A fused gene of nucleoprotein (NP) and herpes simplex virus genes (VP22) induces highly protective immunity against different subtypes of influenza virus

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Abstract

We evaluated the immunogenicity and protective activity of plasmid DNA vaccines encoding the influenza virus NP gene (pNP) alone or in combination with the herpes simplex virus type 1 protein 22 gene (pVP22). Optimal immune responses were observed in BALB/c mice immunized with the combination of pVP22 plus pNP, as assessed by enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot (ELISPOT) and intracellular cytokine staining (ICCS). These mice also showed maximal resistance following challenge with the A/PR/8/34 (H1N1) and A/Udorn/72 (H3N2) strains of influenza virus. The susceptibility of immunized mice to virus infection was significantly increased following depletion of either CD4+ or CD8+ T cells. These results indicate that a plasmid DNA vaccine encoding pVP22 plus NP induces a high level of cross-protective immunity against influenza virus subtypes.

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Introduction

The hemagglutinin (HA) surface protein of the influenza virus is highly immunogenic and thus widely considered to be the antigen of choice for an influenza vaccine. However, HA has a high mutation rate which may reduce its suitability for use in a vaccine designed to provide broad protection. In contrast, the influenza virus nucleoprotein (NP) and M genes have low rates of mutation (Bot et al., 1996; Cox et al., 2002; Donnelly et al., 1997; Epstein et al., 2002, 2005; Heinen et al., 2002; Okuda et al., 2001; Pertmer et al., 1996; Ulmer et al., 1994, 1997). As previously described (Ulmer et al., 1994), DNA vaccines constructed using NP or M genes can induce cross-protective immunity. However, some reports suggest that the synthesis of NP occurs primarily in the nucleus, reducing the immunogenicity of such DNA vaccines (Chen et al., 1999; Kodihalli et al., 2000; Neumann et al., 1997; Robinson et al., 1997; Whittaker et al., 1996). Enhancing the antigenicity of NP might increase the clinical utility of this vaccine strategy.

There are several reports indicating that the tegument protein VP22 of herpes simplex virus 1 (HSV-1) can increase the immunogenicity of antigens to which it is fused (Elliott and O’Hare, 1997; Hung et al., 2001). However, the mechanism by which this improved immunogenicity is achieved is controversial (Perkins et al., 2005). There is some evidence that this involves changes in how the encoded fusion protein spreads between cells (Elliott and O’Hare, 1997; Phelan et al., 1998;
Dilber et al., 1999), although a recent report suggests that increased immunogenicity was achieved in the absence of such intercellular spread (Falnes et al., 2001; Perkins et al., 2005; Lundberg and Johansson, 2001). Despite this uncertainty over mechanism of action, the ability of VP22 to enhance the immunogenicity of a co-delivered antigen is well documented (Dilber et al., 1999; Hung et al., 2001, 2002; Kim et al., 2004; Michel et al., 2002; Wills et al., 2001).

Another strategy for improving the immunogenicity of DNA vaccines involves the use of intramuscular electroporation technology (imEPT) (Babiuk et al., 2002). Results indicate that imEPT reduces the amount of plasmid required to induce an optimal immune response by >20-fold (our unpublished data).

The purpose of this study was to assess whether the immunogenicity of an NP-based DNA vaccine could be augmented by fusion to VP22. Towards that end, BALB/c mice were immunized by imEPT with plasmids expressing genes encoding NP antigen alone, the fused combination of VP22 with NP (VP22/NP), VP22–267/NP or a mixture of NP and VP22. Results demonstrate that VP22/NP DNA immunization induced the highest humoral and cellular immune response and provided strong cross-protection against influenza virus challenge.

Results

Differential expression of the NP gene

To detect the expression of NP, VP22/NP and VP22–267/NP (see Fig. 1 and the Materials and methods section for a description of these recombinant DNA plasmids), each vaccine was transfected into HEK293 cells using lipofectin and protein production analyzed by Western blot. As seen in Fig. 2, the intensity of the VP22/NP band was greater than that observed following transfection with VP22–267/NP or NP alone. No band was observed in HEK293 cells transfected with empty vector alone (data not shown).

Expression of NP protein

To monitor the production of NP protein, transfected cells were stained with a rabbit anti-NP Ab followed by FITC-labeled anti-rabbit Ig. The nuclei of these cells were counterstained with 4,4,6-diamidino-2-phenylindole (DAPI). A high level of NP protein expression was observed in cells transfected with pVP22/NP when compared to the NP or pVP22–267/NP plasmids (Fig. 3).

Humoral response of vaccinated mice

BALB/c mice were immunized with each plasmid construct, and serum anti-NP levels evaluated by ELISA. Mice immunized with pVP22/NP had the highest anti-NP titer of all vaccinated groups (Fig. 4). In contrast, mice immunized with pVP22–267/NP (the truncated form of the VP22 protein, Elliott and O’Hare, 1997) showed no adjuvant activity for antibody production. Our results are consistent with the report of Elliott and O’Hare.

IFN-γ response of vaccinated mice

Spleen cells isolated from immunized mice were re-stimulated in vitro with a peptide encoding an immunodominant MHC binding fragment of NP (Bodmer et al., 1988). The number of antigen-specific IFN-γ-producing cells in the pVP22/NP-immunized group was significantly higher than that in the pNP group (P < 0.05, Fig. 5). The number of antigen-specific IFN-γ-producing cells was slightly increased in recipients of pNP plus pVP22, but not in animals immunized with pVP22–267/NP.
By counterstaining with anti-CD8 Ab, the number of IFN-γ-producing CD8+ cells was found to be significantly increased in the pVP22/NP vs. NP immunized groups (Fig. 6B). This analysis also confirmed that pVP22/NP induced a significant increase in the number of IFN-γ-producing CD8+ cells when compared to pVP22 plus pNP \((P < 0.05)\). pVP22 plus pNP elicited a modest increase in the number of IFN-γ-producing CD8+ cells which did not differ significantly from animals immunized with pNP alone.

**Body weight and survival rate following viral challenge**

Two weeks after the final immunization, vaccinated mice were challenged intranasally (i.n.) with 5LD₅₀ of the A/PR/8/34 (H1N1) strain of flu. All of the control mice died from infection within 8 days of challenge, whereas all of the mice vaccinated with pVP22/NP survived for the 2-week duration of the experiment (Fig. 7A). 33% of mice vaccinated with pNP alone survived, but none of the pVp221-267/NP-vaccinated animals. Similarly, control mice continually lost weight following challenge, whereas the pVP22/NP-immunized group recovered from their acute weight loss by day 10. In the pNP-immunized group, the few mice that survived also showed weight recovery. By comparison, only a slight increase in weight was observed in among surviving pVP221-267/NP-immunized animals (Fig. 7B).

Independent cohorts of vaccinated mice were challenged with the A/Udron/72(H3N2) strain of influenza virus. 80% of

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**Fig. 3.** Expression of the influenza NP and VP22/NP genes in mammalian cells. HEK293 cells transfected with pNP (top), pVP221-267/NP (middle) or pVP22/NP (bottom) gene. Transfected cells were stained by DAPI (left column) and FITC (middle column). These merge figures (right column) were observed under the fluorescence microscope \((×300)\). The FITC-labeled cells containing NP.

**Fig. 4.** Production of IgG against influenza protein in vaccinated mice. In each group, 8–10 mice were immunized twice either with the empty vector, pNP, pVP221-267/NP, pVP22/NP or pVP22 + pNP (mixture) by imEPT. Two weeks after the last immunization, sera were collected. The total IgG titer against the influenza NP protein was determined by ELISA. Data represent the mean ± SE of each group.
the pVP22/NP-immunized group survived, compared to 20% of mice immunized with pNP alone and none of the recipients of the pVP22\textsubscript{1-267}/NP plasmid (Fig. 8A). Changes in body weight consistent with the survival findings were also observed (Fig. 8B). Based on these results, it appears that pVP22/NP vaccination induces significant cross-protective immunity.

**Duration of protective immunity**

To determine whether vaccination elicited long-lasting protective immunity, mice were challenged with A/PR/8/34 6 months after the final immunization. As shown in Table 1, protective immunity persisted even at this late time point in pVP22/NP-vaccinated mice. In contrast, relatively little protective immunity was observed in the pNP immunized group. These findings suggest that pVP22/NP vaccination induces significant cross-protective immunity.

**Mechanism of protection against A/PR/8/34 challenge**

The effect of depleting CD4\textsuperscript{+} or CD8\textsuperscript{+} cells from pVP22/NP-immunized mice on their survival following viral challenge was examined. Vaccinated mice were injected with monoclonal antibodies (mAbs) against CD4 or CD8, based on reports that this completely depleted the relevant cell populations (Dialynas et al., 1983; Epstein et al., 1997, 2000; Sarmiento et al., 1980). Depletion of CD4\textsuperscript{+} cells at the time of pVP22/NP immunization greatly reduced protection against subsequent influenza challenge (Table 2). In contrast, early depletion of CD8\textsuperscript{+} cells had a smaller effect on survival.

These T cell populations were then depleted immediately prior to A/PR/8/34 challenge. Under these conditions, the depletion of the CD8\textsuperscript{+} cells greatly reduced protection. Taken together, these findings suggest that CD4\textsuperscript{+} T cells play an important role during the inductive phase of anti-flu immunity in pVP22/NP plasmid vaccinated mice, whereas CD8\textsuperscript{+} T cells are important during the effector phase of protection.

**Discussion**

Current results clearly demonstrate that a plasmid DNA vaccine encoding the NP protein of influenza fused to the pVP22 protein of HSV-1 induced higher levels of protective immunity against challenge with influenza A/PR/8/34 or A/Udom/72 than pNP alone. Moreover, this protective immunity persisted for at least 6 months after final vaccination.

The induction of cross-protection by pNP was reported by Ulmer et al. (1994). However, the magnitude of this protection was modest, and its durability uncertain. It is reported that the NP tends to remain localized to the nucleus, which might explain its relatively weak immunogenicity (Anton et al., 1999; Biswas et al., 1998; Bullido et al., 2000; Neumann et al., 1997; O’Neill et al., 1998). To overcome this drawback, we fused NP to VP22 (pVP22/NP) and used as a control the truncated form of VP22 (VP\textsubscript{1-267}) that does not enhance immunity (Elliott and O’Hare, 1997). The mechanism by which VP22 enhances immunity is controversial. VP22 is a major tegument protein reported to promote intercellular transport; that is, the protein spreads from the cell in which it is expressed to a large number of surrounding cells (Brewis et al., 2003; Elliott and O’Hare, 1997; Phelan et al., 1998; Stroh et al., 2003; Zender et al., 2002a, 2002b). More recently, this mechanism was challenged and reported to be an artifact of methanol fixation (Lundberg and Johansson, 2001). Whatever the mechanism, VP22 is believed to enhance the ability of transfected antigen to prime antigen-presenting cells (APCs) and to induce cross-protection when released from non-professional APCs (Michel et al., 2002).

In the current study, the expression of NP was enhanced by fusion to VP22. Immunofluorescence studies suggest that this expression was generally limited to the cytoplasm of transfected cells (Fig. 3). In contrast, truncated VP\textsubscript{1-267} failed to enhance the expression of NP (Figs. 2 and 3) and thus did not boost induction of anti-flu immunity. To further improve...
the immunogenicity of this VP22/NP-based DNA vaccine, we administered it by electroporation. There is considerable evidence that electroporation improves the cellular uptake and immunogenicity of DNA plasmids in vivo (Widera et al., 2000).

Results indicate that the pVP22/NP vaccine induced a stronger NP-specific Ab and IFN-γ response and elicited stronger and more durable protection against subsequent influenza challenge than the pNP vaccine alone (Figs. 4–8). In contrast, there was no statistically significant effect of co-administering pNP with pVP22 (Figs. 5 and 6). These findings support the conclusion that simultaneous expression of VP22 and NP in the same cell is critical to increase the immunogenicity of NP.

To clarify the mechanism by which pVP22/NP vaccination induces protection against influenza virus infection, CD4 and CD8 T cell populations were depleted at the time of immunization or at the time of challenge. Results showed that CD4+ depletion at the time of DNA vaccination greatly reduced protective immunity against A/PR/8/34 challenge. Thus, CD4+ T cells appear to play an important role during the inductive phase of immunity. In contrast, CD8+ T cells played an important role in protecting mice during challenge. These findings are consistent with those of Kim et al. (Kim et al., 2004; Ulmer et al., 1998), who showed that depletion of CD8+ T cells caused complete abrogation of protection. In contrast, Epstein et al. (2000) reported that DNA vaccination induced protection from influenza challenge that was independent of CD4+ or CD8+ T cells. In that study, protection was abrogated by in vivo depletion of CD90. While we cannot account for the unexpected findings of Epstein et al., they may reflect differences in the method of immunization (i.m. vs. imEPT) or strain of the challenge virus, but most likely reflected their inclusion of M in addition to NP in their plasmid DNA vaccine, whereas we studied VP22 fused to NP. While further studies into the mechanism of CD90-dependent immunity may clarify these inconsistencies, the current results clearly demonstrated that CD4+ cells are important during the induction phase while

Fig. 7. Survival rate (A) and body weight changes (B) of vaccinated mice after challenge with influenza A/PR/8/34. The empty-vector-vaccinated mice (cross), pVP22/NP-vaccinated mice (closed diamond), pNP-vaccinated mice (open square) and pVP221−267/NP-vaccinated mice (closed triangle) were challenged intranasally (i.n.) with 5LD50 of influenza A/PR/8/34 2 weeks after the last vaccination. The survival rate and body weight changes were monitored for the subsequent 14 days. Each group consisted of 8–12 mice. The data of two other separate experiments showed similar results.

Fig. 6. ICCS assay. Frequency of influenza protein-primed IFN-γ-producing CD8+ T cells in vaccinated mice. In each group, 6–8 mice were immunized either with the empty vector, pNP, pVP22/NP, pVP221−267/NP or pVP22 + pNP (mixture) by imEPT. The splenocytes of these mice were used for an IFN-γ ICCS assay 1 week after the last immunization. The typical percentages of IFN-γ-producing cells in CD8+ T cells are shown in panel A. Panel B represents the mean ± SE of IFN-γ-producing CD8+ T cells of mice immunized with each plasmid. A statistically significant difference (P < 0.05) was observed among these groups.
CD8+ cells are important during the effector phase of protections following pVP22/NP immunization. The results support continued efforts towards optimizing the immunogenicity of therapeutic DNA vaccines.

Materials and methods

Mice

Male BALB/c mice (6 weeks old) were used for vaccination and challenge studies. All mice were maintained with free access to sterile food and water.

Construction of recombinant DNA plasmids

The plasmid encoding the NP gene derived from influenza A/PR8/34(H1N1) was kindly provided by Dr. S. Tamura, National Institute of Infectious Diseases, Japan, and the plasmid encoding the VP22 gene derived from HSV-1 was kindly provided by Dr. Petter O’Hare, Marie Curie Research Institute, UK. PCR amplification was carried out using the following primers: NP sense, 5’ACC GCT CGA GAT GGC GTC TCA AGG CAC CAA AC3’ (containing XhoI site as linker (underlined)) and antisense, 5’GAC GCG TCG ACT TAA TTA TCG TAT TCC TCT GCA TTG3’ (containing SalI site as linker (underlined)); VP22 sense, 5’GAA GAT CTA CCA TGA CCT CTC GCC GCT C3’ (containing BglII site as linker (underlined)) and antisense, 5’CGG GAT CCC TCG ACG GGC CGT GGG CGA GA3’ (containing BamHI site as linker (underlined)). The VP221–267 genes were prepared as described previously (Elliott and O’Hare, 1997; Leslie et al., 1996). To construct the hybrid gene VP22/NP, NP gene fragments were cut by XhoI and VP22 gene fragments were cut by BamHI; the recessed 3 ends were filled using the klenow enzyme and were tandemly ligated. Another hybrid gene VP221–267/NP was prepared in the same manner. To obtain pCAGGS-NP (pNP), pCAGGS-VP22 (pVP22), pCAGGS-VP22/NP (pVP22/NP) or pCAGGS-VP221–267/NP (pVP221–267/NP) constructs, each gene fragment was introduced into the pCAGGS vector, a mammalian expression vector.

Western blotting analysis of expressed protein

HEK293 cells were plated at 1 × 10^5 cells per well into 6-well plates (Corning). The cells were transfected with 1 μg of pDNA by using the transfection reagent PolyFect (Qiagen) according to
Table 1

<table>
<thead>
<tr>
<th>Immunization and in vivo mAb treatment</th>
<th>Number of mice</th>
<th>Survival/total</th>
<th>% survival</th>
</tr>
</thead>
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<tr>
<td><strong>A. Induction phase of immunization</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. pVP22/NP + NRIg*</td>
<td>12/13</td>
<td>92.3</td>
<td></td>
</tr>
<tr>
<td>2. pVP22/NP + anti-CD8 mAb</td>
<td>10/14</td>
<td>71.4</td>
<td></td>
</tr>
<tr>
<td>3. pVP22/NP + anti-CD4 mAb</td>
<td>3/14</td>
<td>21.4</td>
<td></td>
</tr>
<tr>
<td><strong>B. Effector phase of immunization</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. pVP22/NP + NRIg*</td>
<td>13/15</td>
<td>86.7</td>
<td></td>
</tr>
<tr>
<td>5. pVP22/NP + anti-CD8 mAb</td>
<td>2/14</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>6. pVP22/NP + anti-CD4 mAb</td>
<td>11/15</td>
<td>73.3</td>
<td></td>
</tr>
</tbody>
</table>

C. Control

7. empty vector 0/15 0

Mice in group 2 and 3 were injected i.p. with 1 mg each of anti-CD8 or anti-CD4 mAb on days −3 and −1 of the induction phase of immunization. On day 0, all mice were immunized with 50 μg of pVP22/NP by imEPT. The same elimination treatment steps were performed after 4 weeks. On day 43, all mice were challenged i.n. with 5 LD₅₀ of A/PR/8/34. On days 43, 45, 48 and 50, 1 mg of anti-CD8 or anti-CD4 mAb was injected i.p. into mice in groups 5 and 6 and was challenged i.n. with 5LD₅₀ of A/PR/8/34 on day 51. The mortality rate was monitored for 2 weeks.

The manufacturer’s guidelines. The transfected cell lysates were separated using 4–20% polyacrylamide gel electrophoresis (Tris–glycine gel, Invitrogen) by employing Xcell SureLock™ MiniCell apparatus (Invitrogen) according to the manufacturer’s protocol. The protein from the gel was then transferred to nitrocellulose by electroblotting (Invitrogen). An ECL Western blotting kit (Amersham Biosciences) was used with an anti-NP mAb to detect antigen expression from DNA vaccines.

**Immunofluorescence staining of expressed NP gene**

HEK293 cells were plated at 1 × 10³ cells per well into 12-well plates (Nunc, Roskilde, Denmark). The cells were transfected with 5 μg of pNP, pVP22₁₋₂₆⁷/NP or pVP22/NP by using the transfection reagent PolyFect (Qiagen) according to the manufacturer’s guidelines. After 48 h, the transfected cells were fixed by ethanol and rinsed in 2 ml of 0.15 M phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). After rinsing with PBS, the cells were reacted by anti-influenza NP Ab (Dr. T. Tanaka, Jichi Med. Sch., Japan) followed by staining with FITC-labeled anti-rabbit Ig Ab (MBL, Nagoya, Japan). DAPI (Vector Lab, CA, USA) was used for the staining of the nuclei of the cells. After staining, the cells were analyzed under fluorescence microscopy (KEYENCE, Tokyo, Japan).

**Immunization by imEPT**

For DNA immunization, 6- to 8-week-old BALB/c mice (SLC, Shizuoka, Japan) were used; 50 μl of plasmid solution (1 mg/ml in saline) containing either pNP, pVP22/NP, pVP22₁₋₂₆⁷/NP, pVP22 or a mixture of pNP and pVP22 (pNP + pVP22) was injected into the quadriceps muscle. The injection site was electroporated with a constant field strength (30 V/cm, 50 ms × 3 pulses) by using a CUY21 EDIT electroporator (Nepa Gene, Tokyo, Japan). A booster immunization was performed at 4 weeks after the first immunization.

**Enzyme-linked immunosorbent assay**

Enzyme-linked immunosorbent assay (ELISA) was performed as described elsewhere (Okuda et al., 2001). In brief, 96-well microtiter plates were coated with 5 μg/ml of influenza NP, incubated overnight at 4 °C and washed with PBST. They were then treated with 100 μl of serially diluted antisera. The bound immunoglobulin was quantified using an affinity-purified horseradish-peroxidase (HRP)-labeled antimouse Ab (Sigma). The mean Ab titer was expressed as the reciprocal of the serial serum dilution.

**ELISPOT assay**

Two weeks after the final immunization, IFN-γ ELISPOT assay was performed. In brief, MultiScreen-IP plates (Millipore, Bedford, MA) were coated with 50 μl of 10 μg/ml purified rat antismouse IFN-γ antibody (XMG1.2, PharMingen, CA) in PBS overnight at 4 °C. The plate was then blocked with PBS containing 5% BSA and 0.025% Tween 20 for 2 h at room temperature. Lymphocytes (1–10 × 10⁷) that were isolated from the spleen were added to each well in triplicate. The spleen cells were stimulated with or without 10 μg/ml of the influenza peptide (TYQRTALV) for 24 h at 37 °C. After incubation, the cells were removed and incubated with 0.5 μg/ml of biotinylated antismouse IFN-γ antibody (PharMingen) for 2 h at 37 °C followed by the addition of 100 μl/well of 0.2% alkaline phosphatase streptavidin (Vector, CA) in PBS containing 0.05% Tween 20 and 5% BSA for 1.5 h. Finally, the plate was treated with 50 μl/well of BCIP/NBT membrane phosphatase (Kirkgaard and Perry Laboratories, MD) at room temperature for 20 min, and the reaction was terminated by holding the plate under running distilled water. The number of spots was counted using a computer-assisted video image analyzer. The results were expressed as spot-forming cells (SFC) per million cells.

**ICCS assay**

IFN-γ-secreting CD8⁺ cells were detected using the protocol recommended by the manufacturer (Cytofix/CytoPerm Plus Kit, S. Saha et al. / Virology 354 (2006) 48–57 55

Table 2

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Survival no./ Experimental no.</th>
<th>% survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Empty vector # immunized mice</td>
<td>0/15</td>
<td>0</td>
</tr>
<tr>
<td>2. Mice immunized with pVP22/NP</td>
<td>13/15</td>
<td>86.7</td>
</tr>
<tr>
<td>3. Mice immunized with pNP</td>
<td>2/15</td>
<td>13.3</td>
</tr>
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</table>

Six months after the last immunization, each mouse was challenged with 5 LD₅₀ of A/PR/8/34. After 20 days, the percentage of dead mice was calculated. A significant difference was observed between 1 and 2 (P < 0.05) and between 2 and 3 (P < 0.05). Other experimental data showed similar results.
PharMingen, San Diego, CA, USA). In brief, lymphocytes were isolated from the mouse spleen, and a single cell suspension was incubated with 10 μg/ml of the influenza peptide (TYQRTRALV) for 24 h at 37 °C. At 2 h before the end of incubation, 1 μg/ml of GolgiPlug was added. The cells were washed and blocked with 4% normal mouse sera and stained with PE-conjugated antimouse CD8 Ab (Ly-2, PharMingen). The cells were then suspended in 250 μl of Cytofix/Cytoperm solution at 4 °C for 20 min, washed with Perm/Wash solution and stained with antimouse IFN-γ Ab conjugated with FITC (PharMingen) at 4 °C for 30 min followed by flow cytometric analysis.

Virus challenge

The influenza virus strains used here were A/PR/8/34 and A/Udron/72 that were obtained from Dr. S. Tamura (National Institute of Infectious Diseases, Japan). The virus was grown in 10-day-old embryonated chicken eggs and purified by sucrose density gradient ultracentrifuge. Two weeks after the last immunization, the mice were anesthetized with diethyl sulfoxide and infected simultaneously by the intratracheal (i.t.) route with 5 LD50 of influenza A/PR/8/34 or A/Udron/72 in 30 μl PBS by using a 24-gauge stainless steel animal feeding needle (Popper and Sons, New York, NY). These mice were kept under observation from 0 to 14 days, and the body weight and the survival rates were calculated.

Depletion of CD4+ or CD8+ cells

The mAbs GK1.5 used for in vivo depletion were specific for mouse CD4 (Diaynomials et al., 1983), and mAbs 53.72 is specific for mouse CD8. All mAbs were rat IgG2b Abs and had been purified by protein G sepharose. The concentration of rat IgG2b was measured using radial immunodiffusion by employing kits from The Binding Site (Birmingham, UK). The acute depletion protocol has been reported previously (Epstein et al., 1997). In a preliminary experiment, mice were treated with mAb and spleen cells were analyzed by flow cytometry to confirm the depletion.

For the depletion of T cells, mice were injected i.p. with 1 mg anti-CD8 or anti-CD4 mAb on days −3 and −1 of the induction phase of immunization. On day 0, all mice were immunized with 50 μg of pVP22/NP by imEPT. The same depletion treatment steps were performed 4 weeks before the booster immunization. On day 43, all mice were challenged i.n. with 5 LD50 of A/PR/8/34. On days 43, 45, 48 and 50, 1 mg anti-CD8 or anti-CD4 mAb was injected i.p. into mice in groups 5 and 6, and all mice were challenged on day 51 i.n. with 5 LD50 of A/PR/8/34. The percent of dead mice was determined for 2 weeks.

Data analysis

All values were expressed as mean ± SE. Statistical analysis of the experimental data and controls was performed using the two-tailed Student’s t test or one-way ANOVA.

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