Shared modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine viruses

Igor M. Belyakov*†, Patricia Earl‡, Amiran Dzutsev*, Vladimir A. Kuznetsov§, Michael Lemon*, Linda S. Wyatt‡, James T. Snyder*, Jeffrey D. Ahlers*, Genoveffa Franchini¶, Bernard Moss‡, and Jay A. Berzofsky*

*Molecular Immunogenetics and Vaccine Research Section, Metabolism Branch, and ¶Basic Research Laboratory, National Cancer Institute, ‡Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, and §Laboratory of Integrative and Medical Biophysics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

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The concern about bioterrorism with smallpox has raised the possibility of widespread vaccination, but the greater prevalence of immunocompromised individuals today requires a safer vaccine, and the mechanisms of protection are not well understood. Here we show that, at sufficient doses, the protection provided by both modified vaccinia Ankara and NYVAC replication-deficient vaccinia viruses, safe in immunocompromised animals, was equivalent to that of the licensed Wyeth vaccine strain against a pathogenic vaccinia virus intranasal challenge of mice. A similar variety and pattern of immune responses were involved in protection induced by modified vaccinia Ankara and Wyeth viruses. For both, antibody was essential to protect against disease, whereas neither effector CD4$^+$ nor CD8$^+$ T cells were necessary or sufficient. However, in the absence of antibody, T cells were necessary and sufficient for survival and recovery. Also, T cells played a greater role in control of sublethal infection in unimmunized animals. These properties, shared with the existing smallpox vaccine, provide a basis for further evaluation of these replication-deficient vaccinia viruses as safer vaccines against smallpox or against complications from vaccinia virus.

The licensed smallpox vaccine currently in use in the United States, Dryvax, is burdened by a risk of adverse effects and even some mortality (1), and it is considered very risky to immunize the population that is immunocompromised by AIDS, chemotherapy for cancer, or immunosuppression following organ transplant. Such immunosuppressed individuals were much less prevalent before the cessation of universal smallpox vaccination in 1972, making the potential morbidity and mortality rates from vaccination likely to be higher today than during the smallpox eradication campaign. Therefore, a second-generation smallpox vaccine that can be used in the whole population without serious side effects is needed today. The urgency for development of a new more effective vaccine against smallpox is increased today because of the concern about bioterrorism (2). A number of more attenuated vaccinia virus-derived strains have been studied, including modified vaccinia Ankara (MVA), attenuated by passage in chicken cells (3–6); NYVAC, attenuated by deletion of some nonessential genes (7); LC16m8, attenuated by multiple passage through primary rabbit kidney cells at low temperature (8); and attenuated vaccinia virus CVI-78, passaged in chick embryonic tissue (9). Most of these have not been compared side-by-side with each other or with the Wyeth strain used in the licensed Dryvax vaccine. Furthermore, none can be tested directly for efficacy against smallpox, because challenge studies with variola virus in humans cannot ethically be performed, so the efficacy of candidate vaccines can be predicted only by surrogate indicators, such as the level of different immune responses induced and their ability to protect against other orthopoxviruses in animal models. Intranasal (i.n.) infection of the mouse with pathogenic vaccinia virus provides a small animal model well suited for evaluating mechanisms of protection (10). In addition, we have prior experience with MVA and NYVAC as recombinant vaccine vectors in animal studies (4, 11–14). Therefore, we have undertaken to compare MVA, NYVAC, and Wyeth strains of vaccinia virus in a mouse model in which the animals are challenged via the respiratory route (the natural route of smallpox transmission) for protection against a lethal dose of pathogenic vaccinia virus, and also to examine the immunological mechanism of protection to determine whether a replication-defective virus such as MVA will protect by the same types of immune response as the replication-competent Wyeth vaccine strain.

The mechanism of immune protection against smallpox is not completely understood, in part because immunology was in its infancy when the smallpox eradication campaign was completed and routine smallpox vaccination ended (15). Both cellular and humoral immunity (virus-specific antibody) have been thought to play a role in protection against orthopoxviruses. This view is based in part on experience with poor control of vaccinia virus infection in individuals with either humoral or cellular immune defects (16), in part on the known efficacy of vaccinia-immune globulin (17), and in part on studies in mice showing a key role for IFN-γ, probably derived from T cells, in control of vaccinia and ectromelia virus infections (18–21). Several studies have mapped proteins important for the elicitation of neutralizing antibodies (22–27). Also, in a recent trial of dilutions of the licensed smallpox vaccine in human volunteers, formation of a vesicle, indicative of virus replication, was strongly correlated with development of both production of specific antibodies and induction of cytotoxic T lymphocyte (CTL) and IFN-γ T cell responses (28). However, the mechanism of protection against vaccinia virus has not been systematically studied by currently available techniques. In particular, the role of CD8$^+$ CTL and virus-specific antibodies for protection in vaccinia virus-immunized animals and in natural resistance to infection by pathogenic vaccinia virus is poorly characterized. However, it is extremely important to know the mechanism of protective immunity to develop the most effective vaccine against smallpox.

Here, we show that neither CD4 nor CD8 effector T cells were necessary or sufficient to protect mice against disease caused by pathogenic vaccinia virus, whereas an antibody response (which may depend on CD4$^+$ T cell help for its induction) was sufficient. However, antigen-specific CD4 and CD8 cells prevented mortality of infected immunized animals, and T cell immunity was important for natural resistance against vaccinia virus in unimmunized animals. The same types of responses contributed to protection by both MVA and Wyeth strains of vaccinia virus, making comparison of these by surrogate immune response

Abbreviations: MVA, modified vaccinia Ankara; WR, Western reserve; CTL, cytotoxic T lymphocyte; pfu, plaque-forming unit; ELISPOT, enzyme-linked immunospot; NKT cell, natural killer T cell; i.n., intranasal(ly).

†To whom correspondence should be addressed. E-mail: belyakov@mail.nih.gov.

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markers feasible. The results of this study can help in the development of a better vaccine against smallpox.

Methods

Viruses. Vaccinia virus Western reserve (WR) strain is available from the American Type Culture Collection. The Wyeth New York City Board of Health strain of vaccinia virus was obtained from Wyeth Ayerst Laboratories (Marietta, PA). Both were grown in HeLa cells and titered in BS-C-1 cells. Vaccinia virus strain MVA, obtained from Gerd Sutter (GSF; Institute of Molecular Virology, Munich), was propagated and titered in chicken embryo fibroblast cells. NYVAC was a gift of J. Tartaglia (Aventis-Pasteur, Willowdale, Canada) (7). vSC8 is a recombinant thymidine kinase negative WR strain-based vaccinia virus expressing β-galactosidase (29).

Mice, Immunization, and Challenge. Female BALB/c mice (6–10 weeks old) were purchased from Frederick Cancer Research Facility (Frederick, MD). The J_H knockout mouse (on the BALB/c background) carries a target deletion of the J_H locus, such that mice are homozygous for the absence of all four J_H gene segments, resulting in cells that cannot produce a complete, recombined variable region of the heavy chain and are therefore B cell-deficient (Taconic Farms). CD1 $KO^{-/-}$ mice from M. Grusby (30) were a gift of William E. Paul (National Institute of Allergy and Infectious Diseases). For protection studies, BALB/c mice, B cell-deficient mice, and CD1 $KO^{-/-}$ mice were immunized with different doses of MVA i.m. or i.n. One month after immunization, mice were challenged i.n. with 10^6 plaque-forming units (pfu) of WR, and individual body weight was measured daily. Mice with weight loss >25% were required to be killed, generally necessitating termination of the experiments around day 8, when the control group reached this level. Depletion of $CD8^+$ or $CD4^+$ cells was done by i.p. treatment with monoclonal antibody daily for 4 days (clone 2.43; 0.5 mg per mouse per day for CD8 and GK 1.5 ascites, 1 mg per mouse per day for CD4). Depletion was verified by FACScan (Becton Dickinson) analysis of peripheral blood cells to be >98% depleted.

IFN-γ Enzyme-Linked Immunospot (ELISPOT). ELISPOT plates (Millipore) were precoated overnight with anti-IFN-γ antibody (MABTECH, Stockholm). Target cells (P815, a DBA/2 mastocytoma expressing class I but not class II H-2^d molecules) were infected overnight with vSC8 vaccinia virus (29), washed twice, and UV-irradiated for 15 min. Splenic effector cells were mixed with infected target cells and centrifuged together in conical tubes for 3 min at $200 \times g$. Cells were cocultivated for 1 h at 37°C and then transferred to the ELISPOT plate in a volume of 150 μl/well. After 24 h of cocultivation, IFN-γ spot-forming cells were developed by secondary anti-IFN-γ antibody (MABTECH), a Vectastain ABC kit (Vector Laboratories), and an 3-amino-9-ethylcarbazole substrate kit (Vector Laboratories).

Vaccinia Virus ELISA. Reciprocal endpoint binding titers to whole inactivated vaccinia virus strain WR were determined by ELISA as described (31) with the following modifications. Peroxidase-labeled anti-mouse Ig from Nordic Immunological Laboratories was diluted 4,000-fold and incubated at room temperature for 1 h. Substrate BM Blue (Boehringer Mannheim) was used according to the manufacturer's instructions.

Statistical Methods. For statistical analyses, we applied the distribution free longitudinal model (32) and developed our software of a nonparametric repeated-measures ANOVA (33) (available from V.A.K.). Relative treatment effects for two or more experimental groups of animals were estimated by using F statistics and corresponding P values of the nonparametric repeated-measures ANOVA when treatment effect factors and protective dose have been evaluated and compared.

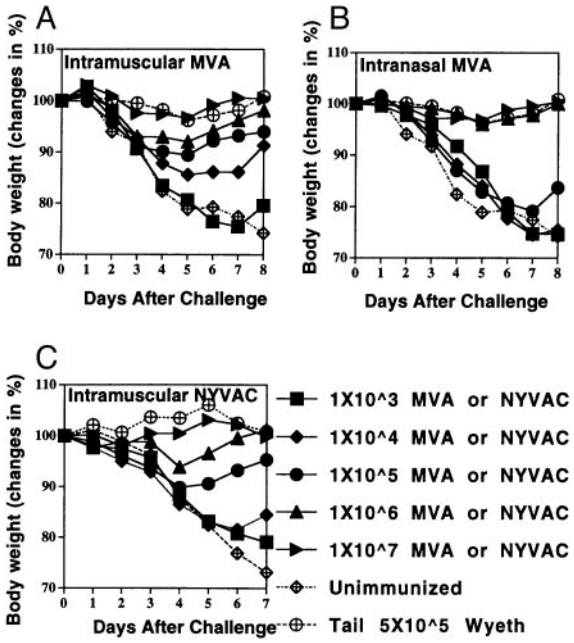

Fig. 1. Protective efficacy of i.m. (*A*) or i.n. (*B*) immunization with MVA, or i.m. immunization with NYVAC (*C*) in BALB/c mice. Groups of mice (five per group) were immunized i.m. or i.n. with 0, 10^3, 10^4, 10^5, 10^6, or 10^7 pfu of MVA or NYVAC, or with 5×10^5 pfu of Wyeth strain. One month later, mice were challenged i.n. with 10^6 pfu of WR vaccinia. Individual weight loss measured daily is presented as means for each group. These experiments were performed twice with comparable results.

Results

We studied the ability of MVA or NYVAC to protect BALB/c mice against WR, a mouse pathogenic strain of vaccinia virus. We immunized BALB/c mice (five mice per group) i.m. or i.n. with MVA or i.m. with NYVAC at doses from 10^3 to 10^7 pfu. For comparison, and as a positive control, we immunized with the Wyeth human vaccine strain of vaccinia virus by tail scratch (corresponding to skin scratch used for human vaccination). One month after immunization, we challenged mice with 10^6 pfu of WR by i.n. inoculation. A dose of 10^6 pfu WR i.n. induced death (increasing weight loss requiring killing) of unimmunized BALB/c mice on days 7–9 after challenge. Clinical protection was measured by prevention of weight loss of immunized animals. We found that i.m. (Fig. 1*A*) or i.n. (Fig. 1*B*) injection with MVA induced protection of immunized animals in a dose-dependent manner. A dose of 10^7 pfu of MVA given i.m. (Fig. 1*A*) and doses of 10^6 pfu and 10^7 pfu of MVA given i.n. (Fig. 1*B*) and 5×10^5 pfu of Wyeth induced complete protection against challenge with WR (Fig. 1 *A* and *B*). Protection at most doses of NYVAC given i.m. was roughly comparable to that produced by the corresponding doses of MVA given i.m., and no statistically significant difference was detected (Fig. 1*C*). Because similar protection was seen with both attenuated strains, subsequent studies were performed with MVA only.

To understand the mode of protection, we asked first what type of immune responses are induced by immunization i.m. with MVA and what type of responses correlate with protection. We detected virus-specific antibody in the sera of animals immunized with 10^6 or 10^7 pfu of MVA but not with lower doses (Fig. 2*A*), correlating with protection. However, the antibody titers

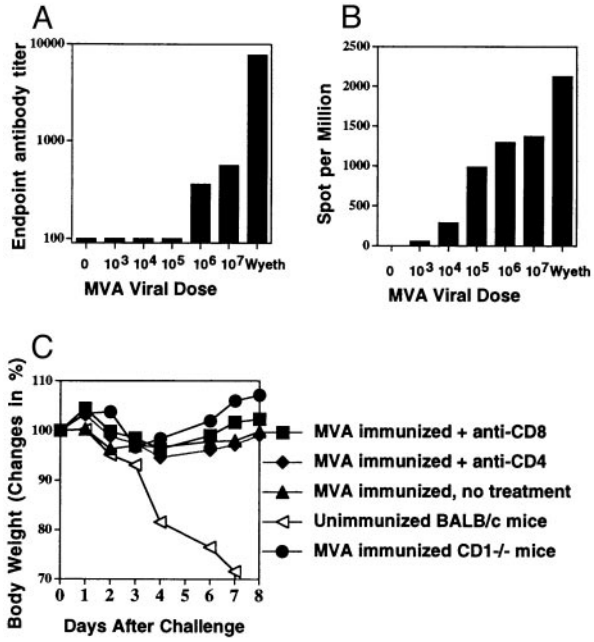

Fig. 2. Mechanisms of protection. Induction of virus-specific antibody (*A*) and IFN-γ-producing cells by ELISPOT (*B*) after i.m. immunization of BALB/c mice with MVA. Groups of mice (five per group) were immunized i.m. with 0, 10^3, 10^4, 10^5, 10^6, or 10^7 pfu of MVA or with 5×10^5 pfu of Wyeth. One month after immunization, mice were bled and then killed, and spleens were harvested. Virus-specific antibody response (end-point titer) was measured by ELISA. The vaccinia virus-specific T cell response was studied by the ELISPOT assay for IFN-γ-producing class I MHC-restricted T cells. (*C*) BALB/c or $CD1^{-/-}$ mice were immunized with 10^6 pfu of MVA i.m. One month later, mice were challenged with 10^6 pfu of WR. Three days before challenge, mice in group 1 ($n = 5$) were treated i.p. with anti-CD8 antibody (clone 2.43; 0.5 mg per mouse per day) daily for 4 days (■); group 2 was treated with anti-CD4 antibody (GK 1.5 ascites) (1 mg per mouse per day) daily for 4 days (◆); group 3 was untreated before challenge (▲); group 4 was unimmunized and untreated (△); and group 5 was $CD1^{-/-}$ mice (●). Individual weight loss measured daily after challenge is presented as means for each group (*C*). Flow cytometric analysis showed that >98% of CD4 and CD8 T cells were depleted by the treatment with the respective Abs. All experiments were performed at least twice with comparable results.

induced by these doses of MVA were a log lower than those induced by Wyeth.

We also asked whether the level of protection correlated with the T cell response. Using an ELISPOT assay, we studied the number of vaccinia-specific IFN-γ-producing cells after immunization i.m. with MVA at doses of 10^3 to 10^7 pfu. The cytokine-producing cells detected were likely to be class I MHC-restricted $CD8^+$ T cells, because they were stimulated with infected P815 mastocytoma cells expressing only class I, not class II, MHC molecules. Moreover, IFN-γ production may be an indicator of a relevant T cell response, because this cytokine has been shown to contribute to control of vaccinia virus infection (18). We found a dose-dependent $CD8^+$ T cell response after immunization with different doses of MVA (Fig. 2*B*).

To understand the role of different immune responses in protection, we studied the correlation between the level of protection and the virus-specific antibody titer, vaccinia-specific T cell response, and doses of MVA in the groups immunized i.m. We observed a correlation between the level of protection on days 4–8 after challenge (the period of substantial weight loss in controls) and the prechallenge titer of virus-specific antibody ($r = 0.657$), the $CD8^+$ T cell ELISPOT response ($r = 0.904$), and the dose of MVA used for immunization ($r = 0.964$). Thus, both cellular immunity (virus-specific T cells producing IFN-γ) and humoral response (virus-specific antibody titer) correlated with the level of protection against WR. However, complete protection (<3% weight loss) was observed only in animals with high antibody titer, whereas high $CD8^+$ T cell number was found also in mice that became ill but then recovered.

Because both antibody and T cell response correlated with the level of protection against vaccinia virus, we asked which arm of the immune response is directly responsible (necessary or sufficient) for this protection. To examine which arm was necessary, we immunized BALB/c mice with 10^6 pfu of MVA (a dose of MVA that induced substantial protection against 10^6 pfu of WR), and 4 days before challenge one group of mice was treated with anti-CD8 antibody (0.5-mg dose per mouse i.p. for 3 days before challenge), and a second group of mice were depleted with anti-CD4 antibody for 3 days (1 mg dose mouse i.p.). Depletion of CD4 or CD8 cells (≥98%) was confirmed by fluorescence-activated cell sorter staining of peripheral blood mononuclear cells. Also, as a control, we used unimmunized animals and animals immunized with 10^6 pfu of MVA but untreated before challenge. Surprisingly, depletion of either CD4 or CD8 cells in MVA-immunized animals was not sufficient to abrogate protection against WR (Fig. 2*C*). MVA-immunized and CD4- or CD8-depleted animals behaved comparably to mice immunized but not depleted ($P > 0.05$, compared with protection in immunized untreated group) (Fig. 2*C*). These data indicate that, for the effector mechanism of protection induced by MVA immunization, neither $CD4^+$ or $CD8^+$ T cells were necessary. Thus, either the protection was not T cell-mediated or there was some redundancy between the subsets (34).

To evaluate the role of natural killer T cells (NKT cells) as a possible source of protective IFN-γ, we immunized CD1 knockout mice (on the BALB/c background) with 10^6 pfu of MVA. CD1 KO mice are deficient in NKT cells because NKT cells are restricted by this nonclassical class I MHC molecule. NKT cell-deficient mice were protected at a level comparable to that of immunized BALB/c mice, and no statistically significant difference was observed ($P > 0.05$), indicating that NKT cells were not required for protection against pathogenic vaccinia in immunized animals. Thus, in MVA-immunized mice, no single arm of cell-mediated immunity (including CD4, CD8, or NKT cells) was necessary to protect against a substantial dose of WR vaccinia virus (Fig. 2*C*).

To determine whether T cells might be sufficient to protect, we carried out a syngeneic cell transfer experiment. We purified CD4 or CD8 cells (or used unseparated immune cells) from the spleens of immunized animals by positive selection using magnetic beads and transferred these cells (5×10^6 in the first experiment and 1×10^7 in the second) to unimmunized mice. We then challenged recipient mice the same day with WR virus and found no protection of mice receiving CD4, CD8, or unseparated spleen cells ($P > 0.05$, compared with unimmunized group) (data not shown). Failure because of blockade by antibodies used for positive selection is unlikely in view of the failure of unselected cells to protect either. Thus, immune $CD4^+$ or $CD8^+$ T cells, or both, at the level transferred were not sufficient to protect against vaccinia virus challenge. However, a negative result does not exclude a role for effector T cells (see below), because the quantity transferred could have been insufficient, or they may not have migrated to the appropriate sites.

To determine the role of vaccinia-specific antibody for protection against pathogenic vaccinia virus, we immunized B cell-deficient mice i.m. with 10^6 pfu of MVA. One month after immunization, mice were challenged i.n. with 10^6 pfu of WR. Immunized B cell-deficient mice were not protected against disease caused by WR and showed a significant weight loss after challenge ($P < 0.0001$, compared with immunized BALB/c mice) (Fig. 3*A*). However, MVA-immunized B cell-deficient

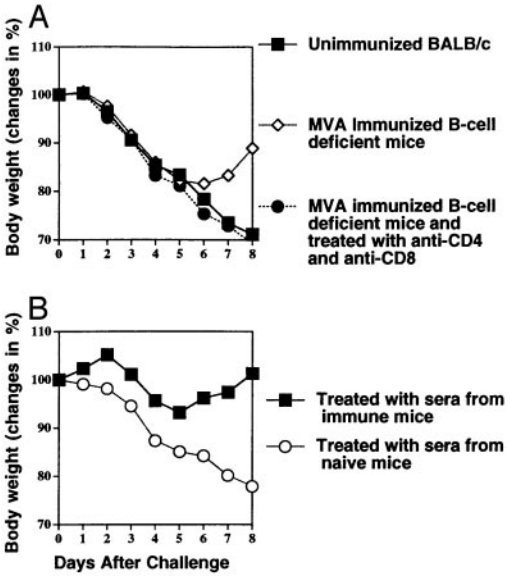

Fig. 3. Vaccinia virus-specific antibody is necessary and sufficient for protection against WR. (*A*) B cell-deficient mice (on the BALB/c background) were immunized with 10^6 pfu of MVA. One month later, mice were challenged with 10^6 pfu of WR, and weight loss was monitored daily. Three days before challenge, group 1 of B cell-deficient mice ($n = 3$) was untreated with antibody, group 2 ($n = 3$) was treated with anti-CD4 and anti-CD8 antibodies, and group 3 ($n = 3$) unimmunized BALB/c mice were left untreated with antibody as a control. Weight loss monitored daily after i.n. challenge with WR is presented as means for each group. (*B*) Protection against WR challenge after syngeneic transfer of immune serum. Immune serum collected 1 month after tail scratch immunization with 5×10^5 pfu of Wyeth was transferred into three syngeneic BALB/c mice per group starting 2 days before challenge (200 μl of serum i.p daily for 7 days). Experiments were performed twice with comparable results.

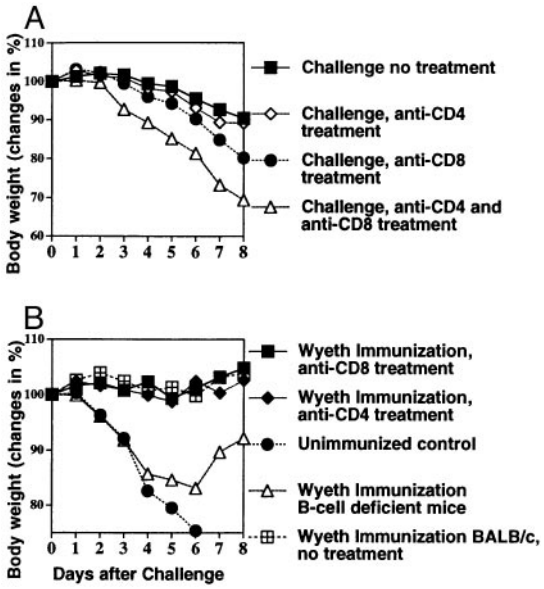

Fig. 4. (*A*) Natural resistance to WR virus depends on CD4 and CD8 cells. Naïve BALB/c mice ($n = 5$) were challenged i.n. with 10^4 pfu of WR. Three days before challenge, BALB/c mice were treated with anti-CD4 antibody (group 1), anti-CD8 antibody (group 2), or anti-CD4 and anti-CD8 antibodies (group 3), or untreated (group 4). After i.n. challenge with 10^4 pfu of WR, weight loss was monitored. (*B*) CD8 CTL and CD4 T cell responses are not required for protection induced by immunization with Wyeth. BALB/c mice were immunized with 5×10^5 pfu of Wyeth by tail scratch. One month after immunization, mice were challenged with 10^6 pfu of WR. Three days before challenge, group 1 ($n = 5$) mice were treated i.p. with anti-CD8 antibody (0.5 mg per mouse per day) daily for 4 days, group 2 was treated with anti-CD4 antibody (ascites) (1 mg per mouse per day) daily for 4 days, and group 3 was untreated before challenge. A fourth group of BALB/c mice was left unimmunized. These were compared with a group of B cell-deficient BALB/c mice that were immunized with Wyeth. Experiments were performed twice with comparable results.

mice survived and recovered after challenge with pathogenic virus, indicating the existence of a secondary (probably cell-mediated) mechanism in protection. This secondary mechanism was present in immunized animals only, whereas unimmunized BALB/c mice did not survive after WR challenge. We hypothesized that antigen-specific CD4 and CD8 cells were involved in recovery of animals in the second phase of viral infection. To test this hypothesis, we immunized three groups of B cell-deficient mice with 10^6 MVA, and 3 days before challenge treated mice with anti-CD4 and anti-CD8 together. Depletion of vaccinia-specific CD4 and CD8 cells in B cell-deficient mice prevented survival of challenged MVA-immunized mice ($P < 0.001$ compared with untreated B cell-deficient mice) (Fig. 3*A*). These results suggest that antibody is necessary for protection against disease, whereas T cells are not sufficient to protect against WR-mediated disease. One caveat is that B-deficient mice may have reduced antigen presentation capability (35, 36). Although T cell responses seem relatively normal in these mice by some measures (37, 38), a decrease in some T cell functions has been observed (39). However, in our case sufficient T cell responses occurred in these B-deficient mice to protect against death, as shown by the abrogation of this protection by T depletion in Fig. 3*A*. Thus, in the absence of antibody, T cells are important for the late recovery of surviving immunized animals.

To further confirm the protective effect of virus-specific antibody and determine whether antibodies are sufficient for protection, we performed a syngeneic transfer of immune serum (from mice immunized with Wyeth 5×10^5 pfu by tail scratch) to naïve recipient mice. Immune serum (200 μl of serum per mouse per day for 7 days) was transferred i.p. starting 2 days before challenge. After challenge, recipients of immune serum were protected ($P = 0.00003$), whereas naïve animals receiving naïve serum remained susceptible to WR vaccinia virus (Fig. 3*B*) (the log-lower antibody titers in animals immunized with these doses of MVA precluded similar serum transfer experiments with volumes of these sera that were obtainable). These data directly demonstrate that transferred antibody can protect animals against lethal challenge with pathogenic WR.

To determine whether there was a difference in mechanism between vaccine-induced protection and natural immune resistance to sublethal challenge doses of virulent WR, we asked the role of CD4 and CD8 cells in the natural resistance of unimmunized animals. For challenge of unimmunized BALB/c mice, we used a sublethal dose of WR (10^4 pfu), which induced no death and only a moderate weight loss in unimmunized animals (up to 10%) (data not shown). Before challenge with the sublethal dose of WR, group 1 was treated with anti-CD4 antibody, group 2 with anti-CD8, and group 3 with anti-CD4 and anti-CD8; group 4 was untreated. On day 8 after challenge, we found that depletion of CD4 cells alone did not substantially affect the course of disease in unimmunized animals ($P > 0.05$), whereas treatment with anti-CD8 antibody permitted a significant weight loss (Fig. 4*A*) ($P < 0.001$). However, the greatest effect after challenge was found when both CD4 and CD8 cells were depleted (Fig. 4*A*) ($P < 0.0001$), suggesting that CD4 and CD8 T cells can each provide some degree of protection. Unimmunized BALB/c mice after depletion of CD4 and CD8 cells did not survive after challenge with a dose of WR that was sublethal in intact unimmunized mice. These data indicate the

importance of CD8 and CD4 cells for natural resistance against vaccinia virus, whereas the mechanism of immune protection in immunized animals is dominated by vaccinia-specific antibody.

Finally, we asked whether the protective immunity induced by MVA given i.m. or i.n. is qualitatively different from immunity induced by Wyeth used to make the Dryvax vaccine for human immunization, given by the conventional skin-scratch route. We immunized normal or B cell-deficient BALB/c mice with 5×10^5 pfu of Wyeth by tail scratch. Three days before challenge, we depleted CD4 and CD8 cells by the protocol described above. After challenge with 10^6 WR, we found that protection was not diminished at all by depletion of CD4 or CD8 cells (Fig. 4*B*). However, B cell-deficient mice were not protected against disease as manifested by weight loss ($P < 0.0001$), although they showed late recovery consistent with a cell-mediated immune clearance of virus, as we saw in MVA-immunized mice (Fig. 4*B*, compare with Fig. 3*A*). Thus, very similar patterns were observed in the mechanisms of protection against vaccinia virus in mice immunized with MVA or Wyeth, with virus-specific antibody dominating in protection against disease but with T cells sufficient to prevent death in antibody-deficient mice. Both vaccines thus recruit a similar variety of protective mechanisms, although the relative contributions of each might differ.

Discussion

This study provides a better understanding of the mechanisms of vaccine protection against vaccinia virus infection and may provide insights for vaccine development against smallpox. Because no true animal model of smallpox is available, i.n. infection of the mouse with vaccinia virus has the merit of a small animal model best suited to revealing the mechanisms of protection. Results of this study suggest that an effective vaccine should induce virus-specific antibody, and not exclusively a $CD8^+$ CTL response. Only antibody was both necessary and sufficient to completely protect against both disease (as measured by weight loss) and mortality in immunized animals. This study does not address the possible dependence of antibody production on $CD4^+$ helper T cells in the afferent limb of the response, but only on the role of T cells in the effector mechanism of protection. Only in the absence of antibody could a role for effector $CD8^+$ T cells be detected, and, under these circumstances, the memory T cell response was not sufficient to prevent disease and substantial weight loss but did prevent mortality and mediate late recovery. The fact that the initial weight loss curve in the B cell-deficient animals was indistinguishable from that of unimmunized mice implies that the T cell response takes time to be reactivated and that it acts only late in the illness, in time to prevent death but not disease. This difference can be explained by the fact that preexisting antibody at high titer induced by vaccination can rapidly neutralize virus, whereas memory T cells take a few days to become reactivated and expanded after reexposure to antigen. Indeed, this same mechanism may explain the shape of the weight loss curves after immunization with lower doses of MVA seen in Fig. 1*A*. For example, at a dose at which antibody production was negligible (e.g., 10^4 or 10^5 pfu, as shown in Fig. 2*A*), the animals lost weight initially with a curve similar to that of unimmunized mice, but then showed late recovery (Fig. 1*A*). Although an anamnestic antibody response cannot be excluded, this result can most likely be interpreted as a memory T cell response induced by low-dose vaccination that took time to be reactivated by the infection. Low-dose immunization has been known to induce T cell responses in the absence of antibody (40). Although for many viral infections both antibodies and T cells are believed to play a role in protection, the relative importance of these is often not well understood, especially in the case of orthopoxviruses, such as vaccinia virus and variola virus, the cause of smallpox. The current study examines this balance between cellular and humoral immunity and shows that replication-deficient vaccines can produce types of protection comparable with those induced by the licensed vaccine strain.

The mechanism by which antibodies protect is presumably through virus neutralization, although other mechanisms may also play a role (22). However, the mechanism of protection by $CD8^+$ T cells is less obvious. It has long been assumed that a major mechanism of protection is lysis of infected cells. However, in the case of vaccinia virus in mice, Karupiah *et al.* (19) and Harris *et al.* (18) have found that IFN-γ plays a key role, acting by inducing nitric oxide, which prevents vaccinia viral replication. In our case, the $CD8^+$ T cell response measured was IFN-γ-producing cells, as enumerated by ELISPOT, which correlated with protection, even though $CD8^+$ T cells were not necessary for protection. Probably the reason that responses in both arms of the immune system correlated with protection is that these all serve to indicate the level of overall immune response induced by the vaccination.

In a recent study by Drexler *et al.* (6), immunization with a peptide vaccine, which included an HLA-A2-restricted immunodominant CTL epitope to induce CTL, was not effective in protecting mice against pathogenic WR vaccinia challenge, in contrast to MVA immunization, which did protect. However, in this study the mechanism of protection was not examined. These results can now be explained in light of our current results, which indicate that a strategy inducing only $CD8^+$ CTL may not prevent vaccinia viral infection or disease, but may reduce mortality.

Both MVA and NYVAC, replication-deficient in mice and primates and thus likely safe to use in immunodeficient individuals, gave roughly comparable levels of protection against pathogenic vaccinia. However, the antibody titers were a log lower than those induced by the currently used Wyeth vaccine strain. Nevertheless, it is important that the mechanism of protection by MVA seemed to involve a set of responses similar in type to those mediating protection by the Wyeth vaccine strain, indicating that the two are qualitatively similar, if not quantitatively equal. This mechanistic similarity is important in evaluating these attenuated viruses as potential smallpox vaccines using surrogate indicators, given that they cannot be tested explicitly for protection of humans against smallpox because experimental challenge is unethical and natural infection with smallpox is no longer known to occur (1, 41). The higher dose required may in part compensate for the lack of replication *in vivo*, and boosting may be required to achieve comparable antibody titers (L.S.W., P.E., and B.M., unpublished observations). In addition, these replication-defective strains might be valuable as a preliminary immunization to reduce the risk of serious adverse effects of conventional smallpox vaccination. Such an approach has been studied by Mayr *et al.* (42) and is the subject of current clinical trials.

It is interesting that the mechanism of natural resistance against low-dose exposure to the virulent strain of vaccinia virus seemed to be somewhat different from the mechanism of protection by a vaccine. This difference may be primarily one of timing. In the vaccine case, prior vaccination allows time for the development of neutralizing antibodies. If these are already present at the time of challenge with the virulent strain, then the antibody is sufficient to protect, and $CD8^+$ T cells are not necessary. In contrast, in the absence of prior immunity, the sublethal infection must induce both T cells and antibodies, but typically the T cell response arises faster than the antibody response. Therefore, T cells are sufficient to start clearing virus before there is any antibody to neutralize virus. Once virus is established, T cells are more effective at clearing the infection than are antibodies, so the antibody response later in the course of infection of a naïve animal will be relegated to a more minor role. An analogous situation has been extensively studied in the

case of influenza virus (34, 43) and might exist in the case of HIV infection. Natural resistance against retroviral infection largely depends on $CD8^+$ CTL. As was shown by Schmitz *et al.* (44) and Jin *et al*. (45), depletion of $CD8^+$ T cells in monkeys infected with simian immunodeficiency virus results in a burst of viremia. Also, the initial peak viremia begins to decline concurrent with the rise of $CD8^+$ T cells but before the appearance of neutralizing antibodies (46). Nevertheless, it is quite possible that preexisting neutralizing antibodies induced by prior vaccination could protect against HIV infection. Therefore, analogous strategies could apply to HIV as well.

Finally, it is of interest to compare the i.m. and i.n. routes of immunization. The dose of MVA required for complete protection in two experiments seemed to be lower when delivered by i.n. than by i.m. immunization, although partial protection may have been achieved at lower doses i.m. These subtle differences may relate to the fact that the challenge was through the respiratory route and that the i.n. immunization was more effective at inducing mucosal immunity than was the i.m. immunization. This interpretation is supported by the finding that local mucosal CTL were necessary for protection against rectal mucosal transmission of a recombinant vaccinia virus expressing the HIV-1 envelope protein (33, 47–51). Given that smallpox is naturally spread through the respiratory mucosal route, we suggest that more attention should be paid to the possibility of delivering a smallpox vaccine, of whatever type, through a mucosal route. The fact that MVA and NYVAC are likely safe to deliver i.n. may make them candidates for such a mucosal immunization against smallpox.

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