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GENERATION OF A RECOMBINANT VACCINIA VIRUS EXPRESSING THE NEW H5 SUBTYPE OF THE INFLUENZA A HEMMAGGLUTININ GENE

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Roland W. Smith

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APPROVED:

Masanori Terijima, Ph.D.
Department of Infectious Disease
and Vaccine Research
Umass Medical Center
Major Advisor

Dave S. Adams, Ph.D.
WPI Project Advisor

ABSTRACT

This project was designed to generate a recombinant vaccinia virus expressing the new H5 subtype of the HA gene as part of eventually defining the CTL epitope on the avian HA protein recognized by this human T cell clone. The result was achieved by first generating a transfer vector for homologous recombination. The vector was used to generate a recombinant vaccinia virus expressing the HA gene. Expression of the HA gene product was confirmed at both transcriptional and translational levels, using RT-PCR and immunoprecipitations, respectively.

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BACKGROUND

Human Influenza A Virus

Influenza A virus infections cause morbidity and mortality on an annual basis (Langmuir and Schoenbaum, 1977; Stuart Harris and Schild, 1976). Excessive mortality is usually observed in the elderly and in individuals with chronic illness. Although older children and adults generally have had previous infections with influenza A viruses, mutations at the major antibody (Ab) combining sites allow reinfections to occur (Webster et al., 1992). These point mutations cause antigenic drift at the Ab combining sites on the external glycoproteins, hemagglutinin (HA) and neuraminidase (NA). In addition, periodically a drastic Ag change occurs that may be due to a reassortment of genes from a human virus strain with genes from a nonhuman virus; this is called antigenic shift. The only times that these shift events have been documented were in 1957 and 1968, and marked increases in mortality and morbidity were noted (Langmuir and Schoenbaum, 1977). The most severe pandemic of influenza A virus that has occurred in modern times was the worldwide pandemic of 1918-1920 when over 20,000,000 deaths occurred (Collins, 1957). The virus that caused the 1918 pandemic has recently been partially characterized as having swine-like H1N1 sequences (Taubenberger et al., 1997), and serologic studies of individuals who were alive during that pandemic also strongly suggest that the virus had a swine-like HA Ag (Masurel and Marine, 1973).

Importance of CD8+ CTL Cells

Influenza virus-specific CTL have been shown in murine studies to limit influenza A virus replication and to protect against lethal influenza A virus challenge (Wells et al., 1981).

CTL have been found in the lungs of influenza A virus-infected mice (Yap and Ada, 1978), and recovery from influenza infection has correlated with clearance by $CD8^+$ CTL (Mackenzie et al., 1989). Virus-specific CTL clones that recognize epitopes on the nucleoprotein (NP), HA, nonstructural 1 (NS1) proteins, and influenza virus-stimulated immune splenocytes adoptively transferred into naive recipients reduced pulmonary virus titers after influenza virus challenge (Wells et al., 1981; Taylor and Askonas, 1986; Kuwano et al., 1988; Kuwano et al., 1990). The Ennis laboratory has shown that active immunization with an HA fusion protein that induced HA-specific CTL but not neutralizing Ab caused a reduction in peak lung virus titers after virus challenge and protection against a lethal challenge dose (Kuwano et al., 1989). Lack of $CD8^+$ CTL delays viral clearance and increases mortality after infection with a virulent strain of influenza virus (Bender et tal., 1992). $CD4^+$ virus-specific T cells may help compensate for the absence of $CD8^+$ CTL because the virus can be cleared in $CD8^+$ CTL-deficient mice; however, mice lacking both $CD4^+$ and $CD8^+$ CTL do not clear virus or survive (Eichelberger et al., 1991). Therefore, CTL seem to be important in both restricting influenza A virus replication and reducing disease severity.

H5N1

In 1997, an H5N1 influenza A virus was isolated from a 3-year-old boy in Hong Kong. This child did not survive the infection, and his was the first of six deaths due to H5N1 influenza A virus infections (Doepel, 1998). The isolated virus was found to be similar to avian H5N1 viruses (Subbarao et al., 1998; Suarez et al., 1998). The Ennis Laboratory speculated that most of the older children and adults living in Hong Kong and elsewhere in the world in 1997 would possess influenza A virus cross-reactive memory T cells, and that some of these clones would

recognize epitopes on the H5N1 avian-derived virus strains that caused human illness possibly providing partial protection from infection. There had been no other reports of the ability of human influenza A virus-specific CTL to recognize epitopes on nonhuman viruses. The Ennis lab developed and characterized a panel of human $CD8^+$ and $CD4^+$ CTL lines from several donors residing in a city in the U.S. (Jameson et al., 1998). These T cell lines were found to be either subtype specific or cross-reactive to the H1N1, H2N2, and H3N2 subtypes of human influenza A viruses (Jameson et al., 1998). They determined whether these human $CD8^+$ and $CD4^+$ CTL lines would recognize epitopes on autologous cells infected with influenza A virus strains of swine and avian species including the H5N1 virus strains recently isolated from patients in Hong Kong. In addition, they tested bulk culture responses of these donors to determine whether CTL cross-reactivity could be detected at the population level and quantitated the precursor frequency of CTL specific for epitopes on the H5N1 virus.

These results demonstrated that adults living in an urban area of the U.S. possess influenza A cross-serotype reactive $CD8^+$ and $CD4^+$ CTL that recognize multiple epitopes on influenza A viruses of other species. Bulk culture cytotoxicity was demonstrated against avian and human influenza A viruses. Enzyme-linked immunospot assays detected precursor CTL specific for both human CTL epitopes and the corresponding A/HK/97 viral sequences. Ennis et al hypothesized that these cross-reactive CTL might provide partial protection to humans against novel influenza A virus strains introduced into humans from other species (Jameson et al., 1999).

The results showed that adult humans with no known exposure to these nonhuman species possess high levels of memory CTL which are readily detected in bulk culture assays following stimulation with a human influenza A virus. The recognition of autologous cells infected with the recent Hong Kong H5N1 isolates or the older avian and swine-derived viruses

was convincing and was similar to the level of lysis of autologous cells infected with human influenza A viruses. These bulk culture results were in accord with the specific CTL lysis of target cells infected with the swine and avian-derived virus strains and the recent H5N1 isolates from Hong Kong by a panel of human $CD8^+$ and $CD4^+$ T cell lines, the majority of which are cross-reactive among the human H1, H2, and H3 subtypes.

The cross-reactive recognition of epitopes by these $CD8^+$ and $CD4^+$ T cell clones fits well with available sequence data. Clones that recognized defined epitopes with no or minimal conserved mutations recognized target cells infected with the swine-derived Hsw1N1, the older avian virus strains H5N2 and H1N1, and the recent H5N1 isolates from Hong Kong similar to target cells infected with human H1N1 virus.

Importance of Cross Reactive Memory T-Cells

What are the effects of these preexisting human influenza A virus cross-reactive memory T cells when a new nonhuman influenza A virus is introduced into humans, as occurred in Hong Kong in 1997 (Webster et al., 1992) and might have occurred in 1918? There was relatively little influenza A virus activity noted in the two decades preceding the 1918 pandemic (Collins, 1957). An epidemic occurred in 1890 which appears to have been caused by a virus with an H3-like HA based on serological evidence of H3-specific Abs found before 1968 in the sera of individuals born before 1890 (Masurel and Marine, 1973). The reasons for the tremendous mortality during the 1918 pandemic are unknown. Young adults, especially the age group of 25-35 years, had very high mortality rates in 1918, which is unusual in influenza; deaths usually occur in the very young and in older individuals (Collins, 1957). Thus, a W-shaped curve in age-related mortality with the peak deaths between the ages of 25-35 was described instead of the usual U-shaped

curve with deaths peaking only in the very young and old (Collins, 19574). In addition to unusual virulence of the 1918 virus, memory immunologic responses, including cross-reactive influenza-specific T cell responses, may have been low or absent in some of the young adult cohort because there was relatively little influenza A activity noted during the two decades immediately before 1918 (Collins, 1957).

The impact of influenza A cross-reactive memory CTL on the morbidity and mortality associated with influenza virus epidemics and the pandemics of 1918, 1957, and 1968 is unknown. In 1957, most adults presumably had cross-reactive CTL memory to epitopes on the H1N1 viruses which circulated widely before the "Asian" pandemic (H2N2) of 1957. Similarly, when the H3N2 "Hong Kong" virus emerged in 1968, adults and older children would be expected to have had cross-reactive memory CTL as a result of infection with the prior H2N2 virus strains, and adults would also have been exposed to the earlier H1N1 strains. Although it is clear that cross-reactive memory T cells did not prevent infections and excess mortality in 1957 and 1968, these memory T lymphocytes may have contributed a degree of partial protection by limiting the degree of viral replication, based on experimental studies of CTL in mouse models (Wells et al., 1981; Taylor and Askonas, 1986; Kuwano et al., 1988; Kuwano et al., 1990).

Overall, those results suggest that adults living in urban areas have $CD8^+$ and $CD4^+$ memory CTL as a result of prior infections with human influenza A viruses, and these CTL are in large part cross-reactive to epitopes on influenza A virus strains derived from swine and avian species. Most of the human CTL clones were able to recognize entirely conserved or mutated viruses. There is considerable conservation among the genes encoding the internal and nonstructural proteins of influenza A virus, which is consistent with those results. The presence of these memory T lymphocytes may play a role in helping to restrict virus replication, thereby

decreasing morbidity and mortality to a degree. These results support the hypothesis that cross-reactive T cells will be activated in humans by infection with a novel influenza A virus derived from another species, and the activation of these memory CTL by infection may result in reduced replication of the new infecting virus. Stimulation of these crossreactive T cells by novel attempts at vaccination including plasmid DNA vaccines may induce protection against novel influenza viruses.

Up to now no T cell epitope has been reported on the Influenza A HA in the human system, although several T cell epitopes were identified on the HA in mice (reviewed in McMichael 1994; Parker and Gould 1996). In The Ennis Laboratory's studies (Jameson et al., 1998; Jameson et al., 1999), they found an HA specific and HLA-B18-restricted CTL line, 10-1G5, that originally recognized HA (H1N1), was crossreactive to H1, H2 and H5 subtypes. B18 is not a common HLA allele, but that crossreactivity is very interesting for vaccine design.

PROJECT PURPOSE

This MQP was designed to generate a recombinant vaccinia virus expressing the new H5 subtype of the influenza virus HA gene as a part of a larger project to eventually define the CTL epitope on the avian HA protein that is recognized by human T cell clones during influenza infection.

MATERIALS AND METHODS

Viruses, Antiserum and Cells

Influenza virus A/Hong Kong/156/97 (H5N1) (A/HK/156/97) and ferret antiserum to this virus were kindly provided by N. J. Cox, World Health Organization Influenza Reference Laboratory, at the Centers for Disease Control and Prevention (Atlanta, GA) and propagated in embryonated chicken eggs. Infected allantoic fluids were harvested 2 days after infection. Wild type vaccinia virus (ATCC #VR1354) was propagated in CV-1 cells. CV-1 cells (ATCC #CCL70) were maintained in complete MEM-10 (minimal essential medium containing 10% FCS, 0.03% glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate). HuTK$^-$143B cells were maintained in complete MEM-10/BrdU (complete MEM with 25 μg/ml of 5-bromodeoxyuridine). Both cells were kept in a humidified 37°C, 5% CO_2 incubator.

Plasmids

The transfer vector, pMJ601, was a gift from Bernard Moss, National Institute of Allergy and Infectious Diseases (Bethesda, MD). The hemagglutinin (HA) gene of the A/HK/156/97 (H5N1) was amplified by the reverse transcriptase-polymerase chain reaction (RT-PCR) and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) in our laboratory.

Generation of the Expression Recombinant Vaccinia Virus

1. *Generation of the transfer vector for homologous recombination*

To eliminate a second ATG sequence which was located within the polylinker upstream of the first ATG of the HA cDNA (different reading frames) , the pCR2.1-HA was double-digested with two restriction enzymes, EcoRV and NotI (New England Biolabs, Beverly, MA) and then treated with the Klenow fragment (New England Biolabs) under the presence of dNTPs to fill-in the 5'-overhang made by NotI digestion. The fragment was gel-purified using the Geneclean II Kit (Bio 101, Vista, CA) following the manufacturer's recommendation. The purified fragment including the vector was self-ligated using the Fast-Link DNA Ligation Kit (Epicentre Tehnologies, Madison, WI) and used to transform the E. coli, INVαF' (Invitrogen). Colonies were picked and cultured overnight in 5 ml of LB media containing 50 μg/ml of ampicillin. Plasmids were prepared using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) following the manufacturer's recommendation, and screened by the NotI and EcoRV digestion. Absence of the ATG sequence before the first ATG of the HA gene was confirmed by nucleotide sequencing. Two unique restriction enzyme sites, XhoI and HindIII (Promega, Madison, WI) were used to cut out the cDNA, and the digested fragment was gel-purified. The transfer vector, pMJ601, was digested by the restriction enzymes, SalI and HindIII (Promega), and gel-purified. The ligation reactions between these two fragments were carried out using the Fast-Link DNA Ligation Kit (the XhoI-digested fragment is compatible to the SalI-digested fragment) and the ligation reaction mixture was used to transform the E. coli, INVαF' (Invitrogen). The colonies were picked up and cultured over night in 5 ml of LB media containing 50 μg/ml of ampicillin (Sambrook et al., 1989). Plasmids were prepared using the QIAprep Spin Miniprep Kit and screened by the EcoRI digestion (Promega). The correct clone, pMJ-HA, was propagated in large scale (500 ml of LB media with ampicillin) and purified using the Wizard Plus Maxipreps DNA Purification System (Promega) following the manufacturer's

recommendation. The quality and quantity of the purified plasmids were determined by measuring optical density at the wavelength of 260nm, and 280nm and 0.8% agarose gel electrophoresis.

2. *Generation of the recombinant vaccinia virus (Earl and Moss, 1993)*

1×10^6 CV-1 cells were seeded in a 25-cm^2 flask the day before transfection. Two hours before transfection CV-1 cells were infected with 1 ml of diluted wild type vaccinia virus (0.05 pfu/cell) in complete MEM-2.5 (2.5% of FCS). Cells were washed with PBS(-) and then 5 μg of pMJ-HA dissolved in 150 μl of TE, pH 8.0 mixed with 30 μl of SuperFect Transfection Reagent (Qiagen) was added to them. Cells were washed with PBS(-) after two hours incubation and cultured in complete MEM-10 for two days. Transfected cells were harvested and resuspended in 0.5 ml of complete MEM-2.5. Cell lysate was prepared by the freeze-thaw cycling and stored at -70°C. Both thymidine kinase (TK) selection and β-galactosidase screenings were used. $HuTK^-$143B cells were plated at 5×10^5/well in 6-well tissue culture dishes the day before infection. Transfected and infected cell lysates were trypsinized with the same amount of 0.25% trypsin, sonicated, and then three 10-fold serial dilutions (10^{-1} to 10^{-3}) were made. Dilutions of 10^{-2} and 10^{-3} were used to infect $HuTK^-$143B cells. After two hours of incubation, inoculum was replaced by complete MEM-10 with BrdU for TK selection. After two days of incubation, basal eagle medium containing melted 1% low melting point agarose, 0.1 mg/ml neutral red, 25 μg/ml BrdU and 0.33 mg/ml X-gal was overlaid onto the cells for β-galactosidase screening. After over-night incubation blue plaques were picked up by sterile, cotton-plugged Pasteur pipets and added into 0.5 ml of MEM-2.5. Cell lysate was prepared by the freeze-thaw cycling and sonication, and 10^{-1} and 10^{-2} dilutions were used to infect $HuTK^-$143B cells for plaque

purification. Plaque purification was repeated three times and then HuTK$^-$143B cells in 12-well dish were infected with a half of cell lysate from the picked-up plaque. After two days cells were harvested, and a half of cell lysate was used to infect two 25-cm^2 flasks of HuTK$^-$143B cells, one of which was for RNA isolation and the other was for large scale culture. After two days of incubation, cells were harvested, and cell lysate or RNA was prepared. One half of the cell lysate was used to infect a 175-cm^2 flask of CV-1 cells, and cells were harvested after three days of incubation, resuspended in 2 ml of complete MEM-2.5, freeze-thawed, sonicated and stored at -70°C. The virus titer was measured by infecting CV-1 monolayers seeded in a 6-well dish with serial dilutions of the virus stock.

Detection of the HA Gene Product

1. RT-PCR

Total cellular RNA was isolated from infected HuTK$^-$143B cells with the recombinant vaccinia virus using the Ultraspec RNA isolation system (Biotecx Laboratories, Houston, TX) following the manufacturer's protocol. Positive control, influenza viral RNA was isolated from infected allantoic fluid using the Ultraspec RNA isolation system. RNAs isolated from infected HuTK$^-$143B cells were treated with DNaseI to eliminate the contaminated vaccinia virus genomic DNA and purified again using the Ultraspec RNA isolation system. To synthesize the cDNA, 2 μg of total RNA was added to the reaction mixture containing 50 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 5 mM DTT, 0.5 mM dNTPs, 100 units of RNAguard (Pharmacia Biotech, Piscataway, NJ), 0.1 A_{260} units of $pd(N)_6$ Random Hexamer (Pharmacia Biotech) and 30 units of AMV reverse transcriptase (Promega) in 100 μl. The RT reaction was performed at 42°C for 60 min followed by heat inactivation at 95°C for 10

min. The HA gene was amplified by a HA specific primer set: upstream primer (HA167192): 5'-GACATACTGGAAAGGACACACAACGG-3'; downstream primer (HA942966): 5'-AGGGGGTGTATGTTGTGGAATGGCA-3'. This primer set produces an 800 bp fragment. The PCR was performed using GeneAmp XL PCR Kit (PE Applied Biosystems, Foster City, CA). One μl of cDNA was added to a PCR reaction mixture containing 1× XL buffer II, 0.2 mM dNTPs, 0.8 mM $Mg(OAc)_2$, 0.25 pmol/μl of each primer and 2 units of rTth DNA Polymerase, XL in 100 μl. PCR was performed in a DNA Thermal Cycler 480 (PE Applied Biosystems) at 94°C for 60 seconds, followed by 30 cycles of 94°C for 30 sec and 65°C for 5 min, and finally 1 cycle of 72°C for 10 min. 10 μl of PCR product was subjected to 1.4% agarose gel electrophoresis and the gel was stained with ethidium bromide.

2. Immunoprecipitation

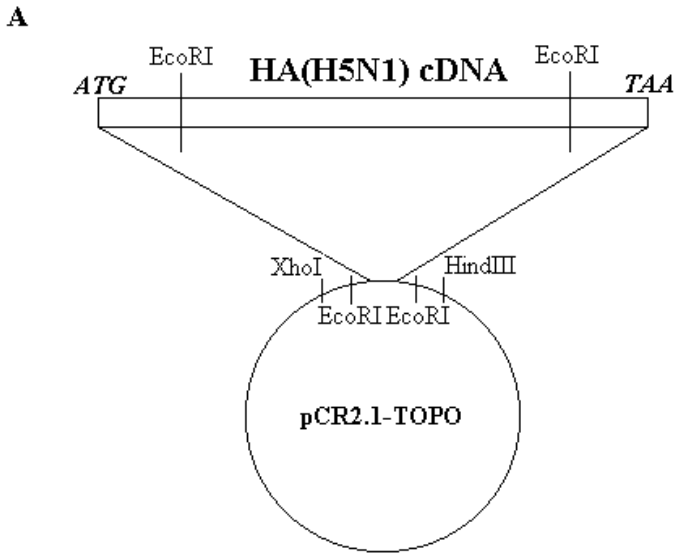
75-cm^2 flask of CV-1 cells were infected with approximately 10^6 pfu of recombinant vaccinia virus or wild type vaccinia virus. After two hours of incubation, CV-1 monolayers were washed with long-term labeling media (9 volumes of methionine-free RPMI medium was mixed with 1 volume of complete serum-free RPMI medium, and fetal calf serum was added to 10%) and cultured over-night. Long-term labeling media contained 0.1 mCi/ml of [^{35}S] methionine (Bonifacino, 1991). Labeled cells were washed with ice-cold PBS twice, then lysis buffer/wash buffer 1 from the Immunoprecipitation Kit (Protein A) (Boehringer Mannheim) was added onto them. Cells were scraped and homogenized. The supernatant (approximately 1 ml) was separated by centrifugation at 12000 × g, 10 min, 4°C and transferred to a new tube. 5 μl of ferret anti-serum raised against A/HK/156/97 (H5N1) was added to the supernatant and the mixture was incubated at 4°C for an hour. 50 μl of protein A-agarose suspension was added to the mixture.

After three hours incubation complexes were collected by centrifugation at 12000 × g for 1 min, and the pellet was resuspended in lysis buffer/wash buffer and incubated 20 min at 4°C for washing. The complexes were collected again by centrifugation at 12000 × g, for 1 min, and then this washing step was repeated. Then the pellet was washed with wash buffer 2 twice in the same way and with wash buffer 3 once. After washing 50 µl of 2×gel-loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue and 20% glycerol) was added to the pellet and 30 µl of the mixture was subjected to 10% SDS-polyacrylaminde gel electrophoresis. After electrophoresis the gel was fixed and analyzed by Bio-Rad Multi-Analyst Version 1.0.2.

RESULTS

Generation of the transfer vector for homologous recombination

Influenza virus A/Hong Kong/156/97 (H5N1) (A/HK/156/97) was propagated in embryonated chicken eggs, and the viral hemagglutinin (HA) gene was amplified by RT-PCR (data not shown) and cloned into plasmid pCR2.1-TOPO to generate plasmid pCR2.1-HA (Figure 1).



CTCGAGCGGCCGCCAGTGTG**ATG**GATATCTGCAGAATTCGCCCTT*CAAA**ATG***
XhoI NotI EcoRV EcoRI

B

CTCGAGCGGCCATCTGCAGAATTCGCCCTT*CAAA**ATG***
XhoI EcoRI

Figure 1. Map of Plasmid pCR2.1-HA. This plasmid contains the H5N1 HA gene amplified by RT-PCR and cloned into vector pCR2.1-TOPO (Panel **A**). The letters immediately beneath the map denote the 5' end of the HA cDNA (CAAAATG in italics) downstream from the EcoRI site within the polylinker. Two ATG sites are bolded, the first one occurs in the polylinker, and the second one within the HA gene. After NotI/EcoRV restriction enzyme digestion, fill-in reaction and self-ligation, the extra ATG within the polylinker was eliminated (**B**) to prevent false translational initiation.

Sequence analysis verified that pCR2.1-HA encodes the same HA amino acid sequence as GenBank Accession AF036356, but differs from Accession AF028709. To eliminate false translation from a second ATG sequence located within the polylinker upstream from the HA ATG, plasmid pCR2.1-HA was double-digested with EcoRV and NotI (Figure 1A) (which flank the ATG to be removed), made blunt by Klenow fill-in, then self-ligated. Self-ligated plasmid was transformed into *E. coli* INVαF', and amp^r plasmids were screened by NotI and EcoRV digestion. Absence of the polylinker ATG sequence was confirmed by nucleotide sequencing.

The HA gene (minus the polylinker ATG) was excised from pCR2.1-HA by digestion with XhoI and HindIII (Figure 1A). This HA fragment was ligated into vaccinia transfer vector pMJ601 (Figure 2) pre-digested with SalI and HindIII (the XhoI-digested fragment is compatible with the SalI-digested fragment). The ligated plasmid was transformed into *E. coli*, and amp^r colonies were screened by EcoRI digestion. The correct clone, pMJ-HA, was propagated large scale, and was used to make recombinant vaccinia virus expressing the HA..

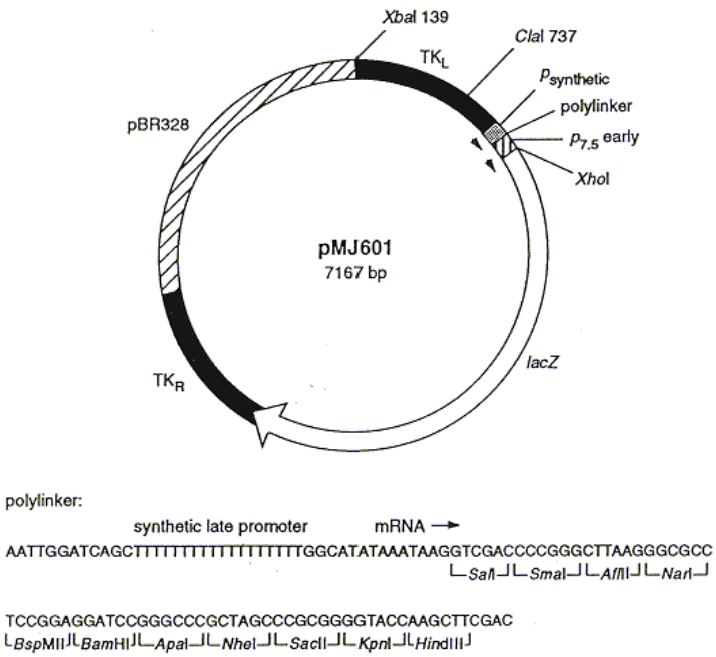


Figure 2. Map of Vaccinia Transfer Vector pMJ601. The synthetic late promoter, multiple cloning sites, $P_{7.5}$ promoter and lacZ gene are flanked by the right (TK_R) and the left (TK_L) half of the thymidine kinase gene for homologous recombination.

Generation of Recombinant Vaccinia Virus Expressing HA

Wild type vaccinia virus was propagated in CV-1 cells, then transfected with plasmid pMJ-HA. Three independent blue plaques were picked and purified by three rounds of plaque purification. These potential positive virus clones were used to infect HuTK-negative cells, and total cellular RNA was screened by RT-PCR for expression of HA mRNA (Figure 3).

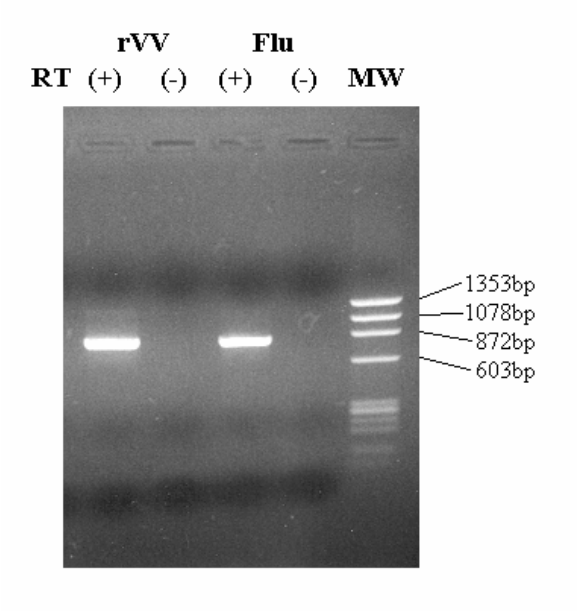


Figure 3. RT-PCR Screen of H5N1 HA mRNA Expression . Total cellular RNA isolated from HuTK$^-$143B cells infected with the recombinant vaccinia virus (rVV) or influenza viral RNA (Positive Control Flu) isolated from infected allantoic fluid were reverse transcribed with (+) or without (-) AMV reverse transcriptase. The strong band at around 800 bp denotes the presence of HA mRNA.

Two of three clones examined were positive for the HA gene expression, and one clone was chosen for large scale propagation. The virus titer of this preparation was 2.2×10^8 pfu/ml (Figure 4) and comparable to wild type or other recombinant vaccinia viruses prepared in our laboratory.

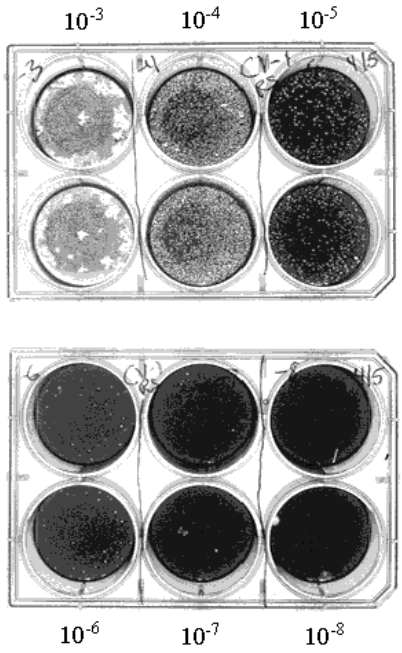


Figure 4. Titration of the Recombinant H5N1 Vaccinia Virus. Viral plaques are seen as round white or transparent spots after crystal violet staining. Duplicate wells of each dilution are shown.

Detection of HA Protein Expression

Immunoprecipitation by antiserum against A/HK/156/97 (H5N1) was performed to detect levels of HA protein from the recombinant virus (figure 5). The expected molecular weights of proteins HA, HA1 and HA2 were approximately 77, 50 and 27 kDa, respectively (Lamb and Chopin, 1983).

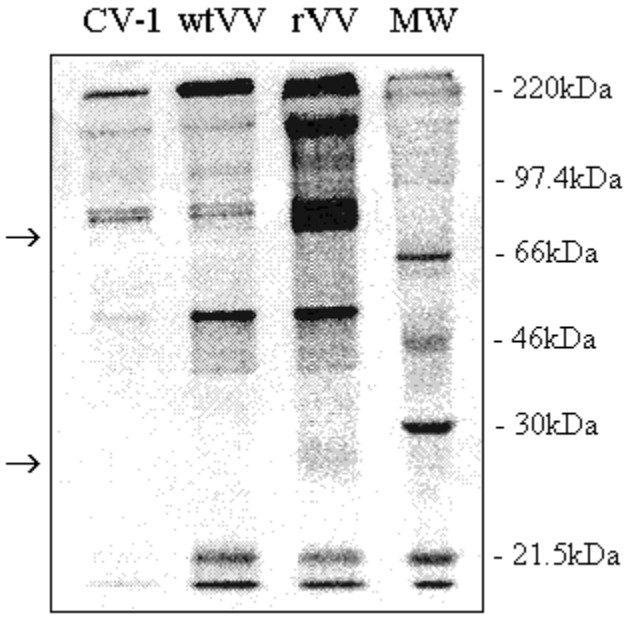


Figure 5. Detection of H5N1 HA Protein by Immunoprecipitation. Cell lysates from uninfected CV-1 cells (CV-1), CV-1 cells infected with wild type vaccinia virus (wtVV) or recombinant vaccinia virus were immunoprecipitated by antiserum against A/HK/156/97 (H5N1). Top arrow shows 77 kDa HA, and bottom arrow shows 27 kDa HA2, respectively.

The SDS-PAGE analysis of uninfected CV-1 cells (left lane) showed some nonspecific bands, but the cell lysate prepared from CV-1 cells infected with the recombinant vaccinia virus (lane 3) had a strong band at 77 kDa whose molecular weight is identical to HA (figure 5, top arrow). The infected cells also showed a faint band about 27 kDa in size (figure 5, lower arrow) which likely corresponds to the HA2 subunit. It was not clear if there was a 50 kDa band because of

the presence of a nonspecific band at 50 kDa, although its intensity increased markedly in vaccinia infected cells as expected for HA1. These results showed that the H5N1 HA gene was indeed expressed, and that a small portion of the HA protein was correctly cleaved into HA1 and HA2.

DISCUSSION

A recombinant vaccinia virus expressing the HA gene of the new influenza virus H5 subtype was generated, and the expression of the HA gene product was confirmed at both transcriptional and translational levels. This recombinant vaccinia virus will be used in the future to confirm that the avian HA protein is recognized by a human T cell clone, 10-1G5, which is the first example of a human CTL specific to the HA. Truncated forms of this recombinant vaccinia virus HA and/or synthetic HA peptides could then be used to precisely identify the T cell epitope on the HA protein. This recombinant vaccinia virus will also be useful to identify and isolate T cell lines specific for H5 HA to determine whether T cell lines exist that are crossreactive to the H5 subtype and to other influenza A virus HA proteins using PBMC obtained from patients and/or healthy volunteers.

The generation and characterization of this recombinant vaccinia / influenza H5 HA virus will be a useful reagent to better identify and define the roles of influenza A virus HA-specific T cells.

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