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# Protection against Lethal Measles Virus Infection in Mice by Immune-Stimulating Complexes Containing the Hemagglutinin or Fusion Protein

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**The importance of each of the two surface glycoproteins of measles virus in active and passive immunization was examined in mice. Infected-cell lysates were depleted of either the hemagglutinin (H) or fusion (F) glycoprotein by using multiple cycles of immunoaffinity chromatography. The products were used to prepare immune-stimulating complexes (iscoms) containing either F or H glycoprotein. Such complexes are highly immunogenic, possibly as a result of effective presentation of viral proteins to the immune system [B. Morein, B. Sundquist, S. Höglund, K. Dalsgaard, and A. Osterhaus, Nature (London) 308:457–460, 1984]. Groups of 3-week-old BALB/c mice were inoculated with the iscom preparations. All animals developed hemolysis-inhibiting antibodies, whereas only sera of animals immunized with the iscoms containing the H glycoprotein had hemagglutination-inhibiting antibodies. Sera from animals immunized with the H or F preparation only precipitated the homologous glycoprotein in radioimmune precipitation assays. The immunized animals were challenged with a lethal dose of the hamster neurotropic variant of measles virus. Of the 7-week-old animals in the nonimmunized control group, 50% died within 10 days after challenge. No animals in the immunized groups showed symptoms of disease throughout the observation period of 3 months. Passive administration of anti-H monoclonal antibodies gave full protection against the 100% lethal acute infection with the hamster neutropic variant of measles virus in newborn mice, whereas anti-F monoclonal antibodies failed to protect the animals. This study emphasizes that both H and F glycoproteins need to be considered in the development of measles virus subunit vaccines.**

Measles virus is a member of the *Paramyxoviridae* family and belongs to the genus *Morbillivirus* (13). Its envelope contains two surface glycoproteins, the hemagglutinin (H) and fusion (F) proteins.

The notion that viral glycoproteins can determine pathogenicity was first established with paramyxoviruses. The F glycoprotein is activated by proteolytic cleavage (11, 31), and this activation is also relevant for the spread of infection and pathogenicity (21). At the same time, it was realized that only antibodies to the fusion protein confine the spread of infection by inhibiting both cell fusion and penetration of virions into the cell (18).

Measles virus is a highly contagious agent and is still responsible for epidemics with high mortality in many developing countries (1). The protective effect of previously produced inactivated measles vaccines proved to be unsatisfactory; in some cases, children developed a serious atypical disease upon exposure to wild measles virus (6). The antigenicity of the fusion glycoprotein in the vaccine products was altered, and as a consequence, no anti-F hemolysis-inhibiting (HLI) antibodies were induced in the vaccines (23). Immunization with live attenuated measles vaccine in the United States has virtually eliminated indigenous measles (5, 10).

We attempted to define in our present study the extent of in vivo protection contributed by the individual surface antigens of measles virus. It was demonstrated that both the H and F glycoproteins separately incorporated into immune-stimulating complexes (iscoms) give rise to protective antibodies, as studied in a mouse model. The present information is relevant for the development of a successful measles subunit vaccine.

## MATERIALS AND METHODS

**Cells, viruses, and monoclonal antibodies (MAbs).** Vero cells (Flow Laboratories, Inc.) were propagated in Eagle minimum essential medium including 5% fetal calf serum. Confluent monolayers of cells in five roller bottles (800 $cm^2$) were infected with the plaque-purified LEC-KI strain of measles virus at a low (0.01 PFU per cell) multiplicity of infection. For identification of the viral surface glycoproteins, 10% of the infected cells were labeled with D-[1,6,-$^3$H(N)]glucosamine hydrochloride at a concentration of 33 μCi/ml of medium at 24 h after infection. For the challenge experiments, the hamster neutrotropic (HNT) strain of measles virus was used. It was originally established and passaged numerous times in hamster brains (4) and was later passaged in brains of newborn BALB/c mice (30). MAb clones to the LEC-KI strain of measles virus were produced and characterized in this laboratory (22, 32).

**Preparation of cell lysates and depletion of one of the surface glycoproteins.** Infected cells, when exhibiting extensive cytopathic effects, were collected by centrifugation in a Sorvall GSA rotor at 3,000 rpm for 10 min and subsequently at 6,000 rpm for 20 min. The pelleted cells were disrupted in 50 ml of lysis buffer (50 mM ammonium acetate, pH 7.8, and 1% *N*-decanoyl-*N*-methylglucamine) (MEGA-10) (9). After one cycle of freezing and thawing, insoluble components were removed from the lysate by centrifugation in a Beckman SW40 rotor at 35,000 rpm for 45 min. The supernatant

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was divided into two portions; one part was mixed with anti-H MAbs (clone no. I-41) coupled to protein A-Sepharose (1 ml of a 50% slurry) and rotated in an end-to-end manner for 2 h at 4°C. The immunoadsorbent beads were separated from the lysate and washed with 15 ml of TDS buffer (10 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]), and the radioactivity of the beads was counted. This procedure was repeated by further identical cycles, each time applying fresh immunoadsorbent. The depletion continued until no radioactivity incorporated in the beads was detected and no H polypeptide band could be observed upon analyzing a large portion of the beads by SDS-polyacrylamide gel electrophoresis (PAGE) and processing the gels for fluorography. The same strategy was applied for removal of the F protein from the other portion of the infected-cell lysate with anti-F MAbs (clone no. 19-FF10) coupled to protein A-Sepharose.

**Preparation of iscoms.** Iscoms were prepared as previously described (20) with slight modifications. MEGA-10 was added to 3 ml of depleted lysate, containing 10 mg of protein, to a final concentration of 2%. In addition, 1 mg of cholesterol and 1 mg of phosphatidylcholine, dissolved in 100 μl of MEGA-10–$H_2O$ (1:5), were also added to the lysate. Before dialysis, a 10% solution of the glycoside Quil A (trade name Spikoside, obtained from Iscotec AB, Luleå, Sweden) was added to the mixture, to a final concentration of 0.1%.

The mixture was then extensively dialyzed against phosphate-buffered saline (PBS), pH 7.2, first overnight at room temperature, and then for another 24 h at 4°C. After dialysis, the mixture was layered on a discontinuous sucrose gradient consisting of 300 μl of 5% sucrose, 0.5% Triton X-100 in PBS, and 10% sucrose in PBS and centrifuged at 28,000 rpm and 20°C for 24 h in a TST 28.38 Kontron rotor to separate the preformed iscoms from free Quil A and lipids. The pellet was dissolved in PBS to a final protein concentration of 1.2 mg/ml, as determined by Bradford (3). The formation of iscoms was monitored by electron microscopy.

**Electron microscopy.** Samples of iscoms were applied to carbon-coated grids rendered hydrophilic by glow discharge. The samples were microdialyzed on droplets of 0.1 M phosphate buffer, pH 7.2, before being contrasted with 2% ammonium molybdate, pH 7.0. Photographs of the specimens were taken in a Philips 300 electron microscope at an accelerating voltage of 60 kV. Photographs were taken in focal series with minimal beam exposure.

**Immunization of mice and subsequent challenge with the HNT strain of measles virus.** Pathogen-free BALB/c mice were bred at our laboratory and used for active immunization studies at 3 weeks of age. The mice were inoculated subcutaneously with 250 μl of physiological NaCl containing either 25 μl of F iscom complex (about 0.25 μg of F protein) or 40 μl of H iscom complex (about 0.1 μg of H protein). The animals were challenged 4 weeks later, together with a nonimmunized control group, by inoculating 30 μl of virus suspension into the midline of the brain under light ether anesthesia. The stock material of the HNT strain of measles virus, titrated in newborn BALB/c mice, contained $10^{5.7}$ 50% intracerebral lethal doses per g of brain (16). For passive immunization experiments, 2-day-old white mice of the NMRI strain and the following ascites fluids were used: three clones of F protein specificity (19-FF10, 19-HB4, and 19-GD6) and seven clones of H protein specificity (I-41, 16-DE6, I-29, I-12, I-44, 7-AG11, and 16-CD11), all clones reflecting distinct epitopes. Ascites fluids (30 μl) were administered to newborn mice intraperitoneally, and 20 μl of HNT stock material, diluted 1:5 in PBS, was simultaneously inoculated intracerebrally.

**Serological tests.** Serological assays were performed on mouse sera that were collected from the animals before challenge with the HNT virus.

Hemagglutination inhibition (HI) and HLI tests were performed as described previously (24).

**Surface immunofluorescence with live cells.** HNT virus-infected Vero cells on glass slides were washed in cold PBS and incubated for 30 min at 4°C with the anti-H MAbs (1:20 dilution) used in the passive immunization experiments. After incubation, the slides were washed three times for 5 min with cold PBS, and fluorescein-labeled goat anti-mouse antibodies (Nordic Immunology, Tilburg, Holland) were added to the preparations and incubated for a further 30 min at 4°C. The samples were washed again in cold PBS, as before, and observed for immunofluorescence.

**Radioimmune precipitation assay (RIPA) and SDS-PAGE.** Twenty microliters of 1:5 diluted mouse sera was added to 40 μl of intracellular D-[1,6,-$^3$H(N)]glucosamine hydrochloride-labeled virus antigens, prepared as described previously, (34) and adjusted to 500 μl with TDS buffer. After standing on ice for 2 h, the immune complexes were immobilized by addition of 100 μl of a 1:1 slurry of protein A-Sepharose in 10 mM phosphate buffer, pH 7.2, and incubated on ice for a further hour with frequent vortexing. The Sepharose beads were collected by centrifugation and washed five times with 1 ml of TDS buffer, and then the immune complexes were disrupted by adding 70 μl of sample buffer, followed by incubation at 100°C for 2 min. The samples were analyzed by SDS-PAGE, by using discontinuous slab gels of 1.5 mm thickness, with a 10% concentration of acrylamide and 0.27% *N,N'*-methylenebisacrylamide (14). After electrophoresis at a constant 70 V for 18 h, the gels were fixed in 10% acetic acid and processed for fluorography as described previously by Bonner and Laskey (2). Fluorography was done with XAR-5 film (Eastman Kodak Co., Rochester, N.Y.).

## RESULTS

**Assaying of lysates depleted of the H or F glycoprotein.** To quantitatively remove either the H or the F protein from the infected-cell lysates, several cycles of immunoaffinity adsorption were performed. Figure 1 shows the depletion of the two glycoproteins from two separate lysates through the successive cycles. In all, six to seven cycles were carried out until neither scintillation counting of the immunoadsorbents nor RIPA (data not shown) could detect a trace of the component subject to removal.

**Characterization of iscoms.** The iscom preparations were analyzed by electron microscopy (Fig. 2). The iscoms appeared as globular structures with a diameter of about 40 nm and consisted of circular subunits with a diameter of 12 nm.

Hemagglutination assay of the three iscom preparations showed titers of 1,280 and 320 for the H iscoms, whereas no hemagglutination activity was found associated with the F iscoms.

**Antibody response induced by the F and H iscoms.** Groups of six and seven animals were immunized once with the H and F iscom preparations, respectively. Antibodies were assayed, with sera taken 4 weeks after immunization, in HI, HLI, and RIPA tests. Results of the HI and HLI tests are shown in Table 1. The mice immunized with the H iscoms had a range of HI antibody titer of 40 to 160. The group immunized with the F iscoms had no demonstrable HI

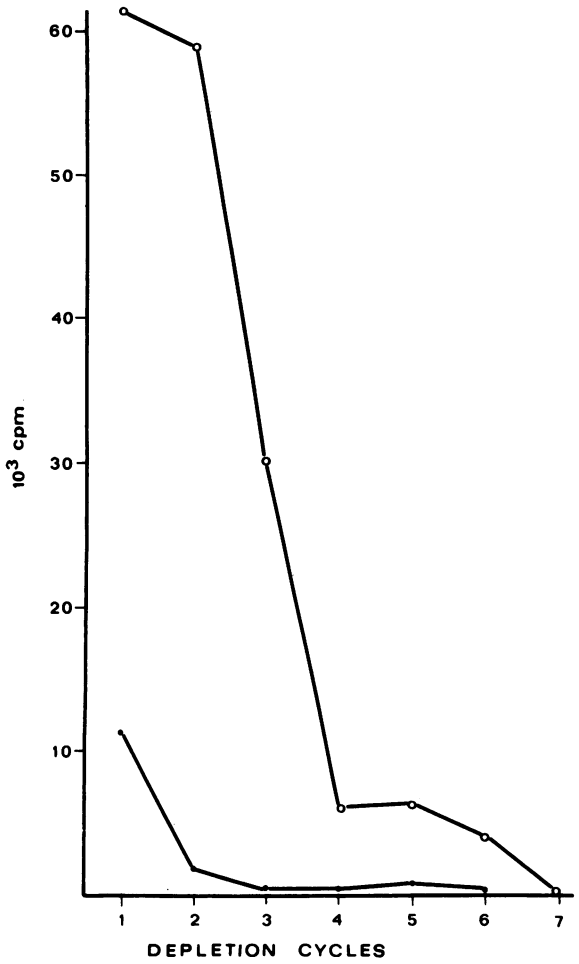


FIG. 1. Consecutive adsorptions of measles virus glycoproteins in infected-cell lysates with MAbs coupled to protein A-Sepharose. Depletion of the hemagglutinin (○) and the fusion protein (●) from LEC-KI strain-infected-cell lysates is illustrated.

antibodies, while the range of HLI antibody titer was 20 to 80. The HLI titer of the H iscom-immunized group was somewhat higher, 80 to 160.

RIPA demonstrated that sera from the iscom-immunized groups exclusively precipitated the homologous protein (Fig. 3).

TABLE 1. Antibody activities of mouse sera from animals inoculated once with iscom preparations containing the H or F glycoprotein

| Animal | Reciprocal of dilution | |
|---|---|---|
| | HI | HLI |
| Immunized with H iscom | | |
| 1 | 80 | 160 |
| 2 | 160 | 160 |
| 3 | 160 | 160 |
| 4 | 40 | 80 |
| 5 | 80 | 160 |
| 6 | 160 | 160 |
| Immunized with F iscom | | |
| 1 | <10 | 30 |
| 2 | <10 | 30 |
| 3 | <10 | 40 |
| 4 | <10 | 20 |
| 5 | <10 | 80 |
| 6 | <10 | 50 |
| 7 | <10 | 50 |

**Challenge of BALB/c mice with the HNT strain.** Four weeks after immunization, the two groups of animals, together with a nonimmunized group of control animals of corresponding age (7 weeks), were challenged by intracerebral inoculation of the HNT strain and were observed for 90 days after challenge. This measles virus strain was described to cause lethal acute encephalopathy in weanling BALB/c mice (30). Both iscom preparations gave complete protection (100% survival of seven and six mice inoculated with F and H iscoms, respectively) against disease in the HNT virus-challenged animals; half (9 of 18 or 50%) of the nonimmunized mice survived. All surviving animals in the nonimmunized group transiently showed severe symptoms.

**Passive immunization with MAbs.** All nonimmunized control animals inoculated with the HNT virus at 2 days of age died within 10 days (Table 2). The seven anti-H MAbs represent four antigenic sites as determined by competitive binding enzyme-linked immunosorbent assay (33). Five MAb clones (I-41, 16-DE6, I-29, I-12, and I-44) gave full

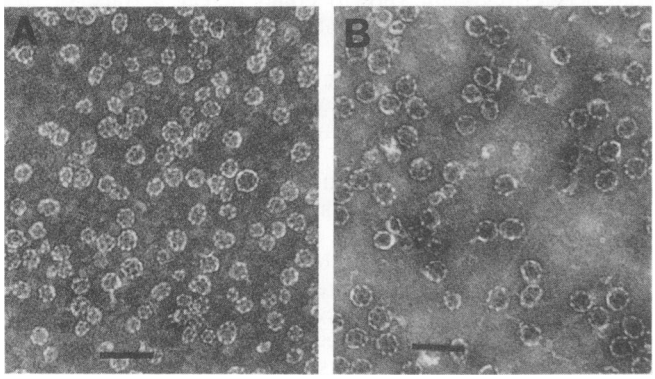


FIG. 2. Electron micrographs of isolated H (A) and F (B) iscoms. Negative contrasting was done with ammonium molybdate. Bars, 100 nm.

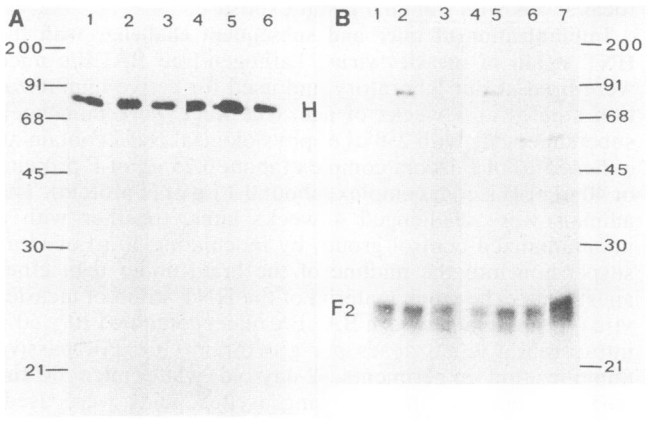


FIG. 3. Immunoprecipitation of [$^3$H]glucosamine-labeled lysates of LEC-KI strain-infected Vero cells with sera from BALB/c mice immunized with H (A) and F (B) iscoms. SDS-PAGE was performed in 10% gels under reducing conditions, and gels were processed for fluorography.

TABLE 2. Effect of passive administration of MAbs to newborn mice on challenge with the HNT strain of measles virus

| MAb designation[a] | No. of animals challenged/no. that survived[b] |
|---|---|
| None (control group) | 21/0 |
| Anti-F MAb clones | |
| 19-FF10 + 19-HB4 + 19-GD6 | 7/0 |
| 19-FF10 | 9/0 |
| Anti-H MAb clones | |
| I-41 | 7/7 |
| 16-DE6 | 7/7 |
| I-29 | 7/7 |
| I-12 | 7/7 |
| I-44 | 8/8 |
| 7-AG11 | 19/0 |
| 16-CD11 | 12/0 |

[a] Ascites fluid (30 μl) was inoculated intraperitonially.

[b] Animals were challenged by intracerebral inoculation of 20 μl ($0.4 \times 10^{2.7}$ 50% lethal doses) of HNT stock virus (1:5 dilution in PBS). Acute deaths, occurring up to 10 days after inoculation, were registered.

protection to the animals, and all of them survived without any symptoms of acute disease (Table 2). However, some of the animals died 2 to 3 months after inoculation. It was shown that MAb to the hemagglutinin was able to change the course of acute encephalopathy to subacute encephalitis in HNT measles virus-infected mice (29). One clone, 7-AG11, did not react with the hemagglutinin of HNT virus in live immunofluorescence (Table 3); thus, protection with this MAb clone was not anticipated. The last anti-H MAb clone (16-CD11) gave no protection, and mortality in this group was 100%. Interestingly, MAb clones 7-AG11 and 16-CD11 reacted with two overlapping sites. Administration of a mixture of three anti-F MAb clones (19-FF10, 19-HB4, and 19-GD6) or a single anti-F MAb clone (19-FF10) did not provide any protection against challenge.

## DISCUSSION

Experience with live and inactivated morbillivirus vaccines has highlighted the separate roles of the two surface antigens in inducing immune protection. Sera from individuals immunized with Formalin- or Tween 80-ether-inactivated measles vaccine exhibited the absence of HLI F-specific antibodies, but HI antibodies induced by the fully immunogenic H component were present (23). The atypical form of disease observed in vaccines after exposure to wild measles virus (6) represents immunopathological disease resulting from an immunity directed primarily against only the H component of the virus. Similar features of disease were observed in trials with inactivated respiratory syncytial virus vaccine (12).

In our present study, we immunized animals with the two types of measles virus surface antigens separated from each other to define the role of individual H and F components in protection. Protection induced by a single type of surface component has also been investigated previously in other morbillivirus systems.

Dogs immunized with affinity-purified canine distemper virus fusion protein and subsequently challenged with virulent canine distemper virus were protected against the development of disease (25). Similarly, cotton rats inoculated with the F glycoprotein of respiratory syncytial virus, expressed by a recombinant vaccinia virus, exhibited significant resistance to challenge with the virus (26). Studies with iscoms using surface structures of several enveloped viruses have been described recently. The hemagglutinin-neuraminidase (HN) and F components of parainfluenza virus type 3 jointly, the H and F spike proteins of measles virus together, and rabies virus glycoprotein G (20) were incorporated into iscoms, as well as surface proteins of human and horse influenza A viruses (B. Sundquist, K. Löfgren, and B. Morein, Vaccine, in press) and glycoprotein gp85 of feline leukemia virus (27). These preparations induced high titers of specific antibodies as judged by enzyme-linked immunosorbent assay, neutralization (NT), HI, and HLI assays, or challenge experiments (19).

It was shown that surface glycoproteins and no other viral components are contained in iscom structures when formed from both solubilized parainfluenza virus type 3 and measles virus particles (19, 20). In the present investigation, the preparation of the two kinds of iscoms was performed in a nearly identical fashion as described in the latter studies, and consequently only one of the glycoproteins among the viral components became incorporated in the purified iscoms.

The incorporation of F and H glycoproteins of measles virus into iscoms in the present experiments also resulted in highly immunogenic structures. This was apparent from the finding that low amounts of glycoproteins applied in immunization (about 0.25 μg of F protein and 0.1 μg of H protein per animal) could evoke a pronounced immune response. These amounts of antigens were comparable with the quantity of influenza hemagglutinin in virosomes (0.25 μg) sufficient to produce a detectable primary immune response (28).

Approximately 1,000-fold higher amounts (unpublished observation) of affinity-purified surface components of measles virus, in the presence of Freund complete adjuvant, were used in past immunization experiments (34). The range of inhibitory activities associated with the H iscom (HI titers of 40 to 160) and F iscom (HLI titers of 20 to 80) preparations (Table 1) is comparable with antibody activities of rabbit hyperimmune sera against the purified H component (HI titer of 80) and F component (HLI titer of 320) in our earlier study (34). These data point to a superior adjuvant function of the iscom structure. Judging from the RIPA and HI assay of mouse sera (Fig. 3 and Table 1), it is evident that the preparations of iscoms elicited a selective antibody response to one of the two glycoproteins.

It has been shown that age has a pronounced influence on symptoms and mortality rate of mice infected with neu-

TABLE 3. Biological activities of anti-H MAbs used in the passive protection experiments

| Clone designation | Titer[a] | | IF |
|---|---|---|---|
| | NT | HI | |
| I-12 | 10[b] | 800 | + |
| I-29 | 28[b] | 1,600 | + |
| 16-DE6 | ND[c] | 1,600 | + |
| I-41 | 480[d] | 3,200 | + |
| I-44 | 480[d] | 12,800 | + |
| 7-AG11 | <8[d] | 6,400 | − |
| 16-CD11 | 10,240[d] | 4,000 | + |

[a] The NT and HI tests were determined with the LEC-KI strain as antigen, and in the immunofluorescence (IF) assay, cells infected with the HNT strain, which lacks hemagglutination activity, were used.

[b] NT titers were determined previously (E. Norrby, unpublished observation).

[c] ND, Not determined.

[d] NT titers were determined previously (32).

rotropic measles virus (8). This fact explains the different mortality rates of control animals in the active and passive immunization experiments found here. Survival of the iscom-immunized animals demonstrates full protection in both groups.

In the passive immunization experiments, none of the anti-F MAbs used gave protection to challenge with the HNT virus. These MAbs do not neutralize the virus in vitro, which however by itself does not exclude the possibility of protection. Both in vitro neutralizing and nonneutralizing MAbs were demonstrated to have the ability to prevent lethal viral infections in mice (15, 17). Of the seven anti-H MAbs used, five antibody clones gave full protection against acute lethal encephalopathy (Table 2). The nonprotecting clone (16-CD11) inhibited hemagglutination to a great extent (Table 3); consequently, protection of the animals can not be correlated to the HI activity of the MAbs. This observation contrasts the results of Giraudon and Wild (7). The present experiments indicate that protection against measles virus infection can be obtained with highly immunogenic complexes of both the H and F subunits. This was demonstrated in a murine model by means of the glycoproteins incorporated into iscoms, a promising application which should be considered for primates, including humans.

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