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Heterologous protection against lethal A/HongKong/156/97 (H5N1) influenza virus infection in C57BL/6 mice

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The continual threat posed by newly emerging influenza virus strains is demonstrated by the recent outbreak of H5N1 influenza virus in Hong Kong. Currently, immunization against influenza virus infection is fairly adequate, but it is imperative that improved vaccines are developed that can protect against a variety of strains and be generated rapidly. Since humoral immunity is ineffective against serologically distinct viruses, one strategy would be to develop vaccines that emphasize cellular immunity. Here we report the successful protection of C57BL/6 mice from a lethal A/HK/156/97 (HK156) infection by immunizing first with an H9N2 isolate, A/Quail/HK/G1/97 (QHKG1), that harbours internal genes 98% homologous to HK156. This strategy also protected mice that are deficient in antibody production, indicating that the immunity is T-cell-mediated. In the course of these studies, we generated a highly pathogenic H5N1 reassortant which implicated NP and PB2 as having an important contribution to pathogenesis when present with a highly cleavable H5. These results provide the first demonstration that protective cell-mediated immunity can be established against the highly virulent HK156 virus and have important implications for the development of novel strategies for the prevention and treatment of HK156 infection and the design of future influenza vaccines.

Introduction

In Hong Kong in 1997, 18 cases of avian-to-human transmission of an H5N1 influenza virus were reported; six of those cases resulted in fatalities (Claas *et al.*, 1998*a*, *b*; Shortridge, 1999; Subbarao *et al.*, 1998; Yuen *et al.*, 1998). There was evidence that some of the infected persons had direct contact with infected poultry but not with each other and that these viruses, although highly pathogenic, were not capable of human-to-human transmission (CDC, 1998). The latter, in conjunction with the slaughter of all the poultry in Hong Kong, prevented further spread of infection. This outbreak emphasized the importance of developing strategies for effective protection against lethal strains of influenza (Belshe, 1998; Webster & Hay, 1998).

It has been established that major histocompatibility complex (MHC) class I-restricted $CD8^+$ cytotoxic T-lymphocytes (CTLs) play a central role in the clearance of primary influenza virus infections (Doherty *et al.*, 1992; Eichelberger *et al.*, 1991; Epstein *et al.*, 1998; Graham & Braciale, 1997). In mouse models, effector CTLs are first detectable in the lung on day 7 and their numbers peak by day 9 or 10 (Allan *et al.*, 1990; Eichelberger *et al.*, 1991; Flynn *et al.*, 1998; Hou & Doherty, 1995). The accumulation of CTLs correlates with clearance of the virus, which occurs by day 8 or 9, and this clearance depends on either Fas or perforin mechanisms (Kagi & Hengartner, 1996; Topham *et al.*, 1997). Antibody is generated late in the primary response and does not play a significant role in clearing primary infection unless the viral dose is high (Graham & Braciale, 1997; Zhong *et al.*, 2000). However, pre-existing neutralizing antibodies are completely protective against secondary challenge with the same virus; this protection is the basis of current influenza vaccines. In general, the presence of virus-specific neutralizing antibodies prevents replication of the virus in the lung and blocks the development of symptoms.

Although humoral immunity provides complete protection against secondary challenge with the same virus, it is ineffective against serologically distinct viruses (Ada & Jones,

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1986; Couch & Kasel, 1983; Gorman *et al.*, 1992). In contrast, cellular responses to cross-reactive epitopes (often from internal viral proteins) provide a substantial degree of protection against serologically distinct viruses (Rimmelzwaan & Osterhaus, 1995; Yewdell *et al.*, 1985). This form of immunity, referred to as heterosubtypic immunity, is unable to prevent reinfection per se, but can reduce the maximal viral load, mediate faster viral clearance, and provide in animal models a substantial degree of protection against challenge with a lethal dose of virus (Anker *et al.*, 1978; Flynn *et al.*, 1998; Liang *et al.*, 1994; Nguyen *et al.*, 1999; Rimmelzwaan & Osterhaus, 1995; Schulman & Kilbourne, 1965; Schulman *et al.*, 1977). Mouse studies indicate that heterosubtypic immunity is mediated by both $CD4^+$ and $CD8^+$ T cells, although the $CD8^+$ subset is generally considered to be more important (Liang *et al.*, 1994; Yap & Ada, 1978). These T cells are primed during the primary response to infection and then persist in the animal after viral clearance and are able to respond more vigorously to a secondary challenge. Although heterosubtypic immunity has been shown to be capable of controlling secondary influenza virus infections, its effectiveness against highly virulent strains of influenza has not been clearly determined.

The A/HK/156/97 (HK156) virus may have resulted from genetic reassortment between cocirculating H5N1 and H9N2 viruses (Guan *et al.*, 1999). One of the H9N2 viruses isolated in the 1997 surveillance of the Hong Kong markets, A/Quail/HK/G1/97 (herein called QHKG1), contains internal genes (i.e. genes that encode internal proteins) that are 98% homologous to those of the HK156 virus. This feature makes QHKG1 a likely candidate with which to study heterosubtypic immunization against HK156. In this report, we show that C57BL/6 mice are protected against a lethal HK156 infection by heterologous immunization with QHKG1. This protection is also observed in μMT knock-out mice that are B-cell-deficient and therefore are incapable of producing an antibody response. These results have important implications for the development of novel strategies against HK156 infection and future influenza vaccine candidates.

Methods

■ **Viruses, mice and infection.** All viruses used were grown in the allantoic cavity of 10-day-old embryonated chicken eggs. HK156, A/SilkieChicken/HK/P21/97 (CHKP21) and A/Goose/HK/437-6/99 (GSHK437) are H5N1 viruses, and A/HK/1073/99 (HK1073) and QHKG1 are H9N2 viruses. The viruses were used at St Jude Children's Research Hospital in the biosafety level 3 containment facility.

C57BL/6 and BALB/c mice were purchased from Jackson Laboratory and the B-cell-deficient μMT mice (Kitamura *et al.*, 1991) were bred in the Animal Resource Center at St Jude Children's Research Hospital (Riberdy *et al.*, 1999). All mice were maintained in specific-pathogen-free conditions prior to infection.

All immunizations were done with live preparations of virus. Briefly, mice were immunized with 4·5 $\log_{10}$ egg infectious doses (EID_{50}) of QHKG1 and challenged with 2·8 $\log_{10}$ EID_{50} of HK156 or 4·2 $\log_{10}$ EID_{50} of R103 (a virus generated in our laboratory by reassortment of GSHK437 and HK1073). All viruses were administered intranasally to mice anaesthetized with Avertin (2,2,2-tribromoethanol). Infection with QHKG1 typically results in loss of up to 15% of the initial weight, but the mice recover and become healthy again. Additional pathological effects are not observed in these mice. Secondary challenge was done 4 weeks after primary infection, and the survival of the mice was monitored daily.

■ **Generation of reassortant virus.** Madin–Darby canine kidney (MDCK) cells were used to generate reassortant viruses. Briefly, the GSHK437 and HK1073 viruses were mixed and incubated for 30 min at 4 °C before infection at 37 °C for 30 min. The cells were washed and incubated overnight at 37 °C. The next day, supernatant was harvested and neutralized with antiserum to H9N2. Virus from the neutralized supernatant was plaque purified twice before it was produced on a large scale. The pathogenic potential of the candidate reassorted viruses was tested by infecting BALB/c mice (4·2 $\log_{10}$ EID_{50}). In addition, haemagglutinin and neuraminidase inhibition assays were performed as previously described (Palmer *et al.*, 1975). To sequence the viral genomes, RNA was obtained with the RNeasy Kit (Qiagen) and reverse transcribed by using AMV reverse transcriptase (Life Sciences). Amplification of cDNA was then accomplished by using TaKaRa Ex Taq (Panvera). Sequencing reactions were performed by the Center for Biotechnology at St Jude Children's Research Hospital. Template DNA was sequenced by using rhodamine or dRhodamine dye-terminator cycle sequencing ready reaction kits with AmpliTaq DNA polymerase FS (Perkin-Elmer, Applied Biosystems), and synthetic oligonucleotides. The samples were analysed on PE/ABI model 373, model 373 Stretch or model 377 DNA sequencers (Perkin-Elmer, Applied Biosystems).

■ **RMA-S stabilization assay.** To determine MHC restriction, peptide binding to K^b or D^b molecules in RMA-S cells was performed as previously described (Cole *et al.*, 1997). These cells internalize MHC molecules very rapidly unless they are bound to a peptide, in which case they become stabilized in the cell membrane. Briefly, RMA-S cells were grown at 31 °C overnight at a density of 5×10^5 cells/ml. The next day, 10^5 cells were seeded per well of a 96-well plate containing different concentrations of the peptide to be analysed. The cells were incubated at 25 °C for 30 min and then at 37 °C for 3 h. After the cells had been stained with monoclonal antibodies to K^b or D^b, cell surface stabilization was analysed by flow cytometry using FACScan and Cell Quest software (Becton Dickinson). The peptides used in this assay were A/PuertoRico/8/34 (PR8) $NP_{366–374}$ (ASNENMETM), HK156 $VA_{366–374}$ (ASNENVEAM), vesicular stomatitis virus $NP_{52–59}$ (RGYVYQGL) and Sendai virus $NP_{324–332}$ (FAPGNYPAL). All peptides were synthesized in a PE/ABI 433 Peptide Synthesizer (Perkin-Elmer, Applied Biosystems) at the Center for Biotechnology, St Jude Children's Research Hospital.

■ **Cytotoxic assays.** Cytotoxic activity was analysed by using the CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega). This assay measures the release of endogenous lactate dehydrogenase enzyme instead of radioactive chromium. Bronchoalveolar lavage (BAL) was performed 10 days after infection, and adherent cells in the specimens were removed by allowing them to adhere to plastic. The percent cytotoxicity was calculated by using the following formula: % cytotoxicity = [(experimental − spontaneous)/(maximal − spontaneous)] × 100.

Results

Heterologous protection against HK156 (H5N1)

Previous studies have shown that the HK156 virus is lethal in mice (Gao *et al.*, 1999; Gubareva *et al.*, 1998; Lu *et al.*,

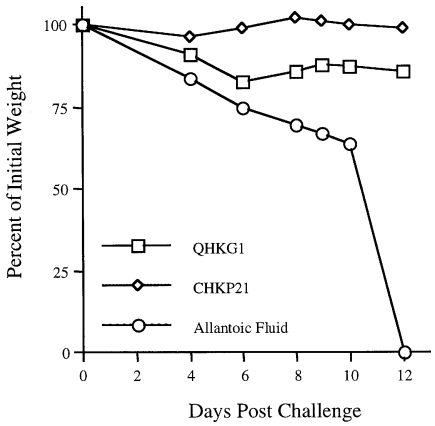

Fig. 1. Heterologous protection against a lethal dose of HK156. C57BL/6 mice were immunized with 4·5 $\log_{10}$ EID_{50} of QHKG1 (H9N2), 3·2 $\log_{10}$ EID_{50} of CHKP21 (H5N1) or 100 µl of allantoic fluid. Four weeks after immunization, mice were challenged with 2·8 $\log_{10}$ EID_{50} of HK156, and their weight and survival monitored daily. Data are representative of three independent experiments and are presented as percent of initial weight for the surviving mice. All of the immunized mice survived HK156 challenge. At day 12 there were no surviving mice in the allantoic fluid group and this is presented as zero percent.

1999; Shortridge *et al.*, 1998). To determine whether C57BL/6 mice could be protected against lethal HK156 infection by immunization with a heterologous virus, we immunized mice with the H9N2 virus, QHKG1. QHKG1 was isolated in the 1997 surveillance of Hong Kong markets and contains internal genes that are 98% homologous to those of HK156 (Guan *et al.*, 1999). As shown in Fig. 1, QHKG1-primed mice were completely protected from death following a lethal challenge with HK156. As expected, control mice primed with the serologically cross-reactive CHKP21 (H5N1) virus, which is non-pathogenic in mice, were also fully protected against a lethal HK156 challenge. These findings suggest that it is possible to induce protective heterosubtypic immunity to HK156. It should be noted that although the QHKG1 priming was fully protective, the animals did exhibit a temporary weight loss (up to 15% of initial weight), indicating that the animals were susceptible to some degree of infection prior to viral clearance. This is typical of heterosubtypic immunity, which is unable to block the initial infection of the animals. In contrast, weight loss was not observed in the CHKP21-primed mice, presumably due to the presence of homotypic antibodies against H5 which prevent infection by HK156 (Kodihalli *et al.*, 1997, 1999).

Generation of reassortant H5N1 virus

To further study the immune response against HK156 infection, we generated variants of H5N1 using classical reassortment techniques (Scholtissek *et al.*, 1985). For this purpose, we used two viruses: A/Goose/HK/437-6/99 (GSHK437), which was isolated during a 1999 poultry surveillance in Hong Kong, and A/HK/1073/99 (HK1073), which was isolated as a clinical specimen (Y. P. Lin and others, unpublished). GSHK437 is an H5N1 goose isolate with a haemagglutinin gene that is 98% homologous to that of HK156, but the other genes differ significantly (Table 1). Although it is known that a highly cleavable haemagglutinin determines pathogenicity [reviewed in Rott (1992) and Steinhauer (1999)], it cannot be the sole determinant because the H5N1 GSHK437 virus is not pathogenic in mice. On the other hand, HK1073 is an H9N2 human isolate whose six internal genes are highly homologous to those of HK156 (Table 1), and HK1073 is also not pathogenic in mice. After coinfection of MDCK cells with these two viruses (see Methods), viral suspensions prepared from purified plaques were used to infect BALB/c mice to assess their pathogenicity. As seen in Fig. 2, neither the GSHK437 nor the HK1073 parental viruses were pathogenic. Interestingly, one of the isolated viruses (isolate R103) was highly pathogenic and was 100% lethal by day 8 post-infection (Fig. 2). This is comparable to the kinetics of animal death following infection by HK156. Other viruses isolated in parallel (such as R102 and R105) showed no increase in either morbidity or mortality (Fig. 2). To determine if isolates R102, R103 and R105 were reassorted viruses, we performed haemagglutination and neuraminidase inhibition assays (HI and NI respectively) to determine their HA and NA identity. In addition, we extracted their RNA and sequenced their six internal genes. The R102 and R103 viruses were H5N1 viruses while R105 was H9N2 (Table 2). In HI experiments, neither anti-H5 nor anti-H9 antisera was cross-reactive, suggesting that the two serotypes are not cross-protective (Table 3). Similar results were obtained with the anti-N1 and the anti-N2 sera in the NI assays (data not shown). After analysing the sequence of the internal genes it was determined that neither R102 nor R105 were reassortant viruses. Nonetheless, they confirmed that passage in MDCK cells did not alter the pathogenicity of the parental GSHK437 and HK1073 viruses. On the other hand, the highly pathogenic R103 virus was a reassortant H5N1, containing both the NP and PB2 genes from the HK1073 parent in the context of the GSHK437 parent (Table 2). Thus, we were successful in obtaining an H5N1 reassortant with restored pathogenic

Table 1. Comparison of genes of Hong Kong isolates with those of HK156

Values are percentage identity at the nucleotide level against HK156 genes. ND, Not done.

Virus	HA	NA	NP	PB1	PB2	PA	M	NS
GSHK437	98	89	83	94	86	89	93	74
HK1073	ND	ND	99	99	98	98	98	98

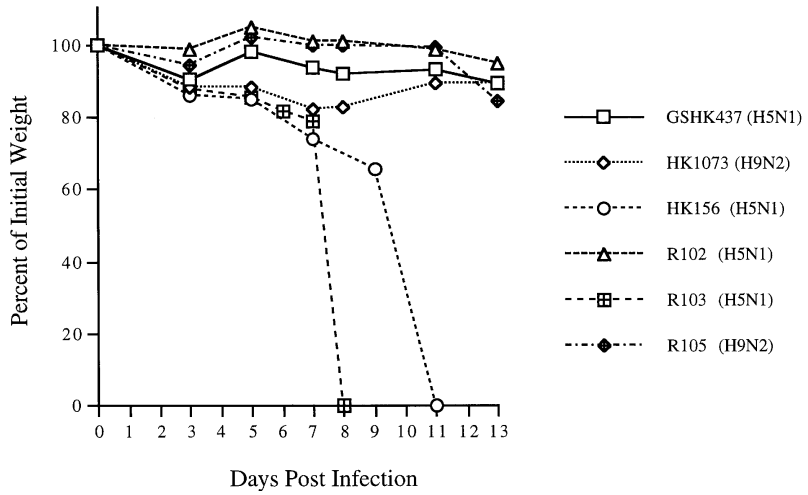

Fig. 2. Generation of a lethal H5N1 reassortant virus. After MDCK cells were coinfected with GSHK437 (H5N1) and HK1073 (H9N2) viruses, plaque-purified isolates were used to infect groups of five BALB/c mice to assess pathogenicity. Data are representative of three independent experiments and are presented as percent of initial weight for the surviving mice. At days 8 and 11 there were no surviving mice in the R103 and HK156 groups respectively and this is presented as zero percent.

Table 2. Gene composition of plaque-isolated viruses

Bold type highlights the contribution of the HK1073 parental virus to the R103 virus.

Virus	HA	NA	NP	PB1	PB2	PA	M	NS
R102	H5	N1	GSHK437	GSHK437	GSHK437	GSHK437	GSHK437	GSHK437
R103	H5	N1	**HK1073**	GSHK437	**HK1073**	GSHK437	GSHK437	GSHK437
R105	H9	N2	HK1073	HK1073	HK1073	HK1073	HK1073	HK1073
HK156	H5	N1	HK1073	HK1073	HK1073	HK1073	HK1073	HK1073

Table 3. Haemagglutination inhibition assay

Data are expressed as the reciprocal titre of highest dilution which inhibited haemagglutination.

Virus	Anti-H5*	Anti-H9
GSHK437	1280	40†
HK1073	80	1280
R102	1280	< 40
R103	> 5120	< 40
HK156	640	40

* The anti-H5 serum was raised against A/Tern/South Africa/61 (H5N3) and the anti-H9 serum against A/Quail/HK/G1/97 (H9N2).
† The lowest dilution used was 1:40.

potential, suggesting that the NP and the PB2 genes from HK1073 (which are HK156-like) are sufficient in combination with a highly cleavable H5 to restore pathogenicity. None of the other plaque-isolated viruses analysed were reassorted viruses (data not shown).

The reassortant R103 virus was selected for further study because it is highly pathogenic (like HK156) and shares only NP and PB2 genes with QHKG1, which we are using as a vaccine strain. Thus, C57BL/6 mice were immunized with QHKG1 or allantoic fluid and then challenged with a lethal dose of R103. Four weeks later, QHKG1-primed mice were completely protected against R103 challenge (data not shown). These studies suggest that heterosubtypic immunity against one or both of the NP and PB2 gene products was sufficient to protect mice from death. Further experiments are under way to characterize the cellular immune response against this reassortant virus.

Role of cellular immunity in the protection against HK156

To formally rule out a role for humoral immunity in protection against HK156 infection after QHKG1 immunization we took advantage of B-cell-deficient mice (μMT), which are unable to make an antibody response (Kitamura *et al.*, 1991; Riberdy *et al.*, 1999). Naïve μMT mice succumbed to HK156 infection in a manner similar to that of wild-type C57BL/6 mice inasmuch as 100% of the mice were dead by day 9. μMT mice that had been previously immunized with QHKG1 were partially protected against HK156 challenge

Table 4. Protection against HK156 challenge in μMT mice

Mice (seven per group) were immunized with 4·5 $\log_{10}$ EID_{50} of QHKG1 or left untreated (naïve), 4 weeks prior to challenge with 2·8 $\log_{10}$ EID_{50} of HK156. Data are expressed as percent survivors.

Day	Naïve	QHKG1
0	100	100
3	100	100
6	100	100
7	100	86
8	43	71
9	0	57
10	0	29
11	0	0

Table 5. Cytotoxic activity of cells from HK156-infected mice

Numerical data are expressed as percent cytotoxicity. Target cells were infected with the indicated virus previous to incubation with effector cells. Effector cells were obtained from BAL of mice 10 days after infection with the indicated virus.

			Effector:target ratio	
Target cells	Infection	Effectors	20:1	5:1
L929-D	HKx31*	HKx31	30	19
L929-K	HKx31	HKx31	9	1
L929-D	HK156	HK156	13	14
L929-K	HK156	HK156	2	0
L929-D	QHKG1	QHKG1	30	0

* HKx31 is an H3N2 laboratory reassortant that contains the surface molecules of A/Aichi/68 and the internal components of A/Puerto Rico/8/34.

inasmuch as there was a significant delay in the death of most of the mice (Table 4). For example, 57% of the QHKG1-immunized μMT mice were still alive at day 9, a timepoint when all of the naïve μMT mice had died. In addition, some of the QHKG1-immunized μMT mice survived until day 10. A log-rank test for difference in survival between both groups revealed that there is evidence to suggest that the QHKG1-immunized mice had better survival than the naïve group. The observation that μMT mice previously immunized with QHKG1 finally succumbed to HK156 challenge was not surprising since these mice are more susceptible to influenza virus infection than wild-type C57BL/6 mice (Graham & Braciale, 1997; Riberdy *et al.*, 1999). However, these data confirm that previous immunization with QHKG1 induces heterosubtypic immunity against the HK156 virus.

We have shown that both C57BL/6 and μMT mice that have been previously immunized with QHKG1 are better able to control a subsequent HK156 infection (Fig. 1, Table 4). Thus, we investigated whether the HK156 and QHKG1 viruses induced CTL responses. C57BL/6 mice were infected with QHKG1 and BAL was obtained at 10 days post-infection. As shown in Table 5, BAL from QHKG1-infected mice showed 30% cytotoxicity against QHKG1-infected L929-D^b target cells. Similarly, BAL from HK156-infected mice was cytotoxic against HK156-infected targets. BAL from control mice infected with HKx31 showed a similar level of cytotoxicity against HKx31-infected targets. These results show that CTL responses are readily generated in the lungs of QHKG1- or HK156-infected mice.

The T cell response to HK156 is not directed to the $NP_{366–374}$ peptide

The previous data show that the QHKG1 immunization protected against both HK156 and R103 infection. In addition, in the case of R103, this protection maps to either the NP or PB2 gene. Interestingly, previous studies have identified an immunodominant, H-2^b-restricted, $CD8^+$ T cell epitope from the nucleoprotein that is present in the A/PR/8/34 virus ($NP_{366–374}/D^b$) and which elicits a strong and dominant CTL response in C57BL/6 mice (Flynn *et al.*, 1998; Townsend *et al.*, 1986; Yewdell *et al.*, 1985). Analysis of the HK156 virus indicated that the nucleoprotein gene of this virus has two amino acid substitutions, $M \rightarrow V_{371}$ and $T \rightarrow A_{373}$ (Subbarao *et al.*, 1998). These substitutions have been observed in those positions for other H-$2D^b$ binding peptides (Rammensee *et al.*, 1995). Therefore we speculated that the protection was being mediated by a $CD8^+$ T cell response to the HK156-$NP_{366–374}/D^b$ epitope (VA peptide). To determine if these substitutions affected presentation of the peptide by D^b we synthesized the VA peptide and determined whether it could bind to either D^b or K^b as a control (Cole *et al.*, 1997). As shown in Fig. 3, the VA peptide binds to D^b but not K^b molecules, as has been described for the PR8 $NP_{366–374}$ peptide, indicating that the two amino acid substitutions on the HK156-NP have little effect on MHC class I binding.

Given that the VA peptide binds to D^b, we next asked whether this peptide was involved in heterosubtypic immunity to HK156. C57BL/6 mice were immunized with the VA peptide (in complete Freund's adjuvant in the base of the tail) and then infected with HK156. However, the mice were unable to mount an effective immune response and succumbed to HK156 challenge (data not shown). These data, in conjunction with the data presented in Fig. 1, suggest that the protection against HK156 infection, achieved by QHKG1-protected mice, is mediated by a different epitope (Cole *et al.*, 1997; Oukka *et al.*, 1996).

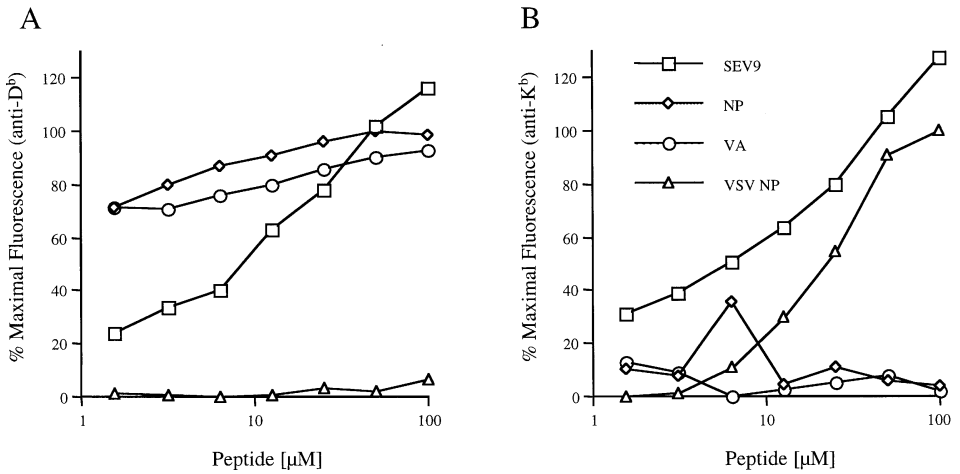

Fig. 3. $VA_{366–374}$ peptide binding to D^b molecules on the surface of RMA-S cells. RMA-S cells were incubated with serial dilutions of Sendai virus $NP_{324–332}$ (SEV9), PR8 virus $NP_{366–374}$ (NP), HK156 virus $VA_{366–374}$ (VA), or vesicular stomatitis virus $NP_{52–59}$ (VSV) at 25 °C for 30 min and then at 37 °C for 3 h. Cells were then stained with anti-D^b (A) or anti-K^b (B) monoclonal antibodies and analysed by flow cytometry. Data are representative of two independent experiments and are expressed as percent maximal fluorescence.

Discussion

The recent emergence of a highly pathogenic H5N1 virus in Hong Kong demonstrates the continual danger posed by newly emerging influenza virus strains. Although current methods of immunization are fairly adequate, it is imperative to develop improved vaccines that can be produced quickly and can protect against a variety of viral strains. One promising strategy is to develop vaccines that emphasize cellular immunity. In this regard, it is essential that studies focus on highly pathogenic strains of virus to develop a real understanding of the ability of cellular immunity to control this type of infection. However, this is particularly problematic due to the dangers of working with such strains. In the current paper, we report the successful protection of C57BL/6 mice from a lethal HK156 infection by immunizing first with an H9N2 isolate, QHKG1, that harbours internal genes 98% homologous to HK156. This strategy also protected mice that are deficient in antibody production, indicating that protection was mediated by a cellular immune response. At this point, we have not identified the cellular mechanism involved in protection. However, it is generally considered that $CD8^+$ T cells are the main mediators of heterosubtypic immunity. In this regard, we demonstrated a cytotoxic response can be established after immunization with QHKG1.

It is generally believed that cellular immune responses have limited capacity to protect animals from a lethal dose of virus. Although a recall T cell response to infection is much faster than a primary response, it nevertheless takes several days to develop, during which time the virus can multiply to high titre. Previous studies have shown that mice can be protected from a lethal challenge with PR8 after priming with x31, a laboratory reassortant that differs from PR8 (H1N1) only in its envelope proteins (H3N2). However, this protection is relatively limited (it does not protect against very high doses of virus) and tends to wane rapidly (Liang *et al.*, 1994). In this regard, it might have been anticipated that heterosubtypic immunity would be ineffective against challenge with a highly virulent virus such as HK156. Therefore, our results are significant since they provide the first demonstration that protective cell-mediated immunity can be established against the highly virulent HK156 virus.

The mechanism of heterosubtypic immunity is poorly understood. $CD8^+$ T cells appear to play a major role in controlling virus replication (Eichelberger *et al.*, 1991). However, it is also apparent that $CD4^+$ cells can also be important (Zhong *et al.*, 2000). Moreover, $CD4^+$ T cells seem to be more important in the immune response after DNA vaccination (Epstein *et al.*, 2000; Ulmer *et al.*, 1998). In the current studies we have not distinguished whether the protective immunity was mediated by either $CD4^+$ or $CD8^+$ T cells due to the difficulties of working with the HK156 virus. However, we did demonstrate that QHKG1 induced a CTL response and postulate that this is a major contributor to the heterosubtypic immunity observed. Very little information regarding cellular immunity against more pathogenic strains such as H5N1 has been reported. To our knowledge, only one group has reported data regarding a cellular response against HK156 and it was an indirect measure of cross-protection in humans that have been previously infected with circulating influenza virus strains (Jameson *et al.*, 1999). In the current studies we were able to determine that heterosubtypic immunity could be generated when only two viral genes were homologous. The fact that this protection can be established despite only two homologous proteins being shared by the viruses used warrants further investigation.

In the course of these studies we generated an H5N1 reassortant that contains six genes from the GSHK437 (H5N1) and two genes (NP and PB2) from the HK1073 (H9N2) non-pathogenic parents. The reassortant is highly pathogenic and implicates NP and PB2 as having an important contribution to pathogenesis when present with the highly cleavable H5 of the GSHK437 isolate. The presence of the highly cleavable H5 is not sufficient to confer high pathogenicity, as the GSHK437 H5N1 virus is not pathogenic. Similarly, the presence of the HK156-like NP and PB2 genes in the absence of the H5 (as in the case of the HK1073 virus) does not result in virulence (Fig. 2). A likely explanation for this observation could be that the combination of NP, PB2 and HA genes from HK156 provides increased replication ability relative to the parental viruses. This point remains to be elucidated. With the advent of new and more efficient methods like the recently described reverse genetics system (Neumann *et al*., 1999), we should be able to generate additional reassortants to further characterize the contribution of each of the HK156-like genes to pathogenesis and the impact of having each one of those genes on the cellular response of the infected host. Nevertheless, this study provides findings that should contribute to the understanding of the pathogenicity of H5N1 influenza viruses.

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References

Ada, G. L. & Jones, P. D. (1986). The immune response to influenza infection. *Current Topics in Microbiology and Immunology* **128**, 1–54.

Allan, W., Tabi, Z., Cleary, A. & Doherty, P. C. (1990). Cellular events in the lymph node and lung of mice with influenza. Consequences of depleting CD4+ T cells. *Journal of Immunology* **144**, 3980–3986.

Anker, W. J., Bakker, A. K. & Masurel, N. (1978). Cross-protection in mice after immunization with H2N2, H3N2, and Heq2Neq2 influenza virus strains. *Infection and Immunity* **21**, 96–101.

Belshe, R. B. (1998). Influenza as a zoonosis: how likely is a pandemic? [comment]. *Lancet* **351**, 460–461.

CDC (1998). Update: isolation of avian influenza A (H5N1) viruses from humans–Hong Kong, 1997–1998. *Morbidity and Mortality Weekly Reports* **46**, 1245–1247.

Claas, E. C., de Jong, J. C., van Beek, R., Rimmelzwaan, G. F. & Osterhaus, A. D. (1998*a*). Human influenza virus A/HongKong/156/97 (H5N1) infection. *Vaccine* **16**, 977–978.

Claas, E. C., Osterhaus, A. D., van Beek, R., De Jong, J. C., Rimmelzwaan, G. F., Senne, D. A., Krauss, S., Shortridge, K. F. & Webster, R. G. (1998*b*). Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus [see comments]. *Lancet* **351**, 472–477. [Erratum **351**, 1292.]

Cole, G. A., Hogg, T. L., Coppola, M. A. & Woodland, D. L. (1997). Efficient priming of CD8+ memory T cells specific for a subdominant epitope following Sendai virus infection. *Journal of Immunology* **158**, 4301–4309.

Couch, R. B. & Kasel, J. A. (1983). Immunity to influenza in man. *Annual Review of Microbiology* **37**, 529–549.

Doherty, P. C., Allan, W., Eichelberger, M. & Carding, S. R. (1992). Roles of alpha beta and gamma delta T cell subsets in viral immunity. *Annual Review of Immunology* **10**, 123–151.

Eichelberger, M., Allan, W., Zijlstra, M., Jaenisch, R. & Doherty, P. C. (1991). Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. *Journal of Experimental Medicine* **174**, 875–880.

Epstein, S. L., Lo, C. Y., Misplon, J. A. & Bennink, J. R. (1998). Mechanism of protective immunity against influenza virus infection in mice without antibodies. *Journal of Immunology* **160**, 322–327.

Epstein, S. L., Stack, A., Misplon, J. A., Lo, C. Y., Mostowski, H., Bennink, J. & Subbarao, K. (2000). Vaccination with DNA encoding internal proteins of influenza virus does not require $CD8^+$ cytotoxic T lymphocytes: either $CD4^+$ or $CD8^+$ T cells can promote survival and recovery after challenge. *International Immunology* **12**, 91–101.

Flynn, K. J., Belz, G. T., Altman, J. D., Ahmed, R., Woodland, D. L. & Doherty, P. C. (1998). Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. *Immunity* **8**, 683–691.

Gao, P., Watanabe, S., Ito, T., Goto, H., Wells, K., McGregor, M., Cooley, A. J. & Kawaoka, Y. (1999). Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. *Journal of Virology* **73**, 3184–3189.

Gorman, O. T., Bean, W. J. & Webster, R. G. (1992). Evolutionary processes in influenza viruses: divergence, rapid evolution, and stasis. *Current Topics in Microbiology and Immunology* **176**, 75–97.

Graham, M. B. & Braciale, T. J. (1997). Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice. *Journal of Experimental Medicine* **186**, 2063–2068.

Guan, Y., Shortridge, K. F., Krauss, S. & Webster, R. G. (1999). Molecular characterization of H9N2 influenza viruses: were they the donors of the 'internal' genes of H5N1 viruses in Hong Kong? *Proceedings of the National Academy of Sciences, USA* **96**, 9363–9367.

Gubareva, L. V., McCullers, J. A., Bethell, R. C. & Webster, R. G. (1998). Characterization of influenza A/HongKong/156/97 (H5N1) virus in a mouse model and protective effect of zanamivir on H5N1 infection in mice. *Journal of Infectious Diseases* **178**, 1592–1596.

Hou, S. & Doherty, P. C. (1995). Clearance of Sendai virus by CD8+ T cells requires direct targeting to virus-infected epithelium. *European Journal of Immunology* **25**, 111–116.

Jameson, J., Cruz, J., Terajima, M. & Ennis, F. A. (1999). Human CD8+ and CD4+ T lymphocyte memory to influenza A viruses of swine and avian species. *Journal of Immunology* **162**, 7578–7583.

Kagi, D. & Hengartner, H. (1996). Different roles for cytotoxic T cells in the control of infections with cytopathic versus noncytopathic viruses. *Current Opinion in Immunology* **8**, 472–477.

Kitamura, D., Roes, J., Kuhn, R. & Rajewsky, K. (1991). A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* **350**, 423–426.

Kodihalli, S., Haynes, J. R., Robinson, H. L. & Webster, R. G. (1997). Cross-protection among lethal H5N2 influenza viruses induced by DNA vaccine to the hemagglutinin. *Journal of Virology* **71**, 3391–3396.

Kodihalli, S., Goto, H., Kobasa, D. L., Krauss, S., Kawaoka, Y. & Webster, R. G. (1999). DNA vaccine encoding hemagglutinin provides protective immunity against H5N1 influenza virus infection in mice. *Journal of Virology* **73**, 2094–2098.

Liang, S., Mozdzanowska, K., Palladino, G. & Gerhard, W. (1994). Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. *Journal of Immunology* **152**, 1653–1661.

Lu, X., Tumpey, T. M., Morken, T., Zaki, S. R., Cox, N. J. & Katz, J. M. (1999). A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. *Journal of Virology* **73**, 5903–5911.

Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D. R., Donis, R., Hoffmann, E., Hobom, G. & Kawaoka, Y. (1999). Generation of influenza A viruses entirely from cloned cDNAs [see comments]. *Proceedings of the National Academy of Sciences, USA* **96**, 9345–9350.

Nguyen, H. H., Moldoveanu, Z., Novak, M. J., van Ginkel, F. W., Ban, E., Kiyono, H., McGhee, J. R. & Mestecky, J. (1999). Heterosubtypic immunity to lethal influenza A virus infection is associated with virus-specific $CD8^+$ cytotoxic T lymphocyte responses induced in mucosa-associated tissues. *Virology* **254**, 50–60.

Oukka, M., Manuguerra, J. C., Livaditis, N., Tourdot, S., Riche, N., Vergnon, I., Cordopatis, P. & Kosmatopoulos, K. (1996). Protection against lethal viral infection by vaccination with nonimmunodominant peptides. *Journal of Immunology* **157**, 3039–3045.

Palmer, D. F., Coleman, M., Dowdle, W. R. & Schild, G. G. (1975). Advanced laboratory techniques for influenza diagnosis. *U.S. Department of Health, Education and Welfare, Immunology Series* **6**, 51–52.

Rammensee, H. G., Friede, T. & Stevanoviic, S. (1995). MHC ligands and peptide motifs: first listing. *Immunogenetics* **41**, 178–228.

Riberdy, J. M., Flynn, K. J., Stech, J., Webster, R. G., Altman, J. D. & Doherty, P. C. (1999). Protection against a lethal avian influenza A virus in a mammalian system. *Journal of Virology* **73**, 1453–1459.

Rimmelzwaan, G. F. & Osterhaus, A. D. (1995). Cytotoxic T lymphocyte memory: role in cross-protective immunity against influenza? *Vaccine* **13**, 703–705.

Rott, R. (1992). The pathogenic determinant of influenza virus. *Veterinary Microbiology* **33**, 303–310. [Erratum **34**, 398.]

Scholtissek, C., Burger, H., Kistner, O. & Shortridge, K. F. (1985). The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. *Virology* **147**, 287–294.

Schulman, J. L. & Kilbourne, E. D. (1965). Induction of partial specific heterotypic immunity in mice by a single infection with influenza A virus. *Journal of Bacteriology* **89**, 170–174.

Schulman, J. L., Petigrow, C. & Woodruff, J. (1977). Effects of cell mediated immunity in influenza virus infection in mice. *Developments in Biological Standardization* **39**, 385–390.

Shortridge, K. F. (1999). Poultry and the influenza H5N1 outbreak in Hong Kong, 1997: abridged chronology and virus isolation. *Vaccine* **17**, S26–S29.

Shortridge, K. F., Zhou, N. N., Guan, Y., Gao, P., Ito, T., Kawaoka, Y., Kodihalli, S., Krauss, S., Markwell, D., Murti, K. G., Norwood, M., Senne, D., Sims, L., Takada, A. & Webster, R. G. (1998). Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. *Virology* **252**, 331–342.

Steinhauer, D. A. (1999). Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology* **258**, 1–20.

Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., Hemphill, M., Rowe, T., Shaw, M., Xu, X., Fukuda, K. & Cox, N. (1998). Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness [see comments]. *Science* **279**, 393–396.

Topham, D. J., Tripp, R. A. & Doherty, P. C. (1997). CD8 + T cells clear influenza virus by perforin or Fas-dependent processes. *Journal of Immunology* **159**, 5197–5200.

Townsend, A. R., Rothbard, J., Gotch, F. M., Bahadur, G., Wraith, D. & McMichael, A. J. (1986). The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* **44**, 959–968.

Ulmer, J. B., Fu, T. M., Deck, R. R., Friedman, A., Guan, L., DeWitt, C., Liu, X., Wang, S., Liu, M. A., Donnelly, J. J. & Caulfield, M. J. (1998). Protective CD4 + and CD8 + T cells against influenza virus induced by vaccination with nucleoprotein DNA. *Journal of Virology* **72**, 5648–5653.

Webster, R. G. & Hay, A. J. (1998). The H5N1 influenza outbreak in Hong Kong: a test of pandemic preparedness. In *Textbook of Influenza*, pp. 561–565. Edited by K. G. Nicholson, R. G. Webster & A. J. Hay. Malden: Blackwell Science.

Yap, K. L. & Ada, G. L. (1978). The recovery of mice from influenza virus infection: adoptive transfer of immunity with immune T lymphocytes. *Scandinavian Journal of Immunology* **7**, 389–397.

Yewdell, J. W., Bennink, J. R., Smith, G. L. & Moss, B. (1985). Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes. *Proceedings of the National Academy of Sciences, USA* **82**, 1785–1789.

Yuen, K. Y., Chan, P. K., Peiris, M., Tsang, D. N., Que, T. L., Shortridge, K. F., Cheung, P. T., To, W. K., Ho, E. T., Sung, R. & Cheng, A. F. (1998). Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus [see comments]. *Lancet* **351**, 467–471.

Zhong, W., Marshall, D., Coleclough, C. & Woodland, D. L. (2000). CD4 + T cell priming accelerates the clearance of Sendai virus in mice, but has a negative effect on CD8 + T cell memory. *Journal of Immunology* **164**, 3274–3282.

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