Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 3009–3013, March 1995
Immunology

Generation of cytotoxic and humoral immune responses by nonreplicative recombinant Semliki Forest virus

XIANZHENG ZHOU*†‡, PETER BERGLUND†‡, HONGXING ZHAO†, PETER LILJESTRÖM†§, AND MIKAEL JONDAL*

*Microbiology and Tumor Biology Center, Karolinska Institute, S-171 77 Stockholm, Sweden; and †Department of Biosciences, Karolinska Institute, S-141 57 Huddinge, Sweden

Communicated by Bernard Moss, National Institutes of Health, Bethesda, MD, December 28, 1994

ABSTRACT **The Semliki Forest virus (SFV) expression system can be used to package recombinant RNA into infectious suicide particles. Such RNA encodes only the SFV replicase and the heterologous protein but no structural proteins of SFV, and it is thus deficient in productive replication. We demonstrate here that infection of C57BL/6 (H-2^b) and BALB/c (H-2^d) mice with recombinant SFV expressing the nucleoprotein (NP) of influenza virus (SFV-NP) resulted in efficient priming of influenza virus-specific CD8$^+$ cytotoxic T-cell (CTL) responses. The generated CTLs lysed both homologous (A/PR/8/34) and heterologous (A/HK/68) influenza virus-infected, or peptide-coated, target cells to a similar degree as CTLs induced by wild-type influenza virus in a major histocompatibility complex class I-restricted fashion. As few as 100 infectious units of virus induced a strong CTL response. Induction of CTL by SFV-NP could also be achieved in CD4 gene-targeted mice, demonstrating the independence of the primary CTL response of CD4$^+$ helper T cells. One immunization generated a CTL response that peaked after 1 week, and an additional booster injection generated a CTL memory, which was still detectable after 40 days. SFV-NP immunizations also generated high-titered IgG humoral responses that remained significant after several months. These results demonstrate that the recombinant SFV suicide system is highly efficient in antigen presentation and suggest that it may have a potential as a recombinant vaccine.**

CD8$^+$ cytotoxic T lymphocytes (CTLs) have been demonstrated to be a major host defense mechanism against many viruses (1–4). During a viral infection, cytosolic antigens are proteolytically degraded into peptides, which are translocated into the lumen of the endoplasmic reticulum (ER) via ATP-dependent transporters. In the ER, peptides associate with class I molecules of the major histocompatibility complex (MHC) and are then transported, along the normal exocytic pathway, to the cell surface for recognition by CD8$^+$ CTLs. In contrast, exogenous antigens such as virus particles, bacteria, or soluble protein subunits are degraded into peptides within the endosomal compartment of the cell. Here they are loaded onto MHC class II molecules for presentation to CD4$^+$ helper T cells (reviewed in refs. 5–8).

Although CTL responses can be generated against a variety of extracellular antigens such as lipopeptides (9, 10), proteins incorporated into immunostimulating complexes (11), and short synthetic viral peptides (12–15), strong cellular and humoral immune responses generally require priming with live virus (9, 16). In effect, live vaccines are the most potent, but their use is often hampered for biosafety reasons. As alternatives, recombinant viral vectors (17–19) and naked DNA (20–23) are gaining increased attention as means of expressing antigens.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Our approach is to use the Semliki Forest virus (SFV) replicon as a basis for developing recombinant RNA vaccines. SFV is an alphavirus consisting of a nucleocapsid with one copy of a single-stranded RNA molecule surrounded by an envelope containing the virus spike proteins (24). SFV has several features that make it well suited for antigen expression. The RNA genome is infectious because of its positive polarity—i.e., naked genomic RNA is able to start an infection when introduced into the cytoplasm of a cell. The infecting RNA is self-replicating by coding for its own replicase, and the efficient replication can result in production of up to 10^8 protein molecules per cell. Replication occurs exclusively in the cytoplasm and cytopathogenic effects arise late in infection, leaving an extensive time window during which a high expression level is combined with negligible morphological changes.

We recently developed a vector system that allows for efficient expression of foreign coding sequences as parts of the SFV RNA replicon (25–27). DNA of interest is cloned into a plasmid vector, which serves as a template for *in vitro* synthesis of recombinant RNA. The recombinant RNA is then cotransfected with helper RNA molecules into cells to become packaged into infectious virus particles (see Fig. 1). Only the recombinant RNA carries an encapsidation signal, and thus, in the absence of the viral structural genes, packaging produces recombinant particles that can infect cells only once, leading to one round of nonproductive replication. By separating the replicase and structural genes of the replicon, a high biosafety level is achieved. However, low levels of recombination between alphaviral RNAs have been observed (26, 28), and therefore a mutant of the SFV helper was made that carries a mutation in the gene encoding one of the spike proteins, resulting in the production of noncleaved forms of that protein (26, 29). In effect, such virus cannot infect new host cells unless first cleaved with exogenous protease. The use of this helper prohibits the spread of stray wild-type SFV and further increases the biosafety of the system (26).

It was recently shown that recombinant Sindbis virus, another alphavirus, can express heterologous antigen to induce cellular and humoral responses in mice (19, 30). In this case, however, the vectors used carried the structural genes of the virus, with the result that several rounds of virus replication occurred *in vivo*. Although Sindbis and SFV are of low pathogenicity to humans, they can cause mild disease, and therefore replication-proficient alphaviruses are not likely to be used for vaccine development. To test the potential of recombinant, nonreplicating SFV suicide particles as vectors for antigen expression, we have cloned the gene coding for the influenza virus nucleoprotein (NP) into SFV. In this report, we present evidence that the SFV system indeed induces strong cellular and humoral immune responses in mice.

Abbreviations: SFV, Semliki Forest virus; NP, nucleoprotein; SFV-NP and SFV-LacZ, recombinant SFV expressing influenza NP and *Escherichia coli* β-galactosidase, respectively; CTL, cytotoxic T lymphocyte; IU, infectious unit; MHC, major histocompatibility complex.

‡X.Z. and P.B. contributed equally to this work.
§To whom reprint requests should be addressed.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The EL4 thymoma and B16 melanoma cells were derived from C57BL/6 mice (H-2^b), and the P815 mastocytoma cell line was from DBA/2 mice (H-2^d). The cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics (penicillin and streptomycin). BHK-21 (baby hamster kidney) cells were grown in BHK medium supplemented with 5% FCS, 10% tryptose phosphate broth, 2 mM glutamine, 20 mM Hepes, and antibiotics.

Production of Infectious Suicide SFV. The plasmid pAPR501 containing the influenza PR8 NP gene was generously provided by P. Palese (The Mount Sinai Hospital, New York). The NP gene was isolated as an *Eco*RI fragment, blunt ended with Klenow fragment, and ligated into the *Sma* I site of pSFV1 (25). Plasmid pSFV3-lacZ has been described (25). *In vitro* made recombinant RNA was packaged into infectious SFV particles as described in detail (25, 26, 31). To determine stock titers, BHK cells grown on coverslips were infected with serial dilutions of activated virus, and expression of antigen was visualized by indirect immunofluorescence (29).

Detection of SFV-Expressed NP. Metabolic labeling of recombinant SFV-infected cells was done as described (31). Labeled cells were scraped off the plate and lysed with Nonidet P-40 (NP-40) buffer (1% NP-40/50 mM Tris·HCl, pH 7.4/150 mM NaCl/2 mM EDTA/1 μg of phenylmethylsulfonyl fluoride (PMSF) per ml and cleared by centrifugation at 6000 rpm in a 5415 Eppendorf centrifuge. Pellet (nuclear) fractions were lysed in SDS lysis buffer (1% SDS/50 mM Tris·HCl, pH 7.4/150 mM NaCl/2 mM EDTA/1 μg of PMSF), sonicated for 15 min at 37°C, and then diluted with NP-40 lysis buffer. Immunoprecipitation was performed as described (29).

Mice, Viruses, and Synthetic Peptides. C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were from B & K Universal (Stockholm). Gene targeted $CD4^-8^+$, $CD4^+8^-$, and $CD4^-8^-$ (H-2^b) mice have been described (32–35) and were generously provided by Tak Mak (University of Toronto). All mice were used at the age of 6–10 weeks. Influenza virus PR8 strain was a gift from A. Douglas (National Institute for Medical Research, London). PR8 was grown in the allantoic cavity of embryonated eggs and used as allantoic fluid for priming and infecting target cells. The mouse-adapted virulent A/HK/68 strain was kindly provided by Innocent Mbawuike (Baylor College, Houston). The influenza H-2D^b binding PR8 NP peptide NP366–374 (ASNENMETM) (36, 37), H-2K^d binding peptide NP147–155 (TYQRTRALV) (37), H-2D^b control peptide from adenovirus E1A protein (SGPSNTPPEI) (38), and H-2K^d tumor antigen of P815 (KYQAVTTTL) (39) were synthesized by B. R. Srinivasa (Astra Research Center, Bangalore, India) and U. Rudén (SMI, Stockholm) by fluorein-9-ylmethoxy carbonyl synthesis (Applied Biosystems). Peptides were purified by reverse-phase HPLC. Stock solutions of peptides were prepared in PBS and stored at −20°C.

CTL Assay. The CTL assay was performed by a standard ^{51}Cr release test. *In vitro* restimulation of immune responding cells has been described (14). Target cells were either infected with virus or incubated with peptide (50 μM) for 1.5 h at 37°C. After virus infection, cells were washed and further incubated for 2–4 h. The cells were then labeled with ^{51}Cr (100 μCi; 1 Ci = 37 GBq; DuPont) for 1 h, washed, adjusted to the appropriate concentration and mixed with titrated effector CTLs in V-bottomed 96-well plates, and incubated for 4–6 h at 37°C. Supernatants were collected and radioactivity was measured in a γ-counter. Results are expressed as percentage of specific lysis according to the formula % specific lysis = [(experimental release − spontaneous release)/(maximal release − spontaneous release)]cpm × 100. In all CTL experiments, one representative experiment of at least three with similar results is shown.

ELISA. Antibodies were detected by ELISA as described (40). The recombinant NP antigen used was produced in the baculovirus expression system and was generously provided by Suezanne Parker (Vical, San Diego). Purified LacZ protein was from Boehringer Mannheim.

RESULTS

Expression of NP from the SFV Vector. We first used pulse-labeling to check expression of recombinant NP from the pSFV1 vector. BHK cells transfected with recombinant SFV expressing influenza NP (SFV-NP) RNA and labeled with [^{35}S]methionine showed a high level of NP expression (Fig. 1*B*). Fractionation of the cell lysate showed that ≈50% of the NP protein had migrated to the nucleus within 60 min (Fig. 1*B*, lanes 2 and 3). The identity of the NP band was verified by immunoprecipitation with a monoclonal antibody (lane 4).

Priming of CTLs *in Vivo* with Recombinant SFV Particles. To determine the priming capacity of recombinant SFV, C57BL/6 mice (H-2^b) were immunized i.v. with 10^6 infectious units (IU) of activated SFV-NP or 10^2 hemagglutination units of PR8. At day 10, immune spleen cells were isolated and restimulated for 5 days with PR8-infected syngeneic spleen cells or with cells loaded with the NP366–374 peptide. When the effector cells were tested on sensitized EL4 target cells, it was found that a strong CTL response had been obtained in both experimental systems (Fig. 2*A*). The same result was obtained when B16 melanoma cells were used as targets (data not shown). The level of CTLs induced by SFV-NP was comparable to that obtained with PR8 virus immunization.

We then used the same experimental setup with different amounts of SFV-NP, using SFV expressing *Escherichia coli* β-galactosidase (SFV-LacZ) virus as a control. C57BL/6 mice

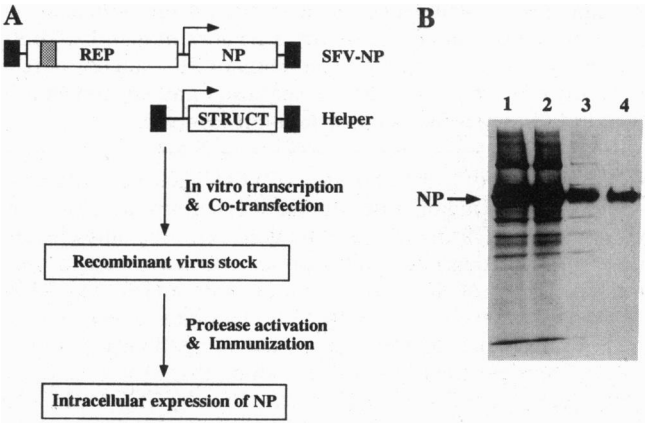

FIG. 1. (*A*) Strategy for production of recombinant SFV particles. The recombinant RNA carries the viral replicase genes (REP) and the influenza NP gene under the viral subgenomic promoter (arrow). The replicase region has been deleted from the helper, which carries only the viral structural genes (STRUCT) under the subgenomic promoter. Both RNA species have 5′ and 3′ replication sequences (solid boxes) recognized by the viral replicase/transcriptase. When these RNAs are cotransfected into cells, the recombinant RNA produces the replicase, which amplifies both species of RNA. The helper produces the viral structural proteins of which the capsid protein is cytoplasmic and complexes with RNAs to form the nucleocapsid. Only recombinant RNAs are packaged, since only this RNA carries the encapsidation signal (stippled box in REP). The two spike proteins are translocated into the endoplasmic reticulum and then migrate to the cell surface where budding of the virus out of the cell is driven by interactions between nucleocapsids and spike complexes. The virus stock produced is activated by chymotrypsin and used directly for immunization, where only the NP protein is expressed in infected cells. (*B*) Pulse-labeling of SFV-NP-infected BHK-21 cells. Lanes: 1, total cell lysate; 2, total NP-40 lysate (all except nuclear material); 3, SDS lysate (nuclear material); 4, immune precipitate from material in lane 1.

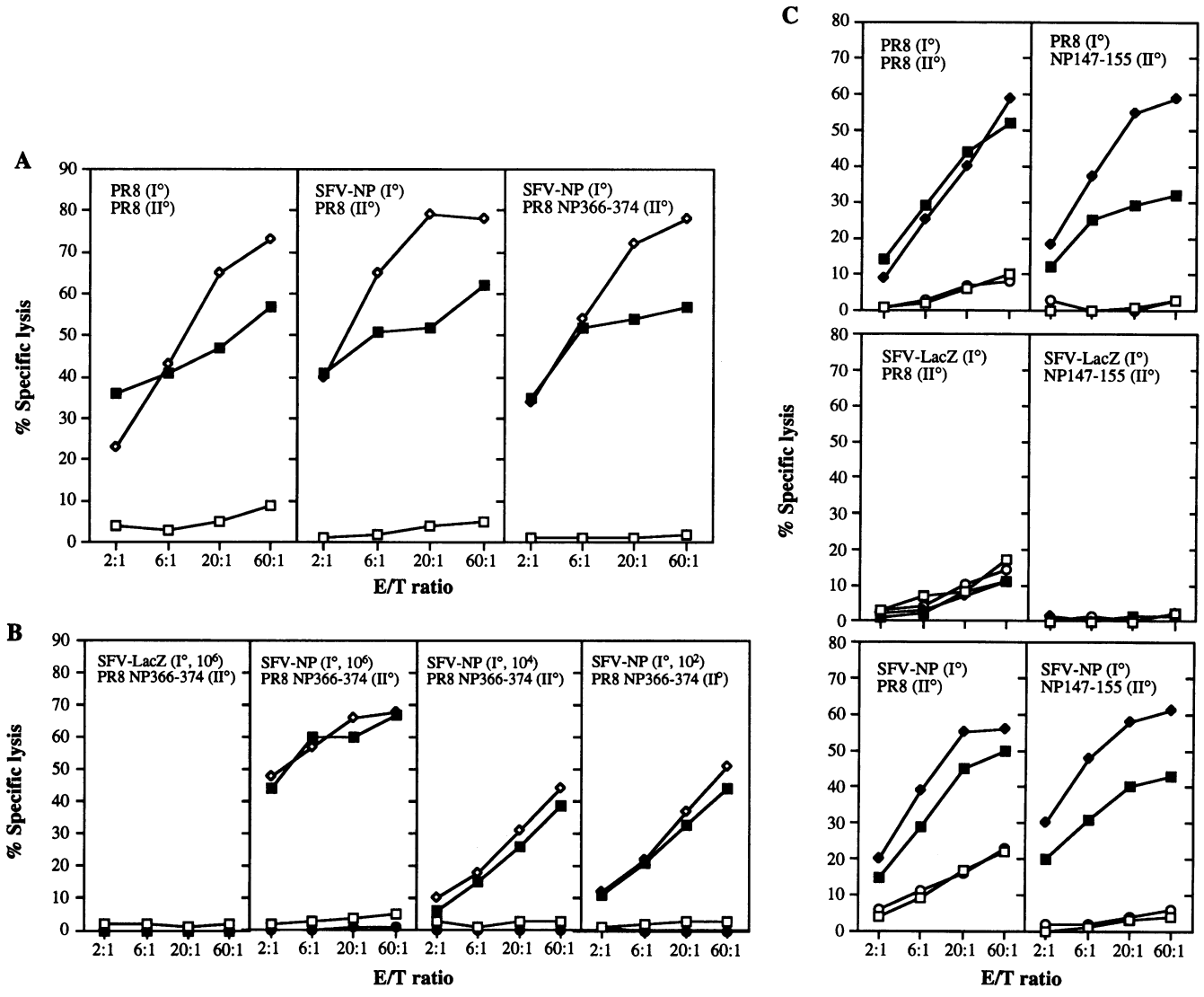

FIG. 2. (*A*) Priming of CTLs in C57BL/6 mice by recombinant SFV-NP. I°, priming *in vivo*; II°, restimulating *in vitro*. EL4 target cells were either untreated (□), infected with PR8 (■), or loaded with NP366–374 peptide (◇). (*B*) Titration of recombinant SFV-NP particles used for priming of C57BL/6 mice. EL4 target cells were untreated (□), infected with PR8 (■), loaded with NP366–374 peptide (◇), or loaded with adenovirus E1A peptide (●). (*C*) Priming of anti-influenza CTLs with SFV-NP in BALB/c mice. P815 target cells were untreated (□), infected with PR8 (■), loaded with NP147–155 peptide (◇), or loaded with P815 peptide (○). ET, effector/target cell.

were immunized i.v. with 10^6 IU of SFV-LacZ or 10^6, 10^4, and 10^2 IU of SFV-NP, and immune spleen cells were restimuated with either PR8-infected or peptide-loaded spleen cells for 5 days. Priming *in vivo* with 10^6 IU of SFV-NP gave the highest specific lysis of target cells expressing endogenous influenza viral antigen or exogenous influenza peptide; 10^4 and even 10^2 recombinant viral particles also generated significant CTL responses (Fig. 2*B*). In the next experiments, 10^6 IU of SFV-NP was used for all subsequent immunizations. It should be noted that when the target cells were coated with an unrelated adenovirus E1A-specific peptide, no lysis occurred, demonstrating that SFV-NP-induced CTLs were specific against NP-expressing EL4 target cells. Similarly, mice primed with activated SFV-LacZ failed to elicit an anti-influenza CTL response (Fig. 2*B*).

To determine whether the CTL response found could be generated in another haplotype strain of mice, BALB/c mice (H-2^d) were infected with SFV-NP, SFV-LacZ, or PR8. As shown in Fig. 2*C*, SFV-NP could prime anti-influenza CTL responses also in the BALB/c strain. Target cells loaded with a control P815 peptide were not lysed. Again, SFV-LacZ did not induce any CTLs against influenza virus-infected or peptide-coated P815 target cells. The priming efficiency by SFV-NP was comparable to that by influenza virus also in this haplotype.

Characterization of the CTL Response. To define the nature of the cellular response, effector cell mixtures from SFV-NP immunizations were depleted of either CD4 or CD8 cells by using lysis by anti-CD4 or anti-CD8 monoclonal antibodies together with complement. Depletion of CD4 cells did not significantly change lysis levels, while these were completely abolished after CD8 depletion (data not shown). It was also interesting to evaluate the importance of $CD4^+$ and $CD8^+$ T cells for the SFV-NP-induced CTL responses. We immunized $CD4^-8^+$, $CD4^+8^-$, $CD4^-8^-$, and control C57BL/6 mice (all H-2^b) with activated SFV-NP. The CTLs generated from $CD4^-8^+$ mutant mice showed a high cytolytic activity against homologous (PR8) and heterologous (HK68) influenza virus-infected or NP366–374-coated EL4 target cells. $CD4^+8^-$ and $CD4^-8^-$ mice gave no CTL response after immunization with SFV-NP (Fig. 3).

CTL Memory. To measure the generation of CTL memory, two groups of BALB/c mice were infected with SFV-NP. The first group received only one priming dose, whereas the other

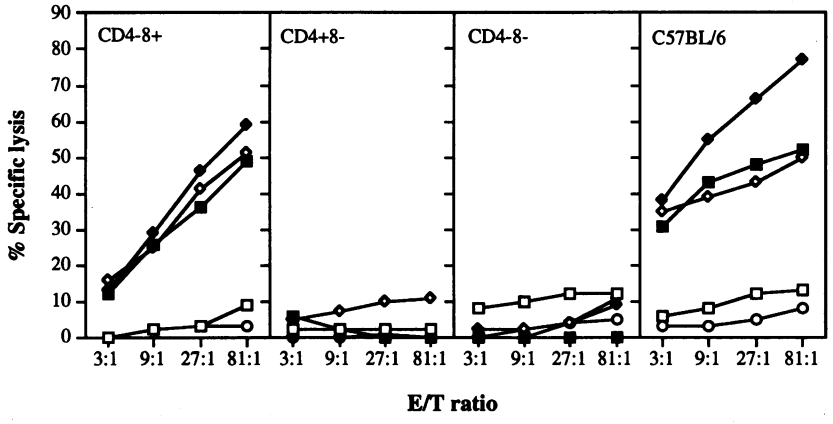

FIG. 3. Generation of CD8$^+$ CTLs in the absence of CD4$^+$ helper T cells by SFV-NP. EL4 target cells were untreated (□), infected with PR8 (■) or A/HK/68 (◇), or loaded with NP366–374 peptide (◆) or adenovirus E1A peptide (○). ET, effector/target cell.

group was given a booster dose at day 14. Immune spleen cells were restimulated with peptide at days 3, 5, 10, 20, and 30 in the former group and at days 20, 30, and 40 in the latter group. The CTL activity elicited by one immunization of SFV-NP peaked at days 5 and 10 and declined close to background level at day 30 (Fig. 4). The booster injection at day 14 resulted in a long-lasting CTL response, which was still significant at day 40.

Induction of a Humoral Response. To test for induction of antibodies, BALB/c mice were immunized at day 0 or at day 0 and day 14 with SFV-NP or SFV-LacZ, and sera were collected at various time points. A single immunization at day 0 resulted in the generation of high levels of anti-NP IgG (Fig. 5). The booster injection enhanced the IgG response to NP 25-fold. Similar results were obtained with SFV-LacZ immunization. Antibodies to NP and LacZ were of the IgG type and were antigen specific as no cross-reactivity between these two antigens was seen (data not shown).

DISCUSSION

In this report, we have shown that recombinant, suicide SFV can be used to efficiently induce cellular and humoral anti-viral responses in two haplotype strains of mice. The generated CTLs were CD8$^+$ and capable of recognizing virus-infected as well as peptide-coated target cells in a MHC class I-restricted manner. As few as 100 particles induced a significant CTL response, and this surprisingly high efficiency may relate to selective accumulation of the virus-expressed material in the immune system.

In the recombinant SFV system, high levels of intracellular antigen are produced. In cell culture, under optimal conditions, a single cell may produce up to 0.1 ng of protein (25). *In vivo*, such a high level is not expected, nor is it likely that all administered particles will infect a cell. Assuming that all particles do infect cells but produce only 10% of antigen compared to the amount seen *in vitro*, a dose of 100 IU could at best produce 1 ng of antigen. *In vivo*, this small amount of antigen must be processed and presented in a highly efficient way in order to generate the high levels of CTL activity and antibody responses seen. For the induction of a cellular immune response, lymphoid cells can respond either to the cells that primarily contain the foreign protein or to antigen presenting cells (APCs) that have taken up and processed material from such cells (41). Both MHC class I and class II pathways can be entered by exogenous antigens, even if the classical MHC class I processing pathway is believed to be the operative only for cytosolic proteins (42–45). Thus, if recombinant SFV directly infects important APCs, then the classical class I pathway might be used for induction of a cellular immune response. Alternatively, material from infected cells might be further processed and presented in professional APCs. At present, we have no information on cells that are the primary targets for SFV after i.v. injection. However, it is likely that these include either important APCs, such as dendritic cells (DCs) or cells in the close proximity of APCs.

SFV-NP also induced memory responses. CTLs could be detected at least 40 days after immunization, and antibodies could be detected for at least 136 days. This may be surprising in view of the fact that the expression of antigen is transient only because of the suicide nature of the system. It has generally been assumed that maintenance of memory is dependent on persistence of antigen (46–48). Since clearance of

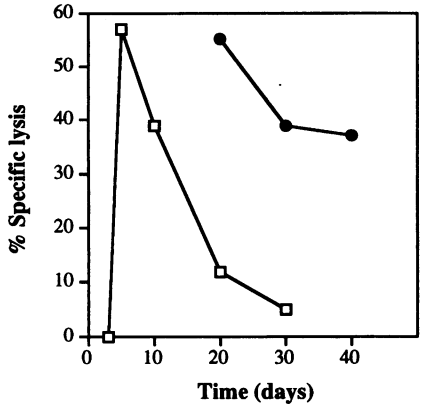

FIG. 4. Memory kinetics of CTLs induced by SFV-NP. P815 cells were used as targets for lysis. Values from an effector/target cell ratio of 60:1 are plotted. Background lysis determined from untreated mice (for approximate levels, see Fig. 2) has been subtracted. □, Mice primed at day 0; ●, mice primed at day 0 and injected at day 14.

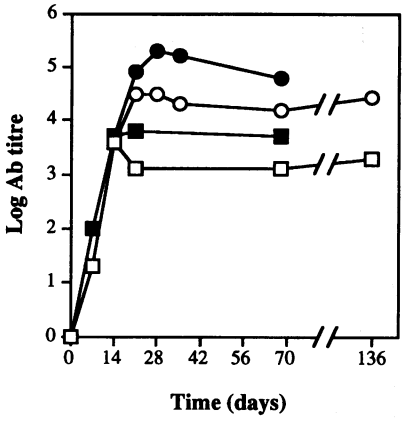

FIG. 5. Antibody (Ab) responses by immunization with the recombinant SFV-NP at day 0 (□) or at day 0 and day 14 (○), or with SFV-LacZ at day 0 (■) or at day 0 and day 14 (●). Every subgroup consisted of four to six mice and results represent the geometric mean titer.

SFV replication will be complete because of the self-destructive features of the system, it is unclear how residual antigen could be sequestered, although antigen on the surface of DCs can persist for long periods of time (49). Interestingly, recent data suggest that long-term T-cell memory may persist even in the absence of the original antigen (50–52).

It has previously been shown that mice lacking $CD4^+$ cells (32, 53) or MHC class II antigens (54) are able to mount virtually normal CTL responses against infecting viruses, although the presence of some helper T-cell function in such cases cannot totally be excluded (55). It has also been shown that CD8 is needed for the maturation and positive selection of class I MHC-restricted CTLs, as cytotoxic responses are dramatically decreased in $CD4^-$ mice (33, 34). Our results suggest that $CD4^+$ T cells do not play a central role for the primary induction of NP-specific CTLs after immunization with SFV-NP. However, clonal expansion and memory development in the absence of T-cell help was not investigated.

The SFV expression system may have several advantageous features useful for immunological stimulation and vaccination purposes. Due to the self-replicating nature of the RNA molecule, high levels of antigen expression can be achieved. This coupled to an efficient *in vivo* expression strategy could mean that only small amounts of virus are needed to achieve a good vaccination effect. Moreover, with the exception of small amounts of viral replicase, no other vector proteins are produced. This is an additional advantage when repeated immunizations with the same vectors are needed. A high level of biosafety is also achieved since the recombinant virus particles infect cells only once. Altogether our results demonstrate that the recombinant SFV suicide system is highly efficient in antigen presentation and suggest that it may have a potential as a recombinant vaccine.

We are indebted to Drs. Tak Mak, Innocent Mbawuike, Peter Palese, and Suezanne Parker for providing materials used in this study. We also would like to thank Dr. Hans-Gustaf Ljunggren for a critical reading of the manuscript and Ms. Anna-Lena Hammarin for growing the influenza virus. This work was supported by the Swedish Cancer Society (M.J.), the Swedish Medical Research Council (P.L.), the Swedish Research Council for Engineering Sciences (P.L.), the Astra Pharmaceutical Company (M.J.), and the Karolinska Institute (M.J. and P.L.). X.Z. and P.B. are recipients of the graduate student scholarship program at the Karolinska Institute.

1. Buchmeier, M. J., Welsh, R. M., Dukto, F. J. & Oldstone, M. B. A. (1980) *Adv. Immunol.* **30,** 275–331.
2. Lin, Y.-L. & Askonas, B. A. (1981) *J. Exp. Med.* **154,** 225–234.
3. Yap, K. L., Ada, G. L. & McKenzie, I. F. C. (1978) *Nature (London)* **273,** 238–239.
4. Kast, W. M., Roux, L., Curren, J., Blom, H. J. J., Voordouw, A. C., Meloen, R. H., Kolakofsky, D. & Melief, C. J. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2283–2287.
5. Germain, R. N. & Margulies, D. H. (1993) *Annu. Rev. Immunol.* **11,** 403–450.
6. Jackson, M. R. & Peterson, P. A. (1993) *Annu. Rev. Cell Biol.* **9,** 207–235.
7. Rammensee, H.-G., Falk, K. & Rötzschke, O. (1993) *Annu. Rev. Immunol.* **11,** 213–244.
8. Barber, L. & Parham, P. (1993) *Annu. Rev. Cell Biol.* **9,** 163–206.
9. Deres, K., Schild, H., Wiesmüller, K.-H., Jung, G. & Rammensee, H.-G. (1989) *Nature (London)* **342,** 561–564.
10. Schild, H., Deres, K., Wiesmüller, K.-H., Jung, G. & Rammensee, H.-G. (1991) *Eur. J. Immunol.* **21,** 2649–2654.
11. Takahashi, H., Takeshita, T., Morein, B., Putney, S., Germain, R. N. & Berzofsky, J. A. (1990) *Nature (London)* **344,** 873–875.
12. Aichele, P., Hengartner, H., Zinkernagel, R. M. & Schulz, M. (1990) *J. Exp. Med.* **171,** 1815–1820.
13. Schulz, M., Zinkernagel, R. M. & Hengartner, H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 991–993.
14. Zhou, X., Berg, L., Motal, U. A. M. & Jondal, M. (1992) *J. Immunol. Methods* **153,** 193–200.
15. Gao, X.-M., Zheng, B., Liew, F. Y., Brett, S. & Tite, J. (1991) *J. Immunol.* **147,** 3268–3273.
16. Kast, W. M. & Melief, C. J. M. (1991) *Immunol. Lett.* **30,** 229–232.
17. Muzyczka, N. (1992) *Curr. Top. Microbiol. Immunol.* **158,** 1–172.
18. Binns, M. M. & Smith, G. L. (1992) *Recombinant Poxviruses* (CRC, Boca Raton, FL).
19. Hahn, C. S., Hahn, Y. S., Braciale, T. J. & Rice, C. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 2679–2683.
20. Fynan, E. F., Webster, R. G., Fuller, D. H., Haynes, J. R., Santoro, J. C. & Robinson, H. L. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11478–11482.
21. Tang, D.-C., DeVit, M. & Johnston, S. A. (1992) *Nature (London)* **356,** 152–154.
22. Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, V. J., Gromkowski, S. H., Deck, R. R., DeWitt, C. M., Friedman, A., Hawe, L. A., Leander, K. R., Mertinez, D., Perry, H. C., Shiver, J. W., Montgomery, D. L. & Liu, M. A. (1993) *Science* **259,** 1745–1749.
23. Wang, B., Ugen, K. E., Srikantan, V., Agadjanyan, M. G., Dang, K., Refaeli, Y., Sato, A. I., Boyer, J., Williams, W. V. & Weiner, D. B. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4156–4160.
24. Schlesinger, S. & Schlesinger, M. J. (1990) in *Virology*, eds. Fields, B. N. & Knipe, D. M. (Raven, New York), 2nd Ed., pp. 697–711.
25. Liljeström, P. & Garoff, H. (1991) *Bio/Technology* **9,** 1356–1361.
26. Berglund, P., Sjöberg, M., Atkins, G. J., Sheahan, B. J., Garoff, H. & Liljeström, P. (1993) *Bio/Technology* **11,** 916–920.
27. Liljeström, P. (1994) *Curr. Opin. Biotechnol.* **5,** 495–500.
28. Weiss, B. G. & Schlesinger, S. (1991) *J. Virol.* **65,** 4017–4025.
29. Salminen, A., Wahlberg, J. M., Lobigs, M., Liljeström, P. & Garoff, H. (1992) *J. Cell Biol.* **116,** 349–357.
30. London, S. D., Schmaljohn, A. L., Dalrymple, J. M. & Rice, C. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 207–211.
31. Liljeström, P. & Garoff, H. (1994) in *Current Protocols in Molecular Biology*, eds., Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G. & Struhl, K. (Greene/Wiley Interscience, New York), pp. 16.20.1–16.20.16.
32. Rahemtulla, A., Fung-Leung, W. P., Schilham, M. W., Kündig, T. M., Sambhara, S. R., Narendran, A., Arabian, A., Wakeham, A., Paige, C. J., Zinkernagel, R. M., Miller, R. G. & Mak, T. W. (1991) *Nature (London)* **353,** 180–184.
33. Fung-Leung, W.-P., Schilham, M. W., Rahemtulla, A., Kündig, M. W., Vollenweider, M., Potter, J., van Ewijk, W. & Mak, T. W. (1991) *Cell* **65,** 443–449.
34. Fung-Leung, W.-P., Kündig, T. M., Zinkernagel, R. M. & Mak, T. W. (1991) *J. Exp. Med.* **174,** 1425–1429.
35. Schilham, M. W., Fung-Leung, W.-P., Rahemtulla, A., Kündig, T., Zhang, L., Potter, J., Miller, R. G., Hengartner, H. & Mak, T. W. (1993) *Eur. J. Immunol.* **23,** 1299–1304.
36. Townsend, A. R., Rothbard, J., Gotch, F. M., Bahadur, G., Wraith, D. & McMichael, A. J. (1986) *Cell* **44,** 959–968.
37. Rötzschke, O., Falk, K., Deres, K., Schild, H., Norda, M., Metzger, J., Jung, G. & Rammensee, H.-G. (1990) *Nature (London)* **348,** 252–254.
38. Kast, W. M., Offringa, R., Peters, P. J., Voordouw, A. C., Meloen, R. H., van der Eb, A. J. & Melief, C. J. M. (1989) *Cell* **59,** 573–577.
39. Wallny, H.-J., Rötzschke, O., Falk, K., Hämmerling, G. J. & Rammensee, H.-G. (1992) *Eur. J. Immunol.* **22,** 655–659.
40. Rhodes, G. H., Dwarki, V. J., Abai, A. M., Felgner, J., Felgner, P. L., Gromkovski, S. H. & Parker, S. E. (1993) *Vaccines 93*, eds. Ginsberg, H. S., Brown, F., Chanock, R. M. & Lerner, R. A. (Cold Spring Harbor Lab. Press, Plainview, NY), 137–141.
41. Huang, A. Y., Golumbek, P., Ahmadzadeh, M., Jaffee, E., Pardoll, D. & Levitsky, H. (1994) *Science* **264,** 961–965.
42. Staerz, U. D., Karasuyama, H. & Garner, A. M. (1987) *Nature (London)* **329,** 449–451.
43. Rock, K. L., Gamble, S. & Rothstein, L. (1990) *Science* **249,** 918–921.
44. Schirmbeck, R., Zerrahn, J., Kuhrober, A., Kury, A., Deppert, W. & Reimann, J. (1992) *Eur. J. Immunol.* **22,** 759–766.
45. Rock, K. L., Rothstein, L., Gamble, S. & Fleischacker, C. (1993) *J. Immunol.* **150,** 438–446.
46. Gray, D. (1993) *Annu. Rev. Immunol.* **11,** 49–77.
47. Mackay, C. R. (1993) *Adv. Immunol.* **53,** 217–265.
48. Sprent, J. (1994) *Cell* **76,** 315–322.
49. Tew, J. G., Kosco, M. H., Burton, G. F. & Szakai, A. K. (1990) *Immunol. Rev.* **117,** 185–211.
50. Müllbacher, A. (1994) *J. Exp. Med.* **179,** 317–321.
51. Hou, S., Hyland, L., Ryan, K. W., Portner, A. & Doherty, P. C. (1994) *Nature (London)* **369,** 652–654.
52. Lau, L. L., Jamieson, B. D., Somasundaram, T. & Ahmed, R. (1994) *Nature (London)* **369,** 648–652.
53. Allan, W., Tabi, Z., Cleary, A. & Doherty, P. C. (1990) *J. Immunol.* **144,** 3980–3986.
54. Bodmer, H., Obert, G., Chan, S., Benoist, C. & Mathis, D. (1993) *Eur. J. Immunol.* **23,** 1649–1654.
55. Locksley, R. M., Reiner, S. L., Hatam, F., Littman, D. R. & Killeen, N. (1993) *Science* **261,** 1448–1451.