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ANTIGENIC AND PHENOTYPIC VARIANTS OF A VIRULENT AVIAN INFLUENZA VIRUS SELECTED DURING REPLICATION IN DUCKS

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ABSTRACT: To evaluate the replication of a highly virulent avian influenza A virus in a potential reservoir host, mallard ducks (*Anas platyrhynchos*) were inoculated with the virulent strain A/Ty/Ont/7732/66 (H5N9). Viruses recovered from the ducks were analyzed by hemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) and found to possess antigenically altered viral hemagglutinins. Plaque formation on the Madin-Darby Canine Kidney (MDCK) cell line and on primary chicken embryo cells was investigated, and isolates recovered from the ducks differed from the wild type by being unable to form plaques on MDCK cells without trypsin. This phenotype did not appear to be due to inefficient cleavage of the hemagglutinin by host cell proteases since hemagglutinin immunoprecipitated from cell lysates was cleaved. Although the plaquing phenotype suggested attenuation of the isolates from the ducks, they were not significantly altered in their virulence for chickens shown by infectivity studies in vivo. These results indicate that replication of influenza A/Ty/Ont/7732/66 virus in ducks can produce antigenic and phenotypic variants which are still highly virulent for domestic poultry.
Key words: Mallard ducks, *Anas platyrhynchos*, avian influenza virus, antigenic variants, phenotypic variants, virulence, replication, experimental study.

INTRODUCTION

Influenza viruses continually circulate among wild waterfowl, particularly migratory ducks. Most subtypes of influenza virus have been isolated from wild ducks, including members of the H5 and H7 hemagglutinin subtypes (Hinshaw et al., 1980, 1985). While virulent strains of H5 or H7 influenza viruses have not been isolated from wild ducks, these subtypes have been associated with devastating outbreaks in domestic birds (Webster and Rott, 1987). Circumstantial evidence indicates that free ranging ducks may be involved in the primary introduction and subsequent spread of highly virulent strains among domestic poultry (Hinshaw et al., 1981; Alexander, 1982), but direct evidence has not been obtained in spite of intensive surveillance efforts on the part of task force members during the 1983–1984 outbreak of H5N2 influenza virus in Pennsylvania, who concluded that no viruses related to the pathogenic strain were present in wild ducks during the outbreak (Nettles et al., 1987).

Little is known about the ability of ducks to replicate and shed highly virulent avian influenza viruses. In one instance, apparently healthy domestic ducks were found to be shedding an H5N8 influenza virus which was highly virulent for chickens and turkeys and closely related to a virus causing disease problems in nearby turkey flocks (McNulty et al., 1985). Experimental inoculation of ducks with highly virulent strains has indicated these viruses are not pathogenic for this host (Kawaoka et al., 1987). However, viruses recovered from the ducks were not examined for potential changes in their virulence and it is possible that replication of these viruses in ducks yields avirulent variants thereby explaining the results. In addition, it has not been asked whether selective pressures imposed by replication in ducks result in antigenic variation of the virus.

Highly virulent avian influenza viruses have a characteristic phenotype with regard to plaque formation on cultured cells: all of such viruses examined to date form plaques with equal titers on cells in the presence or absence of trypsin, whereas less virulent strains form plaques only when trypsin is present. This is believed to be

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due to the efficiency with which the viral hemagglutinin is activated to a fusion competent form by proteolytic cleavage within the host cell (Bosch et al., 1979; Alexander et al., 1981).

This study was undertaken to determine whether an avian influenza virus, A/Ty/Ont/7732/66 (H5N9), which is highly virulent for chickens and turkeys could replicate in mallard ducks, and if so, to determine the consequences to the virus. We have focused on alterations in the viral hemagglutinin, a glycoprotein known to play a pivotal role in the life cycle of the virus by mediating receptor binding and fusion activities (reviewed by Webster and Rott, 1987), as well as being the major target for neutralizing antibodies.

MATERIALS AND METHODS

In vivo studies

Seronegative young adult (6- to 8-mo-old) mallard ducks (*Anas platyrhynchos*) were obtained from a commercial breeding farm (Abendroth's Waterfowl Hatchery, Waterloo, Wisconsin 53594, USA) and inoculated orally or intratracheally with 0.5 ml of infectious allantoic fluid containing approximately 1×10^{8} EID_{50} of wild type influenza A/Turkey/Ontario/7732/66 (H5N9). This virus isolate (A/Ty/Ont/7732/66) was obtained from lyophilized lung tissue from the original outbreak (Lang et al., 1968), and passaged twice in embryonated chicken eggs prior to use. Tracheal and cloacal swabs were collected from the ducks 2 to 4 days postinoculation by inserting dacron swabs (American Scientific Products, McGaw Park, Illinois 60085, USA) into the trachea or cloaca then breaking the head of the swab off into 1 ml of 50% glycerol in phosphate buffered saline (PBS), pH 7.2, supplemented with 1,000 IU/ml penicillin, 200 μg/ml streptomycin, 250 μg/ml gentamicin, and 50 IU/ml mycostatin (all antibiotics from Sigma Chemical Company, St. Louis, Missouri 63178, USA). The medium surrounding the swabs was assayed for the presence of virus by growth in embryonated chicken eggs and detection by hemagglutination. Organs were collected aseptically, and 10% (weight/volume) suspensions were homogenized in a Stomacher (Tekmar Company, Cincinnati, Ohio 45222, USA) and assayed for virus as previously described (Webster et al., 1981). The variants designated 1C and 587C were isolated from cloacal swabs collected from two mallard ducks infected with A/Ty/Ont/7732/66 on days 4 and 3, respectively, and plaque purified on chicken embryo cells.

For pathogenicity studies, 10- to 12-wk-old domestic white leghorn chickens were raised in confinement from hatching, and groups of 10 chickens were inoculated intratracheally with approximately 5×10^{7} EID_{50} of virus. Infected birds were maintained in containment level three isolation and monitored daily over a period of 14 days. Infection of all experimental chickens was verified by virus isolation from tracheal and cloacal swabs collected on the second day after inoculation.

Virus growth and purification

Viruses were grown in 11 day embryonated hen's eggs at 35 C for 30 hr. For purified virus, allantoic fluid was harvested from the eggs, clarified by centrifugation at 25,000 *g* for 15 min, and the virus was pelleted from the clarified fluid by centrifugation through a 10 ml cushion of 30% sucrose (weight/volume) in STE buffer (100 mM NaCl, 50 mM Tris pH 7.2, 1 mM EDTA) at 35,000 *g* for 3 hr in a Beckman SW-28 rotor (Beckman Instruments, Inc., Palo Alto, California 94304, USA). The pellet was resuspended in 0.2 ml of STE and used as antigen in the enzyme-linked immunosorbent assay (ELISA).

Serological methods

Hemagglutination (HA) and hemagglutination inhibition (HI) assays were performed as described by Palmer et al. (1975). Isolation and characterization of a panel of H5 specific monoclonal antibodies will be described in detail elsewhere (M. S. Philpott, B. C. Easterday and V. S. Hinshaw, unpubl. data). Briefly, Balb/c mice (Charles River Laboratories, Inc., Wilmington, Massachusetts 01887, USA) were immunized with A/Ty/Ont/7732/66, then boosted 3 days prior to fusion with A/Ty/Ont/7732/66 or A/Mal/Penn/10218/84 (H5N2). Spleens were removed and fused to NS-1 murine myeloma cells as described by Oi and Herzenberg (1980).

An ELISA binding assay was performed using 200 hemagglutination units (HAU)/well of viral antigen, prepared as described above, which was partially disrupted by pretreatment with 0.05% N-lauroylsarcosine (Sigma Chemical Company, St. Louis, Missouri 63178, USA) and bound to the plates at 4 C in 0.1 M carbonate buffer, pH 9.6. Antibody incubations were conducted under conditions of saturating antibody concentration in phosphate buffered saline supplemented with 0.05% Tween 20 and 0.5% bovine serum albumin (BSA) for 1 hr at room temperature, followed by extensive washing in the same solution without BSA. Following incubation with

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rabbit anti-mouse horseradish peroxidase conjugated second antibody (Zymed Laboratories, Inc., San Francisco, California 94080, USA) at working dilutions recommended by the manufacturer and washes, the enzymatic activity was detected by hydrolysis of hydrogen peroxide in the presence of azino-di-3-ethyl-benzothiazobine-6-sulfuric acid (ABTS, Boehringer Mannheim, Indianapolis, Indiana 46250, USA) in 0.4 M citrate buffer pH 4.0 and quantitated by measuring optical density at 410 nm in an MR600 Microplate Reader (Dynatech Laboratories, Inc., Chantilly, Virginia 22021, USA). Percent binding is calculated by normalizing the optical density of the test wells relative to the amount of antigen and antibody present according to the following equation: Percent Binding = $(B_{xv}/B_{pv})/(B_{xw}/B_{pw}) \times 100$; where B_{xv} = binding of monoclonal x to test virus, B_{pv} = binding of pooled monoclonals to test virus, B_{xw} = binding of monoclonal x to wild type virus, and B_{pw} = binding of pooled monoclonals to wild type virus.

Plaque assays

Chicken embryo cells (CEF) were prepared from 12 day embryos by standard methods (Dulbecco, 1952). Madin-Darby Canine Kidney (MDCK) cells were obtained from American Type Culture Collection (Rockville, Maryland 20852, USA), and used in these experiments between passages 15 and 25. Plaque assays were performed as previously described (Klenk et al., 1975), except that trypsin was used at a concentration of 0.5 μg/ml for CEF monolayers and 1 μg/ml for MDCK, and 0.5% agarose was used instead of agar.

Cleavage for the hemagglutinin

To determine the state of cleavage of the HA of the isolates, labeled cell lysates were prepared from MDCK cells infected at a multiplicity of five and incubated 16 hr in methionine-free minimal essential medium (MEM) supplemented with 200 μCi of L-[^{35}S]methionine (E. I. du Pont de Nemours, Inc., Wilmington, Delaware 19898, USA) with or without 1 μg/ml trypsin. Lysates were prepared from the infected cells, and hemagglutinin was immunoprecipitated from the lysates with a pool of 23 H5 specific monoclonal antibodies as described (Kida et al., 1982) except the washing buffer contained 0.05% Tween 20 and 0.3 M NaCl to reduce background. The immunoprecipitated H5 was subjected to electrophoresis on a 10% acrylamide gel (Laemmli, 1970). The gel was dried and exposed to Cronex 7L X-ray film (E. I. du Pont de Nemours, Inc., Wilmington, Delaware 19898, USA) for 3 days at −70 C.

Table 1. Replication of avian influenza type A/Ty/Ont/7732/66 in ducks.

Route of inoculation[a]	Number positive[b]/total ducks			
	Trachea[c]	Cloaca[c]	Spleen	Lung
Trachea	10/10	3/10	3/3	3/3
Oral	6/10	5/10		

[a] Experimental ducks were inoculated with diluted infectious allantoic fluid as described in the text.

[b] Birds were positive if virus was recovered on one of the four sampling days.

[c] Tracheal and cloacal swabs were collected on days 1 to 4. Three mallards were inoculated tracheally and sacrificed on day 3 for tissue collection, and virus was recovered from the tissues as described (Webster et al., 1981).

RESULTS

In vivo studies and isolation of variants

To determine if ducks could support the replication of a highly virulent avian influenza virus, ducks were inoculated with A/Ty/Ont/7732/66 (H5N9), a virus highly virulent for chickens and turkeys. The inoculated ducks showed no clinical signs of disease, but were infected and shedding virus as determined by virus recovery, as shown in Table 1. Replication of A/Ty/Ont/7732/66 in the mallards was not restricted to the respiratory and intestinal tracts, since virus was recovered from the spleens of infected birds. All isolates were examined by HI serology with a pool of several monoclonal antibodies, and were found to react in a similar manner, but different from the wild type virus (not shown). Two cloacal isolates, designated 587C and 1C, recovered from different mallard ducks on days 3 and 4, respectively, being representative of the viruses shed, were selected for more detailed characterization.

To examine the pathogenicity of isolates 1C and 587C, groups of 10 experimental chickens were inoculated. Virus was recovered on day 2 from each chicken, indicating efficient infection. As shown in Figure 2, isolate 1C resulted in 100% mortality within 4 days, similar to the wild type virus, while isolate 587C killed 80% of the birds but did so more slowly. Both viruses are highly virulent for chickens,

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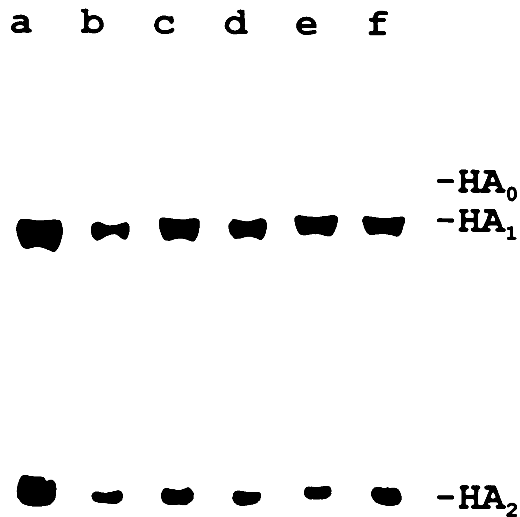

FIGURE 1. Radioimmunoprecipitation of ^{35}S-labeled H5 hemagglutinin from cell lysates infected with the wild type A/Ty/Ont/7732/66 (WT), or variants 1C and 587C. Lysates were prepared from infected MDCK cells incubated in the presence or absence of 1 μg/ml of trypsin in the supernatant. Hemagglutinin was immunoprecipitated with monoclonal antibodies as described in the text. Lane (a) wild type A/Ty/Ont/7732/66 with trypsin, (b) wild type A/Ty/Ont/7732/66 without trypsin, (c) variant 1C with trypsin, (d) 1C without trypsin, (e) variant 587C with trypsin, (f) 587C without trypsin.

TABLE 2. Hemagglutination inhibition titers of variants and wild type influenza A/Ty/Ont/7732/66 using monoclonal antibodies to the H5 hemagglutinin.

Antibodies	Hemagglutination inhibition titers:[a] Viruses		
	A/Ty/Ont/ 7732	1C	587C
Polyclonal	>20,500	>20,500	>20,500
77B1	>20,500	>20,500	>20,500
56G2	>20,500	>20,500	>20,500
42C3	5,120	5,120	10,250
67G1	>20,500	>20,500	>20,500
4C9	5,120	640	1,280
13B8	640	320	640
4D2	320	320	320
1D5	1,280	640	640
1G2	640	160	320
4F10	640	320	640
76E1	5,120	1,280	1,280
24B9	>20,500	5,120	5,120
1D4	640	320	640
2E8	640	640	40
2E3	320	160	160
3B5	1,280	1,280	40
2D6	320	40	320
22D6	80	80	40

[a] Hemagglutination inhibition titers are assayed using four HA units of each virus per well as described by Palmer et al. (1975).

suggesting that replication in ducks did not substantially reduce the pathogenicity of A/Ty/Ont/7732/66.

Serological characterizations

HI serology with polyclonal sera from convalescent chickens and monoclonal antibodies prepared to the H5 hemagglutinin of A/Ty/Ont/7732/66 was used to compare the isolates 587C and 1C to each other and the wild type virus. As shown in Table 2, the different isolates reacted similarly in HI tests with polyclonal sera. However, when individual monoclonal antibodies were used, titers to isolates 587C and 1C were generally reduced relative to the wild type virus. This reduction in HI titer was as high as a 32-fold difference in reactivity between the "wild type" and isolate 587C with monoclonal 3B5. In contrast, this same monoclonal antibody, 3B5, was not significantly reduced in titer to the other isolate, 1C. Likewise, reaction of antibody 2D6 was the same for the wild type and isolate 587C, but was 16-fold lower for the isolate 1C. Thus, these two virus isolates, 1C and 587C, are antigenically distinct from each other as well as from the wild type virus.

To determine whether the observed differences in HI titers were due to reduced ability of the monoclonal antibodies to bind to the variants, each of the monoclonals was tested against partially disrupted viruses in an ELISA binding assay. As shown in Table 3, binding to variant 1C was similar to that of the wild type for all of the monoclonal antibodies. Binding to variant 587C was 30 to 50% that of binding to the wild type for three antibodies, 2E8, 3B5 and 22D6, showing good correlation with the results of HI using these same antibodies. However, for all other monoclonal

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TABLE 3. Percent binding of H5 specific monoclonal antibodies to variants and wild type influenza A/Ty/Ont/7732/66 by ELISA.

Antibodies	Percent binding:[a] Viruses A/Ty/Ont/ 7732	1C	587C
77B1	100	78	90
56G2	100	83	98
42C3	100	82	89
67G1	100	77	84
4C9	100	74	80
13B8	100	78	83
4D2	100	77	90
1D5	100	97	86
1G2	100	75	80
4F10	100	88	94
76E1	100	85	88
24B9	100	77	81
1D4	100	80	83
2E8	100	87	30
2E3	100	74	79
3B5	100	81	50
2D6	100	77	79
22D6	100	112	36

[a] Percent binding is calculated as described in the text such that binding to the wild type virus is defined as 100%.

antibodies the results were similar to those observed in binding to the wild type virus, suggesting that most of the binding sites are intact.

Protease requirements for plaque formation

The ability of the two variants to plaque on both CEF and MDCK cell monolayers with and without trypsin was examined. Table 4 shows that while the wild type virus could efficiently form plaques in the presence or absence of trypsin, both variants did so only on CEF cells. On MDCK cells, both variants required trypsin to form plaques. These results suggest that the hemagglutinins of the duck isolates were altered so that they now required trypsin for plaquing on MDCK.

TABLE 4. Plaque-forming ability of wild type and variants of influenza A/Ty/Ont/7732/66 on CEF and MDCK cells in the presence and absence of trypsin.

Virus	Trypsin[b]	Titer[a] on: CEF	MDCK
A/Ty/Ont/7732	+	1.5×10^7	3.0×10^7
	−	1.6×10^7	2.7×10^7
1C	+	2.0×10^7	6.6×10^6
	−	1.7×10^7	<10
587C	+	1.4×10^7	2.9×10^7
	−	1.8×10^7	<10

[a] Plaque forming units (pfu) per milliliter.
[b] Present in agarose overlay at 0.5 μg/ml for CEF and 1 μg/ml for MDCK cells.

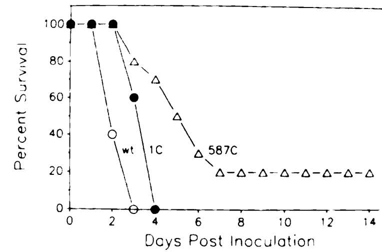

FIGURE 2. Mortality of chickens infected with variants of A/Ty/Ont/7732/66 selected during replication in ducks. The wild type 7732 (open circles), variants 1C (solid circles) and 587C (open triangles) were administered at high dose to groups of 10 white leghorn chickens as described in Materials and Methods.

Cleavage of the hemagglutinin

To determine the state of cleavage of the HA in infected MDCK cells, hemagglutinin was immunoprecipitated from ^{35}S-labeled cell lysates. The results, shown in Figure 1, indicate that the hemagglutinins of these isolates are cleaved in MDCK cells both with and without trypsin.

DISCUSSION

The results of this study indicate that under the conditions used here mallard ducks can act as hosts for a highly virulent avian influenza virus (A/Ty/Ont/7732/66 (H5N9)), although they do this inefficiently. The two isolates shed by the ducks which were characterized had antigenic changes in the hemagglutinin as well as changes which influenced their ability to form plaques on MDCK cells. Possible mechanisms for selection of variants during replication in ducks include immune system pressures, differences in host cell receptors,

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and the substrate specificity of host cell proteases required for cleavage. Whatever the selective pressures imposed on the virus, the two isolates examined in this study are still highly virulent for chickens, indicating that while attenuated variants might occur during circulation of this type of influenza virus in nature, no evidence was found in the present study to suggest that this is a necessary effect of replication in ducks. Three additional passages in mallard ducks did not change the virulence of these isolates for chickens (V. S. Hinshaw, unpubl. data).

Replication of A/Ty/Ont/7732/66 (H5N9) in ducks resulted in antigenic changes in the H5 hemagglutinin of isolates 1C and 587C, which were only detectable using a panel of monoclonal antibodies. When compared with the wild type virus, HI titers for 1C and 587C were considerably lower for many of the monoclonal antibodies, which recognize five neutralizing and an undetermined number of non-neutralizing epitopes (M. S. Philpott, B. C. Easterday and V. S. Hinshaw, unpubl. data). However, an ELISA binding assay using disrupted antigen showed little difference between these isolates and the wild type virus. One possible explanation is that the changes in the hemagglutinin involve changes in the three dimensional structure rather than multiple point mutations. The exact location of the changes on the primary structure has not yet been determined by sequencing.

The wild type A/Ty/Ont/7732/66, like other virulent avian influenza viruses, forms plaques with and without trypsin in the agarose overlay on a permissive cell line such as MDCK, or on primary CEF. Curiously, isolates from the ducks have a unique plaque formation phenotype: they require trypsin for plaquing on MDCK cells yet the viral HA is cleaved in these cells in the absence of trypsin. This observation could result from inappropriate cleavage of the HA in these cells, since highly virulent strains have multiple basic amino acids in this region of the molecule (Bosch et al., 1981; Kawaoka et al., 1987). Inappropriate cleavage of the HA may alter the conformation of the molecule and thereby change its serological reactivity.

Because avirulent strains as a rule require trypsin for plaque formation while virulent strains do not, the requirement for trypsin is sometimes used as an indicator to assess the virulence potential of isolates. In this study, experimental inoculation of chickens with viruses which failed to plaque on MDCK cells without trypsin resulted in rapidly fatal disease, an apparent exception to the general rule. On the other hand, the protease requirements on primary CEF are consistent with the general case and would indicate that CEF are the more appropriate cells to use when assessing the potential virulence of these isolates.

Ducks experimentally infected with A/Ty/Ont/7732/66 did not appear to suffer obvious disease, consistent with the observations of others (Kawaoka et al., 1987). However, recent studies in this laboratory (Cooley et al., 1989) have demonstrated the presence of unusual lesions in the lungs of ducks experimentally infected with A/Ty/Ont/7732/66 as well as other influenza viruses, suggesting that replication of these agents in ducks is not without consequences to their health. In a natural setting, such lesions could potentially affect the health and survival of ducks, emphasizing the need to appreciate the possible exchange of viruses between species. It is therefore advisable that poultry be housed indoors not only to protect domestic birds from viruses potentially shed by free-ranging ducks, but also to protect wild ducks from viruses they might acquire from domestic chickens and turkeys.

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