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Influenza Virus (A/HK/156/97) Hemagglutinin Expressed by an Alphavirus Replicon System Protects Chickens against Lethal Infection with Hong Kong-Origin H5N1 Viruses

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Venezuelan equine encephalitis virus replicon particles (VRP) containing the gene expressing hemagglutinin (HA) from the human Hong Kong Influenza A isolate (A/HK/156/97) were evaluated as vaccines in chicken embryos and young chicks. Expressed HA was readily detected in bird-tissue staining with anti-H5 HA antibody and in chicken cells infected with the replicon preparations following immunoprecipitation with monoclonal antibody. Birds challenged with a dose of the lethal parent virus were protected to different extents depending on the age of the bird. *In ovo* and 1-day-old inoculated animals that received no boost with the VRP were partially protected; birds 2 weeks of age were completely protected with a single dose of VRP. © 2000 Academic Press

INTRODUCTION

Large-scale vaccination of young birds has proven an effective method for control of many viral diseases of poultry (Zander *et al.,* 1997). More recently, vaccination *in ovo* for Marek's disease has become widespread in the U.S. broiler chicken industry (Sharma, 1999), providing an easy, cost-effective method of vaccination against this economically important disease. The alphavirus expression/replicon system (Schlesinger, 1999) has been shown to be effective as a vaccine delivery tool in mammals. In particular, the Venezuelan equine encephalitis (VEE) system (Pushko *et al.,* 1997) has been used to protect mice against a lethal influenza challenge in mice and more recently a lethal SIV challenge in macaques (Davis *et al.,* 2000). The effectiveness of vaccination using the alphavirus vectors, however, has not yet been evaluated in the avian system. With the recent lethal outbreak of H5N1 avian influenza in Hong Kong in 1997 (Subbarao *et al.,* 1998; Claas *et al.,* 1998; Suarez *et al.,* 1998) affecting both birds and humans, it seemed useful to evaluate the efficacy of the VEE replicon system as a vaccine-delivery mode for both mammals and birds. It was of further interest to determine the effectiveness of the vaccine in the late-stage avian embryo, as it was recently demonstrated (Gagic *et al.,* 1999) that multiple live viruses may be used for successful *in ovo* vaccination of chickens.

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RESULTS

When VRP containing a copy of the influenza hemagglutinin RNA were inoculated onto chicken cells at increasing concentrations, expression of the cleaved influenza HA protein was readily detected by immunoprecipitation of the HA using monoclonal antibodies (Fig. 1). Three forms of the HA (see Klenk and Garten, 1994) could be distinguished in the replicon-infected cells: the trimeric form, the noncleaved precursor, and the cleaved monomeric form. In this particular experiment, the concentration of HA appeared to be higher in the cells infected at 0.5 particle/cell than in cells infected with 50 particles (Lanes 3 and 4). This was not consistently seen and was apparently due to variation in our capacity to quantitatively strip and recover the HA from the immune complex. Likewise, though the samples were treated in standard fashion with SDS and mercaptoethanol, the trimeric complex formed again under the electrophoresis conditions employed. It was clear, however, that the HA was being expressed at high levels when cells were infected with 5×10^3 VRP per cell. The noncleaved HA was clearly visible at this level of infection, indicating a level of HA production that apparently exceeded the cells' capacity of proteolytic enzymes to process (Fig. 1, Lane 5, and unpublished observations).

Efficient expression of the H5 HA by the VRPs in chicken cells was also noted both in white leghorn embryos infected at 19 days incubation (Fig. 2) and in 1-day-old hatchlings (data not shown). Tissues were isolated from control (mock-infected) embryos and from VEE-HA inoculated chicken embryos 1-day postinoculation (dpi). Positive immunoreactivity using a mouse monoclonal antibody specific for the H5 protein was

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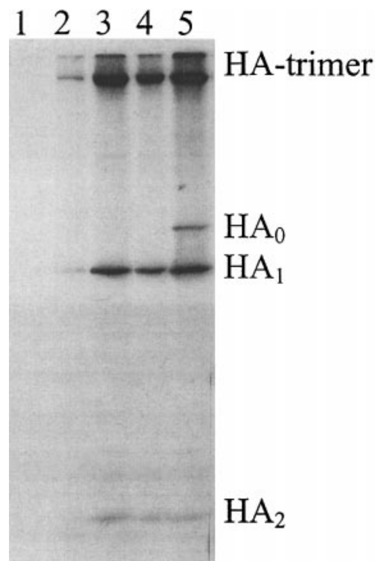

FIG. 1. VEE replicon expression of HA protein in chicken cells. Primary chick-embryo cells were infected with varying amounts of replicon, incubated overnight, and labeled with ^{35}S-methionine. The proteins were then solubilized and precipitated with monoclonal antibody and protein-A agarose. Precipitates were then electrophoresed in SDS-polyacrylamide gels. Lane 1: control (no replicons); Lane 2: 0.005 VRP per cell; Lane 3: 0.5 particles per cell; Lane 4: 50 particles per cell; Lane 5: 5×10^3 particles per cell.

detected in intestines and thymus (Fig. 2), kidney, bursa, heart, proventriculus, intestines, spleen, and liver. The staining patterns suggested systemic distribution of the replicon, with the intestines and thymus appearing to have the most staining both in embryos and 1-day-old birds. No immunoreactivity was observed in mock-infected tissues. These results indicate that embryos inoculated with the VEE-H5 replicon expressed significant levels of the HA protein in a variety of embryo tissues.

In order to determine whether VEE replicons would stimulate an efficient immune response *in ovo,* 19-day-old embryos were inoculated and after hatching, birds were analyzed with and without a subsequent boost with the VRP, for circulating serum antibodies to the HA protein. They were then subsequently challenged with lethal H5N1 virus. In Table 1, the protection levels at the highest input of VEE-HA replicons was less than 100%. Only one of seven birds showed a serum hemagglutination inhibition response to the inoculation and only three of seven birds were protected against the lethal infection. When the birds were boosted at 2 weeks of age with an equivalent dose of replicons, all showed a positive HI response and all were protected from the lethal infection. Likewise, as shown in Table 2, 1-day-old hatchlings were also incompletely protected by VEE-HA replicons alone, while a boost at 14 days yielded 100% protection. In this experiment it was also shown that a single dose of replicons at 14 days of age was capable of providing an equivalent level of protection against lethal challenge.

To determine the extent of reduction of virus in vaccinated birds, *in ovo* vaccinated birds were assayed for virus levels in the oral and cloacal cavities following immunization and challenge. In Fig. 3 are shown the results of these assays. A dose of 10^7 replicons per embryo followed by a boost at 14 days of age provided immunity against lethal infection with A/HK/156/97 (H5N1), and no virus was recoverable at 2 days post-challenge. Mock-vaccinated controls, in comparison, yielded high titers of virus in the oral and cloacal cavities.

DISCUSSION

The alphavirus replicon system for expression of heterologous genes has proven to be a potent method for safe delivery of proteins as immunogens in mammals (Pushko *et al.,* 1997; Davis *et al.,* 2000). This report demonstrates that the system should be equally useful in birds. The results clearly demonstrate that a protective immune response, capable of decreasing challenge virus replication below measurable levels, is mounted in 2-week-old birds inoculated with a single reasonable dose of VEE-HA replicons. The response in hatchlings and *in ovo* was more variable and less efficient unless a boost was given. In general, a measurable HI titer against a given subtype means birds will have low levels or no virus in the oral and cloacal cavities and will be protected from a lethal challenge by the same subtype. In some cases birds are protected from lethal infection even though they do not exhibit a measurable serum HI response. This is not uncommon and has been reported

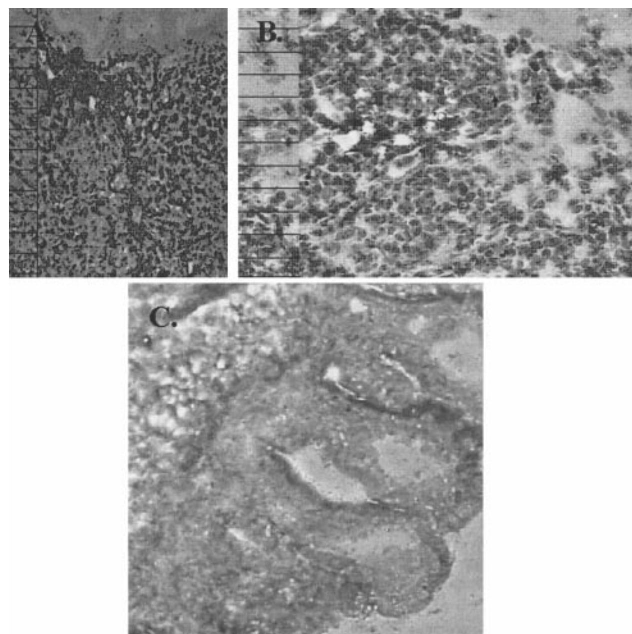

FIG. 2. Expression of H5 HA in chicken embryo tissues. Tissues were removed from chicken embryos infected with the VEE-HA replicons or noninfected controls at 1 day postinfection and stained for the presence of the H5 hemagglutinin. (A) Intestinal tissue from infected embryos, bar = 150 μm. (B) Thymus from infected embryos, bar = 100 μm. (C) Intestinal tissue from control embryos, bar = 250 μm. Arrows (brown staining) indicate positive reactions.

TABLE 1

In Ovo Vaccination and Boost with VEE Replicons Expressing Influenza H5 Hemagglutinin Protein[a]

Inoculum	Number of eggs inoculated	Number of birds with HI response	Range of HI titers–day of challenge	Number of birds surviving challenge	Range of HI titers–10-d postchallenge
PBS + PBS boost	4	0	0	0	NA
	4	0	0	0	NA
10^6 H5HA replicons					
+ 10^6 H5HA boost	7	1	0–4	1	>256
	6	5	0–128	5	>256
10^7 H5HA replicons					
+ 10^7 H5HA boost	7	1	0–4	3	>256
	6	6	32–128	6	>256

[a] Embryos were 19 days old, hatched two days later, and were boosted at 2 wks posthatching.

in other vaccination studies (Crawford *et al.,* 1998, 1999; Stone *et al.,* 1997), as well as in this study. It may reflect a number of possibilities including effects of mucosal immunity or temporally variable low serum HI titers in younger birds.

Many alphaviruses are found naturally in birds (see Perdue, 2000), including Sindbis virus (Frolov *et al.,* 1996), and it is possible that a different alphavirus candidate might be found that would induce a stronger response in the younger birds. The lower levels of antibody seen *in ovo* and in 1-day-old hatchlings may have to do with the status of the still-developing immune system in young birds. A single injection of killed virus in oil emulsion *in ovo* or subcutaneously in 1-day-old hatchlings is capable of eliciting 100% protection in challenges as long as 8–10 weeks following vaccination (Stone *et al.,* 1997; Crawford *et al.,* 1998; unpublished observations). So, at least some elements of the immune system are in place to mount an effective response. Other problems with *in ovo* effectiveness may include the presence of proteases in the allantoic fluid that could affect the efficiency of the infection with the VEE replicons. Since *in ovo* vaccination of chickens has become a routine practice by the poultry industry, it will be well worth pursuing studies that will optimize the immune response in birds by altering the VEE system or perhaps developing another alphavirus replicon system.

The VEE replicon system is certainly effective as a vaccine in the 2-week-old bird. The protection against challenge seen with a single injection is comparable to that seen previously with single injections of either killed virus or HA subunit vaccines against H5 subtypes (Crawford *et al.,* 1998, 1999), though HI titers were lower. It is also possible that including other influenza genes in the vaccination scheme might further enhance the immune response. Initial results with combined vaccination with the VEE replicons expressing the N1 neuraminidase and the H5 HA did not show enhanced protection (data not shown). Including internal genes expressed by the replicons in the combination, however, might enhance the cellular immune response and provide broader protection; these studies are underway.

Preliminary results have also shown the VEE-HA replicons to be effective immunogens in mice (Dybing, unpublished data). It has been previously shown that an H3 subtype HA expressed by an attenuated VEE vector system fully protected mice against a lethal influenza challenge as well (Davis *et al.,* 1995). In a zoonotic outbreak such as the one that occurred in Hong Kong in 1997, it would be useful to devise a vaccine that would have common antigens that would be effective for both humans and nonhumans. The VEE replicon system might provide just such an effective candidate.

TABLE 2

Vaccination of 1- and 14-day-old Chicks with a Single Dose of VEE Replicons Expressing Influenza H5 Hemagglutinin

Inoculum	Number of birds vaccinated	Number of birds with HI titers at day of challenge	Range of HI titer–day of challenge	Number of birds surviving challenge	Range of HI titer–10-d postchallenge
PBS control	8	0	0	0	NA
10^7 H5HA (1 day old)	8	1	0–2	3	128–256
10^7 H5HA (14 days old)	8	8	16–64	8	32–256
10^7 H5HA (1 + 14 days old)[a]	8	8	4–256	8	16–256

[a] Vaccinated at 1 day of age (posthatching) and boosted at 14 days posthatching.

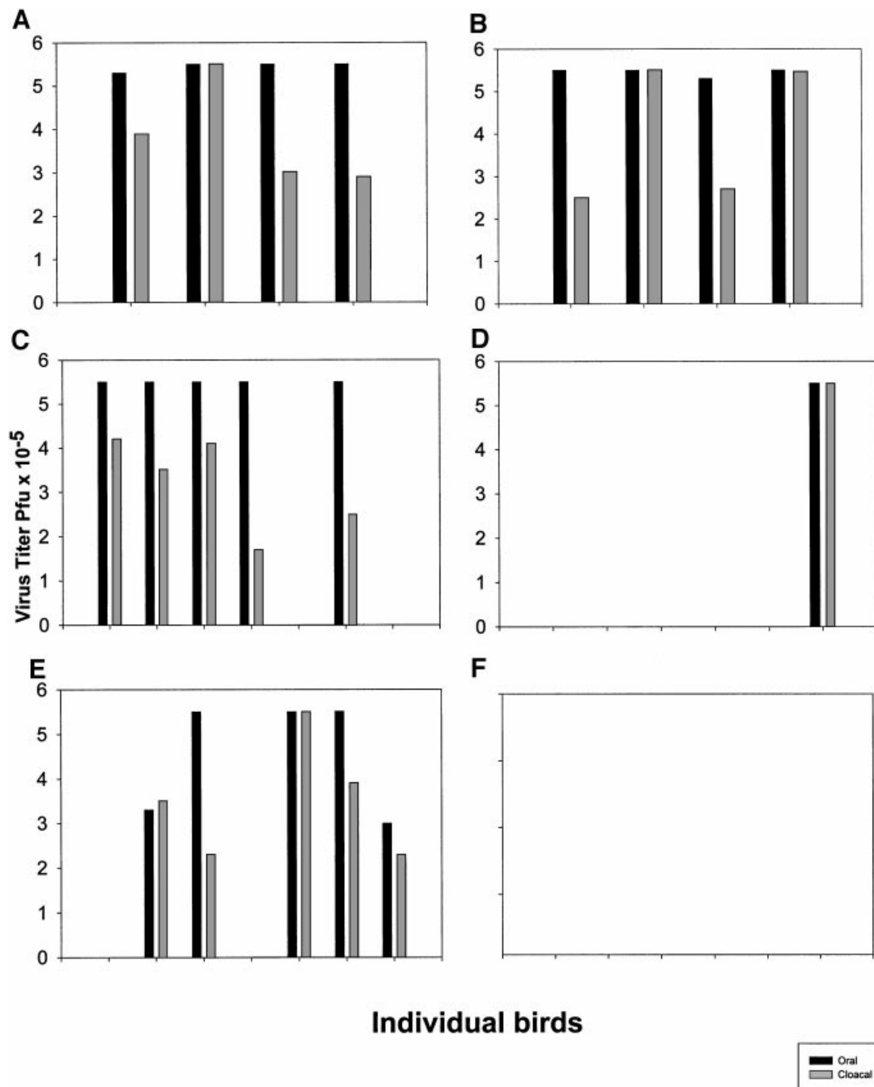

FIG. 3. Virus titers in chickens vaccinated *in ovo* with VEE-HA and challenged with a lethal dose of A/Hong Kong/156/97 (H5N1). Each point on the X axis represents challenge virus titers from the oral cavity and cloacal cavity of the same chicken. (A) Control (mock vaccinated) birds. (B) Control plus boost with PBS. (C) Embryos vaccinated with 10^6 VRP. (D) Embryos vaccinated with 10^6 particles plus a boost at 14 days of age with an additional 10^6 VRP. (E) Embryos vaccinated with 10^7 VRP. (F) Embryos vaccinated with 10^7 particles plus a boost at 14 days of age with an additional 10^6 VRP.

MATERIALS AND METHODS

Vector development. The complete coding sequence of the hemagglutinin (HA) gene from A/Hong Kong/156/97 (H5N1) strain (originally isolated from a 3-year-old child in Hong Kong in April 1997) was RT-PCR amplified, cloned into the pAMP vector (Gibco, Gaithersburg, MD), and sequenced as previously described (Suarez *et al.,* 1998). The plasmid containing the complete HA coding sequence was used as template in a PCR reaction with two primers comprising (for forward, VEE genome-sense, 5′ to 3′) a random 8-mer, a ClaI site, the distal 16 nucleotides of the 26S mRNA untranslated region, and the first nine codons of the HA gene, and (for reverse, complementary, 5′ to 3′) a random 8-mer, a ClaI site, a stop codon, and the 3′-terminal 14 nucleotides of the HA gene. A DNA fragment of the expected size was amplified using 25 cycles of 93°C for 1 min, 55°C for 2 min, and 72C for 1 min with Vent DNA polymerase (New England Biolabs) in a DNA Thermal Cycler (Perkin–Elmer Cetus). The purified DNA fragment was digested with ClaI and inserted at the unique ClaI site of a replicon vector plasmid, pVR2, analogous to that described by Pushko *et al.* (1997). The sequence of the insert was confirmed, and virus replicon particles (VRP) were produced by coelectroporation of baby hamster kidney (BHK) cells with three *in vitro* RNA transcripts, pVR2-HA replicon, VEE capsid helper, and VEE mutant glycoprotein helper, exactly as described (Pushko *et al.,* 1997). Serial dilutions of HA-VRP preparations were used to infect BHK cell monolayers in eight-well chamber slides. VRP-infected single cells were scored by indirect immunofluorescence using anti-influenza virus A/Hong Kong/

156/97 chicken serum, biotinylated anti-chicken IgG (Vector Laboratories, Inc., Burlingame, CA), and avidin FITC.

Immunoprecipitation. Primary chicken-embryo cells were inoculated with varying concentrations of replicons and then incubated for 24 h. The cells were disrupted and processed as previously described in buffer containing Triton X-100 and sodium deoxycholate (Perdue, 1992), and proteins were precipitated using monoclonal antibody specific for the HA of A/Hong Kong/156/97. Gels were run and processed for autoradiography as described (Perdue, 1992).

Vaccination and challenge. Embryos were inoculated as described by Stone *et al.* (1997) at 18 or 19 days of development. Briefly, a $1\frac{1}{2}$ in. 28-gauge needle was inserted full length into a hole drilled in the top of the air sac end of the egg and 0.1 ml of buffer or buffer-containing replicons was injected. The hole was sealed and the embryo hatched. In these experiments hatch rate was always greater than 95%. One-day-old and 2-week-old chickens were vaccinated subcutaneously with 0.2 ml of replicons in the nape of the neck. Vaccinated birds were challenged at 4 weeks of age by intranasal/intratracheal (IN/IT) inoculation with 10,000 egg lethal doses (ELD50) per animal with an embryo-propagated stock of the A/HK/156/97 H5N1 strain. Serum hemagglutination inhibition (HI) titers and titration of virus recovered from oral and cloacal cavities were determined as previously described (Crawford *et al.,* 1998, 1999).

Immunohistochemistry. Briefly, tissues were removed from chicken embryos 1 day postinoculation with PBS or VEE-H5 replicon and snap frozen in liquid nitrogen in OCT freezing compound (Fisher Scientific, Norcross, GA). Frozen tissues were cut into 6-μm sections and thawed on Vectabond-coated microscope slides. The dried tissues were fixed with cold 1:1 methanol/acetone for 3 min at room temperature, blocked with 1% normal horse serum in PBS, and stained with a 1:500 dilution of mouse monoclonal anti-H5 antibody using the Vector ABC alkaline phosphatase staining kit following manufacturer's directions (Vector Laboratories). Fast Red was used as the alkaline phosphate substrate.

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