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Failure to Detect Hemagglutination-Inhibiting Antibodies with Intact Avian Influenza Virions

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Avian influenza viruses replicate in a variety of mammals and birds, yet hemagglutination inhibition tests show that postinfection sera from these animals (e.g., ferrets and ducks) have insignificant levels of antibodies (Hinshaw et al., Infect. Immun. 34:354–361, 1981). This suggested that avian influenza viruses, in contrast to mammalian viruses, may not induce a significant humoral response. Studies reported here indicate that avian influenza viruses do induce high levels of antibodies in ferrets, ducks, and mice and produce long-lived memory for cytotoxic T-cells in mice. The failure to detect hemagglutination-inhibiting antibodies to avian viruses was explained by the finding that antibodies to avian influenza viruses were not detectable in hemagglutination inhibition tests with intact virus yet were readily demonstrable when hemagglutinin subunits were used. In addition, these sera contained high levels of neutralizing antibodies to the avian virus. These findings suggest that the hemagglutinins of avian and mammalian influenza viruses may differ in their accessibility to antibodies or the biological consequence of antibody attachment or both. The practical consequence of these studies is that hemagglutination inhibition tests with intact avian viruses fail to detect antibody and do not correlate with virus neutralization. The avian virus used in these studies, A/Mallard/NY/6870/78 (H2N2), replicated and caused mortality in BALB/c mice, emphasizing that the host range and virulence of avian viruses extends to mammals. The above findings suggest that avian viruses could infect mammals in nature, yet seroepidemiological studies with conventional hemagglutination inhibition tests could give misleading results.

The major reservoir of influenza A viruses in nature is in avian species; 8 of the 13 different hemagglutinin (HA) subtypes are found only in birds, and the remaining 5 subtypes are shared by virus isolates from both birds and mammals (25). Epidemiologically, avian influenza viruses are important, for they have been implicated in the origin of some genes in H2N2 and H3N2 human pandemic strains (18, 22) and have been associated with disease outbreaks in domestic animals (4). The recent appearance of an avian-like influenza virus in seals has suggested that avian viruses can infect and produce disease in mammals in nature (12, 20).

Studies on the replication of avian viruses have shown that these viruses infect and replicate in a variety of lower mammals, e.g., pigs, cats, hamsters, ferrets, squirrel monkeys, and mink, as well as in birds (10, 16, 17). The level of virus replication and disease symptoms varies depending on both the virus and the host involved. One feature that both birds and mammals have in common, however, is a poor antibody response after infection. Studies on the serological response of ducks experimentally infected with avian influenza viruses indicated that little or no antibody was produced, yet the ducks were immune to reinfection (9, 11). The serological response of ferrets after infection with avian influenza virus was also lower than the response after infection with mammalian strains (10). One possible explanation for these findings is that avian viruses induce a restricted humoral response and that protection is due to cell-mediated immunity. Alternatively, the assay system used for measuring humoral antibody may be inadequate. A mouse model system was therefore established to investigate both arms of the immune response to avian influenza viruses.

These studies established that avian influenza viruses do induce humoral antibodies in mice, ferrets, and ducks and also induce long-lived cytotoxic T-cell memory in mice. The failure to detect humoral antibodies was associated with the finding that antibodies to avian influenza viruses frequently do not inhibit hemagglutina-

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tion of intact virions. During development of the mouse model system, it also became apparent that the avian influenza virus used replicated and caused mortality in mice.

MATERIALS AND METHODS

Viruses. The following strains of influenza A viruses were used in these studies: Japan/305/57 (H2N2) (Jap/305), Udorn/307/72 (H3N2) (Udorn), Mallard/New York/6750/78 (H2N2) (Mallard), and Seal/Mass/1/80 (H7N7) (Seal). The recombinant strains, A/Mallard/New York/6750/78 (H2)-Bel/42 (N1) (R) and A/Jap/305/57 (H2)-Bel/42 (N1) (R), were prepared as described previously (19).

The Udorn strain was kindly provided by Brian Murphy, National Institutes of Health, Bethesda, Md., and other strains were from the repository at St. Jude Children's Research Hospital, Memphis, Tenn. The Mallard strain was plaque purified twice in primary chicken kidney cells prepared from Spafas embryos (17) and grown once in 11-day-old chicken embryos. The Seal strain was a second egg passage from the original field isolate. The viruses were grown in 11-day-old chicken embryos and purified by adsorption to and elution from chicken erythrocytes followed by differential centrifugation and sedimentation through a sucrose gradient (10 to 40% sucrose, 0.15 M NaCl) (13). Some of the purified viruses were inactivated with Formalin and standardized in a single radial diffusion system to contain a known amount of HA protein (23).

Isolated HA subunits. HA was isolated from recombinant influenza viruses as described previously (14). Briefly, purified virus was disrupted with sodium dodecyl sulfate, the proteins were separated by electrophoresis on cellulose acetate, and, after elution, the protein was precipitated with ethanol.

Infection of animals. Groups of BALB/c mice (6 to 8 weeks old) were anesthetized with pentobarbital sodium (Abbott Laboratories) and inoculated intranasally with approximately 10^6 50% egg infective dose (EID_{50}) of virus in 0.05 ml. At daily intervals, mice were bled and sacrificed; the lungs were removed, ground in powdered glass, and titrated for virus in 11-day-old embryonated eggs (8). Pekin white ducks (2 months of age) were infected orally with approximately 10^6 EID_{50} of influenza virus (9). Ferrets (4 to 6 months old) were infected intranasally with approximately 10^6 EID_{50} of virus as described previously (10).

Serological assays. Hemagglutination inhibition (HI) tests with receptor-destroying enzyme-treated sera were done as described previously (6). Neuraminidase inhibition assays were done as recommended by the World Health Organization (24). Neutralization tests were done by titrating virus infectivity in the presence of preinfection and postinfection sera. Briefly, serial 10-fold dilutions of virus were mixed with equal volumes of a 1:20 dilution of serum which had been heat inactivated at 56°C for 30 min. After incubation at 4°C for 30 min, the serum-virus mixtures were inoculated into five embryonated eggs per dilution. The eggs were then tested for HA activity after 48 h at 35°C to determine virus titer (EID_{50} per milliliter). Neutralization activities were expressed as neutralization indices, i.e., the difference in virus titers in the presence of preinfection versus postinfection sera.

Cell-mediated immunity assays. BALB/c (H-2d) mice were infected as described above, and cytotoxic T-cells were generated in vitro as previously described (26). In brief, 8×10^5 spleen cells per ml were cultured with 5×10^4 stimulator cells for 5 days in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum (RPMI/10) and 10^{-5} M 2-mercaptoethanol. Stimulator cells were syngeneic lymphoblasts (previously stimulated with 10 μg of *Escherichia coli* lipopolysaccharide per ml in the presence of 5×10^{-5} M 2-mercaptoethanol for 2 to 3 days) infected with 80 HA units of influenza virus per 10^7 cells. After virus adsorption for 60 min in RPMI 1640, lymphoblast cells were washed once in RPMI/10 and added to the induction cultures.

T-cell-mediated lysis was estimated by measuring the release of ^{51}Cr from virus-infected P815 cells. Briefly, 10^7 target cells in 1 ml were infected with 80 HA units of influenza virus. After incubation for 1 h, cells were washed once and cultured in RPMI/10 at 10^6 cells per ml at 33°C overnight. The infected cells were labeled with 100 μCi of $Na_2{}^{51}Cr_2O_4$ for 1 h and were used in the assay after two washes. Assays were done in flat-bottom microtiter plates with 2×10^4 target cells per well and various numbers of immune cells per well in 0.2 ml of RPMI/10. The plates were centrifuged for 30 s at 500 rpm and incubated for 3 h at 37°C in 5% CO_2. After incubation, the plates were centrifuged (5 min at 1,000 rpm), and supernatant samples (0.1 ml each) were assayed in a gamma counter. Background release was determined by incubating target cells alone in RPMI/10. Maximum ^{51}Cr release was estimated by lysis with 2.5% Triton X-100 as follows: percent ^{51}Cr release = (counts released by cytotoxic cells − background release)/(counts released by 2.5% Triton X-100 − background release).

RESULTS

Replication of influenza viruses in mice. To compare the immune response to different influenza viruses after infection, it was first necessary to examine the replication of these viruses in mice. BALB/c mice were infected with Mallard, Seal, and human influenza viruses (Fig. 1). The human and Seal viruses replicated and reached peak titers on day 1 postinfection (p.i.), and shedding continued for the next 7 days. In contrast, Mallard virus grew to a 100-fold lower peak titer, which was reached on day 4 p.i., yet virus was shed for 8 days.

Although the Mallard virus replicated to lower levels than the Seal or Udorn strains, it nevertheless caused significant mortality in mice (Table 1). Examination of the lungs of mice for gross pathological changes revealed that, on day 4 after infection with Mallard virus, the lungs showed at least 50% consolidation. In contrast, mice infected with Seal or Udorn viruses showed no significant consolidation at this time. It could be argued that the high input of infectious virus used in these studies was responsible for the mortality; mice were therefore infected with a 1,000-fold lower dose of Mallard virus, and in this experiment 50% of the mice died on day 4 p.i.

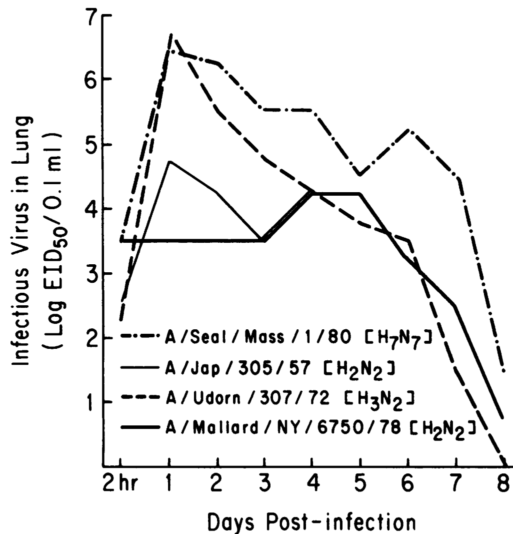

FIG. 1. Replication of influenza viruses in mice. Groups of BALB/c mice were infected as described in the text. Two mice were killed each day, and infectious virus from the lungs was titrated in embryonated eggs.

Although the replication and disease induced by mammalian and avian viruses differed in mice, it was clear that all strains grew; thus, the mouse model was suitable for examining the immune response to different strains.

Serological response of mice infected with influenza viruses. The HI test has traditionally been the most widely accepted assay for detecting antibodies to influenza viruses. This test was therefore used to study the antibody responses of mice infected with avian and human influenza viruses (Table 2). The mice infected with Udorn and Jap/305 viruses produced high levels of antibody, but the HI antibody responses to avian and Seal viruses were so low that they were insignificant (HI titer ≤ 1:20). These results are consistent with the failure to detect HI antibodies in ducks and ferrets after infection with avian strains (9–11) and could be interpreted to mean that antibodies were not induced. However, when sera from mice infected with Mallard virus were tested for HI activity with the antigenically related human strain (Jap/305), high levels of antibodies were detected (Table 3). These studies indicate that mice do produce antibodies to avian influenza viruses, but these antibodies fail to inhibit the homologous avian strain in HI assays.

Detection of antibodies to avian strains with isolated HA subunits. The above studies indicated that the failure to detect antibodies to the avian influenza virus was a property of the assay system. Previous studies have shown that the

TABLE 1. Mortality in BALB/c mice infected with influenza A viruses

Virus	No. of mice inoculated[a]	No. of deaths (days p.i.)	% Mortality
Mallard/NY/6750/78 (H2N2)	78	20 (2–4)	25.6
Seal/Mass/1/80 (H7N7)	35	1 (7)	3
Udorn/1/72 (H3N2)	34	0	0

[a] BALB/c mice were infected as described in the text.

detection of antibodies to some strains of influenza virus, particularly A/equine/Miami/1/63 (H3N8), is increased when isolated HA preparations are used in the HI test (2, 7). To test this possibility, purified HA subunits were isolated from the Mallard virus and used in the HI assay (Table 4). Antisera from mice, ferrets, and ducks that had been infected with Mallard virus or immunized with Formalin-treated virus had low titers of HI antibodies to intact Mallard virus but, when HA subunits were used as antigen, much higher levels of HI antibodies were detected, levels similar to those obtained with the intact human H2N2 strain. In contrast, there was no significant difference in HI antibody titers with intact virus or HA subunits of Jap/305 (H2N2) (Table 4).

These results could be interpreted to mean that antibodies to avian influenza viruses cannot gain access to antigenic determinants on intact virions. This explanation is unlikely, since antibodies to Mallard virus effectively neutralized the infectivity of the homologous intact virus (Table 4). These antibodies also neutralized the related Jap/305 (H2N2) strain, but to much lower titers. The antibodies induced by Formalin-treated avian influenza viruses effectively inhibited the HI activity of isolated HA, but were less

TABLE 2. Homologous HI antibody responses of mice after infection

No. of days p.i.	HI antibody responses to the following influenza A viruses[a]:			
	Mallard/NY/6750/78	Seal/Mass/1/80	Jap/305/57	Udorn/307/72
0	<10	<10	<10	<10
7	<10	<10	120	240
14	10	10	280	320
28	15	10	160	320
42	10	10	160	320
70	10	20		160

[a] Groups of 10 BALB/c mice were infected as described in the text. The values are the HI titers of pooled mouse sera and give the reciprocal of the highest dilution of serum inhibiting four hemagglutinating doses of virus.

TABLE 3. Detection of HI antibody to an avian influenza virus with a serologically related human strain

Virus	No. of days p.i.	HI antibody titer[a]	
		Mallard/NY/6750/78	Jap/305/57
Mallard/NY/6750/78 (H2N2)	7	<10	120
	14	10	120
	28	15	160
	42	10	320
	70	10	160
Jap/305/57 (H2N2)	7	<10	120
	14	<10	280
	28	<10	160
	42	<10	160

[a] Titer was determined as described in footnote *a* of Table 2.

effective in neutralization assays.

These studies indicate that HI tests with intact viruses for the assay of antibodies to avian influenza viruses can give misleading results. Although little or no antibodies were detectable in HI tests, antibodies were obviously present based on the HI activity with HA subunits and the neutralization data.

Cytotoxic T-cell responses of mice to avian influenza viruses. Previous studies (1, 3, 5, 26) have established that influenza A viruses induce specific and cross-reactive populations of H-2 restricted cytotoxic T-cells after infection. Other studies have suggested that these cells play a role in protection against influenza infections (reviewed in reference 1), and recent studies (15) with a cloned T-cell line support this concept. When the present studies were initiated, it seemed possible that the cell-mediated response to avian viruses, rather than humoral antibodies, was responsible for immunity of infected animals. The studies described above established that antibodies were induced after infection and suggested that avian viruses differed from mammalian strains in their presentation of antigenic determinants on the HA molecule. This raised the question as to whether the cell-mediated responses to avian and mammalian viruses might also differ. To examine this possibility, the induction of specific and cross-reactive T-cell populations in mice infected with avian and mammalian viruses was studied. The kinetics of primary cytotoxic T-cell induction in BALB/c mice were the same for the Mallard, Seal, and human strains tested (results not shown). Studies on cytotoxic T-cells from these mice after secondary in vitro stimulation also showed no differences between the responses to avian or mammalian viruses (Fig. 2). Both specific and cross-reactive populations of cytotoxic T-cell lymphocytes were induced and were long lived; high levels of activity were detected 8.5 months after infection (Fig. 2).

TABLE 4. Detection of antibodies to avian influenza virus with HA subunits and by neutralization of infectivity

Immunization[a]				HI titers[b] with the following antigens:				Neutralization indices[b] (log 10)	
				Mallard/NY		Jap/305			
Virus	Animal species	Virus prepn	Route of inoculation	Intact virus	Isolated HA	Intact virus	Isolated HA	Mallard/NY	Jap/305
Mallard/NY/6750/78 (H2N2)	Mouse	Live	IN	<10	80	20	40	>3.0	1.82
		Formalin inactivated (μg)							
		20	IM	40	1,280	600	1,280	—	—
		2	IM	10	320	160	160	—	—
		0.2	IM	<10	80	160	160	—	—
	Duck	Live	Oral	<10	360	—[c]	—	4.85	—
		Formalin inactivated	IV	<10	80	—	—	2.06	—
	Ferret	Live	IN	<10	80	40	40	2.07	0.62
Jap/305/57 (H2N2)	Mouse	Live	IN	<10	40	80	40	—	2.82
		Formalin inactivated (μg)							
		20	IM	20	640	320	320	—	—
		2	IM	<10	160	40	60	—	—
		0.2	IM	<10	10	<10	10	—	—
	Ferret	Live	IN	80	1,280	1,280	1,280	4.2	>5.5

[a] Groups of 10 BALB/c mice, 2 ducks, and 2 ferrets were infected as described in the text. IN, Intranasal; IM, intramuscular; IV, intravenous. Sera were collected 14 to 21 days post inoculation.

[b] HI and neutralization assays were done as described in the text.

[c] —, Tests not done.

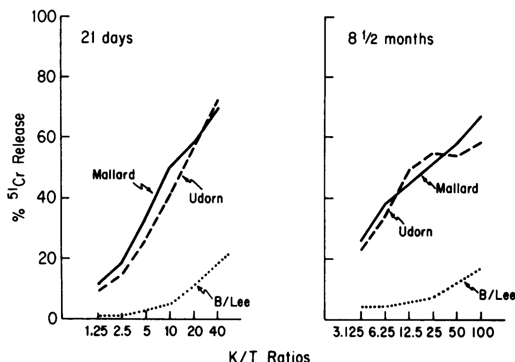

FIG. 2. Cytotoxic T-cell response of mice to avian and human influenza viruses. In vitro secondary cell-mediated cytotoxic responses: 21 days, homologous response or Mallard- and Udorn-infected P815 targets; 8.5 months, homologous (Mallard)- and cross-reactive (Udorn)-infected P815 targets. B/Lee curves give nonspecific lysis. K/T, Killer-to-target cell ratio.

The cross-protection between different subtypes of human influenza viruses which is not due to serological relationships between the glycoproteins has been attributed to cytotoxic T-cell activity (5). Recent studies (reviewed in reference 1) with cloned T-cell lines are in agreement with these findings. In the present study, mice infected with Mallard and Udorn influenza viruses were protected from challenge with human strains (results not shown), indicating that cross-protection between different subtypes of influenza A viruses is not restricted to human strains.

DISCUSSION

Although avian influenza A viruses infect and replicate in both birds and mammals (7, 10, 16, 17), only low levels of HI antibody can be detected. The present studies showed that antibodies to avian influenza viruses are induced in both birds and mammals after infection and can be detected in neutralization tests and by HI tests with isolated HA subunits but not with intact virus. The reasons for this disparity are not known, nor is it known whether the same antibody populations are being measured in each assay. However, recent studies with monoclonal antibodies to the HAs of avian virus A/Dk/Ukr/1/63 (H3N2) (21) and to the Seal virus (H. Kida, unpublished) support the idea that antibodies failing to inhibit hemagglutination by intact virus do nevertheless neutralize infectivity and inhibit the HA activity of subunits. If it is assumed that the same antibodies are being measured in each assay, the question arises as to how antibodies to avian influenza viruses can inhibit isolated HA subunits and yet fail to inhibit intact virus. One explanation for the failure of antibodies to inhibit the HA of intact avian viruses is that the avian virus particles may be larger and more pleomorphic than the human strains, requiring more antibody molecules to inhibit hemagglutination. Electron microscopic examination of the human and avian strains used in this study revealed that they were equally pleomorphic, suggesting that this explanation is untenable. A second possibility is that antigenic determinants on intact virions are masked in some way, e.g., by glycosylation, but this seems unlikely, since isolation of HA subunits with detergent would not be expected to alter the configuration of the carbohydrate residues. A third possibility is that the determinants are located lower down on the stalk of the HA monomer, so that attachment of antibodies to such determinants "in vivo" may require processing of the HA to expose these determinants. In HI tests, these antibodies may combine with the determinants but fail to inhibit binding of erythrocytes. A fourth possibility is that the antibodies may be of low affinity, but this is unlikely, since they efficiently neutralize infectivity. Another possibility is that the lack of HI activity may be related to the species of erythrocytes used in the assay; this will be investigated in later studies. Whatever the mechanism, it is clear that avian influenza viruses differ from mammalian strains in this property. The practical consequence of this finding is that HI assays with intact avian influenza viruses would give erroneous results when used in seroepidemiological studies.

It is apparent that avian influenza viruses do induce both humoral and cell-mediated responses and that cross-protection can be detected between different human and avian subtypes of influenza viruses. In this regard, the response of mice to avian viruses correlates with responses to mammalian strains (5).

The mortality in BALB/c mice caused by the nonadapted avian strain was unexpected, for other studies with the Mallard virus (17) in hamsters, ferrets, and squirrel monkeys have shown that this virus is restricted in its replication in mammals and causes no mortality. The genetic basis for interhost variation is not understood, but it is apparent that differences do exist. This study with mice again emphasizes that the host range of avian influenza virus is not limited to birds and could be important in creating conditions for genetic exchange between influenza viruses.

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