JOURNAL OF VIROLOGY, Sept. 1996, p. 6418–6424 Vol. 70, No. 9
0022-538X/96/$04.00+0

Oral Immunization with a Replication-Deficient Recombinant Vaccinia Virus Protects Mice against Influenza

BRADLEY S. BENDER,[1,2,3]* CHERYL A. ROWE,[4] SCOTT F. TAYLOR,[1] LINDA S. WYATT,[5] BERNARD MOSS,[5] AND PARKER A. SMALL, JR.[4]

Geriatric Research, Education and Clinical Center, Veterans Affairs Medical Center, Gainesville, Florida 32608;[1] Departments of Medicine,[2] Molecular Genetics and Microbiology,[3] and Pathology and Laboratory Medicine,[4] University of Florida College of Medicine, Gainesville, Florida 32610; and Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892[5]

Received 29 February 1996/Accepted 30 May 1996

Mice immunized with two intragastrically administered doses of a replication-deficient recombinant vaccinia virus containing the hemagglutinin and nucleoprotein genes from H1N1 influenza virus developed serum anti-H1 immunoglobulin G (IgG) antibody that completely protected the lungs from challenge with H1N1. Almost all of the mice given two intragastric doses also developed mucosal anti-H1 IgA antibody, and those with high anti-H1 IgA titers had completely protected noses. Intramuscular injection of the vaccine protected the lungs but not the noses from challenge. We also found that the vaccine enhanced recovery from infection caused by a shifted (H3N2) influenza virus, probably through the induction of nucleoprotein-specific cytotoxic T-lymphocyte activity. A replication-deficient, orally administered, enteric-coated, vaccinia virus-vectored vaccine might safely protect humans against influenza.

The standard influenza virus vaccine induces serum immunoglobulin G (IgG) antibody but is much less effective at inducing secretory IgA antibody and cellular immunity, both of which are important in host defense against the influenza virus. The serum neutralizing antibodies protect the lungs from viral infection and thereby save lives, but the inconsistent induction of mucosal anti-influenza virus IgA antibody—which prevents infection of the upper respiratory tract (15, 46, 47)—explains the inability of the killed vaccine to prevent infection and stop the spread of the infection (22, 53). The usefulness of this vaccine is further limited by the continual antigenic changes of the influenza virus envelope proteins, which may result in the inability of the induced serum antibodies to protect against new circulating strains. Once infection occurs, there is a redundancy in recovery mechanisms. Recovery is primarily mediated by $CD8^+$ class I major histocompatibility complex (MHC)-restricted cytotoxic T-lymphocyte (CTL) activity (29, 30, 38, 64, 67). However, transgenic mice that lack β_2 microglobulin (β_2m−/− mice) and therefore class I CTLs can also recover from an influenza virus challenge (17), though at a slower rate than heterozygous controls (3). Influenza virus-infected SCID mice can also clear their infection by the transfer of neutralizing anti-influenza virus antibodies (44, 51).

Heterotypic immunity (also referred to as heterosubtypic immunity) is another important aspect of influenza virus host defense. Mice that have recovered from an infection caused by an influenza A virus (e.g., H1N1) are significantly protected from a subsequent challenge with a shifted influenza A virus (e.g., H3N2) (6, 52, 68). This heterotypic immunity can be mediated by cross-reactive CTLs directed against the nucleoprotein (NP) (63, 70), a conserved gene product (21, 23), but it can also be generated in β_2m−/− mice (2, 18). Studies of heterotypic immunity in humans have clearly shown that infection with one strain of influenza A virus may not prevent infection with a shifted influenza A virus (57); however, the subsequent disease course in individuals infected during the same season may be less severe (20, 56).

Recombinant vaccinia viruses are potential human and veterinary vaccines. A foreign gene is flanked with nonessential vaccinia virus DNA sequences and introduced via a plasmid into a vaccinia virus-infected cell in which recombination occurs (42, 45). The foreign proteins synthesized by the recombinant vaccinia virus vectors are processed (including post-translational modifications) by the host cells and are similar to the proteins synthesized following infection with the parental organism (42). Animals vaccinated with recombinant vaccinia viruses produce neutralizing serum antibodies and specific class I MHC-restricted CTLs and are protected from subsequent challenge with the relevant pathogen (4, 8, 9, 54, 55, 71).

Safety issues are of concern when recombinant vaccinia viruses are used as human vaccines. Because of the risk of dissemination, during the Smallpox Eradication Programme, vaccination was contraindicated in infants with eczema or anyone suffering from immune dysfunction (14, 19). There should be negligible risks with an avian host-restricted poxvirus, such as canarypox virus, which had no significant side effects in a phase I study (13), or modified vaccinia virus Ankara (MVA). MVA was derived for use as a smallpox vaccine by repeated passaging in chicken embryo fibroblasts (25). Genetic analysis revealed that MVA had suffered six major deletions of its genome, resulting in the loss of 30,000 base pairs (15% of its genome), so that it became host restricted and replication deficient in mammalian cell lines (41). MVA was found to be avirulent in both normal and immunocompromised animals, and during the Smallpox Eradication Programme it was given to 120,000 people, many at high risk for complications from administration of the standard vaccine, without significant side effects (25, 31–33, 58, 65). The block in replication of MVA in human cells occurs at a step in virion assembly (rather than at an early stage of infection), so recombinant gene expression is unimpaired (59). Mice immunized with recombinant MVA that expresses the genes of influenza virus H1N1 hemagglutinin (HA) and NP (referred to as MVA HA-NP) developed

* Corresponding author. Mailing address: GRECC-182, Veterans Affairs Medical Center, Gainesville, FL 32608-1197. Phone: (352) 374-6114. Fax: (352) 374-6142. Electronic mail address: bbender@qm.server.ufl.edu.

anti-H1 serum IgG antibodies and CTL activity and were protected from a lethal challenge with a homologous influenza virus (60).

Another potential disadvantage of vaccinia virus vectors was noted with the demonstration that while parenteral immunization stimulates protective levels of serum IgG antibody and cellular immunity, it does not induce mucosal immunity (54). Mucosal administration of a recombinant vaccinia virus, however, induces solid mucosal, humoral, and cellular immunity (27, 40), and vaccinia virus-rabies virus recombinants given as oral bait have successfully immunized animals in the wild, probably as a result of viral replication in the tonsils (10, 49, 50). We report here that enteric administration of a recombinant replication-deficient vaccinia virus to mice effectively induces mucosal IgA, serum IgG, and CTL activity and protects them from a homotypic and heterotypic influenza virus challenge.

i.g. administration of MVA HA-NP induces serum and mucosal anti-influenza virus antibody. Female BALB/c mice, 5 to 6 weeks of age (Taconic Laboratories, Germantown, N.Y.), were housed in specific-pathogen-free conditions. One hour before intragastric (i.g.) inoculations, each mouse received an intraperitoneal injection of a combination of 3 mg of cimetidine HCl (SmithKline Beecham, Philadelphia, Pa.) and 0.02 μg of sincalide (the C-terminal octapeptide of cholecystokinin) (Squibb, Princeton, N.J.) in 100 μl of phosphate-buffered saline (PBS) to inhibit stomach acid secretion and empty the gallbladder. Mice received 200 μl of MVA HA-NP (59, 60) in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (Mediatech, Washington, D.C.) containing 10^8 PFU via a 1-in. (2.54-cm) feeding needle. For some mice, i.g. inoculation was repeated 5 weeks later. For intramuscular (i.m.) inoculations, 100 μl of wild-type MVA or MVA HA-NP, containing 0.5×10^8 PFU, was injected into each quadriceps muscle. Stock influenza viruses used for intranasal (i.n.) inoculation or challenge were grown in the allantoic cavities of 10-day-old embryonated chicken eggs for 3 days at 35°C, harvested, and clarified (68). Mice used as convalescent controls were infected i.n., without anesthesia, with 20 μl of influenza virus A/Puerto Rico/8/34 (PR8; an H1N1 virus) containing $10^{7.1}$ 50% tissue culture infective doses ($TCID_{50}$). For challenge studies at 9 weeks post-initial immunization, mice were anesthetized with sodium pentobarbital and inoculated i.n. with H1N1.

High levels of serum anti-H1 IgG antibody, as determined by enzyme-linked immunosorbent assay (ELISA) (39), were found in all mice receiving MVA HA-NP (either i.m. or i.g.) or H1N1 influenza virus (Fig. 1A), though levels were about fivefold lower in the i.g. immunized mice.

Vaginal-wash samples were frozen at −20°C and later tested by ELISA for anti-H1 IgA antibodies. To dissolve mucous strands, an equal quantity of 0.01 M dithiothreitol (Sigma, St. Louis, Mo.) was added and the samples were vortexed, incubated at room temperature for 30 min, and centrifuged for 1 min at $15{,}000 \times g$. Duplicate twofold dilutions of samples were placed in wells precoated with influenza virus HA and in wells not containing antigen, followed by addition of goat anti-mouse IgA (Sigma). The labelling reagent was alkaline phosphatase linked to rabbit anti-goat IgG (ICN Immunobiologicals, Irvine, Calif.), and *p*-nitrophenyl phosphate (Sigma) was used as the substrate. Color was allowed to develop for 45 min at room temperature, and the A_{405} was read on a Titertek Multiscan (Flow). Each titer was expressed as the highest dilution for which the optical density of the positive (antigen-containing) well divided by the optical density of the respective negative (control) well gave a ratio greater than or equal to 2 (27).

Mucosal anti-H1 IgA antibody was detected in the vaginal secretions of 31 of 33 mice that received MVA HA-NP i.g. and in 9 of 9 positive controls (having recovered from an H1N1 infection). At 8 weeks, vaginal IgA anti-H1 titers of mice receiving two i.g. doses of MVA HA-NP were about twofold lower than those of mice that had recovered from an influenza virus infection (Fig. 1B). In another set of experiments, two i.g. doses of MVA HA-NP induced anti-influenza virus serum neutralizing antibody, nasal-wash IgA antibody, and gut wash IgA antibody while two i.m. doses of MVA HA-NP induced only serum antibody (data not shown). Furthermore, three i.g. doses of MVA HA-NP induced serum anti-H1 IgG antibody levels (as determined by ELISA) that were essentially identical to three doses given i.m. (data not shown).

For i.g. administration to be effective, we found that the vaccine must elude the stomach acid and bile. We and others have found that bile and gastric acid can inactivate vaccinia virus (1a, 24), so direct surgical implantation of a replicating recombinant vaccinia virus into the jejunum was more immunogenic than i.g. administration (40). We also studied the effect of pretreatment of mice with cimetidine and cholecystokinin and found that this pretreatment enhanced the proportion of animals responding to i.g. administration of MVA HA-NP from ~60 to ~100% (data not shown). The appearance of anti-influenza IgA in vaginal and nasal washes following i.g. administration is consistent with the induction of the immune response in the intestine and migration of the immunocytes to other mucosal sites, a phenomenon recognized as part of the common mucosal immune system (12, 36, 37). We therefore believe that the induction of mucosal IgA antibodies was due to abortive infection of the epithelial cells of the small intestine with MVA HA-NP.

i.g. administration of MVA HA-NP prevents influenza virus infection of the nose and lungs following a homotypic challenge. To evaluate the functional significance of these antibodies, vaccinated mice with a wide range of antibody titers were chosen for challenge with H1N1 influenza virus. Following this challenge, the noses of naive mice shed influenza virus on days 1 and 3 postchallenge at titers of ~3 and ~4 $\log_{10}$ $TCID_{50}$, respectively (Fig. 2A). The noses were protected only in those mice receiving i.g. doses of MVA HA-NP and in convalescent mice (Fig. 2A). Five of the 12 mice receiving two i.g. doses of MVA HA-NP shed no virus from their noses on day 1 postchallenge, and the mean titer (± the standard error) of the total group (0.7 ± 0.2) was significantly lower than that of naive mice (3.0 ± 1.2), mice vaccinated i.m. with MVA HA-NP (2.5 ± 0.4), or mice vaccinated i.m. with MVA (2.5 ± 0.4). All five mice immunized twice with MVA HA-NP i.g. that were sacrificed 3 days postchallenge had high prechallenge vaginal IgA antibody titers and shed no virus from their noses. Protection of the MVA HA-NP i.g.-immunized mice correlated strongly with the vaginal-wash anti-HA titer; 6 of 6 mice with titers of ≤1/8 shed virus, compared with 1 of 11 mice with titers of ≥1/16 ($P = 0.0004$, Fisher's exact test). As expected from the low anti-H1 IgA titers induced by one i.g. dose of MVA HA-NP, the noses of these mice were not protected from H1N1 challenge (1.8 ± 0.4).

Pulmonary virus shedding was ~4.5 $\log_{10}$ $TCID_{50}$ on days 1 and 3 postchallenge in the naive or i.m.-immunized MVA mice (Fig. 2B). Two i.g. doses of MVA HA-NP completely protected the lungs of 12 of 12 mice on the day following the challenge (Fig. 2B), as did i.m. MVA HA-NP in 8 of 9 mice. One i.g. dose provided partial, but significant ($P < 0.001$), protection of the lungs on day 1 following challenge (1.1 ± 0.3)

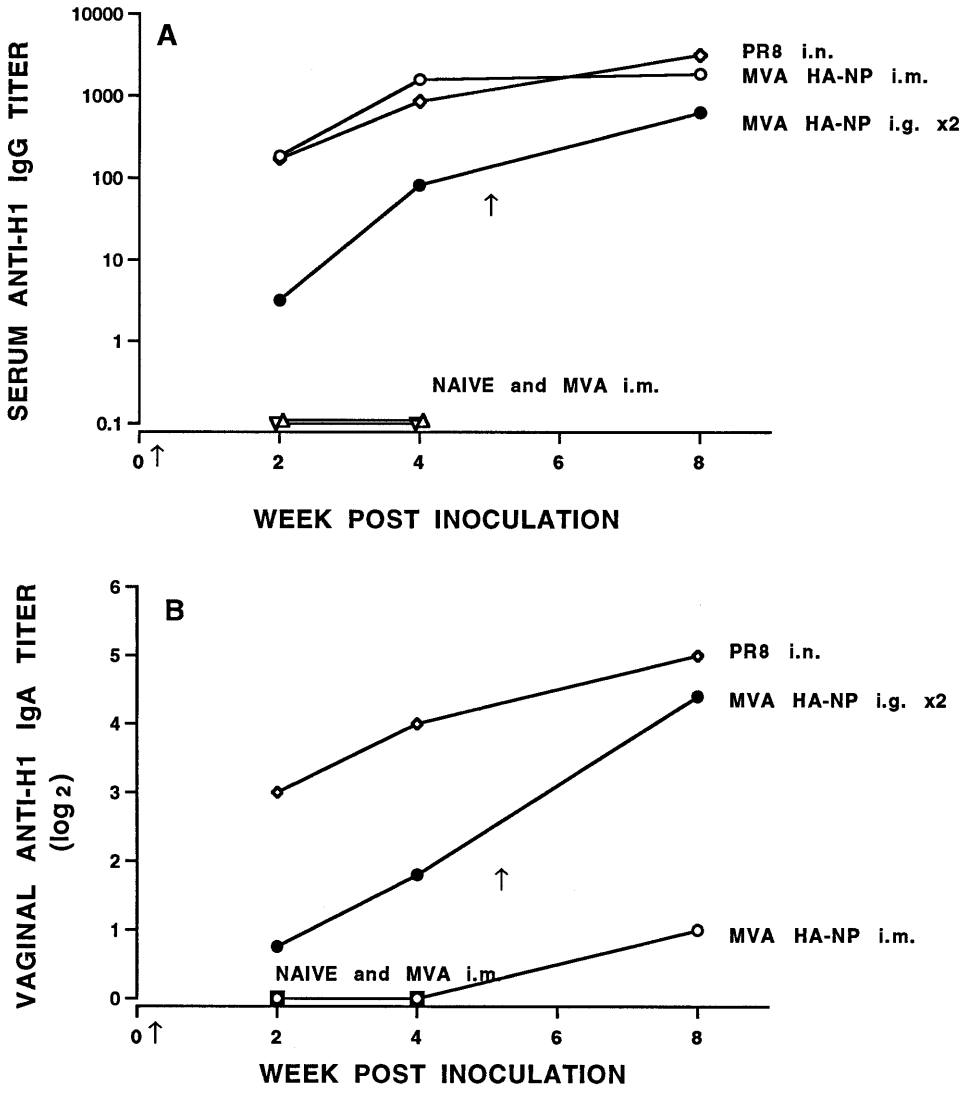

FIG. 1. Production of serum IgG (A) and vaginal IgA (B) anti-H1 antibodies. Mice were given 10^8 PFU of MVA HA-NP in each of two i.g. inoculations (●) at weeks 0 and 5 (↑). Control mice were either inoculated i.n. with influenza virus H1N1 (PR8) (◇) or i.m. with MVA (△) or MVA HA-NP (○) or were naive (▽). Serum was obtained from the tail vein, and vaginal wash fluid was obtained by flushing the vagina six to eight times with the same 80 μl of PBS during weeks 2, 4, and 8. The results are plotted as the geometric means of the ELISA values (n = 5 to 6 per group) versus time after the first inoculation.

compared with naive (4.7 ± 0.3) or i.m.-vaccinated MVA (4.6 ± 0.3) mice.

i.g. administration of MVA HA-NP induces in vitro and in vivo heterotypic immunity to influenza. To assess anti-influenza virus CTL activity, mice were immunized with MVA HA-NP i.g. and 2 weeks later were challenged with influenza virus A/Port Chalmers/1/73 (an H3N2 virus). Primary anti-influenza virus CTL activity was evaluated using pulmonary lymphocytes (7), and secondary CTL activity was evaluated using splenic lymphocytes and an in vitro culture system (5, 6). Table 1 demonstrates that one i.g. dose of MVA HA-NP primed for levels of splenic CTL activity similar to the levels achieved by a prior H1N1 infection. Primary pulmonary CTL activity, however, was significantly lower in the MVA HA-NP-immunized mice, suggesting that i.g. immunization may not be as efficient in inducing migration of these lymphocytes to the target organ. However, unlike killed vaccines, which are poor inducers of CTL activity because $CD8^+$ CTLs are typically generated via intracellularly processed peptides (43, 62, 69), MVA HA-NP clearly induces CTL activity.

As a measure of the efficacy of this cell-mediated immune response, MVA HA-NP-immunized mice were challenged with influenza virus H3N2. As these mice did not have protective antibodies, initial (day 1) virus titers of the nose and lung were statistically indistinguishable in the immunized and control mice (data not shown). Enhanced recovery of mice that had been immunized with one or two i.g. doses of MVA HA-NP was apparent from both the nasal and pulmonary influenza virus titers 5 days postinfection (Fig. 3). Mice immunized i.g. twice with MVA HA-NP had nasal titers (1.1 ± 0.3) that were significantly lower than mice immunized i.m. with MVA HA-NP (2.2 ± 0.3) (Fig. 3A). Although not as low as lung titers in mice immunized with MVA HA-NP i.m. (2.0 ± 0.4), lung virus titers of mice immunized with MVA HA-NP i.g. (2.3 ± 0.3) were significantly lower than negative control (naive or MVA-immunized [i.m.]) mice (Fig. 3B). Our finding

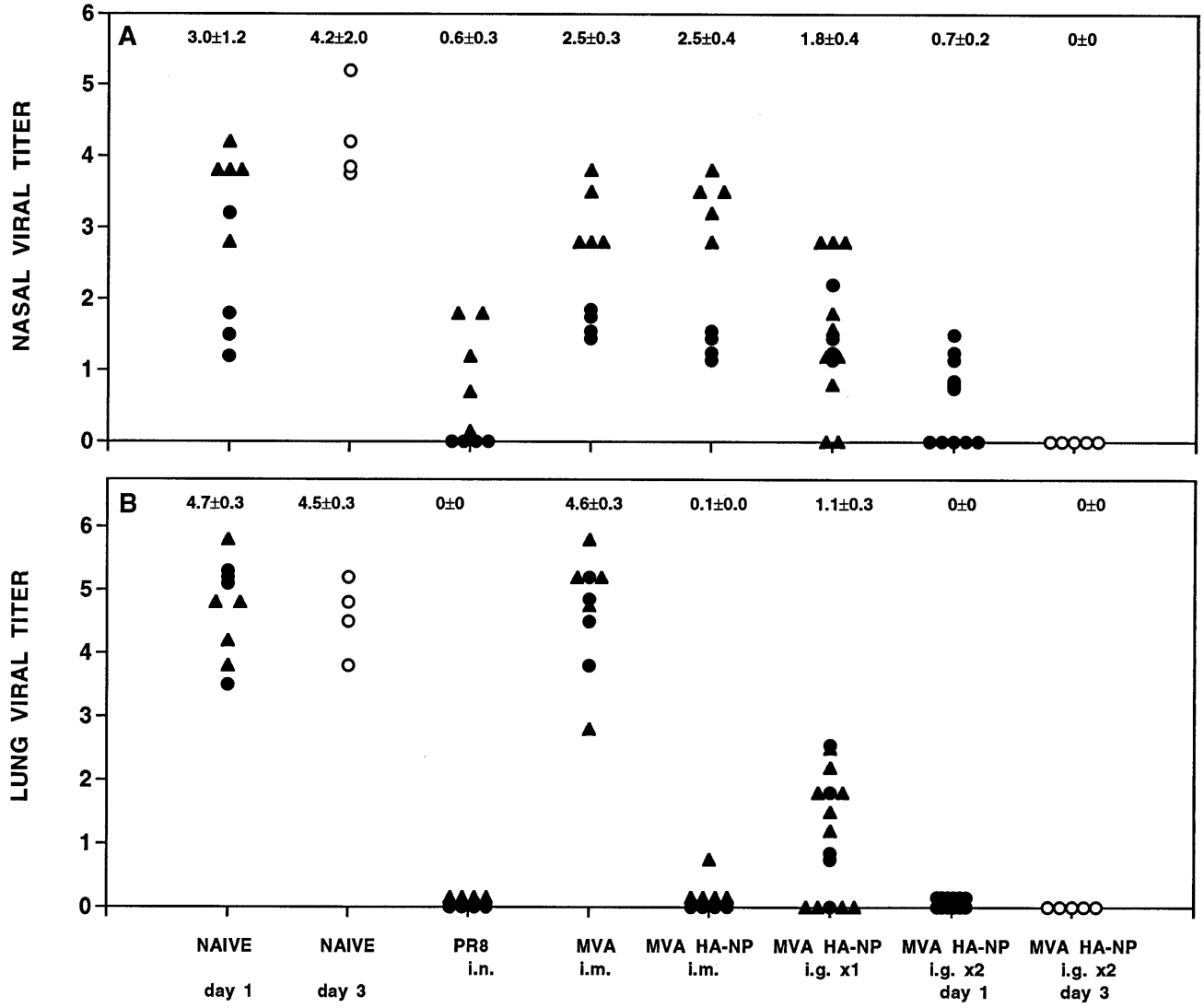

FIG. 2. Nasal (A) and pulmonary (B) influenza virus titers of mice following challenge with influenza virus H1N1 (PR8). Twenty-four days after the second inoculation with MVA HA-NP, mice were challenged, under anesthesia, with H1N1 (PR8) and then sacrificed 1 day (closed symbols) or three days (open symbols) later. The data are a summary of two experiments; experiment 1 used $10^{7.0}$ $TCID_{50}$ (▲) and experiment 2 used $10^{4.1}$ $TCID_{50}$ (●, ○) of H1N1 for the challenge. Virus titers were determined by a tissue culture microassay (5). Log_{10} of undetectable virus was defined as 0. Results obtained in a separate experiment after i.g. administration of MVA were virtually identical to those obtained for naive mice or for mice administered MVA i.m. and are not shown. Day 1 nasal viral titers were significantly lower than titers of control (naive and MVA i.m.) mice administered MVA HA-NP i.g. ($P < 0.001$, experiment 1; $P < 0.05$, experiment 2) and PR8-convalescent mice ($P < 0.001$, experiment 1; $P < 0.05$, experiment 2) but not mice immunized with MVA HA-NP i.m. ($P > 0.05$, experiments 1 and 2). Day 1 pulmonary viral titers were significantly lower than titers of control mice inoculated with MVA HA-NP i.g. ($P < 0.001$, experiments 1 and 2), PR8-convalescent mice ($P < 0.001$, experiments 1 and 2), and mice administered MVA HA-NP i.m. ($P < 0.001$, experiments 1 and 2). The numbers at the top of each group are the mean log_{10} $TCID_{50}$ viral titers (± standard error).

that i.g. administration of MVA HA-NP enhanced recovery of both the upper and lower respiratory tracts while i.m. administration enhanced recovery of only the lower respiratory tract is consistent with the observation that recovery mechanisms differ in these two sites (28a).

Although recovery correlated with anti-NP CTL activity, other factors may be involved. For example, heterotypic immunity can be induced in $\beta_2m-/-$ mice (2, 18), so the mechanism of protection in these animals cannot involve class I MHC-restricted T cells. Further, other experiments have demonstrated that vaccinia virus constructs that induce anti-NP CTL activity are not protective (1, 28). The protection may have been due to induction of class II MHC-restricted $CD4^+$ T cells or mucosal IgA (2); recent data show that IgA can neutralize intracellular viruses (34, 35).

Several areas of research need to be evaluated prior to further promotion of MVA HA-NP as a vaccine. First, the doses used in these experiments, $\sim 10^8$ PFU per animal, may be more than is required to induce immunity. While the majority of mice given 10^4 PFU of MVA HA-NP by the i.m. route were protected from a lethal challenge with influenza virus and 10^5 PFU was 100% successful, higher doses were needed for effective i.n. administration (60). Second, the duration of the immune response needs to be determined. We challenged the animals 3 to 4 weeks after inoculation, when protection (especially that mediated by IgA) would be ex-

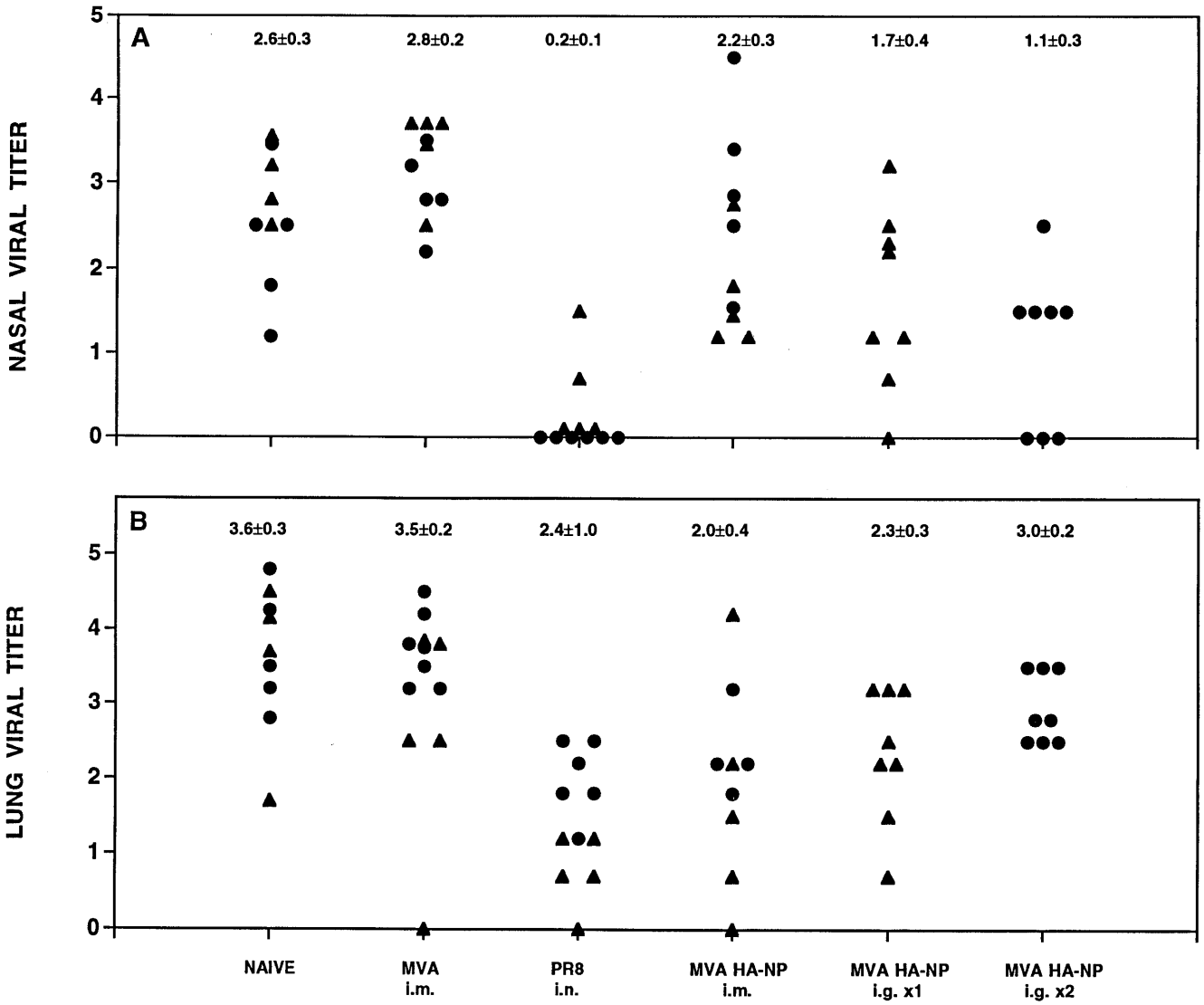

FIG. 3. Nasal (A) and pulmonary (B) viral titers of mice 5 days following challenge with H3N2 influenza virus. Four weeks after the second inoculation with MVA HA-NP, mice were challenged with H3N2 and sacrificed 1 and 5 days later. The data are a combination of two separate experiments; experiment 1 used $10^{6.5}$ (▲) and experiment 2 used $10^{4.6}$ (●) $TCID_{50}$ of H3N2 for the challenge. Day 1 titers (data not shown) varied from 1.8 to 2.5 $\log_{10}$ $TCID_{50}$ in the nose and from 3.0 to 3.6 $\log_{10}$ $TCID_{50}$ in the lungs and were not significantly different between groups. Day 5 nasal viral titers were significantly lower than titers of control (naive and MVA i.m.) mice inoculated with MVA HA-NP i.g. ($P < 0.05$, experiments 1 and 2) and PR8-convalescent mice ($P < 0.01$, experiments 1 and 2) but not mice immunized with MVA HA-NP i.m. ($P > 0.05$, experiments 1 and 2). Day 5 pulmonary viral titers were significantly lower than titers of control mice administered MVA HA-NP i.g. ($P < 0.05$, experiments 1 and 2), PR8-convalescent mice ($P < 0.001$, experiments 1 and 2), and mice inoculated with MVA HA-NP i.m. ($P < 0.05$, experiment 1; $P < 0.01$, experiment 2). The numbers at the top of each group are the mean $\log_{10}$ viral titers (± standard error).

pected to be at its maximum. Third, the results of experiments with MVA HA-NP need to be directly compared with those of experiments using other host-restricted poxviruses so that the most promising vector can be evaluated in clinical trials.

The additional studies seem to be worth pursuing because oral immunization with a replication-deficient recombinant vaccinia virus such as MVA offers many potential advantages over other vaccine candidates. First, as demonstrated here, this method is not only effective in inducing immune response in all three arms of the immune system—serum IgG antibody, mucosal IgA antibody, and cell-mediated immunity—but is also efficacious. Use of an oral rather than a parenteral vaccine may enhance patient (and/or parental) acceptance and would obviate the need for syringes and needles. Second, MVA is an extremely safe vector that has undergone extensive safety testing in humans and animals (24, 25, 31–33, 58, 61, 65). Third, multivalent recombinant MVA can be constructed. Even if it proves to be too difficult to transfer more than a few genes into MVA or have their products expressed in appropriate concentrations, a cocktail of recombinant MVA viruses, each containing several genes, might also work. Fourth, lyophilized vaccinia virus is extremely heat stable. Heating it to 100°C for 2 hours led to a loss of only 1 log of infectivity, and after storage at 45°C for 2 years, it was still 100% successful in vaccination of volunteers (16). On the basis of these data, one would also expect MVA to be heat stable. These four properties also suggest that oral recombinant MVA might meet all the requirements of the Children's Vaccine Initiative (11, 48, 66). It could provide children in the developing world with an ideal

TABLE 1. Pulmonary and splenic CTL activity of mice convalescent from H1N1 infection or vaccinated i.g. with MVA HA-NP or MVA[a]

Day post-challenge with H3N2	Immunizing virus and mode of inoculation	CTL activity			
		Primary pulmonary		Secondary splenic	
		H3N2	B/Georgia	H3N2	B/Georgia
3	H1N1, i.n.	0 ± 0	0 ± 0	78 ± 2	2 ± 1
	MVA HA-NP, i.g.	0 ± 0	0 ± 0	50 ± 10	4 ± 2
	MVA, i.g.	0 ± 0	0 ± 0	13 ± 5	3 ± 1
4	H1N1, i.n.	43 ± 9	0 ± 0	78 ± 6	6 ± 2
	MVA HA-NP, i.g.	18 ± 8	0 ± 0	70 ± 5	7 ± 4
	MVA, i.g.	3 ± 1	1 ± 0	10 ± 5	7 ± 2
5	H1N1, i.n.	60 ± 5	0 ± 0	63 ± 1	6 ± 1
	MVA HA-NP, i.g.	25 ± 4	0 ± 0	52 ± 1	10 ± 1
	MVA, i.g.	2 ± 1	0 ± 0	12 ± 3	7 ± 2

[a] Data are mean percentages (± standard error) of ^{51}Cr released from P815 cells sensitized to H3N2 or B/Georgia at an effector-to-target ratio of 30:1. Data are from two experiments, each with a total of five to six mice per day per group on days 3, 4, and 5 post-H3N2 challenge.

vaccine and save children throughout the developed world from the fear of shots. We will ". . .continue to prosecute this inquiry, encouraged by the hope of its becoming essentially beneficial to mankind" (26).

We thank R. Cottey and R. Hankison for technical assistance.

This work was supported by grants from the Department of Veterans Affairs (B.S.B.) and Connaught Laboratories (P.A.S., B.S.B., and C.A.R.).

REFERENCES

1. **Andrew, M. E., and B. E. H. Coupar.** 1988. Efficacy of influenza haemagglutinin and nucleoprotein as protective antigens against influenza virus infection in mice. Scand. J. Immunol. **28:**81–85.
1a. **Bell, W., P. A. Small, Jr., and B. S. Bender.** Unpublished data.
2. **Bender, B. S., W. E. Bell, and P. A. Small, Jr.** 1994. Class I major histocompatibility complex-restricted cytotoxic T-lymphocytes are not necessary for heterotypic immunity to influenza. J. Infect. Dis. **170:**1195–1200.
3. **Bender, B. S., T. Croghan, L. Zhang, and P. A. Small, Jr.** 1992. Transgenic mice lacking class I major histocompatibility complex-restricted T-cells have delayed viral clearance and increased mortality after influenza virus challenge. J. Exp. Med. **175:**1143–1145.
4. **Bender, B. S., M. P. Johnson, C. Flexner, B. Moss, and P. A. Small, Jr.** 1990. Cytotoxic T-lymphocyte activity induced by influenza vaccination in young and aged mice, p. 69–73. *In* F. Brown, R. Chanock, H. S. Ginsberg, and R. A. Lerner (ed.), Vaccines 90. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Bender, B. S., M. P. Johnson, and P. A. Small, Jr.** 1991. Influenza in senescent mice: impaired cytotoxic T-lymphocyte activity is correlated with prolonged infection. Immunology **72:**514–519.
6. **Bender, B. S., and P. A. Small, Jr.** 1993. Heterotypic immune mice lose protection against influenza infection in senescence. J. Infect. Dis. **168:**873–880.
7. **Bender, B. S., S. F. Taylor, D. Zander, and R. Cottey.** 1995. Pulmonary immune response of young and aged mice following influenza challenge. J. Lab. Clin. Med. **126:**169–177.
8. **Bennink, J. R., J. W. Yewdell, G. L. Smith, C. Moller, and B. Moss.** 1984. Recombinant vaccinia virus primes and stimulates influenza haemagglutinin-specific cytotoxic T cells. Nature (London) **311:**578–579.
9. **Bennink, J. R., J. W. Yewdell, G. L. Smith, and B. Moss.** 1987. Anti-influenza virus cytotoxic T lymphocytes recognize the three viral polymerases and a nonstructural protein: responsiveness to individual viral antigens is major histocompatibility complex controlled. J. Virol. **61:**1098–1102.
10. **Blancou, J., M. P. Kieny, R. Lathe, J. P. Lecocq, P. P. Pastoret, J. P. Soulebot, and P. Desmettre.** 1986. Oral vaccination of the fox against rabies using a live recombinant vaccinia virus. Nature (London) **322:**373–375.
11. **Bloom, B. R.** 1989. Vaccines for the third world. Nature (London) **342:**115–120.
12. **Brandtzeag, P.** 1989. Overview of the mucosal immune system. Curr. Top. Microbiol. Immunol. **146:**13–25.
13. **Cadoz, M., A. Strady, B. Meignier, J. Taylor, J. Tartaglia, E. Paoletti, and S. Plotkin.** 1992. Immunisation with canarypox virus expressing rabies glycoprotein. Lancet **339:**1429–1432.
14. **Centers for Disease Control.** 1991. Vaccinia (smallpox) vaccine. Recommendations of the Immunization Practices Advisory Committee (ACIP). Morbid. Mortal. Weekly Rep. **40**(RR-14)**:**1–10.
15. **Clements, M. L., R. S. Betts, E. L. Tierney, and B. R. Murphy.** 1986. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. J. Clin. Microbiol. **24:**157–160.
16. **Cross, R. M., C. Kaplan, and D. McClean.** 1957. The heat resistance of dried smallpox vaccine. Lancet **i:**446–448.
17. **Eichelberger, M., W. Allan, M. Zijlstra, R. Jaenisch, and P. C. Doherty.** 1991. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. J. Exp. Med. **174:**875–880.
18. **Epstein, S. L., C.-Y. Lo, J. A. Misplon, C. M. Lawson, B. R. Murphy, and E. K. Subbarao.** 1995. Mediators of heterosubtypic immunity to influenza virus infection in mice, p. 393–397. *In* R. M. Chanock, F. Brown, H. S. Ginsberg, and E. Norling (ed.), Vaccines 95. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Fenner, F., D. A. Henderson, I. Arita, Z. Jezek, and I. D. Ladnyi.** 1988. Smallpox and its eradication. World Health Organization, Geneva.
20. **Frank, A. L., L. H. Taber, and J. M. Wells.** 1983. Individuals infected with two subtypes of influenza A virus in the same season. J. Infect. Dis. **147:**120–124.
21. **Gammelin, M., J. Mandler, and C. Scholtissek.** 1989. Two subtypes of nucleoproteins (NP) of influenza A viruses. Virology **170:**71–80.
22. **Ghendon, Y.** 1989. The immune response of humans to live and inactivated influenza vaccines. Adv. Exp. Med. Biol. **257:**37–45.
23. **Gorman, O. T., W. J. Bean, Y. Kawaoka, I. Donatelli, Y. Guo, and R. G. Webster.** 1991. Evolution of influenza A virus nucleoprotein genes: implications for the origins of H1N1 human and classical swine viruses. J. Virol. **65:**3704–3714.
24. **Hochstein-Mintzel, V., H.-C. Huber, and H. Stickl.** 1972. Die orale und nasale Immunisierung mit Poxvirus Vacciniae. III. Mitteilung: tierexperimentelle Untersuchungen. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig. B. **156:**30–96.
25. **Hochstein-Mintzel, V., H.-C. Huber, and H. Stickl.** 1972. Virulenz und Immunogenität eines modifizierten Vaccinia-Virus (Stamm MVA). Z. Immununitaetsforsch. **144:**140–145.
26. **Jenner, E.** 1798. An inquiry into the causes and effects of the variolae vaccinea. Sampson Low, London. (Reprint, Classics of Medicine Library, Birmingham, Ala., 1978.)
27. **Kanesaki, T., B. R. Murphy, P. L. Collins, and P. L. Ogra.** 1991. Effectiveness of enteric immunization in the development of secretory immunoglobulin A response and the outcome of infection with respiratory syncytial virus. J. Virol. **65:**657–663.
28. **Lawson, C. M., J. R. Bennink, N. P. Restifo, J. W. Yewdell, and B. R. Murphy.** 1994. Primary pulmonary cytotoxic T lymphocytes induced by immunization with a vaccinia virus recombinant expressing influenza A nucleoprotein peptide do not protect mice against challenge. J. Virol. **68:**3505–3511.
28a. **Liang, S., K. Mozdzanowska, G. Palladino, and W. Gerhard.** 1994. Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. J. Immunol. **152:**1653–1661.
29. **Lin, Y., and B. A. Askonas.** 1981. Biological properties of an influenza A virus-specific cytotoxic T lymphocyte clone. J. Exp. Med. **154:**225–234.
30. **Lukacher, A. E., V. L. Braciale, and T. J. Braciale.** 1984. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. J. Exp. Med. **160:**814–826.
31. **Mayr, A., and K. Danner.** 1979. Bedeutung von Tierpocken für den Menschen nach Aufhebung der Pflichtimpfung gegen Pocken. Berl. Muench. Tieraerztl. Wochenschr. **92:**251–256.
32. **Mayr, A., V. Hochstein-Mintzel, and H. Stickl.** 1975. Abstammung, Eigenschaften und Verwendung des attenuierten Vaccinia-Stammes. Infection **3:**6–14.
33. **Mayr, A., H. Stickl, H. K. Muller, K. Danner, and H. Singer.** 1978. Der Pockenimpfstamm MVA: Marker, genetische Struktur, Erfahrungen mit per parenteralen Schutzimpfung und Verhalten im abwehrgeschwächten Organismus. Zentralbl. Bakteriol. Parasitenkd. Infektionskd. Hyg. Abt. I Orig. B **167:**375–390.
34. **Mazanec, M. B., C. L. Coudret, and D. R. Fletcher.** 1995. Intracellular neutralization of influenza virus by immunoglobulin A anti-hemagglutinin monoclonal antibodies. J. Virol. **69:**1339–1343.
35. **Mazanec, M. B., C. S. Kaetzel, M. E. Lamm, D. Fletcher, and J. G. Nedrud.** 1992. Intracellular neutralization of virus by immunoglobulin A antibodies. Proc. Natl. Acad. Sci. USA **89:**6901–6905.
36. **McGhee, J. R., and J. Mestecky.** 1990. In defense of mucosal surfaces. Infect. Dis. Clin. N. Am. **4:**315–341.
37. **McGhee, J. R., J. Mestecky, M. T. Dertzbaugh, J. H. Eldridge, M. Hirasawa, and H. Kiyono.** 1992. The mucosal immune system. From fundamental concepts to vaccine development. Vaccine **10:**75–88.
38. **McMichael, A. J., F. M. Gotch, G. R. Noble, and P. A. Peare.** 1983. Cytotoxic

T-cell immunity to influenza. N. Engl. J. Med. **309:**13–17.
39. **Meitin, C. A., B. S. Bender, and P. A. Small, Jr.** 1991. Influenza immunization: intranasal immunization with a live vaccinia recombinant contrasted with parenteral inactivated vaccine. Vaccine **9:**751–756.
40. **Meitin, C. A., B. S. Bender, and P. A. Small, Jr.** 1994. Enteric immunization of mice against influenza with recombinant vaccinia. Proc. Natl. Acad. Sci. USA **91:**11187–11191.
41. **Meyer, H., G. Sutter, and A. Mayr.** 1991. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. J. Gen. Virol. **72:**1031–1038.
42. **Moss, B.** 1991. Vaccinia virus: a tool for research and vaccine development. Science **252:**1662–1667.
43. **Nuchtern, J. G., J. G. Bonifacino, W. E. Biddison, and R. D. Klausner.** 1989. Brefeldin A implicates egress from endoplasmic reticulum in class I restricted antigen presentation. Nature (London) **339:**223–226.
44. **Palladino, G., K. Mozdzanowska, G. Washko, and W. Gerhard.** 1995. Virus-neutralizing antibodies of immunoglobulin G (IgG) but not of IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice. J. Virol. **69:**2075–2081.
45. **Paoletti, E., B. R. Lipinskas, C. Samsonoff, S. Mercer, and D. Panicali.** 1984. Construction of live vaccines using genetically engineered poxviruses: biological activity of vaccinia virus recombinants expressing the hepatitis B virus surface antigen and the herpes simplex virus glycoprotein D. Proc. Natl. Acad. Sci. USA **81:**193–197.
46. **Renegar, K. B., and P. A. Small, Jr.** 1991. Immunoglobulin A mediation of murine nasal anti-influenza virus immunity. J. Virol. **65:**2146–2148.
47. **Renegar, K. B., and P. A. Small, Jr.** 1991. Passive transfer of local immunity to influenza virus infection by IgA antibody. J. Immunol. **146:**1972–1978.
48. **Robbins, A., and P. Freeman.** 1988. Obstacles for developing vaccines for the third world. Sci. Am. **259**(5):126–133.
49. **Rupprecht, C. E., and M. P. Kieny.** 1988. Development of a vaccinia-rabies glycoprotein recombinant virus vaccine, p. 335–364. *In* J. B. Campbell and K. M. Charlton (ed.), Developments in veterinary virology series, vol. C. Rabies. Kluwer Academic Publishers, Boston.
50. **Rupprecht, C. E., T. J. Wiktor, D. H. Johnston, A. M. Hamir, B. Dietzchold, W. H. Wunner, L. T. Glickman, and H. Koprowski.** 1986. Oral immunization and protection of raccoons (*Procyon lotor*) with a vaccinia-rabies glycoprotein recombinant virus vaccine. Proc. Natl. Acad. Sci. USA **83:**7947–7950.
51. **Scherle, P. A., G. Palladino, and W. Gerhard.** 1992. Mice can recover from pulmonary influenza virus infection in the absence of class I-restricted cytotoxic T cells. J. Immunol. **148:**212–216.
52. **Schulman, J. L., and E. D. Kilbourne.** 1965. Induction of partial specific heterotypic immunity in mice by a single infection with influenza A virus. J. Bacteriol. **89:**170–174.
53. **Small, P. A.** 1990. Influenza: pathogenesis and host defense. Hosp. Pract. **25**(11):51–62.
54. **Small, P. A., Jr., G. L. Smith, and B. Moss.** 1985. Intranasal vaccination with recombinant vaccinia containing influenza hemagglutinin prevents both influenza virus pneumonia and nasal infection: intradermal vaccination prevents only viral pneumonia, p. 175–176. *In* R. A. Lerner, R. M. Chanock, and F. Brown (ed.), Vaccines 85. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
55. **Smith, G. L., B. R. Murphy, and B. Moss.** 1983. Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. Proc. Natl. Acad. Sci. USA **80:**7155–7159.
56. **Sonoguchi, T., H. Naito, M. Hara, Y. Takeuchi, and H. Fukumi.** 1985. Cross-subtype protection in humans during sequential, overlapping, and/or concurrent epidemics caused by H3N2 and H1N1 influenza viruses. J. Infect. Dis. **151:**81–88.
57. **Steinhoff, M. C., L. F. Fries, R. A. Karron, M. L. Clements, and B. F. Murphy.** 1993. Effect of heterosubtypic immunity on infection with attenuated influenza A virus vaccines in young children. J. Clin. Microbiol. **31:**836–838.
58. **Stickl, H., V. Hochstein-Mintzel, A. Mayr, H. C. Huber, H. Schafer, and A. Holzner.** 1974. MVA-Stufenimpfung gegen Pocken. Dtsch. Med. Wochenschr. **99:**2386–2392.
59. **Sutter, G., and B. Moss.** 1992. Nonreplicating vaccinia vector efficiently expresses recombinant genes. Proc. Natl. Acad. Sci. USA **89:**10847–10851.
60. **Sutter, G., L. S. Wyatt, P. L. Foley, J. R. Bennink, and B. Moss.** 1994. A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity to influenza virus. Vaccine **12:**1032–1040.
61. **Taylor, S. F., P. A. Small, Jr., and B. S. Bender.** Unpublished observations.
62. **Townsend, A., C. Öhlen, J. Basten, H.-G. Ljunggren, L. Foster, and K. Kärre.** 1989. Association of class I major histocompatibility heavy and light chains induced by viral peptides. Nature (London) **340:**443–448.
63. **Townsend, A. R. M., A. J. McMichael, N. P. Carter, J. A. Huddleston, and G. G. Brownlee.** 1984. Cytotoxic T cell recognition of the influenza nucleoprotein and hemagglutinin expressed in transfected mouse L cells. Cell **9:**13–25.
64. **Wells, M. A., F. A. Ennis, and P. Albrecht.** 1981. Recovery from a viral respiratory infection. II. Passive transfer of immune spleen cells to mice with influenza pneumonia. J. Immunol. **126:**1042–1046.
65. **Werner, G. T., U. Jentsch, E. Metzger, and J. Simon.** 1980. Studies on poxvirus infection in irradiated animals. Arch. Virol. **64:**247–256.
66. **World Bank.** 1993. Investing in health. Oxford University Press, London.
67. **Yap, K., L. G. L. Ada, and I. F. C. McKenzie.** 1978. Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. Nature (London) **273:**238–241.
68. **Yetter, R. A., S. Lehrer, R. Ramphal, and P. A. Small, Jr.** 1980. Outcome of influenza infection: effect of site of initial infection and heterotypic immunity. Infect. Immun. **29:**654–662.
69. **Yewdell, J. W., J. R. Bennink, and Y. Hosaka.** 1988. Cells process exogenous proteins for recognition by cytotoxic T lymphocytes. Science **239:**637–640.
70. **Yewdell, J. W., J. R. Bennink, G. L. Smith, and B. Moss.** 1985. Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. USA **82:**1785–1789.
71. **Yilma, T., D. Hsu, L. Jones, S. Owens, M. Grubman, C. Mebus, M. Yamanaka, and B. Dale.** 1988. Protection of cattle against rinderpest with vaccinia virus recombinants expressing the HA or F gene. Science **242:**1058–1061.