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Induction of Adult-like Antibody, Th1, and CTL Responses to Measles Hemagglutinin by Early Life Murine Immunization with An Attenuated Vaccinia-Derived NYVAC(K1L) Viral Vector

Jiri Kovarik,*[,1] Muriel Gaillard,* Xavier Martinez,* Paola Bozzotti,* Paul-Henri Lambert,* T. Fabian Wild,† and Claire-Anne Siegrist*

**World Health Organization Collaborating Centre for Neonatal Vaccinology, Departments of Pathology and Pediatrics, University of Geneva Medical School, Geneva, Switzerland; and †Inserm Unit 404, Immunity and Vaccination, CERVI, 69365 Lyon Cedex 07, France*

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Although initially developed in adult animals, novel viral vectors expressing recombinant measles antigens must eventually prove their success in the early life setting, where the efficacy of the currently used live-attenuated measles virus vaccine is limited. The immunological requirements for vaccine candidates include the generation of protective antibody responses as well as the induction of Th1 and cytotoxic T lymphocytes (CTL) responses, which is challenging in the neonatal setting. Here, we report that young BALB/c mice immunized with a single dose of a vaccinia-based NYVAC(K1L) vector generate adult-like antihemagglutinin (HA) antibody responses as well as adult-like Th1 and CTL responses. Despite this strong immunogenicity in early life, antibody responses (but not T-cell responses) to a single dose of NYVAC(K1L)-HA remained susceptible to inhibition by preexisting measles antibodies, calling for use of prime-boost strategies. NYVAC(K1L)-HA is the first attenuated live viral vector demonstrated as capable of inducing adult-like antibody, Th1, and CTL responses against measles in an early life murine immunization model, a capacity previously only reported for measles DNA vaccines.

Key Words: measles; vaccination; neonates; Th1; CTL; vaccinia vector; murine.

INTRODUCTION

Measles disease causes approximately 1.0 million deaths every year and its eradication remains a global challenge despite the introduction of live-attenuated measles vaccine in 1963 (Arvin, 2000). Although highly effective in children and adults, this vaccine is weakly immunogenic in infants less than the age of 6–9 months (Redd *et al.*, 1999). This limited efficacy was long considered to essentially result from inhibition of infant responses by persistent measles-specific maternal antibodies during the first months of life (Albrecht *et al.,* 1977). However, it was recently recognized that the immaturity of the infant immune system is responsible for weak or absent antibody responses to live-attenuated measles virus even in the absence of maternal antibodies (Gans *et al.,* 1998). As a consequence, infants younger than 12 months of age account for a significant fraction of measles-associated fatalities worldwide. As infant protection through herd immunity requires a very high level of measles vaccine coverage that may not be within reach of many countries, this disease burden justifies the attempts to develop a measles vaccine which could already be protective early in life.

Initial attempts to use Formalin-inactivated, alum-precipitated MV vaccines were abandoned due to the induction of short-lived immunity and increased risk for atypical measles disease upon exposure to wild-type virus, possibly through preferential induction of Th2-like responses (reviewed in Redd *et al.,* 1999). Increasing the titers of live-attenuated measles vaccines resulted in improved infant immunogenicity, but unexpected excess mortality in vaccinated girls precluded the implementation of this alternative vaccine strategy (Garenne *et al.,* 1991) (reviewed in Wild, 1999). Use of alternative strains of live-attenuated measles vaccines yielded some interesting results, although none appears capable of fully circumventing the limitations of early life responses to measles vaccine (Pabst *et al.,* 1999). Thus, efforts are ongoing to develop novel vaccines/immunization strategies that would be both safe and effective already very early in life.

In addition to DNA immunization (Martinez *et al.,* 1997; Polack *et al.,* 2000; Schlereth *et al.,* 2000a), a series of recombinant live viral vectors are under preclinical evaluation in various rodent and nonhuman primate models. This includes replication-deficient vectors, such as the

[1] To whom correspondence and reprint requests should be addressed at W.H.O. Collaborating Centre for Neonatal Vaccinology, Department of Pathology, C.M.U., Rue Michel Servet 1, CH-1211 Geneva 4, Switzerland. Fax: (+41)-22-702 57 46. E-mail: jiri.kovarik@medecine.unige.ch.

[2] *Abbreviations:* ALVAC, recombinant canarypox virus vector; HA, measles-hemagglutinin; MV, measles virus; MVS, measles vaccine (Schwarz strain); NYVAC, attenuated vaccinia virus vector; VV, vaccinia virus.

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ALVAC[2] canarypox (Taylor *et al.,* 1992), modified vaccinia virus Ankara (MVA) (Stittelaar *et al.,* 2000), or an engineered adenovirus vector (Fooks *et al.,* 1998), as well as replication-competent vectors such as vesicular stomatitis virus (VSV) (Schlereth *et al.,* 2000b) and human parainfluenza virus type 3 (PIV3) (Durbin *et al.,* 2000), considered for mucosal immunization. Most of these strategies are based on measles-hemagglutinin (MV-HA), which contains virus-neutralizing B- as well as T-cell epitopes, and is considered as an essential measles vaccine antigen (Tamin *et al.,* 1994). However, these novel live viral vectors developed for intended use in young infants have not yet been tested in early life models.

Murine models of neonatal immunization have recently proven useful to study the impact of immune immaturity on vaccine responses to tetanus, respiratory syncytial virus, and measles vaccine antigens (reviewed in Siegrist, 2000, 2001). As observed in infants, immunization with live-attenuated measles vaccine (MV-Schwarz, MVS) of 1-week-old BALB/c mice resulted in weaker antibody responses than those elicited later in life (Barrios *et al.,* 1996) and was susceptible to inhibition by maternal antibodies (Siegrist *et al.,* 1998a). Early life murine immunization was associated with limited Th1 responses, which was also recently reported in infant studies (Gans *et al.,* 1999), and to weak cytotoxic T lymphocytes (CTL) responses (Barrios *et al.,* 1996). Thus, this murine model appears useful to characterize factors limiting early life responses and strategies able to circumvent these limitations.

Based on numerous observations suggesting the central role of antigen-presenting cells (APC) in the shaping of early life T-cell responses, we postulated that a vaccine capable of replication within APC could result in a more optimal activation of neonatal APC than replication-defective vaccines. The vaccinia-based NYVAC strain was derived from the Copenhagen strain by the precise deletion of 18 open reading frames encoding functions implicated in the pathogenicity of Orthopoxviruses as well as host-range regulatory functions governing the replication competency of these viruses on cells derived from certain species, including human and mouse (Tartaglia *et al.,* 1992). By the reintroduction of the K1L host-range gene (Goebel *et al.,* 1990) into the NYVAC vector, the capacity of the virus to replicate in human and rodent cells is restored (Perkus *et al.*, 1989). Nevertheless, the absence of the thymidine kinase gene in the NYVAC(K1L) construct ascertains a highly reduced neurovirulence compared to the Western reserve (WR) vaccinia strain (Buller *et al.,* 1985; Wild *et al.,* 1992). In the present study we inserted the MV-HA gene (Wild *et al.,* 1992) under the control of the VV-modified early/late H6 promoter, into the NYVAC(K1L) vector to generate the NYVAC(K1L)-HA vaccine construct. This NYVAC(K1L) vector encoding for several different circumsporozoite proteins was reported able to protect BALB/c mice from challenge in the *Plasmodium berghei* rodent malaria model (Lanar *et al.,* 1996). In this report, we assessed the early life immunogenicity of the novel NYVAC(K1L)-HA construct, as compared to other MV-HA delivery systems, in our murine models of early life immunization.

RESULTS

Induction of adult-like CTL and Th1-like responses by early life immunization with NYVAC(K1L)-HA

BALB/c mice were immunized at 1 week of age or as adults with a single dose of NYVAC(K1L)-HA, or with ALVAC-HA (controls). Mice were sacrificed 3 weeks after immunization for evaluation of T-cell responses. MV-HA-specific CD8 responses were first assessed by measuring CTL activity following *in vitro* restimulation with the MV-HA 544–552 immunodominant CTL peptide (Barrios *et al.,* 1996). In adult mice, NYVAC(K1L)-HA and ALVAC-HA both induced considerable CTL responses, measured by lysis of MV-HA-transfected P815 targets cells (Fig. 1). Similarly strong CTL responses were elicited by a single dose of NYVAC(K1L)-HA given to 1-week-old mice (Fig. 1A), which contrasted with a lack of CTL induction following early life ALVAC-HA immunization (Fig. 1B). When the capacity of NYVAC(K1L)-HA to induce IFN-γ-secreting MV-HA CTL peptide-specific $CD8^+$ T cells was assessed by ELISPOT, similar high frequencies of antigen-specific IFN-γ-producing $CD8^+$ T cells were observed in mice immunized with NYVAC(K1L)-HA at 1 week of age or as adults (Fig. 2). This was again in contrast to immunization with ALVAC-HA, which only induced antigen-specific $CD8^+$ IFN-γ-secreting cells in adult, but not in infant mice (Fig. 2).

Analysis of CD4 T-cell responses to a single vaccine dose indicated significant production of IFN-γ and almost complete absence of IL-5 secretion by splenocytes of mice immunized at 1 week of age with NYVAC(K1L)-HA. Indeed, NYVAC(K1L)-HA immunization resulted in comparable cytokine secretion following adult or early life immunization, with similar IFN-γ levels (1836 ± 645 pg/ml in adults and 1353 ± 1213 pg/ml in 1-week-old mice) and no detectable IL-5 production in either age groups (Fig. 3 and data not shown). This again contrasted with the high levels of IL-5 observed following early life immunization with either MVS or ALVAC-HA (Fig. 3). Thus, in contrast to both MVS and ALVAC-HA, NYVAC(K1L)-HA immunization proved capable of inducing adult-like early life Th1, and CTL responses even in Th2-prone 1-week-old BALB/c mice.

Antibody responses to NYVAC(K1L)-HA following early life or adult immunization

Total IgG antibodies to MV-HA, measured by ELISA, indicated similar IgG antibody responses following early

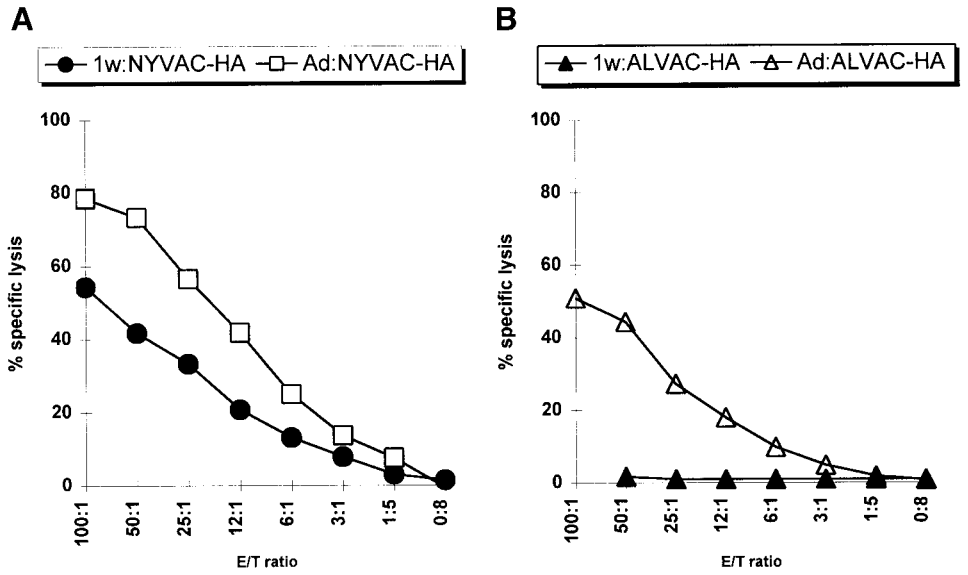

FIG. 1. Specific lysis of ^{51}Cr-labeled P815-HA target cells by spleen cells harvested from mice immunized 3 weeks previously with (A) NYVAC(K1L) or (B) ALVAC-HA. Spleen cells of mice immunized either at the age of 1 week (1w) or in adulthood (Ad) were restimulated for 7 days with the CTL-HA peptide (see Materials and Methods) prior to the incubation with P815-HA targets. Results shown are representative of three independent experiments.

life or adult immunization with NYVAC(K1L)-HA, both in terms of kinetics and in antibody titers (Fig. 4A). In mice primed at 1 week of age, IgG antibody responses were elicited earlier than following DNA immunization, but reached a plateau similar to those induced by MV-DNA-HA (Fig. 4B) or ALVAC-HA (Fig. 4C). As previously observed following MV-DNA-HA immunization of adult mice, antibodies elicited by NYVAC(K1L)-HA remained weaker than those induced by ALVAC-HA (Fig. 4C and Martinez *et al.* (1997)). NYVAC(K1L)-HA induced a similar IgG2a (2.4–2.5 $\log_{10}$)/IgG1 (1.8–2.1 $\log_{10}$) subclass distribution of MV-HA antibodies in both adult and young mice, as observed following DNA immunization (not shown) and in contrast to preferential induction of IgG1 antibodies in mice immunized at 1 week of age with either ALVAC-HA or MVS (Barrios *et al.,* 1996).

As antibody-mediated protection not only relies on induction of initial antibody responses but also on priming efficacy, mice primed at 1 week of age with NYVAC(K1L)-HA received a second dose of vaccine, either NYVAC(K1L)-HA or ALVAC-HA. Efficient early priming was demonstrated by the 10-fold or 100-fold increase of

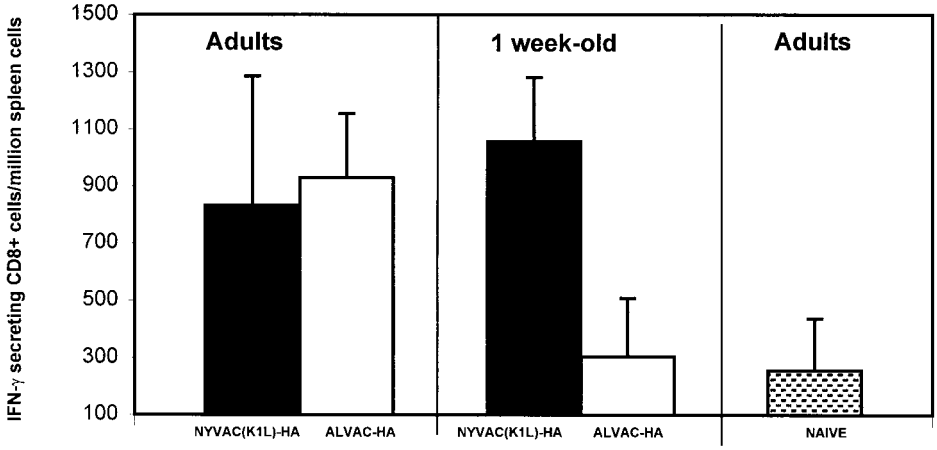

FIG. 2. Enumeration of CTL-HA peptide-specific CD8$^+$ T cells secreting IFN-γ by ELISPOT assay. Spleen cells of mice immunized with viral vectors either at 1 week of age or as adults were harvested 3 weeks postimmunization and restimulated for 7 days with the CTL-HA peptide. Defined numbers of cells were then incubated on anti-IFN-γ-coated nitrocellulose plates and the plates further processed to obtain specific spots, as described under Materials and Methods. Indicated are the mean number of spots per million spleen cells incubated for every experimental group ($\pm$SD), as detected from wells with various concentrations of effector cells (see Materials and Methods). Indicated also is the respective background value of naïve adult mice. Similar results were generated in a repeat experiment.

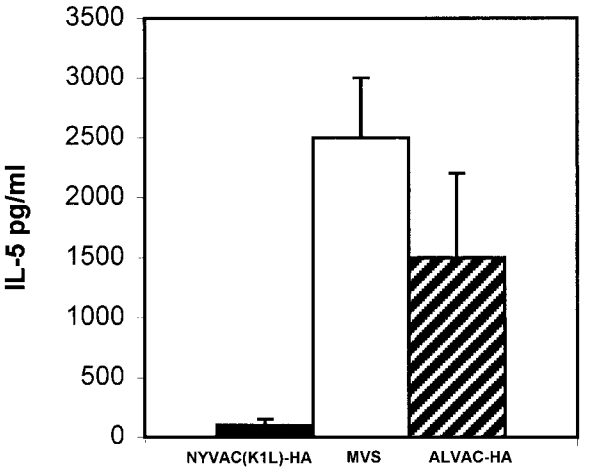

FIG. 3. IL-5 content of culture supernatants from splenocytes harvested from HA-immune mice restimulated with HA antigen for 72 h. One-week-old mice were immunized with NYVAC(K1L)-HA, ALVAC-HA, or MVS and sacrificed 3 weeks later to harvest their spleen cells. Cytokine levels were measured in culture supernatants from cells of individual mice (n = 5 to 8 mice per group) using capture ELISA. Results are expressed as means (±SD) for each experimental group. Results shown are representative of several (n = 3) independent experiments.

IgG antibodies following NYVAC(K1L)-HA or ALVAC-HA injection, respectively (Fig. 5). The relative increase of MV-HA IgG (Fig. 5), IgG1, and IgG2a (not shown) antibody titers was similar following boosting with either vaccine.

Influence of antibodies of maternal origin on antibody and T-cell responses to NYVAC(K1L)-HA

Given the capacity of NYVAC(K1L)-HA to induce adult-like early life Th1, CTL, and antibody responses, we asked whether it could also overcome the inhibition of antibody responses by preexisting measles-specific antibodies. Two-week-old BALB/c mice were thus immunized 48 h after passive ip transfer of serum from MVS immune adult females, a procedure previously demonstrated as mimicking transfer of antibodies of maternal origin to infant pups (Siegrist *et al.,* 1998a). The presence of relatively low titers of passively transferred HA-specific IgG (4.5 $\log_{10}$), measured by preimmunization bleeding, completely inhibited early life MV-HA antibody response to 1×10^6 PFU of NYVAC(K1L)-HA (Fig. 6A). Next, in the presence of even lower titers of passively transferred HA-specific IgG (<4.0 $\log_{10}$), a second immunization of NYVAC(K1L)-HA (1×10^6 PFU) was given 1 week after primary immunization, a strategy previously successfully applied to circumvent the inhibition of antibody responses mediated by passively transferred IgG in various immunization models including MVS (Martinez *et al.,* 1999; Siegrist *et al.,* 1998a, 1999). This early two-dose strategy did allow induction of antibody responses to HA, but only in four of seven pups and at a late time point (Fig. 6B).

To define whether the failure of NYVAC(K1L)-HA to induce MV-HA antibodies in the presence of passively transferred MV-HA antibodies was due to neutralization of the vaccine load or to another HA-specific immune mechanism, IgG responses to the NYVAC(K1L) vector were measured. Failure to induce MV-HA antibody responses in the presence of passively transferred MV-HA antibodies was correlated with the absent or very low antibody responses to the NYVAC(K1L) vector, both at early (day 12) and at late (day 97) time points after immunization (not shown). In contrast, significant anti-NYVAC(K1L) antibodies were present in pups that had escaped inhibition of MV-HA antibody responses by MV-HA antibodies following use of the early prime-boost strategy. Thus, passively transferred MV-HA antibodies inhibited induction of both anti-HA and anti-NYVAC(K1L) antibody responses. In contrast, a single dose of NYVAC(K1L)-HA elicited similarly strong CTL responses (Fig. 7) and CD4 T-cell-dependent IFN-γ secretion (1282 ± 530 vs 1742 ± 674 pg/ml (controls)) whether

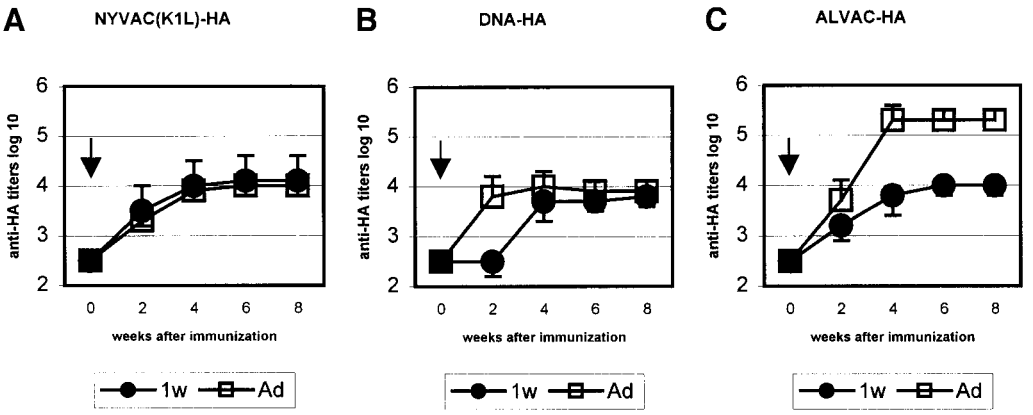

FIG. 4. Anti-HA IgG responses to immunization of 1-week-old mice with NYVAC(K1L)-HA (A), DNA-HA (B), or ALVAC-HA (C). Measured were total IgG titers in sera from individual mice bled at several time points postimmunization. Results are expressed as mean IgG ELISA titers per group (n = 6–8 mice) and SD in reference to a serum standard (see Materials and Methods).

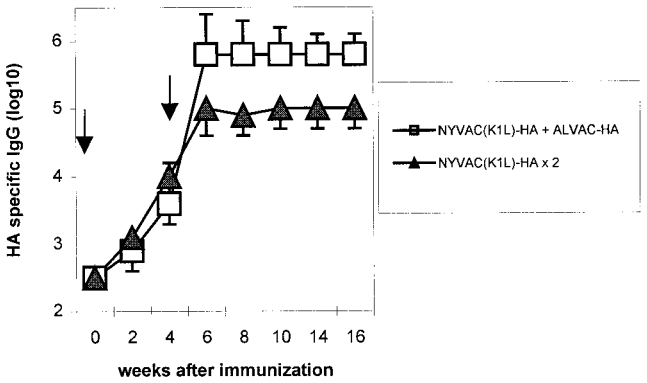

FIG. 5. Anti-HA IgG responses after primary immunization (day 0) of 1-week-old mice with NYVAC(K1L)-HA and secondary immunization (day 28) with either NYVAC(K1L)-HA or ALVAC-HA. Measured were total IgG titers in sera from individual mice bled at several time points post primary and secondary immunization. Results are expressed as mean IgG ELISA titers per group (n = 6–8 mice) and SD in reference to a serum standard (see Materials and Methods).

administered in the presence or the absence of passively transferred HA-specific IgG (4.1 $\log_{10}$). Thus, induction of CD8 and CD4 responses remained unaffected by the presence of HA-specific IgG at titers which completely inhibit the generation of infant mice antibody responses.

DISCUSSION

The challenge for a new measles vaccine is to be able to induce adult-like antibodies, Th1-like and CTL responses at an early time in life where the currently used live-attenuated vaccines are of insufficient immunogenicity (Gans *et al.,* 1998). We show here that this may be achieved in infant mice by immunization with a new viral vector, the vaccinia-derived NYVAC(K1L) vector. In contrast to immunization with live-attenuated MVS and live-recombinant ALVAC-HA, a single dose of NYVAC(K1L)-HA induced adult-like $CD8^+$ responses already in 1-week-old mice, an age previously experimentally defined as best correlating with the stage of immune maturation of human neonates (reviewed in Siegrist, 2000, 2001). These CD8 responses are associated with CD4 T cells secreting high levels of IFN-γ and no IL-5, both in adult and in young mice, despite the use of the Th2-prone BALB/c mouse strain. This exclusive Th1 pattern of early life measles responses contrasts with the preferential induction of Th2 early life responses to MVS or to ALVAC-HA (Barrios *et al.,* 1996).

The observation that two poxvirus-derived vaccines (ALVAC-HA and NYVAC(K1L)-HA) encoding the same measles antigen and showing similar T-cell responses in adult mice exhibit very distinct immunogenic properties in early life is of interest. Induction of adult-like neonatal Th1 and CTL responses is currently considered as dependent on the extent of neonatal APC activation, and thus of APC-T cell interactions (Adkins, 1999). The difference in the early life immunogenicity of ALVAC-HA and NYVAC(K1L)-HA could thus reflect differences in their tropism for neonatal vs adult APC. This may not be monitored *in vivo*, as no virus can be recovered from tissues of mice inoculated with strongly attenuated, nonvirulent vaccinia-derived vaccine strains (not shown).

Distinct early life immunogenicity patterns could, however, directly result from differences in the capacity of the two vectors to optimally activate neonatal APC. The capacity of single-step replicating viral vectors (Sendai TR-5 vaccine (Siegrist *et al.,* 1998b) and DISC herpes

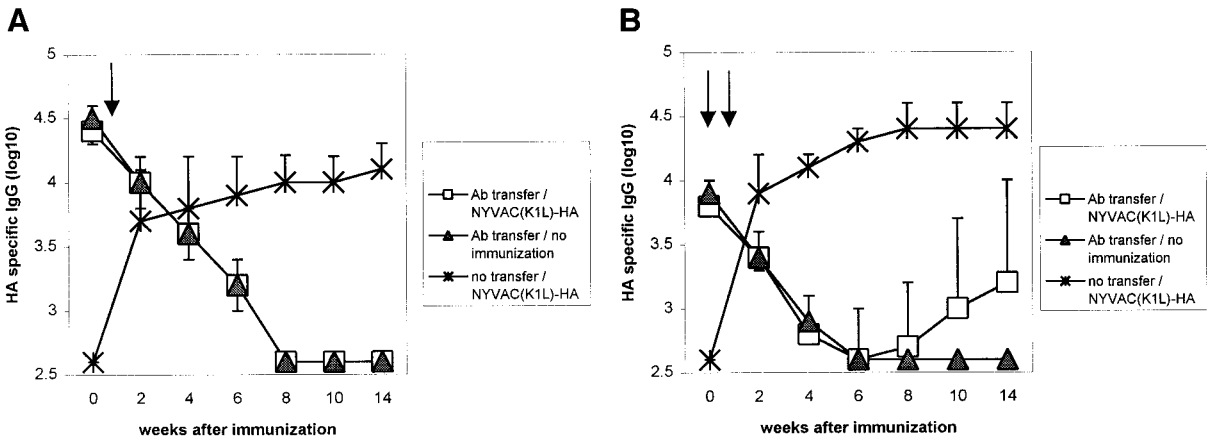

FIG. 6. Induction of anti-HA antibody responses to NYVAC(K1L)-HA immunization of 2-week-old mice in the presence of anti-HA IgG passively transferred 2 days prior to immunization. (A) Time course of anti-HA antibody responses to a single immunization of 1×10^6 PFU NYVAC(K1L)-HA in the presence of moderate titers of preexisting anti-HA IgG. (B) Time course of anti-HA antibody responses to two subsequent immunizations (day 7 and 14) of 1×10^6 PFU NYVAC(K1L)-HA in the presence of low titers of preexisting anti-HA IgG. The control groups for these experiments included mice that were passively transferred with anti-HA IgG but immunized with PBS only (natural decline of the transferred anti-HA IgG antibodies) and mice that did not receive anti-HA IgG prior to the immunization with NYVAC(K1L)-HA (positive controls). ELISA was used to measure titers in sera from individual mice bled at various time points after immunizations. Results are expressed as mean IgG ELISA titers per group (n = 6–8 mice) and SD in reference to a serum standard.

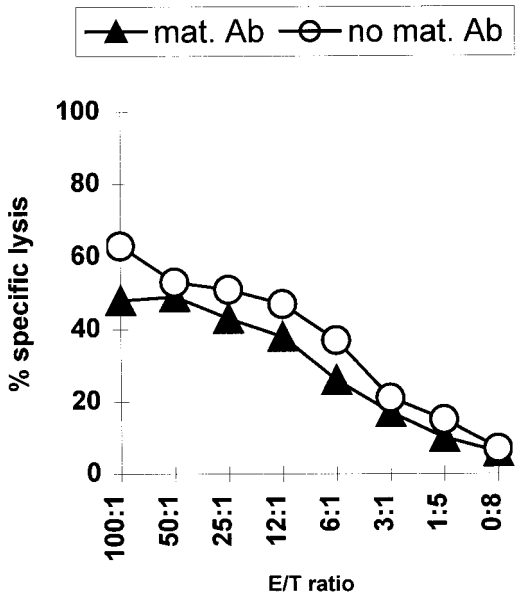

FIG. 7. Specific lysis of ^{51}Cr-labeled P815 target cells pulsed with CTL-HA peptide at different effector:target ratios. Spleen cells were harvested 3 weeks after immunization of 2-week-old mice with a single dose of NYVAC(K1L)-HA in the presence or absence of transferred HA-specific IgG (4.1 log10). Spleen cells of mice were restimulated for 7 days with the CTL-HA peptide as indicated under Materials and Methods.

vaccine (Franchini *et al.,* 2001)) to induce adult-like Th1 and CTL responses in neonatal mice supports this hypothesis, and viral replication is known to lead to strong IFN-α production and subsequent IL-18 up-regulation (Sareneva *et al.*, 2000).

The antibody response generated by NYVAC(K1L)-HA is strikingly similar to responses elicited by DNA-HA immunization: both delivery systems induced low but adult-like responses of similar kinetics, antibody titers, and isotype distribution regardless of age at immunization. The failure of NYVAC(K1L)-HA and of DNA-HA to induce higher antibody responses than those elicited in infant mice by live-attenuated MVS could be of concern given the importance of antibodies for protection against measles, and the limited capacity of human infant antibody responses even to MVS (Gans *et al.,* 1998). Importantly, however, strong antibody responses were elicited when NYVAC(K1L)-HA priming was followed by early boosting (Fig. 5), indicating that induction of antivector responses does not suppress vaccine uptake and immunogenicity. This was confirmed by demonstrating that priming with a NYVAC(K1L)-NP vaccine did not affect subsequent responses to NYVAC(K1L)-HA (data not shown). As previously observed following DNA priming (Martinez *et al.,* 1999), use of heterologous prime-boost strategies (Fig. 5) may elicit yet higher antibody responses.

Despite the capacity of NYVAC(K1L)-HA to induce adult-like antibody, Th1, and CTL responses in young mice, it remains susceptible to inhibition by passively transferred antibodies of maternal origin. These results are in accordance with results obtained in adult mice as well as in adult monkeys using the HA-expressing VV recombinant derived from the Copenhagen strain (Galletti *et al*., 1995; van Binnendijk *et al.,* 1997), as well as in other experimental models using vaccinia-derived vectors (Murphy *et al.,* 1988). Inhibition of anti-HA responses by passively transferred MV-HA antibodies was associated with inhibition of antibody responses to the NYVAC(K1L) vector, suggesting neutralization of the NYVAC(K1L)-HA vaccine and preventing *in vivo* replication. However, the unaffected induction of HA-specific CD4 and CTL responses indicates that NYVAC(K1L)-HA replication did occur at levels sufficient to allow antigen presentation at the APC surface and T-cell activation. The amount of viral antigen sufficient for APC-T cell activation may thus be significantly lower than the one required for B-cell activation. The observation that maternal antibodies may inhibit infant antibody responses to a novel vaccine candidate, be it NYVAC(K1L)-HA or DNA (Siegrist *et al.,* 1998a), should thus not lead to the conclusion that such a vaccine cannot be used in early life. CTL responses in mice (Galletti *et al*., 1995) and proliferative T-cell responses in monkeys (van Binnendijk *et al.,* 1997) to HA delivered by VV recombinants of the Copenhagen strain were not inhibited in the presence of passively transferred anti-MV polyclonal serum. Moreover, maternal antibodies did not inhibit T-cell vaccine responses to conventional, canarypox, or DNA vaccines in mice (Siegrist *et al.,* 1998a), monkeys (Stittelaar *et al.,* 2000), and humans (Gans *et al.,* 1999), even if some reduction of CTL activity was recently reported in infant monkeys that received measles-specific immune globulin prior to vaccination with MVA or WR vaccines expressing MV-HA and MV-fusion proteins (Zhu *et al.,* 2000). Measles-specific T cells are considered as capable of limiting disease severity should early exposure occur. Thus, to immunize very early in life, so as to initiate T-cell responses as early as possible, and to rely on an early prime-boost approach to increase antibody responses and to circumvent maternal antibody-mediated inhibition of antibody responses appears as the most promising vaccination strategy. Here, a second dose of NYVAC(K1L)-HA given as early as 1 week after priming circumvented passive antibody-mediated inhibition and allowed antibody responses in 70% of infant mice.

Although mice cannot be used for measles respiratory challenge studies, they have proven valuable models for measuring the immunogenicity of measles vaccines, demonstrating similar comparative immunogenicity profiles of live-attenuated MVS and pox-derived vectors as cotton rats and monkeys (Barrios *et al.,* 1996; Stittelaar *et al.,* 2000; Wyde *et al.,* 2000). Despite the risk of overinterpreting the infant mouse model, the results continue to

show consistency and appear to correlate with the recently described nature of human infant responses to measles vaccine (Gans *et al.,* 1999). Whether the infant mouse model may also be useful to compare the neonatal safety of the novel measles vaccine candidates in various prime-boost combinations, or whether this will have to be addressed directly in nonhuman infant primates, is now open for studies.

MATERIALS AND METHODS

Mice

Specific pathogen-free adult BALB/c inbred mice were purchased from Biological Research Laboratories (Füllinsdorf, Switzerland) and kept under specific pathogen-free conditions. Breeding cages were checked daily for births. Pups were kept with mothers until weaning at the age of 4 weeks. Adult mice were used at 8–12 weeks of age.

Vaccines and immunizations

Recombinant VVs expressing MV-HA were constructed by using the host range selection system described by Perkus *et al.* (1989). The VV-attenuated NYVAC (vP866) strain was originally derived from the Copenhagen strain by the precise deletion of 18 open reading frames (Tartaglia *et al.,* 1992). To generate NYVAC(K1L)-HA, the MV-HA gene (Wild *et al.,* 1992) was inserted into the plasmid containing the vaccinia-K1L gene and the modified vaccinia early/late H6 promoter (Goebel *et al.,* 1990; Lecouturier *et al.,* 1996). The K1L gene, H6 promoter, and polylinker region are located in this construct within flanking Copenhagen vaccinia arms, replacing the ATI region (ORFs A25L, A26L) (Goebel *et al.,* 1990). For immunizations, NYVAC(K1L)-HA was used at 1×10^5 PFU per mouse (1-week-old mice) or at 1×10^6 PFU per mouse (adults) unless indicated otherwise in the text. The immunization of 1-week-old mice with 1×10^5 PFU or 1×10^6 PFU generated similar T-cell and antibody responses, whereas 1×10^6 PFU was required to induce substantial antibody responses in adult mice (data not shown). Live-attenuated measles virus (Schwarz strain (MVS), 5×10^5 CCID50 per mouse) was obtained from Pasteur Mérieux Connaught, Marcy l'Etoile, France. Live-recombinant canarypox virus expressing the measles virus HA (vCp85, ALVAC-HA, 5×10^7 PFU per dose) (Taylor *et al.,* 1992) was generously provided by Dr. J. Tartaglia, Virogenetics Inc., Troy, NY. These vaccines were injected ip in a total volume of 100–200 μl. The DNA vaccine encoding the membrane-bound MV-HA subcloned into the pV1J plasmid was characterized previously (Martinez *et al.,* 1997). The DNA plasmid was injected im in each quadricep at a total dose of 100 μg in a volume of 25 μl. In experiments with passively transferred anti-HA antibodies, 2-week-old mice were immunized 48 h after ip transfer of 200 μl of immune serum raised by repeat MVS immunization of adult mice. This was previously demonstrated to result in titers of MV-HA antibodies similar to those obtained in pups of immune mothers (Siegrist *et al.,* 1998a). To achieve lower MV-HA-specific titers prior to immunization, MVS immune serum was diluted in PBS prior to ip transfer.

Quantification of vaccine-specific antibodies

Mice were bled at regular intervals for the determination of vaccine-specific serum antibodies. Serum MV-HA antibodies were measured by using plates coated with Ltk-HA-transfected fibroblasts (Barrios *et al.,* 1996). Incubations were performed with serial serum dilution starting at 1/100. After washing, the relevant isotype-specific peroxidase-conjugated goat or rabbit anti-mouse antibody (Zymed Laboratories Inc., San Francisco, CA) was incubated for 2 h at 37°C prior to washing, incubation with ABTS substrate, and reading. The results were generated by reference to serial dilution of an out-titrated serum pool from measles-HA-immunized adult mice. Antibody titers below the cutoff of the assay were given an arbitrary titer of one-half the cutoff to allow calculation of geometric mean antibody titers. To detect antibodies against the viral vector NYVAC(K1L) in mice immunized with NYVAC(K1L)-HA, 96-well plates were coated with 0.5×10^7 PFU/ml of NYVAC(K1L)-NP (50 μl per well) at 4°C overnight. After washing, blocking was performed with 1% BSA in PBS/0.05% Tween 20 for 1 h at 37°C. Incubations were performed with serial serum dilutions starting at 1/50. After washing, peroxidase-conjugated goat or rabbit anti-mouse total IgG antibody (Zymed Laboratories Inc.) was incubated for 2 h at 37°C prior to washing, incubation with substrate, and reading.

Quantification of lymphokines in supernatants of *in vitro* cultures

Spleens were harvested 3 weeks after immunization. Splenocytes were incubated in a humidified incubator in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) at 37°C in 5% CO_2, with 1.5 mM L-glutamine, 50 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 mg/ml streptomycin, essentially as previously described (Barrios *et al.,* 1996). Cells were cultured in 24-well plates and coincubated with vaccine antigens (MVS) or with DMEM–10% FCS only (control wells). Cell supernatants were collected after 48 or 72 h to measure IL-5 and IFN-γ contents by capture-ELISA (Barrios *et al.,* 1996). Values for IL-5 and IFN-γ were expressed by reference to a standard curve constructed by assaying serial dilution of the respective mouse cytokines. Values below the cutoff of the assay (IL-5: 25 pg/ml; IFN-γ: 80 pg/ml) were given the concentration of one-half the cutoff. Antigen-specific cytokine secretion was obtained by

subtracting the cytokine content of the supernatant from splenocytes incubated with DMEM–10% FCS alone.

Determination of CTL using ^{51}Cr-release assay

Splenocytes were harvested 3–4 weeks after immunization. Identical numbers of splenocytes from immunized mice were pooled and cultured as bulk as described (Barrios *et al.,* 1996). Cells were cultured in DMEM or RPMI with 10% FCS containing 20 μg/ml MV-HA 544–552 CTL peptide (Barrios *et al.,* 1996) and the cytolysis assay was performed on day 7 of culture. Varying numbers of effector cells were added to ^{51}Cr-labeled, HA-transfected, MV-HA CTL peptide pulsed or nonpulsed (control) P815 target cells (5×10^3 per well). After 5 h of incubation at 37°C, cell supernatants were harvested for the quantification of ^{51}Cr content using in a Minaxi 5000 gamma counter (Packard). The percentage of specific lysis was calculated as [(experimental c.p.m. − spontaneous c.p.m.)/(total c.p.m. spontaneous c.p.m.)] × 100. Spontaneous release and total release were determined from target cells incubated with medium alone and after the addition of 100 μl of 1 M HCl, respectively.

ELISPOT assay of HA-specific CD8$^+$ cells producing IFN-γ

This assay for the detection of CTL epitope-specific IFN-γ-secreting T cells was adapted from Miyahira *et al.* (1995). Splenocytes from immunized mice were pooled per group and cells were grown in bulk cultures in DMEM/10% FCS containing 20 μg/ml MV-HA 544–552 CTL peptide (Barrios *et al.,* 1996). The ELISPOT assay was performed on day 7 of culture. Multiscreen 96-well nitrocellulose plates (Millipore, Molsheim, France) were coated overnight with 10 μg/ml rat anti-mouse IFN-γ monoclonal antibody (clone R46A2) (Pharmingen, San Diego, CA). Cells were washed and placed at various dilutions into the wells containing complete medium supplemented with 30 U/ml IL-2 (EL-4 supernatant). Then, 10^5 irradiated (8000 Rad), MHC class II negative P815 cells expressing HA or P815 control cells were added as APC to each well and the plates were incubated for 24 h at 37°C and 5% CO_2. The plates were then washed and incubated overnight at 4°C together with 5 μg/ml biotinylated anti-mouse IFN-γ antibody (clone XMG) (Pharmingen), washed, and then incubated with peroxidase-conjugated ExtrAvidin (Sigma, St. Louis, MO). Spots were developed by adding freshly prepared substrate buffer (0.3 mg/ml of 3-amino-9-ethyl-carbazole and 0.03% H_2O_2 in 0.1 M sodium acetate (pH 4.8)). The number of spots (per well) was counted using the KS ELISPOT Reader System (Zeiss, Hallbergmoos, Germany) and expressed as spots/million spleen cells/experimental group.

Statistical analysis

Significant analysis between results obtained from various groups of mice was performed by using the Mann–Whitney U test. Probability values > 0.05 were considered insignificant.

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