virus strain was kindly provided by Dr V. ter Meulen, Institut für Virologie und Immunbiologie, Würzburg, F.R.G. (Liebert & ter Meulen, 1987). Surviving mice were recorded daily.

RESULTS

Purification and incorporation of the F protein into iscoms

The F protein was purified from a detergent-solubilized crude MV preparation by affinity chromatography using a monoclonal antibody ($F_1$7-21) with specificity for the F protein. Analysis of the purified protein by SDS–PAGE under reducing conditions showed an M_r 40000 protein whereas under non-reducing conditions an M_r 55000 protein was observed (Fig. 1), corresponding to the F_1 component and the disulphide-linked F_1–F_2 complex of the F protein respectively (Hardwick & Bussell, 1978; Varsanyi *et al.*, 1984). The protein observed at M_r 110000 in the gel under non-reducing conditions is probably a dimer of the 55000 M_r F protein, since it also reacted with monoclonal antibody $F_1$7-21 in a Western blotting experiment (not shown). An average yield of about 500 µg purified F protein could be recovered from a 1·0 ml affinity column containing 1·0 mg coupled monoclonal antibody.

Attempts to incorporate the purified F protein into iscoms according to the standard procedure described by Morein *et al.* (1984) resulted in the formation of micellar structures with the characteristic club-like form of the individual peplomers (Fig. 2a, b; Varsanyi *et al.*, 1984). However, the addition of a mixture of lipids, consisting of at least phosphatidyl ethanolamine and cholesterol in a molar ratio of 1:2, to the purified protein prior to centrifugation of the protein over the Quil A-containing gradient resulted in the formation of characteristic iscom-like structures in the fractions corresponding with a peak of the F protein (Fig. 2a, c). F iscoms proved stable after centrifugation over a sucrose gradient to remove free Quil A molecules.

Antibody response induced by F iscoms and MV iscoms

The development of MV-specific antibodies in monkeys immunized with F iscoms or F–Quil A micelles was monitored at weekly intervals by ELISA and all samples were also tested for the presence of antibodies inhibiting biological activities of the virus in VN, HI, HLI and FI tests. In none of the serum samples taken before immunization were MV-specific antibodies demonstrated by these assays. The two monkeys immunized once with 10 or 50 µg F iscoms developed ELISA and HLI antibody titres within 2 weeks, and the two monkeys immunized with 10 or 50 µg F–Quil A micelles developed lower ELISA and no HLI antibody titres (Fig. 3). After a second immunization 4 weeks later, with either the F iscom or the F–Quil A micelle
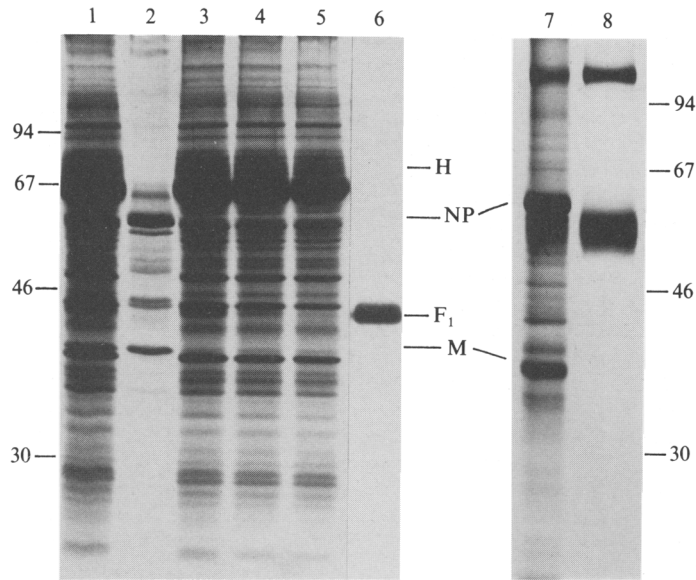
Fig. 1. Analysis of viral and cellular polypeptides at various stages of the F protein purification by SDS–PAGE (12·5%) under reducing (lanes 1 to 6) or non-reducing (lanes 7 and 8) conditions followed by silver staining. Molecular weight markers are indicated ($\times 10^{-3}$). Lane 1, concentrated MV culture supernatant prior to purification; lane 2, pellet after solubilization of the membrane proteins with Triton X-100 and ultracentrifugation; lane 3, supernatant after ultracentrifugation, used as IAC starting antigen; lane 4, proteins not bound to the anti-F column prepared with monoclonal antibody $F_1$7-21 after one cycle of adsorption; lane 5, as lane 4 after four cycles of adsorption; lane 6, protein fraction bound to the anti-F column and eluted by 5 M-NH_4SCN in TEN buffer; lane 7, MV; lane 8, as lane 6.

Table 1. *MV antibody titres in four monkeys, 14 weeks or 1 year after the last immunization with F iscoms or F–Quil A micelles*

	ELISA		HLI	
	14 weeks	1 year	14 weeks	1 year
10 μg F iscoms	340	130	100	30
10 μg F micelles	100	32	20	< 16
50 μg F iscoms	590	290	170	160
50 μg F micelles	720	590	80	80

preparation, ELISA, HLI and FI antibody titres increased in all immunized monkeys within 1 week. Monkeys immunized with the F iscom preparation developed in both cases higher titres than those immunized with the F–Quil A micelle preparation. MV-specific antibody titres were still observed 1 year after the last immunization in all four monkeys (Table 1). Immunization of rabbits or BALB/c mice with either of the F protein preparations showed similar results: within 2 weeks the F iscom preparation induced higher HLI and ELISA antibody titres than the F–Quil A micelle preparation (data not shown). No HI or VN antibody titres (> 2), the latter even after the addition of complement, were observed during the course of the experiments in the monkeys, rabbits or mice. Two other monkeys were immunized with 10 μg of the MV iscom preparation, which apart from F protein contained less than 0·5 μg H protein as estimated by SDS–PAGE and densitometry. Both monkeys developed VN, HI and (non-HI) HLI antibody titres within 4 weeks (Fig. 4).
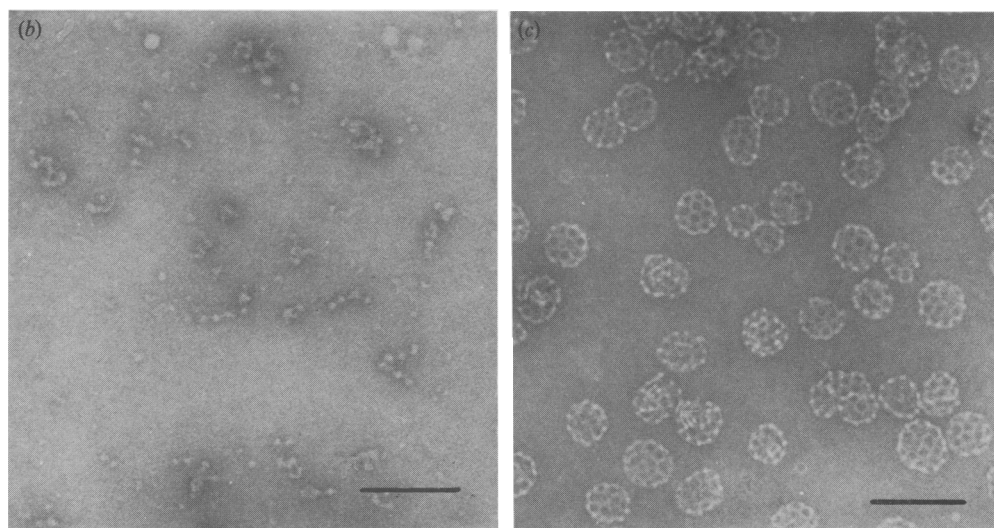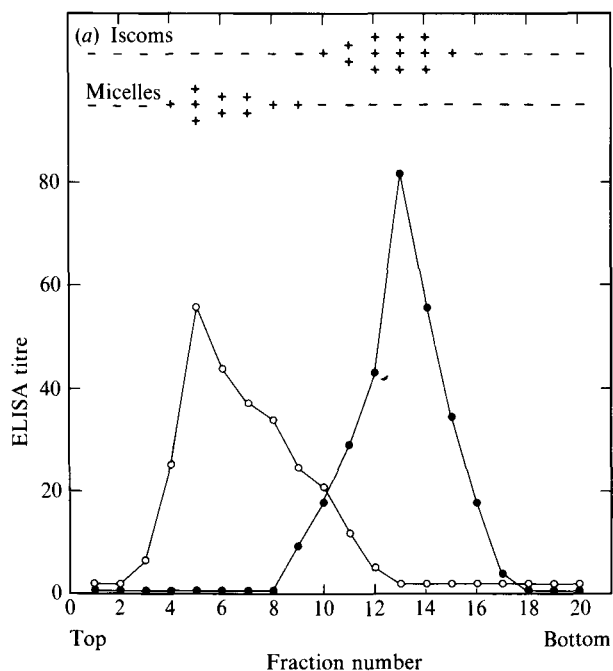
Fig. 2. Transfer of MV F protein in the presence (●) or absence (○) of a lipid mixture from Triton X-100 to a Quil A solution by sedimentation into a 10 to 60% sucrose gradient containing 0·1% Quil A (*a*). The presence of F protein in the gradient fractions was quantified by antibody sandwich ELISA (*a*) and the presence of protein–micelle structures and iscoms was analysed by electron microscopy (*b* and *c* respectively). The number of + symbols above the fractions indicate arbitrary units for the relative amounts of iscoms or micelles in the corresponding fractions. Bar markers represent 100 nm.

DTH response induced by F iscoms

The induction of antibody responses to viral glycoproteins has been shown to be T cell-dependent in many viral systems. We demonstrated the activation of MV-specific T cells by the F protein preparations in a MV-specific DTH response.

After immunization of BALB/c mice with 50 µg u.v.-inactivated MV a DTH response was induced by challenge 7 days later with 10 µg of the same preparation. A maximal reaction at
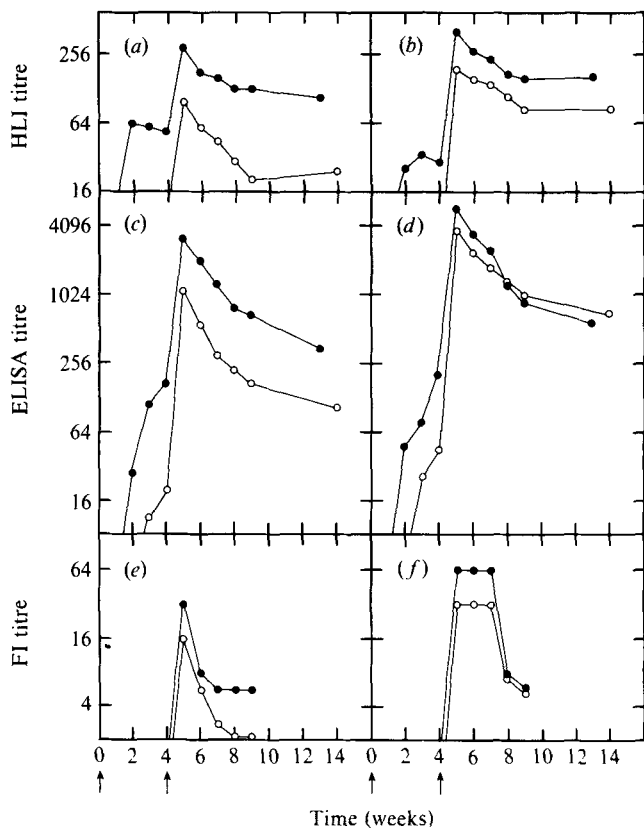
Fig. 3

Fig. 4

Fig. 3. Development of HLI, ELISA and FI antibody titres in four monkeys after immunization with 10 μg (*a, c, e*) or 50 μg (*b, d, f*) doses of the F iscom preparation (●) or with 10 μg (*a, c, e*) or 50 μg (*b, d, f*) doses of the F-Quil A micelle preparation (○). All monkeys were immunized twice with the same dose and preparation at an interval of 4 weeks (as indicated by arrows).
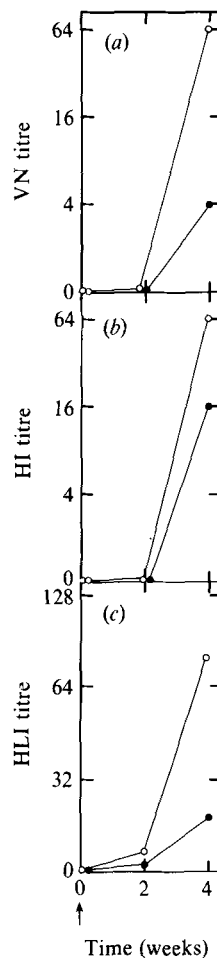
Fig. 4. Development of (*a*) VN, (*b*) HI and (*c*) FI antibody titres in two monkeys (● and ○) after immunization with a 10 μg dose of the MV iscom preparation.

about 48 h after challenge showed a specific ear swelling of up to 270 μm. The DTH reaction was clinically characterized by a red induration and histologically by a dense dermal infiltrate of lymphocytes and monocytes (data not shown). Background swellings measured in sham-immunized mice after challenge with MV, or in MV-immunized mice challenged with control Vero cell antigen, never exceeded 70 μm.

Priming of BALB/c mice with the F iscom or F–Quil A micelle preparations resulted in an MV-specific DTH reaction upon challenge 7 days later with 10 μg of inactivated MV (Fig. 4). Immunization with 0·25 or 1·0 μg of the F iscom preparation resulted in a specific ear swelling of a maximal 76 μm or 153 μm above background values respectively, whereas swellings up to about 25 or 75 μm above background values were observed after immunization with 0·25 and 1·0 μg of the F–Quil A micelle preparation respectively.
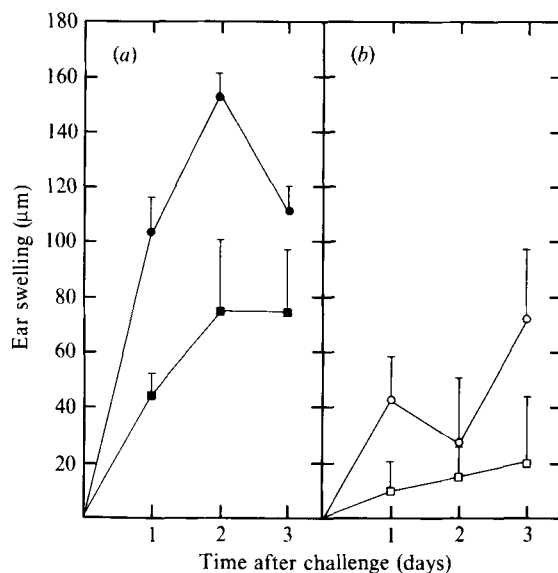
Fig. 5. Development of the DTH response in BALB/c mice after priming with 1 μg (●) or 0·25 μg (■) of the F iscom preparation (a) or 1 μg (○) or 0·25 μg (□) of the F–Quil A micelle preparation (b) and a challenge immunization 7 days later with 15 μg u.v.-inactivated MV. Ear swelling is expressed in μm as the mean value ± s.d. of independent measurements of three mice within each group.

Table 2. *Antigen specificity in the proliferative response of T cell clones obtained from F iscom-immunized mice**

	T cell clones				
Antigen	20F1	20F3	0·5E10	0·5F1	0·5F5
MV	5375 ± 516	5710 ± 1930	6529 ± 242	6869 ± 1460	10832 ± 1304
F	2679 ± 249	2505 ± 390	824 ± 127	1226 ± 85	7699 ± 633
H	166 ± 69	771 ± 435	416 ± 115	492 ± 90	416 ± 147
NP/M	143 ± 13	1175 ± 147	198 ± 49	402 ± 92	377 ± 322
Vero	117 ± 53	327 ± 218	249 ± 91	236 ± 52	175 ± 18

*Cells (1×10^4) from clones 20F1, 20F3, 0·5E10, 0·5F1 or 0·5F5 were co-cultured with 3×10^5 irradiated syngeneic spleen cells, briefly incubated with MV, purified F protein or H protein, a mixture of NP and M protein or Vero antigen. Stimulation was assessed 3 days later by measuring the incorporation of [³H]thymidine added 18 h before termination of the cultures. Results are expressed as mean c.p.m. ± s.d. of triplicate cultures.

Table 3. *Protection of mice after i.c. challenge with measles virus CAM/RBH, by immunization with F iscom or MV iscom*

Immunization dose	Expt. 1 0·1 μg	Expt. 2 0·3 μg	Expt. 3 1·0 μg
F iscom-immunized	4/7*	9/10	NT†
MV iscom-immunized	4/6	9/10	8/8
Non-immunized	4/8	5/9	2/7

*Number of mice surviving/number of mice challenged.
†NT, Not tested.

Isolation of T cell clones from F iscom-immunized mice

The antigen specificity of five T cell clones isolated from F iscom-immunized BALB/c mice is shown in Table 2. All T cell clones tested were shown to be specific for u.v.-inactivated MV and purified F protein. The majority of cells (>90%) from each T cell clone stained for Thy-1.2,

Lyt-1 and L3T4 markers. However, no significant staining for Lyt-2 antigens was detected in either cell population. Thus, the five T cell clones revealed a surface phenotype of Thy-1$^+$, L3T4$^+$, Lyt-2$^-$, a pattern characteristic of murine T helper cells (Wilde *et al.*, 1983).

Protection of mice by F iscoms and MV iscoms

To test whether F or MV iscoms would protect mice from lethal MV challenge, mice were vaccinated twice at the ages of 12 and 18 days with 0·1, 0·3 or 1·0 µg of F iscom or MV iscom and were challenged i.c. at the age of 3 weeks with measles virus CAM/RBH (Table 3). In non-immunized mice a survival rate of 30 to 55% was observed in three independent experiments. Control animals inoculated i.c. with control suckling rat brain homogenate remained healthy (not shown). Mice immunized with F iscoms or MV iscoms clearly showed increased survival rates (up to 100%), dependent on the dose of iscom administered.

DISCUSSION

The importance of a proper immune response to the F protein of paramyxoviruses for the induction of protective immunity has been documented extensively for MV and several other members of this family (Norrby *et al.*, 1975, 1986; Merz *et al.*, 1980). In the present study we showed that highly purified MV F protein presented in a new antigenic form, the iscom, induced biologically active anti-F antibody titres in monkeys, and these levels persisted for more than 1 year. The F iscom preparation induced an MV-specific DTH response in BALB/c mice, from which MV-specific T cell clones were also isolated. We also showed that mice vaccinated with MV iscoms or F iscoms were protected from lethal challenge. The F protein was highly purified by affinity chromatography using monoclonal antibody F$_1$7-21, selected from a panel of six F protein-specific monoclonal antibodies on the basis of its high binding capacity in a one-cycle purification procedure. For the incorporation of purified F protein into iscoms, lipids were shown to play a hitherto undefined role. The addition of a lipid mixture consisting of at least phosphatidyl ethanolamine and cholesterol to the purified F protein resulted in the formation of iscoms instead of the F–Quil A micelles which were formed in the absence of lipids (Fig. 2).

It is important to note that, although the amount of the adjuvant Quil A (Flebbe & Braley-Mullen, 1986) in the F iscom preparation was about 50 times lower than that present in the F–Quil A micelle preparation, F iscoms induced higher titres of biologically active HLI and FI antibodies in monkeys (Fig. 3), and in rabbits and mice (data not shown). This observation supports previous findings (Morein *et al.*, 1984), i.e. that the immune-potentiating properties of iscoms cannot be attributed to a direct adjuvant effect of Quil A.

In none of the animals, including rabbits and mice, inoculated with the F iscom or F–Quil A micelle preparations, were HI or VN antibodies observed. This finding is in contrast with the results of others, who showed that purified F protein of MV or canine distemper virus could induce low titred VN antibodies in rabbits or dogs respectively (Varsanyi *et al.*, 1984; Norrby *et al.*, 1986). Although this absence of VN antibodies in the post-inoculation sera may have been a reflection of the highly purified nature of our F protein preparations, indicating that the F protein may not harbour epitopes inducing these antibodies; we cannot exclude the possibility that during the affinity purification such epitopes have been destroyed. Epitopes inducing HLI antibodies however were shown to be preserved. Also, destruction of possible VN-inducing epitopes on the F protein by Quil A may be considered unlikely, since the MV iscom did induce VN antibody titres. For vaccine development it is important to note that the presence of a low amount of H protein in iscom preparations resulted in the induction of VN antibody titres (Fig. 4).

Apart from the important role that the F protein plays in the induction of a biologically active antibody response, it could well be argued that for protection against MV infection the generation of a cell-mediated immune response against the F protein may be of crucial importance. Since T cell-specific differentiation markers of *M. fascicularis* have not been well characterized so far, we have chosen BALB/c mice to study the induction of cell-mediated immunity by F iscoms. We showed that F iscoms induced an MV-specific DTH response in mice and that MV-specific T cell clones with the T helper phenotype could be generated from F

iscom-immunized mice. At least two of the five T cell clones (20F1 and 0·5E10) were able to assist B cells in the production of MV-specific antibodies *in vitro*, when cultured in the presence of MV and antigen-presenting cells (data not shown). To our knowledge, this is the first description of murine T cell clones specific for MV proteins.

To evaluate further the potential of F iscoms and MV iscoms in terms of induction of protective immunity *in vivo*, we have conducted a series of protection experiments in a rodent MV infection model. Like in the MV rat model described previously by Liebert & ter Meulen (1987), mice can be infected lethally with CAM/RBH only by the i.c. route before 24 days of age (unpublished observation). This leaves only 10 to 12 days for the induction of a protective immune response in immune-competent animals. We have been able to show that juvenile mice are protected against i.c. infection as early as 9 days after the first vaccination with either F iscoms or MV iscoms (Table 3). This also confirms the suggestion by Norrby *et al.* (1975) that the F protein may play an important role in the induction of protective immunity.

Taken together the data on the induction of both antibody- and cell-mediated immunity on the one hand and the data on the induction of protection on the other hand clearly indicate the potential of the iscom antigen presentation form as a vaccine against measles.

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