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Priming with Cold-Adapted Influenza A Does Not Prevent Infection but Elicits Long-Lived Protection against Supralethal Challenge with Heterosubtypic Virus


We show in this study several novel features of T cell-based heterosubtypic immunity against the influenza A virus in mice. First, T cell-mediated heterosubtypic protection against lethal challenge can be generated by a very low priming dose. Second, it becomes effective within 5–6 days. Third, it provides protection against a very high dose challenge for >70 days. Also novel is the finding that strong, long-lasting, heterosubtypic protection can be elicited by priming with attenuated cold-adapted strains. We demonstrate that priming does not prevent infection of the lungs following challenge, but leads to earlier clearance of the virus and 100% survival after otherwise lethal challenge. Protection is dependent on CD8 T cells, and we show that CD4 and CD8 T cells reactive to conserved epitopes of the core proteins of the challenge virus are present after priming. Our results suggest that intranasal vaccination with cold-adapted, attenuated live virus has the potential to provide effective emergency protection against emerging influenza strains for several months. The Journal of Immunology, 2007, 178: 1030–1038.

Current immunization strategies against influenza virus seek to generate neutralizing Abs to hemagglutinin (HA) and neuraminidase (NA) molecules on the viral surface. These Abs block attachment of the virus to airway epithelium and abort infection. The problem, however, is that the influenza viruses undergo variation of coat proteins by antigenic drift and by antigenic shift, and new strains emerge that do not react with the Ab. New vaccines must be made for each new wave of virus at the start of each flu season. This strategy may prove difficult or impossible for some pandemic outbreaks with unfamiliar H and N types because of the long lead time before new vaccine production. Investigators have sought to produce vaccines that elicit T cell immunity to internal gene products that are less subject to this hypervariability.

Immunization with viruses of a different subtype that do not share coat protein Ags does not elicit neutralizing Abs to the challenge strain, but does elicit heterosubtypic T cell immunity. In this case, the virus infects airway epithelial cells and viral replication proceeds before the virus is cleared, albeit more rapidly than in unimmunized controls. Heterosubtypic immunity has been demonstrated in mice by numerous investigators (1). It provides protection from otherwise lethal challenge and is correlated with activated memory T cells in the lung (2). Other studies have suggested that multiple factors, including CD4 and CD8 T cells, non-neutralizing IgA Abs, NKT cells, and γδ T cells also contribute to heterosubtypic protection (3).

Heterosubtypic immunity has been shown to persist in mice for >50–100 days but declines to a relatively modest level at later times. Some level of protection from heterosubtypic infection following vaccination or natural infection has been observed in humans (4–7). The lack of protection from infection and the somewhat limited duration of protection have generally been considered inadequate as an outcome for any program of immunization (8). The key issue with respect to this report, whether heterosubtypic priming with attenuated live vaccines can provide protection against lethal challenge in humans or even early viral clearance, has not been directly addressed.

We demonstrate in this study that there is, in mice, a very effective level of protection against lethal challenge that it is present for a considerable period after priming. Protection against lethal challenge is effective against heterosubtypic influenza A virus expressing H and N subtypes that do not cross-react at the level of neutralizing Abs but not against viruses other than influenza A. The protection persists at a very high level for >70 days. It can be elicited by cold-adapted, attenuated heterosubtypic virus and is effective against lethal challenge with distantly related heterosubtypic strains. The important point to emphasize in this study is that we have demonstrated that low-dose priming leads to early viral clearance and dramatically enhanced survival after otherwise lethal challenge even though infection has not been prevented.

Priming with wild-type (WT) or cold-adapted viruses induces vigorous CD4 and CD8 T cell responses to epitopes that are shared with human outbreak strains of influenza. We suggest, based on these animal studies, that intranasal immunization of humans with live cold-adapted viruses (9) should provide significant short-lived protection against the lethal effects of such viruses and should be explored as an emergency prophylactic strategy under conditions in which conventional vaccines cannot be made available quickly.
Mice were challenged by intranasal inoculation with 10^4–10^6 50% egg infectious dose (EID_{50}) at 4°C with 100 μl. Multiscreen mixed ester nitrocellulose plates (Millipore) were coated overnight at 4°C with influenza peptides in a standard ELISPOT assay (15). Briefly, 96-well plates were coated with 10 μg/ml peptides. Plates were then incubated overnight at 37°C and developed overnight with a biotinylated detection Ab, rat anti-mouse IFN-γ (BD Pharmingen). The plates were then incubated with streptavidin—alkaline phosphatase substrate (Sigma-Aldrich) for 2 h at room temperature, developed with 5-bromo-4-chloro-indolyl phosphate substrate solution prepared as per the manufacturer’s instructions (Sigma-Aldrich). Visible spots of IFN-γ-secreting cells were counted in an ImmunoSpot reader (Cellular Technology).

Results

Rapid induction of protection against high doses of heterosubtypic challenge virus following intranasal priming with low doses of live virus

We conducted an extensive series of experiments to define the parameters of protection, defined as survival after lethal heterosubtypic challenge. We determined how low a dose could be used to prime, how quickly protection developed, and how high a dose of virus of another subtype could be protected against. Low-dose A/Alaska stock in the plaque assay. Titers of cold-adapted viruses on Madin-Darby canine kidney cells are not strictly comparable to similar titers of WT viruses because of temperature sensitivity and we have listed them as 50% tissue culture infectious dose (TCID_{50}) instead of PFU in recognition of this point. We made two batches of ca.A/Alaska with similar TCID titers, but batch 2 used only in the experiment presented in Fig. 1 was 5-fold less effective at eliciting protection from heterosubtypic challenge. In subsequent experiments, we used 5-fold less-dilute doses of batch 2 and have seen the same levels of protection as seen with batch 1.

Ab depletion of T cells or T cell subsets

Mice were injected with two doses of 0.5 mg Ab in PBS, 1 day before and 1 day after challenge. Abs used were anti-Thy-1.2 (30H12), anti-CD8 (2.43), or anti-CD4 (GK1.5). Isotype-matched control Ig was IgG2b (clone LTF-2).

T cell responses to A/PR8 epitopes

T cell responses were sacrificed by cervical dislocation at various time points before and after exposure to virus, and spleen, lymph nodes, and lungs were taken. Lungs were removed following perfusion with 5 ml of PBS via the left ventricle of the heart. Single-cell suspensions were prepared using 70-μm nylon filters (Falcon).

Tetramer staining

The cells were washed and resuspended in FACS buffer (PBS with 2% w/v BSA; InterGen) and 0.1% (w/v) sodium azide (Sigma-Aldrich), then incubated with 1 μg anti-FcR (clone 2.4G2, produced by Trudeau Institute) followed by anti-CD8-PerCP (53-6.7), anti-CD44 (IM7) PE, and anti-Thy-1.2 FITC obtained from BD Biosciences, and nuclear protein (NP), acid phosphatase (PA), and basic polymerase 1 (PB-1) tetramers. PA224–233 (D^b), NP366–374/D^b, and PB-1(903–711/K^b) tetramers contained the peptides derived against a challenge of 2, 20, or 200 LD_{50} (10^4, 10^5, or 10^6 EID_{50}) A/PR8 viruses 6 days later as shown in Fig. 2. There was little weight loss following the priming infection and no additional weight loss was seen after challenge with either of the lower doses. Mice challenged with 10^6 EID_{50} lost weight initially but started to regain weight 8 days after challenge and four of five survived. Control, unprimed mice exposed only to intranasal PBS lost weight rapidly following even the lowest challenge dose, and all had died by 8 days after challenge. In other experiments, protection against 20 LD_{50} challenge was seen at 5 days after priming but was not present at day 4 (data not shown), and protection persisted for >100 days (see below).

Cold-adapted, attenuated viruses can also prime against high-dose challenge

We next investigated whether attenuated, cold-adapted viruses, in which infection is limited to the nasal passages, could protect against lethal challenge with heterosubtypic virus. BALB/c mice were primed with one or two doses (10 days apart) of 250 TCID_{50}
or 2500 TCID₅₀ ca.A/Alaska/72 CR9 (H3N2) and were challenged with 10⁶ EID₅₀ A/PR8 on day 20 (Fig. 1). (We have designated the viral amounts of the cold-adapted virus in tissue culture infective dose rather than PFU because the measurements with cold-adapted virus are not strictly comparable to the measures of WT virus.) Mice given a single priming dose at 250 TCID₅₀ at day 0 did not survive challenge with 10⁶ EID₅₀ A/PR8, but mice primed with a higher dose or two lower or two higher doses all survived and there was little or no weight loss following priming with two higher doses. Similar groups of primed mice challenged with 10⁵ EID₅₀ A/PR8 all survived (data not shown). All of the PBS-primed mice died on challenge with 10⁵ PR8 (data not shown). In other experiments, protection against lethal challenge was seen at day 8 (data not shown) and persisted for 70 days (see below).

ca.A/Alaska-primed mice are protected for up to 70 days after priming and ca.A/Alaska priming is as protective as A/X31

We next wanted to compare the efficacy of the cold-adapted virus with that of A/X31 virus in priming for protection against lethal challenge. Fig. 3 shows the weight loss curves following priming with a single dose of 50 EID₅₀ A/X31, (left panels) using BALB/c mice, or a single dose of 2500 TCID₅₀ ca.A/Alaska/72 (right panels) using C57BL/6 mice. (We have seen no differences in the protection generated in BALB/c and C57BL/6 mice in a large number of experiments.) All mice were challenged with 10⁵ EID₅₀ A/PR8 at days 30, 50, and 100 for A/X31-primed mice or on days 30, 50, and 70 for ca.A/Alaska/72-primed mice. In all cases, the primed mice lost weight upon challenge, but in all cases 100% of the primed mice survived. Unprimed BALB/c and C57BL/6 mice challenged with 10⁵ EID₅₀ A/PR8 (20 LD₅₀) have invariably died (data not shown). These results show that priming with ca.A/Alaska/72 was equally as effective as A/X31 at days 30 and 50 and that ca.A/Alaska/72 was as effective at day 70 as A/X31 at day 100. There was no evidence that protection against was waning at day 70, as judged by the response to 10⁵ EID₅₀ A/PR8, but the level of protection afforded by ca.A/Alaska priming was declining, because some mice died after challenge with 10⁵EID₅₀, a dose 10-fold higher than that shown in Fig. 3, whereas all survived this dose at day 50.

ca.A/Alaska priming generates T cell responses to core protein epitopes

Naive C57BL/6 mice or mice primed with one or two intranasal infections, 10 days apart, with 2500 TCID₅₀ ca.A/Alaska/72 were challenged with 10⁵ EID₅₀ A/PR8 10 days after the last priming dose. C57BL/6 mice were used rather than BALB/c so that we could take advantage of the tetramers and peptides that we had previously developed for the dominant epitopes recognized by CD4 and CD8 T cells from H-2Kb mice (14). Spleens, lymph nodes, and lungs were harvested from naive mice, from naive mice after challenge, or from the primed mice after priming (10 days after single priming or 5 days after double priming) and 4 days after lethal A/PR8 challenge. Cell suspensions were prepared, counted, and stained with fluorescent-labeled Abs to Thy-1.2, H-2Kb.

It can be seen in Fig. 4A that the lungs of naive mice contained no measurable tetramer-positive cells before challenge and only low numbers of any of the CD8+ CD44bright tetramer-positive cells following challenge with a lethal dose of A/PR8 (naïve column). The lungs of primed mice contained substantial numbers of PA and PB-1 and lesser numbers of NP tetramers after intranasal exposure to a single dose of 2500 TCID50 ca.A/Alaska/72 (Fig. 4, Alaska d0 column). Similar numbers of tetramer-positive cells were seen in lungs harvested 4 days after lethal challenge. A somewhat larger number of tetramer-positive cells was seen in lungs taken from mice after two priming doses and after lethal A/PR8 challenge (Fig. 4, Alaska d0 + d10 column).

Cells were also harvested from spleen and lymph nodes at all of the same points and a similar pattern of response was observed (data not shown).

ELISPOT analyses for IFN-γ-secreting cells were performed on the same cell suspensions. In the ELISPOT analyses (Fig. 4B), we used the PA224–233, and NP366–374 peptides recognized by CD8 T cells (upper panels) and the NP261–275 and NP311–325 peptides recognized by CD4 T cells (lower panels). NP311–325 is also recognized by CD8 T cells (6). The overall pattern seen with ELISPOTs paralleled the findings obtained with tetramers, but showed that strong CD4 T cell responses were also elicited in twice-primed mice.

These results demonstrate that strong CD4 and CD8 T cell responses are made to epitopes of the core proteins of the challenge virus in mice primed with the cold-adapted A/Alaska virus (even when the sequences of the prime and challenge virus were not identical) but not in the control unprimed mice. Similar numbers of reactive cells were seen 4 days after PR8 challenge. We wanted to be sure that no Abs were generated that cross-reacted with the challenge virus. We took spleen and lymph node populations from mice primed with ca.A/Alaska in the experiment shown in Fig. 4 and found that the number of ELISPOTs against PR8 were less than or equal to the background levels in naive mice for IgM and essentially zero for IgG, IgB2a, and IgA. We also tested A/Alaska batch 2 to confirm that it was not contaminated with PR8 virus to address a concern raised by the reviewer. The serum of mice harvested days after priming with A/Alaska, batch 2, had no measurable HA inhibition against the A/PR8 virus.

Conservation of core protein sequences

We have developed a visual comparison of the protein sequences of all available influenza isolates ranging from the 1918 virus, through the intervening outbreaks, up to the recent H5N1 bird flu sequences as listed in Fig. 7. In Fig. 5, the sequences for HA and NA show only 31 and 34% conservation, whereas those for the core proteins, NP, PA, and PB-1, range from 80–84% in all outbrak strains. Fig. 6 shows a more detailed picture of the sequence conservation for a key T cell Ag NP, which includes sequences for A/Ann Arbor 60/66, A/NT/6/60, two PR8s, X-31, H5s (Hong Kong, Hayt, Vietnam, and Thailand, one sequence each), and Brevig Mission, 1918. Many of the individual NP epitopes recognized by C57BL/6 H2b or BALB/c H-2b mice are completely conserved or differ by changes that are still recognized by the same T cell (Fig. 6). It is therefore likely that vaccination with cold-adapted virus will elicit responses that will react with epitopes from any new outbreak strain regardless of subtype.

Mechanisms of protection

We conducted a limited number of analyses to establish the mechanism in this model of heterosubtypic protection. The intranasal
route was key to successful priming. Priming by i.p. injection was much less effective but still provided some protection (data not shown). T-deficient, BALB/c nu/nu mice were not protected after intranasal priming when challenged at day 8. Nude mice lost weight following the priming dose, and all five of the primed and unprimed control nude mice died on challenge (data not shown), suggesting a requirement for T cell immunity. In contrast, a similar experiment using B cell-deficient, BALB/c JhD mice showed that

FIGURE 5. Conservation of core proteins NP, PA, and PB1, variability of coat proteins HA and NA. The black bars represent the completely conserved sequence of the individual protein and the white notches indicate variability, the depth of the notch being proportional to the amount of variability. To evaluate the conservation of the internal and external proteins of influenza A virus, full-length protein sequence alignments were generated using the CLC Free Workbench software package version 2.01 (T. Knudson, CLC bio, Aarhus C, Denmark). All available virus protein sequences were selected for analysis from the GenBank database, with only one sequence per isolate included to minimize bias. A line graph depicting the relative conservation of each amino acid within the protein sequence was generated within the CLC Free Workbench, and known CD4 and CD8 T cell epitopes were mapped on the consensus sequence. The number of protein sequences aligned were as follows: HA, n = 30; NA, n = 98; NP, n = 130; PA, n = 62; and PB1, n = 65. Phylogenetic trees were created from the protein alignments with a CLC Free Workbench using the Neighborjoin algorithm.

FIGURE 6. A more detailed portrayal for the NP core protein and the locations of the class I and class II epitopes for BALB/c and C57BL/6 mice. A phylogenetic tree showing the human viral isolates used for the compilation of this data is shown in Fig. 5.
FIGURE 7. Human influenza isolates used in the construction of the diagram of NP sequence variation shown in Fig. 6.
Mice were primed with a single low dose of 250 TCID<sub>50</sub> ca.A/Alaska on day 0 or two high doses of 2500 TCID<sub>50</sub> ca.A/Alaska on days 0 and 10 and were challenged with 10<sup>4</sup> EID<sub>50</sub> A/PR8 on day 20. Mice primed with the lower dose lost weight after challenge but recovered and all survived (Fig. 9A). Mice primed with the high dose lost little, if any, weight after challenge and all survived. Unprimed mice all died after challenge.

Additional mice subjected to the same conditions were sacrificed, their lungs were harvested on days 2, 4, and 7 after challenge, and the lung viral titers were determined. It can be seen (Fig. 9B) that neither the low nor the double high-priming dose prevented infection. Viral titers at days 2 and 4 after challenge in the low-dose primed mice were comparable to those seen in the unprimed control mice, whereas those in the twice-primed mice were one log lower than in the unprimed control mice. However, the virus was cleared by day 7 in both primed groups and no longer detectable (below 10<sup>-2</sup> PFU/lung), whereas the titer in the control mice, although falling, was still more than four logs higher at 10<sup>6</sup> PFU/lung.

**Discussion**

Heterosubtypic immunity in mice has been recognized and characterized for >40 years (16). The level of protection afforded by classic heterosubtypic immunity declines after the first 50-100 days (1) but then stabilizes at a lower level that persists for many months. In most previous studies, investigators used only a single priming and challenge dose, did not determine when protection first became apparent, and did not determine how high a challenge dose could be resisted after priming. What is novel in the data presented here is that protection was completely lost upon depletion of both CD4 and CD8 T cells before challenge. Mice that were primed, challenged, and given two injections of 0.5 mg anti-Thy1.2 (30H12), a third group with two injections of 0.5 mg anti CD8 (2.43), a fourth group with two injections of control 0.5 mg IgG2b (clone LTF-2), and a fifth group with two injections of 0.5 mg anti CD4 (GK1.5). Injections of depleting Abs were on days 7 and 9. Survival and weight loss were monitored. Values shown are percentage of initial weight ± SD. Survival in each group is noted next to the respective line.

To further investigate the role of T cells in protection against lethal challenge with heterosubtypic strains of virus, we determined the effect of depleting various T cell populations from the primed BALB/c mice at the time of lethal challenge. All of the mice (Fig. 8) were primed with 2500 TCID<sub>50</sub> ca.A/Alaska and challenged with 10<sup>6</sup> EID<sub>50</sub> A/PR8 on day 8. Control mice that were primed and then challenged, but received PBS instead of depleting Abs, lost weight until day 12, and then recovered rapidly, and five of five survived. Protection was completely lost upon depletion of both CD4 and CD8 T cells before challenge. Mice that were primed, challenged, and given two injections of 0.5 mg anti-Thy-1.2, one at day 7 and one at day 9, continued to lose weight, and all had died by day 14. Protection was also lost on depletion of CD8 T cells. Thus, two injections of 0.5 mg anti-CD8 also prevented recovery and all died by day 14. In contrast, mice depleted of CD4 T cells upon receiving two injections of anti-CD4 (GK1.5) lost weight initially but all were protected and alive at day 22. The recovery of lost weight was slower than that seen in the control groups, suggesting that CD4 T cells made some contribution to the protection. To confirm that the CD4 T cells were indeed depleted, blood was taken from the GK1.5-treated mice at day 15 and found to contain <0.03% CD4<sup>+</sup> T cells using Ab (RM4H4; BD Pharmingen) that does not compete with GK1.5 (data not shown). Mice receiving two injections of 0.5 mg IgG2b as control for the depleting Abs were still protected and all survived. Protection was strongly dependent on CD8 T cells, but CD4 T cells appeared to have some role.

An identical pattern of responses was seen when mice were challenged at day 110 (data not shown).

**FIGURE 8.** Heterosubtypic protection from lethal challenge is abrogated by depletion of CD8 T cells. Groups of five BALB/c mice were primed with 2500 TCID<sub>50</sub> ca.A/Alaska and challenged with 10<sup>4</sup> A/PR8 on day 8. Group with two injections of 0.5 mg anti Thy-1.2 (30H12), a third group with two injections of 0.5 mg anti CD8 (2.43), a fourth group with two injections of control 0.5 mg IgG2b (clone LTF-2), and a fifth group with two injections of 0.5 mg anti CD4 (GK1.5). Injections of depleting Abs or PBS were on days 7 and 9. Survival and weight loss were monitored. Values shown are percentage of initial weight ± SD. Survival in each group is noted next to the respective line.

**FIGURE 9.** Neither low- nor high-dose ca.A/Alaska priming prevents infection, but it does lead to early viral clearance and protects against lethal challenge. A, BALB/c mice were primed with PBS, a single dose of 250 TCID<sub>50</sub>, or two doses of 2500 TCID<sub>50</sub> ca.A/Alaska, and challenged with 10<sup>6</sup> A/PR8 on day 20. Survival and weight loss were monitored until days 27–33. Values shown are percentage of initial weight ± SD. Survival in each group is noted next to the respective line. B, Groups of mice subjected to the same protocols were sacrificed on days 2, 4, and 7 after challenge, and the viral titers in the lung were determined by the Madin-Darby canine kidney plaque assay as described in Materials and Methods. (BDL, below limit of detection at 10<sup>2</sup> PFU/lung).

Primed with cold-adapted virus does not prevent infection, but the virus was cleared early and all of the mice survived lethal challenge

Mice were primed with a single low dose of 250 TCID<sub>50</sub> ca.A/Alaska on day 0 or two high doses of 2500 TCID<sub>50</sub> on days 0 and 10 and were challenged with 10<sup>4</sup> EID<sub>50</sub> A/PR8 on day 20. Mice priming with the lower dose lost weight after challenge but recovered and all survived (Fig. 9A). Mice primed with the high dose lost little, if any, weight after challenge and all survived. Unprimed mice all died after challenge.
presented in this study is that heterosubtypic protection can be elicited by a very low priming dose, is already effective very shortly after priming, provides protection against a high challenge dose of virus, and persists for >70 days. All four of these features could prove invaluable in the face of an emergency situation. Most importantly, we have shown that low-dose priming can lead to enhanced survival after otherwise lethal challenge, even though infection has not been prevented, and that such protection can be elicited by cold-adapted viruses.

The mutation rates for the genes that code for the HA and NA coat proteins appear to be only modestly greater than that for the core proteins (17), but the selection pressure from Ab responses drives a much more rapid selection of mutants. Abs recognize the tertiary structure of the HA and NA protein and are independent of the HLA type of the responding person. Thus, Abs in separate individuals can collectively select against the initial sequences present at the start of each flu season. T cell responses in humans are HLA specific and the individual responses of the persons with varying HLA types cannot exert the same uniform selective pressure on mutants of the core proteins. CTL escape mutants have been shown to occur, but the requirement for function limits the range of possible mutants (18).

The low-dose priming is probably effective because even a very low dose of virus replicates enormously before being cleared and generates a robust T cell response (13). The protection against heterosubtypic challenge can be expected to be strongest when internal proteins are identical between priming and challenge virus, as is the case with A/X31 and A/PR8. Under these conditions, low-dose priming protects against 20 LD<sub>50</sub> (10<sup>5</sup> EID<sub>50</sub>) at day 5 and 200 LD<sub>50</sub> at days 6–100. Protection against weight loss is less marked by day 50 following 20 LD<sub>50</sub>, but still assures 100% survival.

The protection is also achieved when the priming virus does not have identical internal proteins and a single priming dose of A/NT60/68, which was isolated 34 years later than A/PR8/34, can still give 100% protection against 2 LD<sub>50</sub> challenge at day 8 (data not shown). As expected, mice primed with the unrelated Sendai virus were not protected at day 8 or day 14, whereas priming with influenza B gave only very low levels of protection at day 8 (data not shown).

Cold-adapted attenuated viruses, which only infect the upper respiratory tract, have been shown to be less immunogenic than WT strains (19) as judged by the ability to elicit serum Ab formation. This was true in our hands with a low frequency of Ab-forming cells in the spleen after priming (data not shown). It was thus all the more striking that a single exposure to 250 TCID<sub>50</sub> ca.A/Alaska/72 (H3N2) was able to protect against 20 LD<sub>50</sub> A/PR8 on day 20, and higher doses or multiple doses could protect against 200 LD<sub>50</sub> with no discernable weight loss. The higher single dose of 2500 TCID<sub>50</sub> ca.A/Alaska/72 was as effective as priming with A/X31 and the protection lasted for at least 70 days.

Our investigation of the mechanism of the protection is enough to establish that we are dealing with the well-studied classical heterosubtypic protection, even at 10 days after priming, and not some nonspecific effect.

We have shown previously that small numbers of activated CD8 T cells are present already in the lung and bronchoalveolar lavage 4 days after priming and increase to high numbers by day 6 and could be available for protection (20). We did, however, initially consider a number of other possibilities including IFN-α, TNF, collectins, and sticky mucus as explanations of the early protection, but our demonstration of the fact that Sendai and influenza B were not protective at day 8 argued against these possibilities. We harvested the bronchoalveolar lavage at day 6 and showed that the neutralizing activity present was specific only for the priming virus and did not provide heterosubtypic protection (data not shown). The T cell depletion experiments in both X31-primed (data not shown) and in ca.A/Alaska-primed mice (Fig. 8) indicated that the CD8 T cells were the major contributors to protection measured at the day 8 challenge but that CD4 T cells made some contribution. These findings are in accordance with those of Gerhard and colleagues (1), although other investigators (3) have provided evidence that a number of other mechanisms may also operate in heterosubtypic protection to influenza virus.

We conclude that our studies, although far from exhaustive, argue against innate immune mechanisms and more in favor of adaptive T cell immunity as the mechanism of early protection, as seen in classic heterosubtypic immunity.

cA.A/Alaska/72 priming elicited strong T cell responses as seen by tetramer and ELISPOT analyses. These responses were seen in cell suspensions prepared from the lungs (Fig. 4) but also in spleen and lymph nodes (data not shown). It is interesting to note that tetramer-specific and peptide-specific T cells could be detected in cells from cA.A/Alaska-primed mice even though the peptide sequences used were those of the challenge rather than the priming virus. There are sequence differences in A/Ann Arbor/6/60 (used in the construction of cA.A/Alaska/72) vs A/PR8 in the PA<sub>224-233</sub>, SCLENFRAYV vs SSSLFRAYV, and the NP<sub>366-374</sub> ASNEN MTDM vs ASNENMTDM, but not in the PB<sub>1703-711</sub> SYR RPVGI for both. Underlined letters are the amino acids that differ between viral strains.

A comparison of the sequences of the proteins of all available influenza isolates, ranging from the 1918 virus through the intervening outbreaks up to the recent H5N1 bird flu sequences, the sequences for HA and NA (Figs. 5 and 6) suggest that T cell responses to core proteins can be expected to provide sufficient cross-reactivity to influenza A viruses of all pandemic subtypes that might emerge, including H5N1 bird flu.

The question, then, is what level of protection can such T cell responses provide. The data from this and other studies in mice suggest that T cell immunity can provide strong protection in the mouse against lethal challenge with influenza virus of a different subtype and that protection can last for a significant period of time.

Heterosubtypic immunity is believed to be weak in humans, but data from human studies is fragmentary and the data collected are not comparable in nature to that collected in mice. Thus, one study, using cold-adapted virus (4), showed little or no significant effect of previous exposure to influenza on the response to vaccines in very young children, as judged by viral shedding and rates of seroconversion, but for obvious reasons did not provide information on protection against lethal challenge. Two other studies (5, 6) did show significant reductions in the incidence of natural infection in groups of older children if they had been previously infected with a heterosubtypic virus. A very recent retrospective analysis of data collected in the Cleveland Family Study provided further evidence of heterosubtypic protection in adults in the H1N1 to H2N2 shift that occurred in the 1957 pandemic (7).

In none of these studies was the kinetics of viral clearance or the reduction in the severity of the disease determined, or whether there was any decreased mortality. This is crucial because our findings in mice demonstrate that although cold-adapted attenuated virus may not prevent infection by heterosubtypic strains, they might well provide crucial amelioration of the infection, earlier viral clearance, and protection from what might otherwise be a lethal outcome.

Influenza pandemics have the potential to wreak enormous medical, social, and economic damage, and every avenue that can lead to protection should be explored. We suggest that immunization
with live attenuated influenza vaccine (9, 21) should be considered as a rapid emergency strategy under conditions in which conventional vaccines to new viruses are not yet available to a community currently in the throes of a lethal outbreak of infection. Our data suggest that although it may not prevent infection it might nevertheless give a high level of protection against an otherwise lethal outcome following infection with distantly related heterosubtypic A influenza strains.

Further studies should explore the possibility of intensifying the response and extending the window during which protection is provided.

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The authors have no financial conflict of interest.

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