Intranasal Immunization with Mumps Virus DNA Vaccine Delivered by Influenza Virosomes Elicits Mucosal and Systemic Immunity

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To improve the efficiency of liposome-mediated DNA transfer as a tool for gene therapy or vaccinology, we have further developed a new delivery system based on the modified immunopotentiating reconstituted influenza virus (IRIV). In this study, we engineered a plasmid DNA vector expressing the mumps virus hemagglutinin or the fusion protein. The administration of this DNA vaccine delivered by influenza virosomes, in combination with the mucosal adjuvant Escheriagen via the intranasal route, was efficient for inducing an immune response, both mucosally and systemically, in mice. The production of IgG2a mumps virus-specific antibodies and the secretion of interleukin 10 (IL-10) by antigen-specific T cells indicated that not only Th1 but also Th2 responses were induced by this DNA vaccine formulation. These results suggest that cationic virosomes in combination with Escheriagen may have great potential as an efficient delivery system for intranasal DNA immunization and provide an immune barrier at the mucosal sites. © 2000 Academic Press

Key Words: influenza virosome; mumps virus; DNA vaccine.

INTRODUCTION

Mumps virus is a member of the Paramyxoviridae family of the paramyxovirus genus. Parotitis is the most common symptom of the disease, but the incidence of meningitis following natural mumps infection has been estimated to occur in about 10% of all cases (Furesz and Contreras, 1990). Beginning in 1968, the widespread use of the live attenuated mumps virus vaccine was followed by a decrease in the incidence of mumps; however, cases of infection after vaccination have still been occurring (Boulianne et al., 1995; Brown et al., 1991; Forsey et al., 1992; German et al., 1996; Jonville-Bera et al., 1996; Nalin, 1992; Sugiuara and Jamada, 1991). Lack of clinical protection has been attributed to primary vaccine failure, which occurs in persons who do not seroconvert after vaccination or to a waning vaccine-induced immunity (Briss et al., 1994).

In the present study we investigated the efficacy of a mumps DNA vaccine intranasally delivered by virosomes and administered to mice. Virosomes comprising cationic lipids and fusion active influenza hemagglutinin protein appear to be a very efficient delivery system for nucleic acid vaccines (Cryz and Glück, 1998; Waelti and Glück, 1998). The use of DNA vaccines associated with virosomes avoids the complex physicochemical problems associated with the use of adjuvants and may also result in in vivo antigen presentation of the encoded epitopes in a manner similar to the presentation of the epitope that would follow natural infection. In this investigation we combined the virosomal carrier system with the mucosal adjuvant Escheriagen (E. coli heat-labile toxin; Swiss Serum and Vaccine Institute, Berne). Here we show that the priming of mice with influenza virosomes and Escheriagen before vaccination enhanced the humoral response. The results obtained show that specific s-IgA antibodies are produced in the mucosal layer of the respiratory tract and, in addition, circulating IgG antibodies are also produced, indicating that both systemic and distal mucosal responses are induced in intranasally immunized mice.

RESULTS AND DISCUSSION

Plasmid immunogens and antigen expression

We engineered two plasmids, GC9 (MuV HN) and GC23 (MuV F), to determine whether they could elicit protective immunity against MuV. They were designed to express the full-length, membrane-anchored MuV hemagglutinin and the fusion protein. Before testing them in mice, they were tested in transfection assays in vitro. Naked DNA, or DNA entrapped in influenza virosomes, was able to express the antigens in Vero cells, as shown by immunofluorescence tests (Fig. 1). The increased expression of MuV proteins in vitro by transfection of mixtures of DNA with virosomes as compared with transfection of DNA alone was then confirmed by immunoprecipitation assay, as shown in Fig. 2.

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Influenza "priming" effect

Some groups of mice received the influenza priming because we wanted to mimic the epidemiological situation existing among humans. As shown in Table 1, the "priming" of mice with influenza vaccine (Swiss Serum and Vaccine Institute, Berne, Switzerland) and Escheriagen before immunization increased the humoral response of either IgG and s-IgA in the nasal cavity, increasing the antigen uptake and/or antigen presentation (De Haan et al., 1996b; Douce et al., 1995; Verweij et al., 1998; Walker and Clements, 1993) (Table 1). Escheriagen is known to be a very potent mucosal immunogen and has also proved to be a strong adjuvant in humans (Glück et al., 1999). In the present study this priming, administered intranasally, significantly stimulated IgG responses and induced a local s-IgA response in mice. It is

<table>
<thead>
<tr>
<th>Mice</th>
<th>Serum IgG</th>
<th>BAL IgA</th>
<th>NW IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (GC9)</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>B (GC9)*</td>
<td>268 ± 164</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>C (GC9/virosome)</td>
<td>35 ± 13</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>D (GC9/virosome)*</td>
<td>1132 ± 600</td>
<td>NEG</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>E (GC23)</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>F (GC23)*</td>
<td>519 ± 437</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>G (GC23/virosome)</td>
<td>100 ± 43</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>H (GC23/virosome)*</td>
<td>1393 ± 358</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>I (virosome alone)</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>L (pcDNA)</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>M (live MuV)</td>
<td>2270 ± 1100</td>
<td>NEG</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>

Note. Animals were administered with 5 μg of DNA/virosome or with 5 μg of naked DNA ± Escheriagen. NEG, negative. The concentration of the specific IgA (μg/mg of total IgA) in each NW sample was normalized to the total IgA concentration, and reported as means ± SD. The IgG values are given as antibody GMT ± SD tested by ELISA.

* Group of mice that had received the influenza "priming."

Table 1

**Immunological Response of Mice Immunized by Intranasal Administration of MuV Vaccines at One Month after the Last Immunization**

![Image](99x148 to 243x722)

![Image](361x587 to 509x722)
interesting to note that, when priming was given with
Escheriagen alone or with influenza virosomes alone,
the adjuvant activity was suppressed. Moreover, Esche-
riagen did not show any adjuvant activity when admin-
istered with naked DNA (data not shown). Influenza HA
and Escheriagen appear to be effective in our system
only if they are administered together. The influenza
priming effect has also been observed using virosomes
as an antigen carrier system (Zurbriggen and Glück,
1999). It is possible that during the priming, Escheriagen
supports the action of HA on the virosomes that bind
macrophages and other immunocompetent cells through
sialic acid-containing receptors. The mechanism of the
influenza priming is not yet known. It is possible that the
synergic effect of the two factors promotes the produc-
tion of cytokines, which, during the booster may have a
positive influence on the DNA immunization. Conse-
quently, the stimulated cells determine an enhanced im-
mune response whenever virosomes associated with
DNA plasmid are administered.

Humoral response to MuV antigens elicited by DNA
immunization

The efficacy of intranasal (i.n.) immunization with plas-
mid DNA encoding the HN was evaluated at the mucosal
site, with respect to the protocol of vaccination. Table 1
shows that i.n. immunization with DNA-virosomes after
priming resulted in a significant stimulation of the serum
IgG response with respect to the response induced by
i.n. immunization with naked DNA (P = 0.03). It is gen-
erally assumed that the immunostimulating effect of li-
posomes primarily involves the uptake of liposome-as-
associated antigen by cells of the mononuclear phagocytic
system (De Haan et al., 1996a; Lasic, 1998; McCluskie et
al., 1998; Wheeler et al., 1996). In our study, the influenza
HA on the virosomes facilitates the targeting of plasmid
DNA to antigen-processing and -presenting cells. The
increased uptake of DNA would result in an improved
expression of the vaccine antigen (Templeton et al.,
1997). Virosomes may also improve transfection effi-
ciency by increasing the retention time and reducing the
rate of DNA degradation by extracellular nucleases
(Dzau et al., 1996; Meyer et al., 1995). The MuV HN and
F antigens are both capable of inducing specific anti-
MuV antibodies. No cross-reaction was revealed be-
tween anti-MuV and anti-influenza antibodies as demon-
strated by immunoenzymatic assays. The influenza prim-
ing had a positive effect on the humoral immune
response of mice immunized with naked DNA (groups B
and F), particularly with DNA-virosomes (groups D and
H). These groups developed a response similar to that of
mice immunized with the live MuV (Urabe Am9 strain)
(P = 0.09).

This vaccine formulation also induced a neutralizing
activity. The sera from mice which had received priming
and were immunized with GC9 (MuV HN) developed a
neutralizing response (GMT 8.3 ± 2.1) against the
mumps virus, as did mice immunized with the live MuV
(GMT 16). We did not find neutralizing antibodies in mice
immunized with GC23. This result confirms other previ-
ously published data (Kövamees et al., 1990; Örvell, 1984;
Örvell et al., 1997), which assess the presence of neu-
tralizing epitopes in the mumps virus HN (Cusi et al.,
submitted). The lack of an animal model suitable to show
the MuV pathogenesis has slackened the molecular
study of this virus. However, since the HN protein repres-
sents the major target to induce a protective immune
response to mumps virus (Houard et al., 1995), these
results could be a step toward a genetically engineered
vaccine against mumps virus infections.

Mucosal antibody response

By analyzing the mucosal immunity we found that,
among the primed mice, only those immunized with GC9-
virosomes developed an amount of IgA in NW samples
comparable to the IgA level obtained in mice immunized
with a high dose of live MuV (P > 0.05). Moreover, when
mice were immunized i.n. with a lower dose of live MuV
(1000 TCID<sub>50</sub>/mouse), no IgA were detected in NW sam-
plies. However, this response was short-lived. IgA anti-
odies were also detected in feces drawn 7–10 days
after the last immunization, indicating that i.n. adminis-
tration of DNA also induces a mucosal immunity at the
enteric site (data not shown) (Asakura et al., 1997; Okada
et al., 1997). The presence of antibodies to the HN of
mumps virus in the mucosa is very important, since they
represent the barrier immunity and can prevent the bind-
ing of the virus to the specific receptor. It is unclear why
mice immunized with naked GC23 or GC23-virosomes
did not develop an IgA response; likely, the processing of
the two MuV antigens, HN and F, inside the cells is
different; in fact, they induce a different Th response in
immunized animals, which could be responsible for pro-
motion of IgA synthesis.

It is worth noting that IgA was revealed in the BALs
only when mice had been immunized with GC9-viro-
somes and when the samples were drawn 2 weeks after
the last immunization (Table 2). It is possible that a
shorter-interval period between immunizations is neces-
sary for inducing IgA in lungs, since the turnover is less
than 1 week for bronchial epithelial cells; moreover, it
has been suggested that alveolar macrophages sup-
press immune responses in lung by inhibition of T-cell
proliferation and down-regulation of antigen-presenting
functions of pulmonary dendritic cells (De Haan et al.,
1996a). The results demonstrate that virosomes admin-
istered with DNA have the capacity not only to induce
IgG response but also to stimulate a significant local
s-IgA response in the secretory tract.
Cell-mediated immune response in immunized mice

Th play an important role in eliciting both humoral and cellular immune responses. Th subsets, such as Th1 and Th2, are critical components of the immune response. Th1 cells are associated with a dominant cellular response, while Th2 cells are associated with a dominant humoral response. The balance between these two subsets can influence the overall immune response. For example, a Th1 response is often associated with a stronger cellular immune response, whereas a Th2 response is associated with a stronger humoral immune response.

Intranasal administration of mumps virus vaccines has been shown to induce a Th1 response. This can be explained by the nature of the vaccine or the route of administration. For example, intranasal administration of vaccines can lead to the induction of a Th1 response, while intramuscular administration can lead to a Th2 response.

The induction of a Th1 response is important in the prevention of mumps virus infection. Th1 cells are known to be effective in clearing viral infections, and the induction of a Th1 response can help to control the spread of the virus. In contrast, a Th2 response is less effective in clearing viral infections and can lead to the persistence of the virus.

In conclusion, the induction of a Th1 response is important in the prevention of mumps virus infection. This can be achieved through the use of intranasal administration of vaccines, which can help to control the spread of the virus and prevent infection.

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**TABLE 2**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Serum IgG</th>
<th>BAL IgA</th>
<th>NW IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (GC9)</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>B (GC9)</td>
<td>25 ± 7</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>C (GC9/virosome)</td>
<td>30 ± 5</td>
<td>8 ± 7</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>D (GC9/virosome)</td>
<td>356 ± 115</td>
<td>11 ± 6</td>
<td>10 ± 6</td>
</tr>
<tr>
<td>E (GC23)</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>F (GC23)</td>
<td>22 ± 18</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>G (GC23/virosome)</td>
<td>28 ± 9</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>H (GC23/virosome)</td>
<td>160 ± 110</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>I (virosome alone)</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>L (pcDNA)</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>M (live MuV)</td>
<td>1565 ± 566</td>
<td>20 ± 10</td>
<td>NEG</td>
</tr>
</tbody>
</table>

* Group of mice that had received the influenza “priming.”

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**TABLE 3**

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-2</th>
<th>IFN-γ</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC9</td>
<td>0</td>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>GC9-IRIV</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>GC23</td>
<td>500</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GC23-IRIV</td>
<td>250</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IRIV</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pcDNA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>live MuV</td>
<td>150</td>
<td>0</td>
<td>900</td>
</tr>
</tbody>
</table>

*Note. Values are given in pg/ml. Cells were stimulated with 10 μg/ml of live MuV.*

---

**TABLE 4**

<table>
<thead>
<tr>
<th>Group</th>
<th>IgG</th>
<th>IgG 1</th>
<th>IgG 2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC9</td>
<td>212</td>
<td>85.5</td>
<td>14</td>
</tr>
<tr>
<td>GC9-IRIV</td>
<td>323</td>
<td>105</td>
<td>9.5</td>
</tr>
<tr>
<td>GC23</td>
<td>163</td>
<td>8</td>
<td>84</td>
</tr>
<tr>
<td>GC23-IRIV</td>
<td>267</td>
<td>24.5</td>
<td>73.5</td>
</tr>
<tr>
<td>IRIV</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pcDNA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>live MuV</td>
<td>1862.5</td>
<td>235</td>
<td>1563</td>
</tr>
</tbody>
</table>

*Note. Values are given in ng/ml.*
immunity. The induction of mucosal IgA and neutralizing antibodies by immunization with GC9-virosomes could be very important to block the entry of MuV into the host. However, a combination of the two vaccines containing either the MuV HN or the MuV F proteins could represent a good candidate to obtain both humoral and cellular immune response.

A balanced Th1/Th2 cytokine profile, characterized by a significantly higher IL-10 response and the predominance of IgG2a Abs, was observed in mice immunized i.n. with the live MuV. In our study, HN and F proteins appear to stimulate a different Th response; thus, the presence of both of the antigens against the mumps virus could be necessary for a vaccine capable of inducing cellular and humoral immunity (Staats et al., 1994).

Safety of the vaccine

Little is known about how long the antigen is produced after mucosal plasmid DNA administration. To address this issue, lung tissue and lymphomonocytes were examined by PCR to reveal the presence of the plasmid. Only 2% of the lymphocyte samples from the group of mice immunized with DNA-virosomes revealed the presence of plasmid DNA 1 month after the last immunization. No plasmid DNA was detected in lungs. It seems that lymphomonocytes could be the vehicle for DNA-virosomes. The persistence of the plasmid over a month after immunization could be useful for an adequate expression of the recombinant vaccine protein. It would be interesting to study the period of MuV genes expression after immunization. Studies have shown liposome-formulated DNA to be safe and nontoxic in animal models at doses producing immunological responses (Caplen et al., 1995; Porteous et al., 1997; Tsan et al., 1997). Influenza virosomes were also shown to be nontoxic in mice; moreover, this system has the advantage of using as little as 5 μg of DNA to induce a good response and to provide an immune barrier at the mucosal sites, since it can be administered by the natural route of infection. It should also be important to transfer the technology from small animal models to nonhuman primates. The virosomes appear to be an effective tool for targeting and gene delivery (Cusi and Glück, 2000), providing a novel promising approach for the development of an efficacious human vaccine.

MATERIALS AND METHODS

Preparation of DNA plasmid-virosome complexes

The hemagglutinin (HN) and the fusion (F) genes of the Urabe Am9 strain of the mumps virus were amplified by RT-PCR (Cusi et al., 1995, 1996), digested with BamHI and BgIII, respectively, and ligated into the pcDNA3 expression vector (Invitrogen, San Diego, CA) to obtain the recombinant plasmids GC9 (MuV-HN) and GC23 (MuV-F). Primers used for HN amplification were 5’-AACG-GATCCAGATGGACCCCTGAAA-3’ and 5’-AGGGATCC-CTTATCAAGTGATGTCACCT-3’. Primers used for F amplification were 5’-ACAGATCTGAGTACGTCGAA-3’ and 5’-ACAGATCTCAGGAGTTACCT-3’. The constructs were grown in DH5α cells and plasmid DNA was purified by Qiagen EndoFree plasmid Kit (Qiagen, Chatsworth, CA) as described by the manufacturer. Influenza virosomes were prepared as described elsewhere (Mengiardi et al., 1995; Waelti and Glück, 1998). IRIVs are spherical, unilamellar vesicles which are prepared by a mixture of natural and synthetic phospholipids containing egg yolk phosphatidylcholine, phosphatidylethanolamine, N-1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate (DOTAP), and 10% envelope phospholipids originating from influenza A/Singapore/6/86 and influenza surface glycoproteins. Briefly, 1 ml of DOTAP virosomes was added to 31 μg plasmid (1.3 μmol). Nonencapsulated plasmids were separated by gel filtration on a High Load Superdex 200 column (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was equilibrated with sterile PBS. The void volume fractions containing the virosomes with encapsulated plasmids were eluted with PBS and collected.

Transfection of Vero cells in vitro

About 10⁵ Vero cells were grown on coverslips at 37°C and infected with 0.3 μg of DNA-virosomes or transfected with 1 μg of plasmid DNA using the Effectene Transfection reagent (Qiagen) as described by the manufacturer. After 2 days, mumps antigen expression was analyzed by immunofluorescence. The cells were washed twice with PBS, fixed with cold methanol/acetone, and treated with either anti-MuV HN or anti-MuV F MAb (kindly provided by Prof. J. Wolinsky), followed by FITC-conjugated goat anti-mouse immunoglobulin G (1/100; Sigma, St. Louis, MO). The coverslips were mounted on slides and examined using a Diaplan microscope (Leitz, Wetzlar, Germany). Positive control (mumps virus-infected cells) and negative control (mock-infected cells) were included in each test.

Assessment of protein expression by RIPA

Vero cells (2 × 10⁵) were plated onto a 35-mm tissue-culture dish, incubated for 1 h at 37°C, and transfected as described above. At 24 h after transfection, the cells were washed with PBS three times, incubated in methionine-free Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Life Technologies, MI, Italy) for 1 h at 37°C, and radiolabeled for 4 h at 37°C in methionine-free DMEM containing 100 μCi [³⁵S]methionine. The labeled cells were washed with PBS and lysed with the lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-P40 [NP-40]) at 4°C for 30 min. The cells debris was pelleted by centrifugation at 15,000 g for 5 min at 4°C. The lysate was mixed with 30 μl of anti-MuV HN or
Immunization of mice

Four-week-old female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA) were used. Each experiment (n = 6) was repeated three times to ensure the reproducibility of results. Mice were anesthetized with ketamine-xylazine and were immunized by i.n. instillation of 5 μg of DNA/mouse in a volume of 20 μl, resulting in deposition of the inoculum throughout the respiratory tract. Groups A–B and C–D were immunized with naked GC9 plasmid and GC9-virosome complex, respectively; groups E–F and G–H were immunized with naked GC13 plasmid and GC13-virosome complex, respectively. Booster immunizations were given 4 and 8 weeks after primary immunization. Groups B, D, F, and H received an intranasal priming with influenza virus vaccine (20 μl containing 0.6 μg of HA and 40 ng of Escherichia coli) 10 days before the first immunization. Control groups consisted of mice administered viromes alone (group I), or pcDNA3-virosome (group L), or 5 × 10⁶ TCID₅₀ of live mumps virus (Urabe Am9 strain) (group M), and their lymphocytes were isolated and tested for T-cell proliferation. Groups I and L represent the negative control of mice immunized with influenza viromes and pcDNA3, respectively.

incubation at 37°C for 3 h, 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma) was added and allowed to react at room temperature for 30 min, and the reaction was stopped with 100 μl of 0.5 N H₂SO₄. Colorimetric conversion for the substrate was measured in a microplate spectrophotometer at 450 nm (Behring).

For the determination of mumps virus-specific IgG and IgA antibodies, purified viromes of mumps virus were diluted in coating buffer (0.05 M NaHCO₃/Na₂CO₃, pH 9.6) to 1 μg of proteins per ml, and dispensed to a 96-well plate at 100 μl/well. After allowing them to absorb overnight at 4°C, the wells were washed with PBS–0.05% Brij 35 and blocked for preventing nonspecific binding by incubation with 5% heat-inactivated fetal calf serum (FCS) in PBS–Brij 35 for 2 h at room temperature. A 100-μl aliquot of samples was diluted twofold in the plate and allowed to react for 1 h at 37°C. The plate was then washed and 100 μl of goat horseradish peroxidase-labeled anti-mouse IgG (γ) antiserum (1/8000; Bio-Rad, Richmond, CA) for IgG ELISA or goat anti-mouse IgA (α) antiserum (1/6000; Southern Biotechnology Associates) for IgA ELISA was added and the plate was incubated for 1 h at 37°C. After washing, the substrate was added and allowed to react at room temperature for 30 min. Colorimetric conversion for the substrate was measured in a microplate spectrophotometer at 450 nm (Behring). IgG titers of samples were calculated from endpoint dilutions showing an optical density at least twice the value of the background represented by a pool of negative control sera. The concentration of total and MuV-specific IgA was calculated against a standard curve of mouse myeloma IgA (Cappel Laboratories, Cochranville, PA) determined on the same plate. Results were expressed as micrograms of MuV-specific IgA mg⁻¹ of total IgA.
Neutralization assay

Virus neutralization was carried out on Vero cells in a 96-well microplate (Örvell et al., 1997). Briefly, serial two-fold dilutions of immunized mice serum were added to an equal volume of mumps virus (wild type MuV strain, isolated and sequenced in the laboratory of the Microbiology Section, University of Siena) containing 100 TCID₅₀ in 50 μl and incubated for 90 min at 37°C. A 50-μl sample of cells (10⁶/ml) was suspended in MEM with 5% FCS and added to each well. Five days after incubation at 37°C, the cultures were examined microscopically for the presence of the cytopathic effect.

Cytokine assay

Splenocytes were drawn from immunized mice and lymphocytes were collected by Ficoll–Hypaque (Pharmacia) gradient. About 100 μl of 2 × 10⁸ unfractonated cells per ml in a complete RPMI 1640 plus 10% FCS were cultured in a total volume of 200 μl with 10 μg/ml of purified mumps virus or phytohemagglutinin (PHA, 5 μg/ml; Sigma) in a 96-well flat-bottomed plate. Control wells received cell suspension only. After 24 h in culture, cell-free supernatants were harvested for the presence of IL-2 and, after 48 h, for the presence of IFN-γ and IL-10. Samples were stored at −80°C. Briefly, microtiter plates were coated overnight at 4°C with 100 μl of anticytokine capture MAb (Pharmingen, San Diego, CA) at 1 μg/ml. The plates were washed twice with PBS–Tween and blocked with 100 μl of 10% FCS in PBS per well per 2 h at room temperature. The plates were then washed twice and incubated with duplicates of serially diluted samples and standards (Sigma) overnight at 4°C. Then 100 μl of the biotinylated anticytokines (Pharmingen) MAb at 1 μg/ml was added to each well and the mixture was incubated at room temperature for 1 h. The plates were then washed three times, 100 μl of streptavidin-peroxidase (1/1000; Sigma) was added, and the mixture was incubated at room temperature for 30 min. Following multiple final washings, the color was developed with TMB (Sigma) and stopped with 100 μl of 10% FCS in PBS per well per 2 h at 37°C, the cultures were examined microscopically for the presence of the cytopathic effect.

Lymphocyte proliferation assay

To determine whether MuV lymphoproliferative responses were induced in immunized animals, their spleens were removed 4 weeks after the last immunization to make a single cell suspension. A 100-μl sample of splenocytes (2 × 10⁷/ml) in complete RPMI 1640 was added to each well in 96-well flat-bottomed plates. Stimulated cells received purified MuV at a concentration of 10 μg/ml; transferrin served as a negative control (120 μg/ml; Sigma) and PHA (5 μg/ml; Sigma) as a positive control. Control wells received cells only. The cells in the well were cultured in 200 μl of medium. After 4 days in culture, the cells were pulsed with [³H]thymidine (1 μCi/well) for 18 h and harvested with FilterMate (Packard, Downers Grove, IL) and the incorporated radioactivity was determined by TopCount (Packard). The stimulation index was calculated as the mean cpm of the stimulated wells divided by the mean cpm of the control wells.

PCR

PCR was performed on DNA extracted from the lymphocytes and the lung tissue (2 mg) of immunized mice by using the QiAamp tissue Kit (Qiagen), as described by the manufacturer. Each sample was subjected to 40 cycles at 94°C for 1 min, 56°C for 40 s, and 72°C for 90 s. The primers used to detect GC9 were the sense 5'-TCCA-GATGGAGCCCTCGAAA-3' and the antisense 5'-TTAT-CAAGTGATAGCTAATC-3'. The primers used to detect GC23 were the sense 5'-CCGCGATCAGTAATCATGAA-3' and the antisense 5'-GCCGCTGAGGAGTTTACCTT-3'.

Statistical analysis

Antibody titers are presented as geometric means ± SD. The Mann–Whitney rank sum test was used to analyze changes in the level of total and specific IgG and IgA. *P < 0.05 was considered significant.

REFERENCES


