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Immunization with Plasmid DNA Encoding for the Measles Virus Hemagglutinin and

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We have evaluated the DNA vaccination strategy for measles virus (MV) hemagglutinin (HA) and nucleoprotein (NP) genes. Plasmids encoding either the MV, HA, or NP proteins inoculated intramuscularly into Balb/c mice induced both humoral and CTL class I restricted responses. Antibody responses were not increased by multiple inoculations. The major antibody isotype induced by both the HA and NP was IgG2a consistent with a Th1 response. In contrast, immunization with a plasmid which directed the synthesis of a partially secreted form of HA gave mainly IgG1 antibodies. When the amount of DNA was reduced for the HA plasmid (1 or 10 μg/animal), although the antibody was not induced, a CTL response was observed.

INTRODUCTION

The attenuated measles vaccine has been used for more than three decades with a high degree of success in the West, but in the developing countries where measles infections occur at an earlier age, protection at these younger ages is difficult to achieve due to the presence of maternal antibody. One of the measures taken to try to overcome this problem has been to use a high-titer measles vaccine (Garenne *et al.,* 1991). However, although this resulted in higher sero-conversion rates, long-term mortality actually increased. Such observations underline our present lack of understanding of the immunobiology of measles infections.

To study which measles virus (MV) proteins contribute to protection against infection, cloned MV genes have been expressed using a number of vectors and the immune responses provoked by the individual proteins assessed. The two envelope glycoproteins, the hemagglutinin (HA) and fusion (F) proteins, both protect against infection (Drillien *et al.,* 1988). Similar results have been obtained after passive administration of monoclonal antibodies (anti-HA or anti-F), suggesting that the humoral response to these proteins contributes to protection (Giraudon and Wild, 1985; Malvoisin and Wild, 1990). In contrast, fewer studies have examined the cell-mediated immune response, although it has been shown that T-helper cells specific for several MV proteins protect rats against intracerebral challenge (Reich *et al.,* 1992).

In developing new vaccine strategies for measles, it will be necessary to determine which antigens give the optimum protection. Furthermore, the response to the individual antigens should be balanced so as to avoid possible vaccination problems as observed with the formaldehyde-inactivated and high-titer vaccines (Fulginiti *et al.,* 1967). Future vaccines should have an optimal antigen composition that also induces the correct Th1/Th2 balance.

The observation that inoculation of plasmid DNA expression vectors into the muscle of animals can induce an immune response to the encoded proteins showed the potential of this technology in the field of vaccinology (for review see Donnelly *et al.,* 1993). Using this system, individual viral proteins can be expressed and processed in conditions similar to these of the natural infection. As a first step to assessing its applicability to measles vaccination, we have immunized mice with DNA encoding the MV-HA and nucleoprotein (NP) genes and have studied the immune response. We show that for both viral proteins, humoral and cytotoxic T lymphocyte (CTL) responses were induced.

MATERIALS AND METHODS

Construction of vaccine DNAs

cDNAs encoding the MV-HA (Gerald *et al.,* 1986) and MV-NP (Buckland *et al.,* 1988) were subcloned into the *Bgl*II site of the plasmid pV1J (Montgomery *et al.,* 1993), which is driven by the CMV promoter. To facilitate these constructions, site-directed mutagenesis (Kunkel, 1985) was used to introduce *Bam*HI sites at the 5′ and 3′ ends of the MV-HA cDNA and *Bgl*II sites at the 5′ and 3′ ends of the MV-NP cDNA. Furthermore, site-directed mutagenesis was used to optimize the kozak sequences of the

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ATGs in both cDNAs in order to optimize the expression of the encoded proteins (Kozak, 1986).

Cells

CTL assays were performed in P815 (H-2^d) cells (which express MHC class I but not class II molecules) and derivatives of these cells expressing high levels of the MV antigens H and NP (P815-H and P815-NP, respectively) as described in Beauverger *et al.* (1993a). For ELISA assays, Ltk^- and Ltk H- or NP-expressing cells were used (Beauverger *et al.,* 1993b).

Mice

BALB/c (H-2^d) female mice were obtained from IFFA-CREDO (France). Five- to 6-week-old mice were immunized with 1 to 100 μg of DNA in 50 μl of PBS in the quadricep muscles (im). The mice were boosted at 3-week intervals.

Antibody assays

MV-neutralizing antibodies were measured using two-fold dilutions of serum against 25 PFU of virus in 96-well microtiter plates. Vero cells were used as indicator cells as previously described (Wild and Huppert, 1980). Antibody responses to the MV-HA and MV-NP proteins were measured by ELISA. Ltk^- cells constitutively expressing the HA or NP proteins (Beauverger *et al.,* 1993b) were grown in 96-well microtiter plates. Antisera dilutions were incubated with the intact cells (for HA) or acetone (80%) fixed cells (for NP) for 90 min at 37°. After the HA-antibody step, the complexes were fixed with a mixture of acetone/water (80:20) for 10 min at −20°C. The specific antibody response was developed with biotinylated anti-mouse IgG1, IgG2a, or IgG(γchain) and the streptavidine-phosphatase alkaline system (Sigma, France). Results are expressed with reference to control anti-HA or anti-NP antibodies which were used to standardize assays for IgG1 and IgG2a. Titers were calculated using the SOFTmax programme (Molecular Device Corp., Menlo Park, CA). Data are given as relative ELISA units.

Cytotoxic T-lymphocyte (CTL) assays

Spleens were removed from immunized animals and, after elimination of red blood cells by perfusion with DMEM, cocultivated with P815(H-2^d) cells expressing the corresponding MV protein (HA or NP) (Beauverger *et al.,* 1993b), which had been incubated with mitomicin C (25 μg/ml) for 30 min at 37°. The ratio of spleen to stimulator cells was 10:1. The cocultures (11×10^6 cells/well) were distributed in 24-well plates and incubated in DMEM-10% FCS containing 5×10^{-5} M-2-mercaptoethanol. On Day 5, half the medium was changed and the cytotoxic activity was tested on Day 7.

For the CTL assays P815, P815-H, or P815-NP cells were radiolabeled with 50 μCi ^{51}Cr for 90 min at 37°, washed twice in DMEM-1% FCS, incubated for 1 hr in DMEM-10% FCS, and washed once before using in a 4 hr ^{51}Cr release assay. The percentage of cell lysis was calculated as [(experimental c.p.m. − spontaneous c.p.m.)/(total c.p.m. − spontaneous release)] × 100. Non-specific percentage of lysis, obtained with P815 target cells was subtracted. Results are expressed as percentage of specific lysis. Spontaneous release and total release were determined from target cells incubated with medium alone or after the addition of 100 μl of 1 *M* HCl, respectively. Normally the background was less than 10%, but in exceptional cases, individual mice had up to 30% in 30/1 E/T ratio.

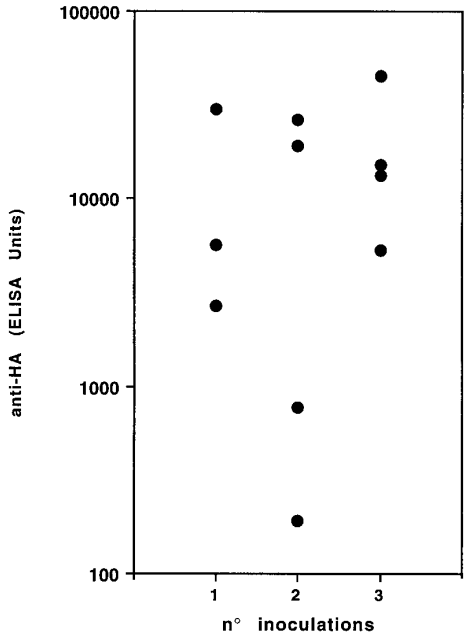

FIG. 1. Anti-MV HA antibody response as measured by ELISA in mice injected im with 100 μg of pV1J-HA. Sera were collected 3 weeks after the last immunization. Data represent individual animals. Sera from mice immunized with a control pV1J had a mean ± SD value of 127 ± 360 anti-HA ELISA units (n = 8).

RESULTS

Immune response to MV-HA DNA

The cDNA for the MV-HA (Gerald *et al.,* 1986) was subcloned into the vector pV1-J. Transfected into murine L cells, this gave a high level of expression of HA as measured by immunofluorescence (data not shown). To test the ability of pV1J-HA to induce an immune response, Balb/c mice were inoculated im 1–3 times at 3-week intervals with 100 μg of plasmid and the antibody levels measured by ELISA (Fig. 1). At the concentration of plasmid used (100 μg/mouse), high levels of antibody were obtained irrespective of the number of inoculations. In a small number of cases, mice failed to seroconvert.

Examination of the serum for MV neutralization showed titers up to 320 after a single inoculation of 100 μg of DNA (Table 1). Studies on the CTL activity of the immunized mice confirmed the results obtained for the humoral response, i.e., pV1J-HA induced good CTL responses even with a single inoculation (Fig. 2). When P815-NP cells were used as negative control targets, no cross-reactive lysis was observed, thus confirming the antigen specificity of the CTLs. Furthermore, no NK activity could be detected in these effectors, since they could not lyse the NK-sensitive YAC targets (data not shown). Measurement of antibody levels up to 8 $\frac{1}{2}$ months after immunization showed the antibody levels to be maintained throughout this period (Fig. 3).

In contrast to the results obtained after inoculating 100 μg of pV1J-HA, when lower doses were used (1, 10, or 50 μg/dose/mouse), even after three inoculations we failed to detect anti-HA antibodies. However, CTL activity was observed in the majority of cases (Fig. 4).

Targeting MV-HA to other tissues

To investigate if it would be advantageous to target antigens to other cells for immune responses, since muscle cells are not considered as antigen presenting cells, an HA protein was engineered in which the first ATG was in the middle of the transmembrane region. Transfection studies *in vitro* using this plasmid (pV1J-sol-HA) established that 10% of the total HA protein synthesized was excreted into the medium (data not shown).

Balb/c mice were immunized im with either the standard or pV1J-sol-HA plasmids and the antibody (Fig. 5) and CTL (Fig. 6) responses examined. Although the overall humoral and CTL responses were similar for the two HA molecules, there was a switch in the antibody isotype produced. The standard HA induced mainly an IgG2a response which is indicative of a Th1-type response, whereas the sol-HA induced IgG1 which is associated with a Th2 response.

TABLE 1

Induction of Measles Virus Neutralizing Antibodies after Intramuscular Inoculation of pV1J-HA

Plasmid	Mouse	Neutralizing titer
pV1J-HA	1	<20
	2	320
	3	160
	4	<20
	5	80
pV1J-sol-HA	6	<20
	7	20
	8	<20
	9	20
	10	<20

Note. Balb/c mice were immunized im with 100 μg of pV1J-HA DNA and the MV serum neutralizing antibodies were measured 4 weeks later.

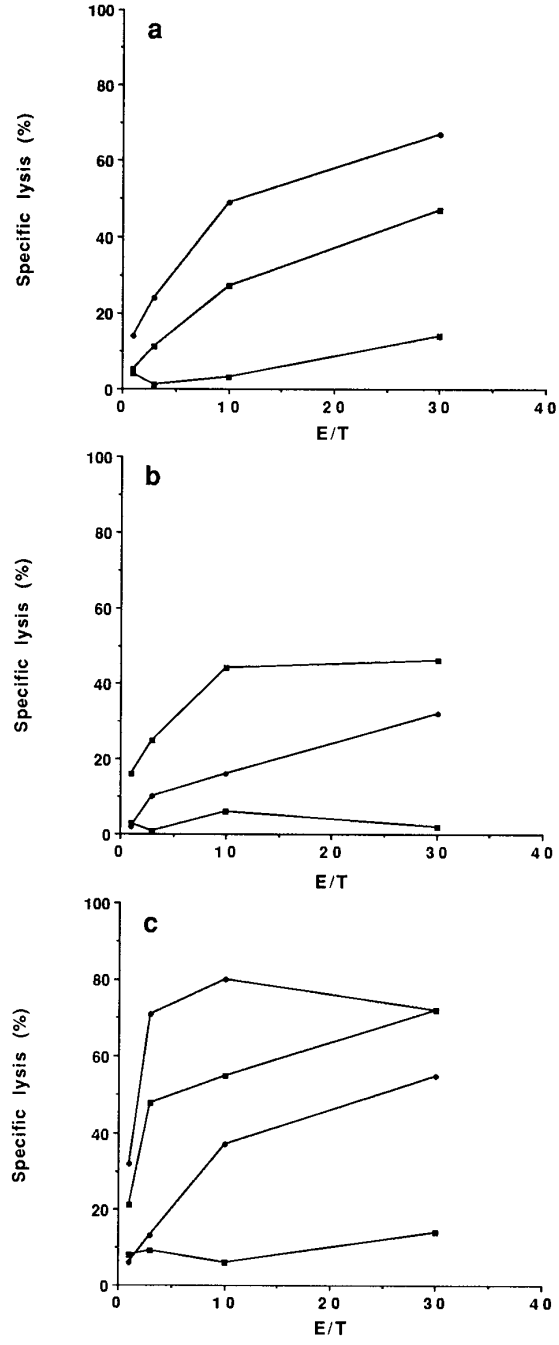

FIG. 2. Anti-MV HA CTL response after immunization with pV1J-HA. Balb/c mice were injected im with 100 μg of pV1J-HA once (a), twice (b), or three times (c) at 3-week intervals. The spleen cells were removed 3 weeks after the last immunization. After *in vitro* stimulation with P815-HA cells lysis was measured on P815-HA and P815 (control) cells. The results show the specific lysis of individual mice at graded E/T ratios.

Immune response to MV-NP DNA

The cDNA for MV-NP (Buckland *et al.,* 1988) was subcloned into the pV1J plasmid and after transfection of

murine L cells was shown to give a high level of expression of the MV protein (data not shown). To study the immune response, Balb/c mice were inoculated im 1–3 times with 100 μg of pV1J-NP. Similar to the HA study, a good humoral response was obtained after a single immunization (Fig. 7) and the antibody isotype produced was predominantly of the IgG2a class (Fig. 8). A strong CTL response was also obtained (Fig. 9). When P815-H cells were used as negative control target, no cross-reactive lysis was observed, thus confirming the antigen specificity of the CTLs. Furthermore, no NK activity could be detected in these effectors, since they could not lyse the NK-sensitive YAC targets (data not shown).

DISCUSSION

Natural infection by measles gives life-long immunity. The attenuated virus vaccine has now been used for three decades and although the antibody titers induced are lower than in natural infections, specific MV antibody has been detected more than 20 years after vaccination

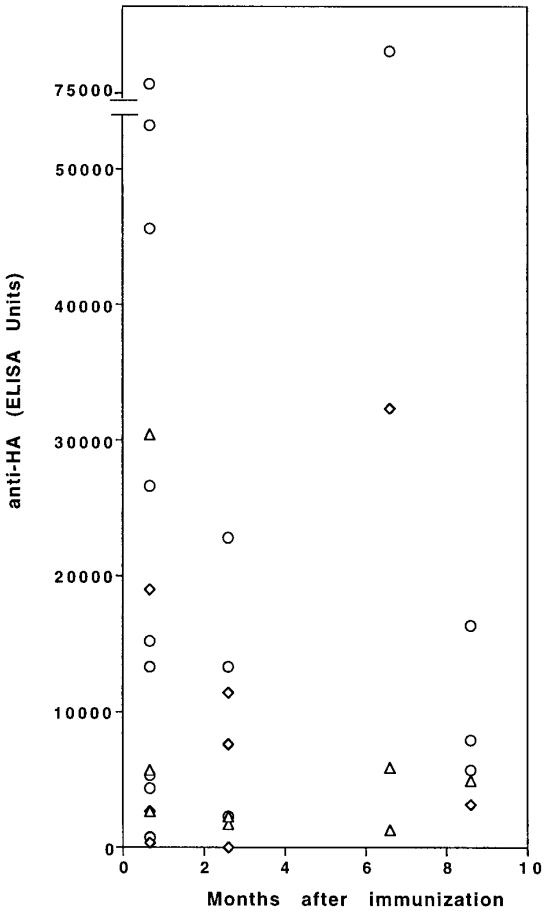

FIG. 3. Persistence of anti-MV HA antibodies after immunization with pV1J-HA. Balb/c mice were injected im with 100 μg pV1J-HA once (△), twice (◇), or three times (○) at 3-week intervals. The antibody response was measured by ELISA and the results are expressed for individual mice.

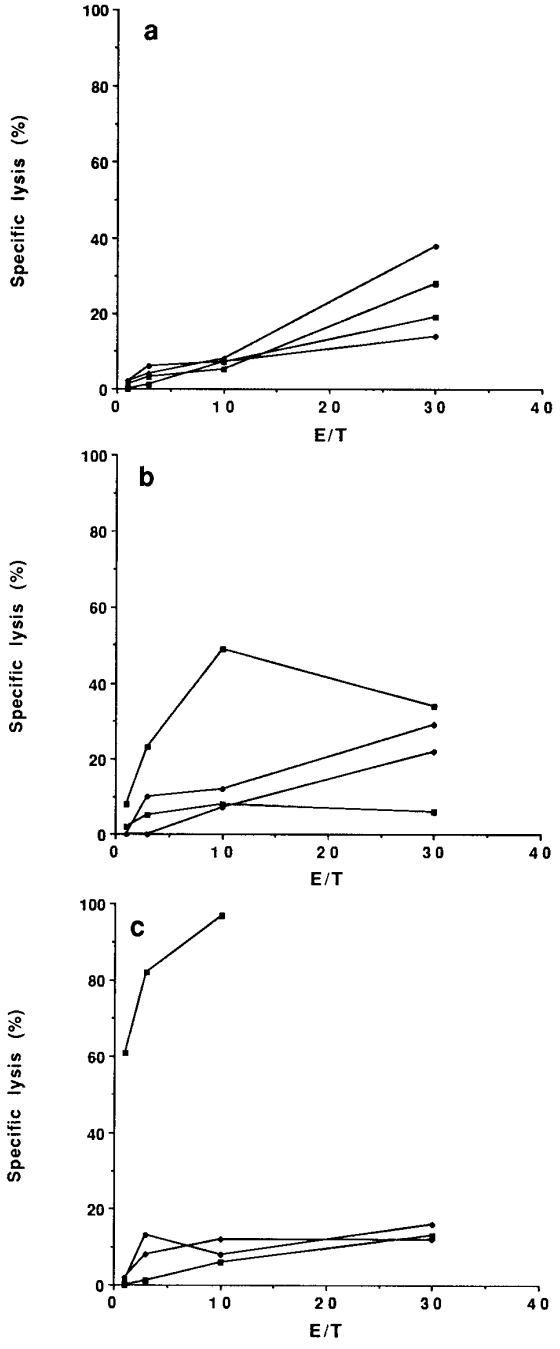

FIG. 4. Anti-MV HA CTL response in Balb/c mice injected im with 1 (a), 10 (b), or 50 μg (c) pV1J-HA. The spleens were removed 3 weeks after immunization. After *in vitro* stimulation with P815-HA cells lysis was measured on P815-HA and P815 (control) cells. Each curve shows the specific lysis of individual mice at graded E/T ratios.

(Miller, 1987). In the developing countries, children become infected at an earlier age, but the persisting level of maternal antibody reduces the efficiency of the vaccine. There is therefore, a need for a vaccine which can be used early in life and if it is a vectored vaccine, maternal antibodies to the vector should be absent. If DNA vaccines can express antigen over a period of time as has been reported for a construct encoding a reporter gene

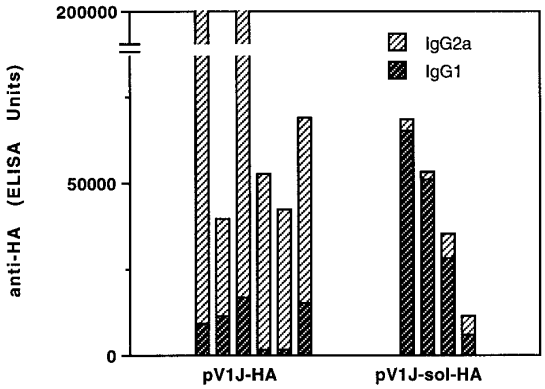

FIG. 5. Isotype of antibodies induced after immunization (im) with the standard pV1J-HA or a secreted version (pV1J-sol-HA). Balb/c mice were injected twice (100 μg DNA) at an interval of 3 weeks. Data represent individual animals. Sera from mice immunized with a control pV1J had mean $\pm$ SD values of 94 $\pm$ 220 (n = 10) and 192 $\pm$ 441 (n = 11) anti-HA ELISA units for IgG1 and IgG2a, respectively.

(Wolff *et al.,* 1990), a DNA vaccine may offer utility in infants who still have maternal antibodies to measles. Additionally, in view of this, we have examined the possibility of DNA vaccination as a means of inducing a balanced immune response.

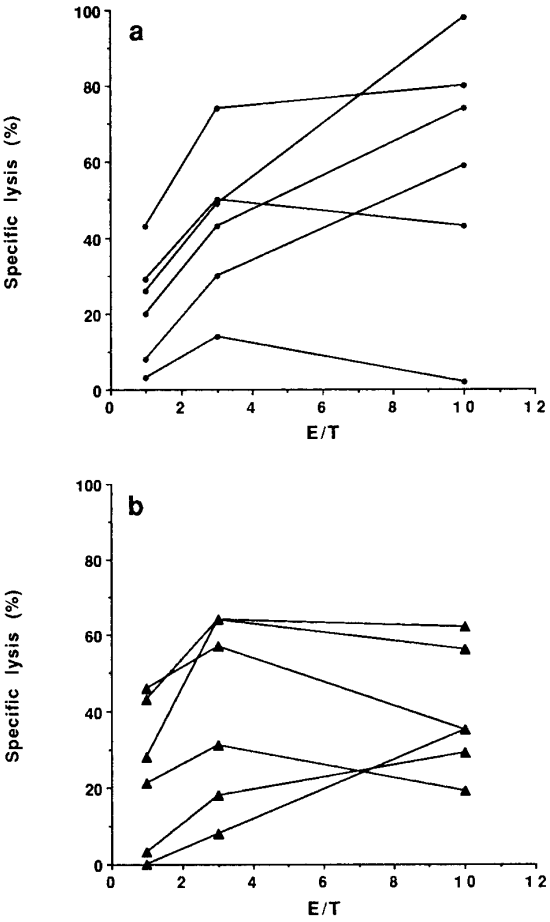

FIG. 6. Anti-MV HA CTL response in Balb/c mice immunized with pV1J-HA (a) or a secreted version (pV1J-sol-HA) (b). Immunization as described in the legend of Fig. 5. The results show the specific lysis of individual mice at graded E/T ratios.

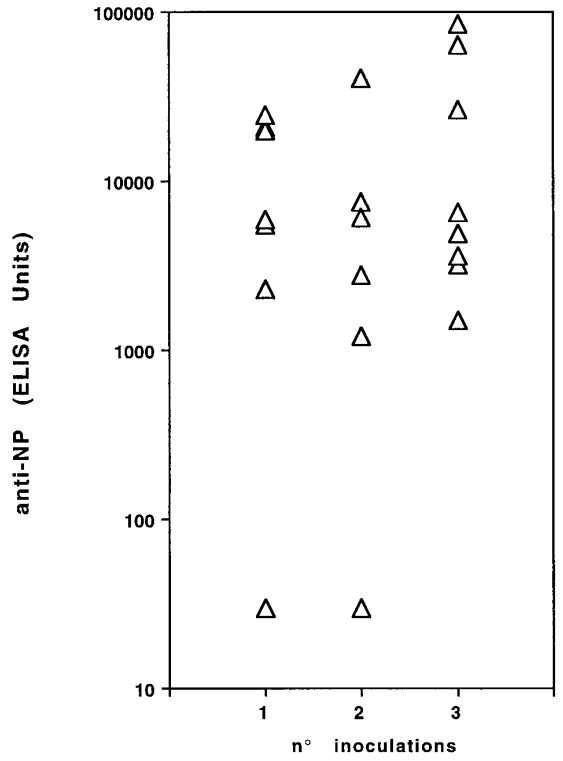

FIG. 7. Anti-MV NP antibody response as measured by ELISA in Balb/c mice injected im with 100 μg of pV1J-NP. Sera were collected 3 weeks after the last immunization. Data represent individual animals. Sera from mice immunized with a control pV1J had a mean $\pm$ SD value of 196 $\pm$ 237 anti-NP ELISA units (n = 8).

Protection against measles infection is observed after the passive transfer of γ-globulin to children. The specificity of this protection has been confirmed using anti-MV-HA and anti-MV-F monoclonal antibodies in a mouse model (Giraudon and Wild, 1985; Malvoisin and Wild, 1990). In contrast although there is no direct evidence for the involvement of cell-mediated immunity, children with T-cell deficiencies suffer complications in measles infections (Burnett, 1968). More recently, using a murine model we have shown that despite the inability of MV-specific CTLs to block infection, mice were protected from a lethal challenge (Beauverger *et al.,* 1996).

In the present study, MV genes expressed from a cytomegalovirus promoter in the plasmid pV1J induced both humoral and CTL responses. The HA and NP were chosen as previous studies established that immunization of Balb/c mice with vaccinia recombinants expressing these proteins induced both humoral and CTL immunity. Intramuscular inoculation of 100 μg of DNA of either pV1J-HA or pV1J-NP induced both humoral and CTL responses. The magnitude of the response was not increased by multiple inoculations when given during a 6-week period and there was no correlation between CTL and antibody level for individual animals. Even after a

single inoculation, both humoral and CTL responses could be detected more than 8 months later. In contrast, when lower quantities of DNA were inoculated (1 or 10 μg) only CTL responses could be detected.

In murine models, the antibody isotype induced is predominantly IgG2a with a 10- to 100-fold excess over IgG1 (Coutelier *et al.,* 1987). Compared to IgG1, IgG2a has the advantage that it can activate both the classical and the alternative pathways of complement fixation (Klaus *et al.,* 1979). It has been postulated that the antibody isotype is a reflection on the Th1/Th2 response. Interferon-γ which is associated with the Th1 cells has been shown to upregulate IgG2a production (Snapper and Paul, 1987). In contrast, IgG1 synthesis has been associated with Th2 cells (Mosmann and Coffmann, 1989). Intramuscular immunization with either HA or NP DNA induced IgG2a.

A number of hypotheses have been forwarded to explain the mechanism of the immune response following im inoculation. One possibility is that the antigen is released from the muscle cells and captured by antigen-presenting cells. To investigate this, we used a DNA which encodes an HA which is partially secreted from cells. The antibodies induced by this mixture of secreted and membrane bound proteins were of the IgG1 isotype and typical of a Th2 response. This would suggest that when at least a portion of the antigen is released from the cell, the antigen presentation may differ sufficiently to alter the type of immune response. The present study shows that intramuscular immunization with MV DNA induces an immune response fulfilling certain criteria required by a potential vaccine.

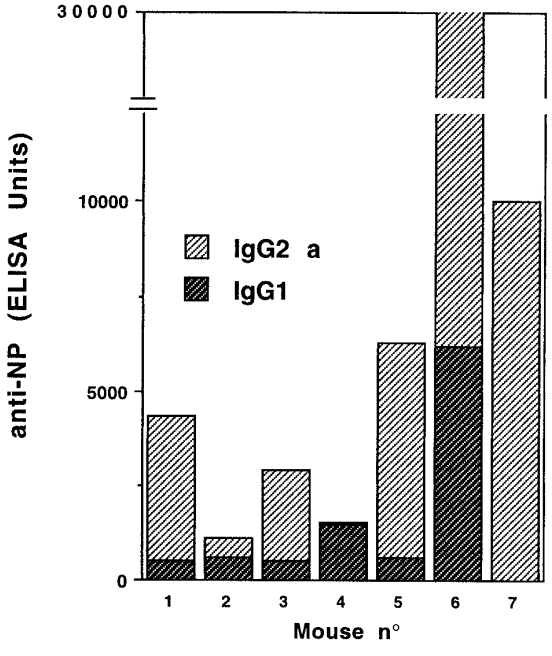

FIG. 8. Isotype of antibodies induced after immunization (im) with pV1J-NP. Balb/c mice were injected three times with 100 μg pV1J-NP. Each bar represents individual animals. Sera from mice immunized with a control pV1J had mean $\pm$ SD values of 147 $\pm$ 211 (n = 9) and 121 $\pm$ 196 (n = 10) anti-NP ELISA units for IgG1 and IgG2a, respectively.

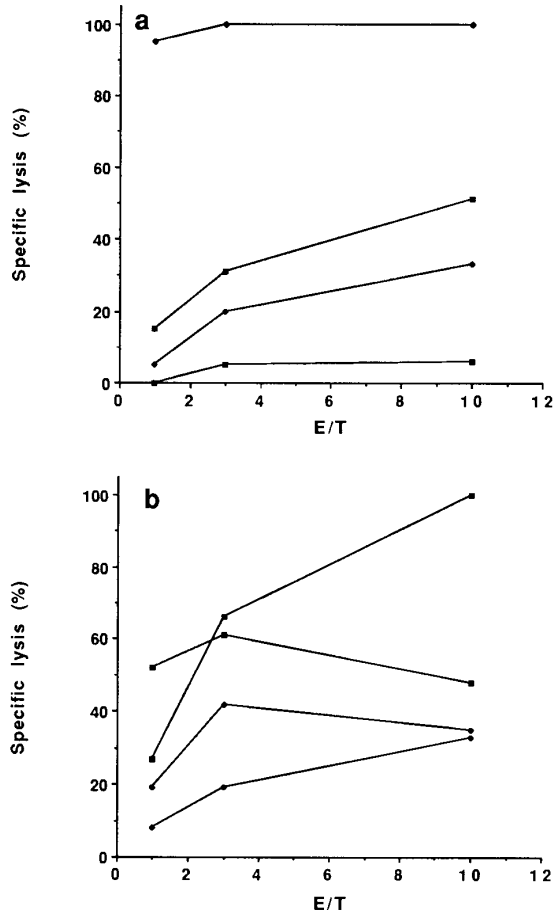

FIG. 9. Anti-MV NP CTL response in Balb/c mice injected im with 100 μg of pV1J-HA either once (a) or three times (b). The spleen cells were taken 9 weeks (1 inoculation) or 3 weeks (3 inoculations) after the last inoculation. After *in vitro* stimulation with P815-NP cells, lysis was measured on P815-NP and P815 (control) cells. The results show the specific lysis of individual mice at graded E/T ratios.

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