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Antibody Response in Humans to Influenza Virus Type B Host-Cell-Derived Variants after Vaccination with Standard (Egg-Derived) Vaccine or Natural Infection

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Hemagglutination inhibition (HI) and neutralization tests were used to determine antibody responses to egg-derived and Madin-Darby canine kidney (MDCK)-derived influenza B virus (B/England/222/82) in paired sera from persons naturally infected with influenza B and in persons vaccinated with standard egg-derived inactivated influenza vaccine. When tested by HI, the MDCK-derived antigen gave significantly higher (8- to 12-fold) geometric mean titers (GMT) in convalescent-phase sera from persons naturally infected during community outbreaks, as well as more 4-fold titer rises, than did tests with egg-derived antigen. When tested by neutralization, however, the convalescent-phase sera GMTs were only threefold higher with the MDCK-derived antigen and an equivalent number of fourfold titer rises were detected with both antigens. With postvaccine sera, the MDCK-derived antigen gave GMTs that were threefold higher than those obtained with egg-derived antigen in both the HI and neutralization tests and both antigens detected an equivalent number of fourfold titer rises in HI and neutralization tests. Sucrose gradient-fractionated egg-derived antigen showed a single peak of hemagglutinin activity corresponding to whole virions, whereas MDCK-derived antigen contained two distinct peaks of hemagglutinin activity, one of which had a lower sedimentation rate. The overall findings indicate that the egg-derived antigen in the vaccine induced HI and neutralizing antibody to both egg- and MDCK-derived variants and suggest that titers of antibody to MDCK-derived virus may be affected by the physical form of the hemagglutinin antigen.

Influenza viruses characteristically exhibit antigenic drift of the surface glycoproteins, the neuraminidase and the hemagglutinin (HA). The accumulation of point mutations in the HA gene continually alters the antigenic specificity of the HA and allows the virus to escape neutralization by antibody induced in the population against strains that circulated earlier.

Other mechanisms have been reported whereby influenza virus undergoes changes which affect the properties of its HA. Host cell selection of variants was originally described as the O-D variation that occurred upon adaptation to eggs (2). Since then, inhibitor-sensitive and inhibitor-resistant variants have been described by Choppin and Tamm (3), and Kendal et al. (7) reported the isolation of two subpopulations of swine influenza with distinguishable HA antigens from single specimens. Kilbourne (8) demonstrated the same phenomenon, a finding which could be attributed to host cell selection. A more recent example has been the report by Schild et al. (11), using influenza type B viruses from the 1980-to-1982 period, which demonstrated that antigenically distinguishable viruses may be selected by passage in embryonated eggs and Madin-Darby canine kidney (MDCK) cells. Two monoclonal antibodies, 238 and 209, were shown to react exclusively with egg- and MDCK-derived viruses, respectively, in hemagglutination inhibition (HI) tests. A potential glycosylation site at amino acid residue 196 on the HA of MDCK-derived virus was lacking in the egg-derived virus, and the HA of the MDCK-derived virus migrated slower in polyacrylamide gel electrophoresis, consistent with the presence of an additional carbohydrate side chain (10). The extra carbohydrate possibly interferes with the ability of the MDCK-derived virus to attach to and infect allantoic or amniotic cells in embryonated eggs, as the virus subpopulation specifying the glycosylation site was always lost when MDCK-derived type B virus was subpassaged in eggs.

The observation that selected human sera apparently had lower inhibition titers in HI tests with the egg-derived virus than with the MDCK-derived virus has suggested that the latter virus most closely resembles the virus which replicates in humans. The relevance of these findings to vaccine composition has thus become a contemporary issue (11). To further examine this issue, we have tested paired sera from persons naturally infected with influenza B and paired sera from persons who received the standard (egg-derived) inactivated influenza vaccine for antibody to the egg- and MDCK-derived type B virus variants previously studied by Schild et al. (11). HI tests were performed, including the use of ether-treated virus antigen, which is known to increase the sensitivity of antibody detection with recent egg-grown influenza B virus. In addition, a neutralization test yielding results in less than 24 h was developed to provide supporting data.

MATERIALS AND METHODS

Virus strains. The egg- and MDCK-derived strains of B/England/222/82 were kindly provided by G. Schild (London, United Kingdom). On the basis of reciprocal HI tests with ferret sera in our laboratory, the egg-derived B/England/222/82 is antigenically similar to B/Singapore/222/79, the strain recommended and used as the type B component of inactivated influenza vaccine from 1981 to 1984. HI titers of ferret sera to reference influenza B strains were severalfold higher to the MDCK-derived B/England/222/82

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than to homologous virus used to infect the ferrets, so that strain identity could not reliably be determined. Vaccine responses were also measured with egg-derived whole virus B/Singapore/222/79 which had the egg-derived phenotype determined with monoclonal antibodies 209 and 238 (see below), as well as B/Singapore/222/79 treated with ether, as previously described, to increase sensitivity in HI tests (9).

HI test. The HI tests were performed by a standard microtiter method after treatment with receptor-destroying enzyme (4).

Neutralization test. A rapid neutralizing antibody assay for influenza virus strains was developed, modified from a neutralization assay for the characterization of respiratory syncytial virus strains (1). Sera were heat inactivated at 56°C for 30 min, and twofold dilutions in 50 μl were prepared with Dulbecco minimal essential medium in flat-bottom 96-well tissue culture plates (Costar, Cambridge, Mass.) by using a Titertek multichannel pipetter (Flow Laboratories, McLean, Va.). An equal volume of Dulbecco minimal essential medium containing 50 to 100 50% tissue culture infective doses of the appropriate challenge virus was added to each serum dilution, and the plates were covered and incubated at 37°C for 1 h. After this incubation, 100 μl of a suspension of freshly trypsinized MDCK cells in Dulbecco minimal essential medium containing 4% fetal bovine serum at a concentration of 3×10^6 cells per ml was added, and the plates were incubated for 16 h at 37°C in a 5% CO_2 atmosphere.

After this incubation period, medium was removed and the cells were fixed with 80% acetone in phosphate-buffered saline (100 μl) and held at 4°C for 15 min. The acetone solution was then aspirated, and the plates were allowed to air dry at room temperature. Plates were then washed three times with PT (phosphate-buffered saline containing 0.05% Tween 20) by using an automatic plate washer (Skatron, Norway). The wells were preincubated with 50 μl of PTS (PT plus 1% fetal bovine serum) for 30 min at 4°C, followed by the addition of 50 μl of monoclonal antibody specific for the nucleoprotein of influenza B (13) in PTS, and incubated for an additional hour at 4°C. The final dilution of ascitic fluid containing the monoclonal antibody in these studies was 1:250. Unbound monoclonal antibody was removed with three washes with PT, and bound antibody was detected by the addition of 100 μl of goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Tago, Inc., Burlingame, Calif.) diluted 1:4,000 in PTS. The conjugate was allowed to react for 30 min at 4°C before being removed by three PT washes. Substrate was prepared immediately before use by dissolving 20 mg of *o*-phenylenediamine and 10 μl of 30% H_2O_2 in 50 ml of citrate-phosphate buffer (0.05 M citric acid, 0.01 M Na_2PO_4, pH 5). Substrate (100 μl) was added to each well and allowed to react for 20 min at room temperature before the reaction was stopped by the addition of 50 μl of 8 M H_2SO_4. The A_{490} was read with an automated EIA reader (Dynatech, Alexandria, Va.). Wells having absorbance readings that were greater than three standard deviations below the mean absorbance value for the virus control wells were considered positive for inhibition of virus growth. Neutralization titers were reported as the reciprocal of the highest serum dilution that inhibited virus growth. Comparison of this rapid neutralization test with a standard microneutralization test (5) indicated a high degree of correlation (M. W. Harmon, P. A. Rota, and A. P. Kendal, unpublished results).

Human sera. Acute- and convalescent-phase serum pairs were selected from cases of naturally acquired influenza infection. Sera had been sent to the Centers for Disease Control by several state health departments for confirmation of HI or complement fixation antibody responses detected after infection occurring among the general public during the 1982 season, including both miscellaneous cases and cases among a group of nursing home residents. Pre- and postvaccine sera were from vaccinees who received standard trivalent inactivated influenza vaccine which contained B/Singapore/222/79 along with A/Brazil/11/78 (H1N1) and A/Bangkok/1/79 (H3N2) antigen components. A sample of the B/Singapore/222/79 component of the vaccine from that year was tested with monoclonal antibodies 209 and 238 (see below) and was determined to have the egg phenotype. Vaccinees included nursing home residents (not the same population as that described above) and Air Force recruits.

Monoclonal antibodies. Monoclonal antibodies 209 and 238 were provided by G. Schild (London, United Kingdom) and were used to confirm that the antigenic properties of MDCK- and egg-derived antigens used in the antibody assays corresponded to those reported by Schild et al. (11). The influenza B HA- and nucleoprotein-specific monoclonal antibodies have been previously reported by this laboratory (13).

Sucrose gradients. Viruses were grown in egg or MDCK cells, frozen and thawed once, and clarified before loading onto continuous 30 to 60% sucrose gradients prepared in Tris (20 mM)-buffered saline containing 2 mM EDTA. Gradients were centrifuged in an SW41 rotor at 24,000 rpm for 3 h at 4°C. Fractions of 0.5 ml were collected and assayed for HA activity. Fractions were also diluted 1:1,000 and added to microtiter plates, and the reaction of absorbed antigen was measured with monoclonal antibodies specific for the HA and nucleoprotein (NP) antigens of influenza B virus by a standard indirect enzyme immunoassay (13).

RESULTS

The HI and neutralizing antibody geometric mean titers (GMTs) in acute- and convalescent-phase sera and pre- and postvaccine sera are given in Table 1. When tested by HI with egg- or MDCK-derived antigen, the sera from persons naturally infected with influenza B gave significantly higher (8- to 12-fold) convalescent-phase GMTs with the MDCK-derived antigen. The results with postinfection sera are consistent with findings by Schild et al. (11). Significantly more fourfold titer rises were also detected with the MDCK-derived antigen. When these same sera were tested by neutralization, the GMTs with the MDCK-derived virus were no more than about threefold higher than the GMTs with the egg-derived virus, and an equivalent number of titer rises was noted.

With pre- and postvaccine sera from nursing home residents or young adults, the GMTs in the HI test were no more than about threefold higher with the MDCK-derived antigen than with the whole virus egg-derived antigen. The numbers of fourfold titer rises observed with both antigens were comparable. Similar results were obtained with the neutralization test. The highest titers were obtained when ether-treated egg-derived virus was used as the HI test antigen, a finding which is consistent with previous results (9). The overall findings showed that the egg-derived antigen in the vaccine induced antibody to both egg- and MDCK-derived variants, but that unless treated with ether, the egg-derived virus had the lowest sensitivity for detecting the antibody.

Figure 1 compares the fold rise in HI and neutralizing antibody titers achieved in individual serum pairs with egg- and MDCK-derived antigens. Outbreak sera tested by HI showed generally higher rises when the MDCK-derived

TABLE 1. Antibody responses after natural infection or administration of influenza B vaccine to MDCK- and egg-derived influenza B virus antigens

Antigen exposure	Population (no.)	Test antigen source[a]	HI				Neutralization			
			GMT			No. of fourfold rises (%)	GMT			No. of fourfold rises (%)
			Pre	Post	Rise (fold)		Pre	Post	Rise (fold)	
Natural infection	Miscellaneous serum pairs[b] (50)	B/Eng-Egg	5	15	3	18 (36)	17	348	20	46 (92)
		B/Eng-MDCK	10	113	11	45 (90)	53	1,114	21	48 (96)
	Nursing home[c] (17)	B/Eng-Egg	6	13	2	6 (35)	46	236	5	12 (71)
		B/Eng-MDCK	17	160	9	15 (88)	75	434	6	13 (77)
Vaccination	Nursing home[d] (20)	B/Eng-Egg	6	14	2	8 (40)	32	113	4	6 (50)[e]
		B/Eng-MDCK	13	50	4	12 (60)	36	143	4	7 (58)[e]
		B/Sing-Egg	10	63	6	14 (70)				
	Adults[d] (70)	B/Eng-Egg	6	26	4	44 (63)	42	222	5	47 (67)
		B/Eng-MDCK	16	63	4	41 (59)	183	800	4	42 (60)
		B/Sing-Egg	16	97	6	50 (71)	57	368	6	51 (74)

[a] B/Eng-Egg, B/England/222/82 egg-grown antigen; B/Eng-MDCK, B/England/222/82 MDCK-grown antigen. B/Singapore/222/79 egg-grown antigen (B/Sing-Egg) was disrupted with ether for HI testing. Non-ether-treated, egg-grown virus was used for the neutralization assay.

[b] Serum pairs with fourfold HI rises to whole virus B/Singapore/222/79 (44 of 50) or ether-treated B/Singapore/222/79. All sera were not tested with ether-treated antigen.

[c] Serum pairs with fourfold HI rises to B/Singapore/222/79 (10 of 17) or complement fixation rises to influenza B.

[d] Nursing home residents and young adults given standard influenza inactivated vaccine containing B/Singapore/222/79 as the influenza B component. A sample of the vaccine from that year contained egg-derived phenotype influenza B component.

[e] Only 12 sera available for testing by neutralization.

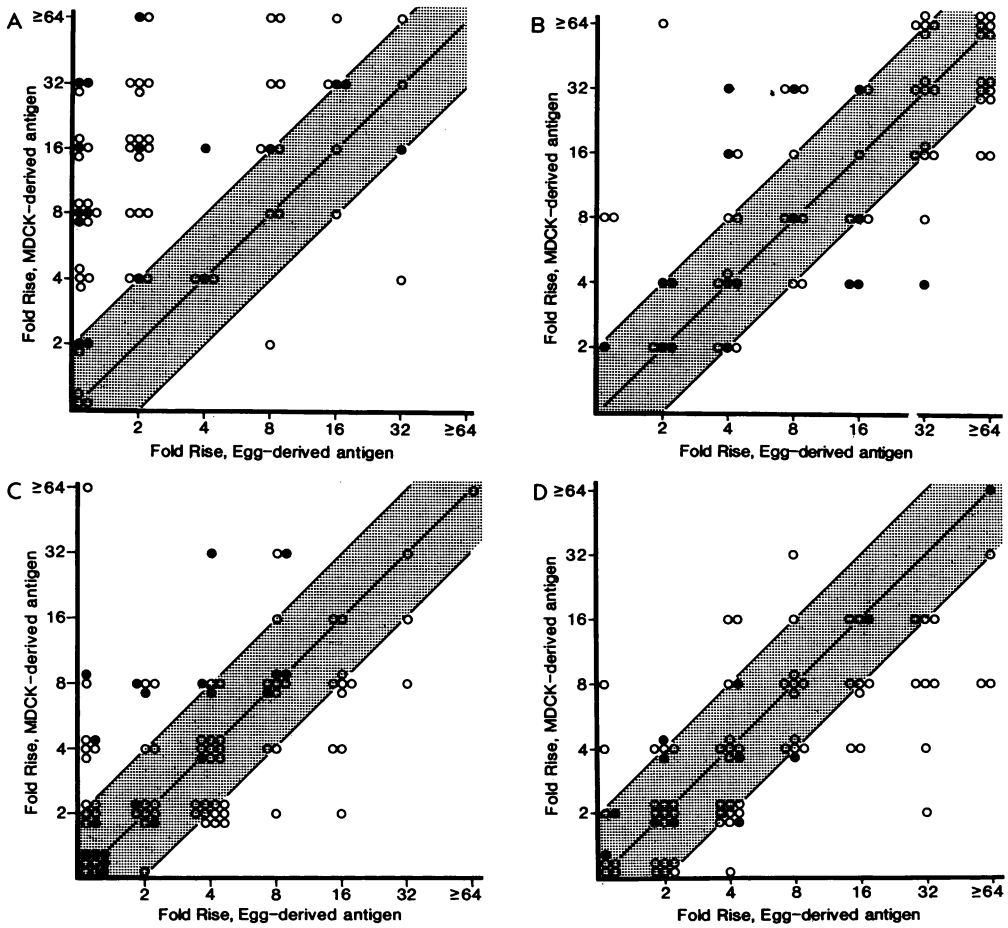

FIG. 1. Comparison of the fold rise in HI and neutralizing antibody titers achieved with egg- and MDCK-derived B/England/222/82 antigens. Shaded area indicates serum pairs in which the fold rises for egg and MDCK antigens were within fourfold of each other. (A) Fold rises in 50 serum pairs from various outbreaks (○) and in 17 serum pairs from an outbreak in a nursing home (●) tested by HI. (B) Fold rises in 50 serum pairs from various outbreaks (○) and in 17 serum pairs from an outbreak in a nursing home (●) tested by neutralization. (C) Fold rises in 70 serum pairs from vaccinated young adults (○) and 20 nursing home residents (●) tested by HI with whole virus (non-ether-treated) antigen. (D) Fold rises in 70 serum pairs from young adults (○) and 12 nursing home residents (●) tested by neutralization.

antigen was used than when the egg-derived antigen was used (panel A). Of 50 serum pairs, 31 gave rises that were fourfold higher with the MDCK-derived antigen than with the egg-derived antigen, and 9 of 17 serum pairs from a nursing home outbreak gave rises that were fourfold higher with the MDCK-derived antigen in the HI test. However, when these sera were tested by the neutralization assay (panel B), both antigens gave comparable results. The fold rises in pre- and postvaccine serum pairs were comparable when tested with either the MDCK- or the egg-derived antigen in both HI (panel C) and neutralization (panel D) tests.

Sucrose gradient-fractionated egg- and MDCK-derived viruses are shown in Fig. 2. A single peak of HA antigen and HA activity, cosedimenting with a peak of NP, was seen with the egg-derived virus. However, two distinct peaks of HA antigen and HA activity were observed with the MDCK-derived antigen, of which only the faster-sedimenting peak comigrated with the NP antigen. The slower-sedimenting peak of HA antigen overlapped a peak of NP, which corresponded to a peak of free (presumably soluble) NP in the egg-derived virus preparation.

DISCUSSION

Previous reports measured antibody titers with egg- and tissue culture-derived viruses in unvaccinated populations (11, 12). This is the first investigation of the human antibody response to MDCK- and egg-derived influenza B antigens after vaccination with standard inactivated influenza vaccine, including sera from high-risk elderly persons, who are one of the main target groups for vaccination (6). Because of the known relatively poor performance of the HI test for influenza B virus, we also used an ether-treated antigen in HI tests and developed a rapid neutralizing antibody assay to supplement the HI results. The neutralization assay gave higher titers than the HI test and in most cases detected additional fourfold titer rises.

Turner et al. (12) were the first to observe an increased GMT and seroconversion rate to influenza B when tissue culture-derived antigen was used in place of egg-derived antigen for the HI test. They found the main difference to be in the convalescent-phase sera, a finding reproduced here. The magnitude of the difference they found was a 2- to 3-fold increase in GMT, whereas we observed differences on the

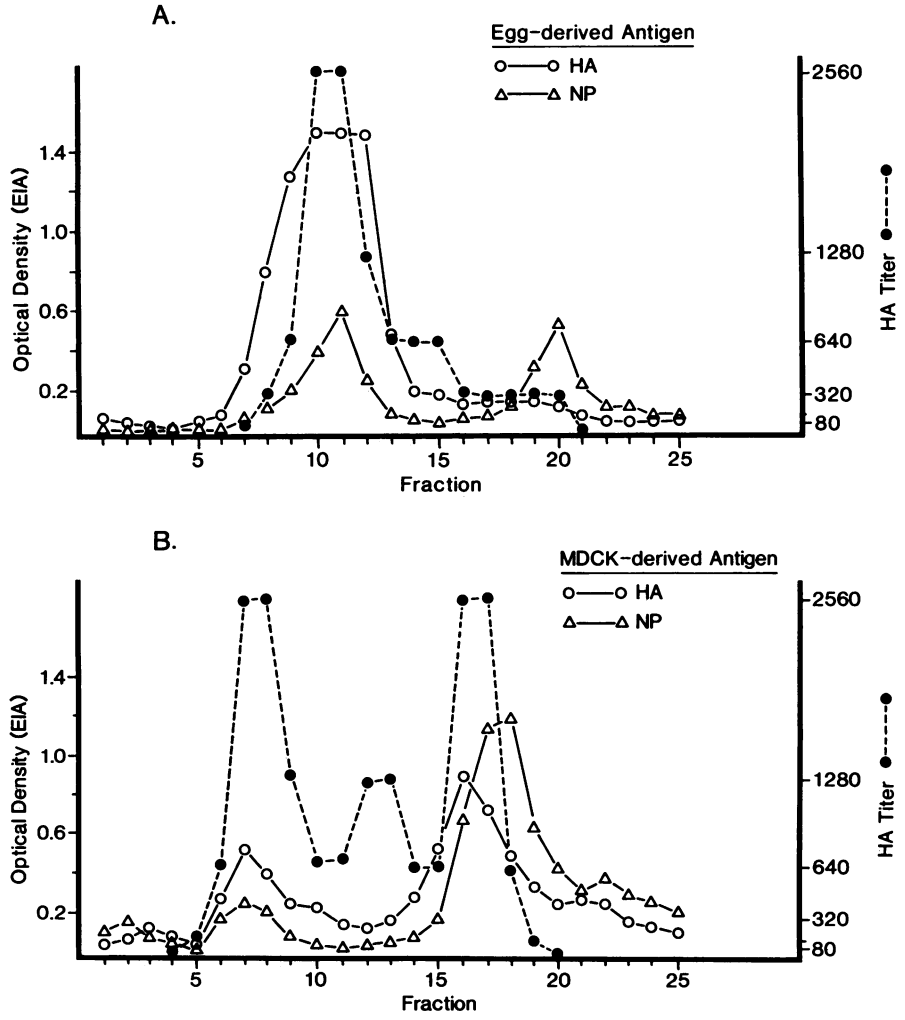

FIG. 2. Egg (A)- and MDCK (B)-derived viruses fractionated on continuous 30 to 60% sucrose gradients. Gradients were centrifuged in an SW41 rotor at 24,000 rpm for 3 h at 4°C. Fractions of 0.5 ml were collected and assayed for HA activity. Fractions were also diluted 1:1,000 and added to microtiter plates, and the reaction of adsorbed antigen was measured with monoclonal antibodies specific for the HA and NP proteins of influenza B.

order of 8- to 12-fold with convalescent-phase sera from natural infection. The increase in seroconversion rate when tissue culture-derived antigen was substituted for whole virus egg-derived antigen in the HI test in both studies was approximately twofold. The use of a neutralization assay in the present study confirmed a higher GMT in sera when MDCK-derived virus antigen was used in the test with sera from naturally infected persons. However, the difference in neutralization titers was much less than in HI titers.

The higher titers (and greater number of rises) observed with HI when MDCK-derived antigens for naturally acquired antibody in human sera are used has been suggested to occur as a result of a true antigenic difference between egg-derived and MDCK-derived virus, with the assumption that the virus that circulates in humans resembles the MDCK virus more than the virus strain selected for in eggs (11). In these studies, however, only a single antigenic variant derived from eggs was used in the tests with human sera, and its antigenic relationship to reference strains defined by the World Health Organization (on the basis of antigenic comparisons of hundreds of egg isolates) was not described. In our studies, the modest differences in titers of human serum antibody against MDCK- and egg-derived strains in neutralization assays, which normally closely correlate with HI tests, tend to argue against the above explanation for low antibody titers in human serum to egg-derived influenza B/England/222/82 virus. Alternative explanations that should be considered include the possibility that the avidity of the MDCK-derived and egg-derived viruses differ in the HI test performed with avian erythrocytes, because of selection in eggs for virus that strongly binds to avian cell receptors, and the possibility that the physical form of virus produced in eggs and MDCK cells is different.

Data based on studies of virus centrifuged through sucrose gradients indicate that egg-derived HA antigen is nearly all in the form of complete virions of a uniform size, whereas MDCK-derived HA antigen contains much HA in a low-molecular-weight form as well as in complete particles. Such a difference in physical form of the virus antigens would certainly influence the performance of the HI test in a way leading to higher HI titers with MDCK-derived virus but should have a much lesser effect in neutralization tests. The observation that ether-treated influenza B virus antigen also greatly increases sensitivity in the HI test further supports the need to examine the role of antigen structure, as well as avidity for cell receptors, as factors in HI test results.

To obtain definitive data on the significance of the antigenic difference between MDCK- and egg-derived HA antigens, Rota et al. (P. A. Rota, M. W. Shaw, and A. P. Kendal, Virology, in press) cloned DNA copies of the respective antigens and expressed them in vaccinia virus vectors. Cross-protection studies in mice have shown that immunization with the egg-derived HA induced high levels of antibody to both egg- and MDCK-derived antigens and resulted in good cross-protection. That data, and the human serology presented here, indicate that the egg-derived antigen induces HI and neutralizing antibody to both egg- and MDCK-derived viruses and that the induced antibody is protective. Thus, previously raised concerns that the use of embryonated eggs for the selection and production of influenza vaccine for human immunization might result in vaccines being poor inducers of antibodies to an alternative (MDCK-derived) form of the virus have not been substantiated in either animal model studies with genetically defined vaccines or in human vaccination studies. In addition, future studies comparing the biologic significance of antigenic differences between egg- and MDCK-derived viruses should clearly relate the antigenic specificity of the antigens used in antibody detection assays to internationally recognized reference strains used for vaccine production.

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