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Selection of a Single Amino Acid Substitution in the Hemagglutinin Molecule by Chicken Eggs Can Render Influenza A Virus (H3) Candidate Vaccine Ineffective

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This study investigated whether a single amino acid change in the hemagglutinin (HA) molecule influenced the efficacy of formalin-inactivated influenza A (H3N1) vaccine candidates derived from high-growth reassortants between the standard donor of high-yield genes (A/PR/8/34 [H1N1]) and host cell variants generated from the same clinical isolate (A/Memphis/7/90 [H3N2]) by passage in embryonated chicken eggs. Two clones of the isolate generated by growth in eggs differed from the parent virus (represented by an MDCK cell-grown counterpart) solely by the presence of Lys (instead of Glu) at position 156 or Ile (instead of Ser) at position 186 in the HA1 subunit. The protective efficacy of egg-grown HA Lys-156 and HA Ile-186 reassortant variants was compared with that of the MDCK cell-grown reassortant vaccine. Classically, antibody titers in serum have been used to demonstrate vaccine efficacy. Here, parameters of B-cell responsiveness were monitored, including the kinetics, character, and localization of the primary antibody-forming cell (AFC) response and the development of B-cell memory in lymphoid tissues associated with the priming site (spleen) and responsive to pulmonary challenge with infectious virus (upper and lower respiratory tract lymph nodes). We show that the egg-grown HA Lys-156 variant induced an AFC profile vastly different from that elicited by the other two reassortant vaccines. The vaccine was poorly immunogenic; it induced antibodies that were cross-reactive prior to challenge but which, postchallenge with a lethal dose of the MDCK cell-grown reassortant virus, were targeted primarily to the HA Lys-156 variant, were of the immunoglobulin M isotype, were nonprotective, and were derived from the spleen. In contrast, the egg-grown HA Ile-186 variant was remarkably like the MDCK cell-grown virus in that protective immunoglobulin G antibodies were unaffected by the Ile-186 substitution but poorly recognized HA with Lys-156. Furthermore, memory AFC responsiveness was localized to regional lymphoid tissue in the upper respiratory tract, where challenge HA was found. Thus, it is recommended that in the selection of vaccine candidates, virus populations with the egg-adapted HA Lys-156 substitution be eliminated and that, instead, egg-grown isolates which minimally contain Ile-186 be used as logical alternatives to MDCK cell-grown viruses.

Antigenic variation in influenza viruses is due to extensive variation in the antigenic properties and structure of their hemagglutinins (HAs). Distributed on the surface of HA molecules are the epitopes to which the antibody repertoire of the host immune system generates neutralizing and protective antibodies. There are three kinds of antigenic variation in influenza viruses. Two well-characterized mechanisms of variations are known as antigenic drift and shift. Antigenic drift involves a series of amino acid sequence changes primarily in the globular head region of the HA molecule. On the other hand, antigenic shift is the result of more extreme change, by which a new influenza virus subtype emerges in the population, with surface antigens (HA and neuraminidase [NA]) unlike those that immediately preceded it.

Evidence of a third form of antigenic variation has been reported for influenza viruses (reviewed in reference 50). This variation results from the selection of virus subpopulations by the host cells in which the virus is cultivated. Cultivation of influenza A or B virus in embryonated chicken eggs results in selection of an HA that differs antigenically and structurally from that derived in Madin-Darby canine kidney (MDCK) cells (51, 58) and is distributed on progeny virus with a decided growth advantage. The molecular changes associated with host cell variation surround the receptor-binding site located on the membrane-distal region of the HA molecule. Selective pressures within embryonated eggs are believed to be of at least two types, to be mutually exclusive, and to operate by restricting the growth of some variant(s) by (i) favoring virus binding to cell surface sialyloligosaccharides and internalization into cells of the chorioallantoic membrane and (ii) suppressing the replication of others by contact with extracellular inhibitory molecules in amniotic and/or allantoic fluid (22a).

Embryonated chicken eggs are currently the only host in which sufficient quantities of virus can be cultivated economically and within the short time necessary to ensure a vaccine supply. However, protective studies with animal models of inactivated vaccines harboring several egg-selected amino acid substitutions in their HA have indicated a reduced capacity to protect against challenge with native virus (32, 74). These findings also raise important questions about the optimal substrate for the propagation of influenza viruses to be used for epidemiological surveillance and vaccine production. Since two or three different egg-grown variants can be isolated from a single individual (34, 49, 53), it is possible that among these variants

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there is one that is antigenically identical to its mammalian cell-grown counterpart.

In the present study, we selected two egg-grown variants, each with a single amino acid change in the HA1 region, Ser→Ile at position 186 or Glu→Lys at position 156, to examine the immunological basis for differences in the efficacy of influenza vaccines. The substitutions at positions 186 and 156 are two of the most prevalent egg-selected changes associated with H3N2 viruses (32, 38, 45, 50) and are independently selected by the chorioallantoic membrane and egg fluids, respectively (22a). Both changes are located within antigenic site B (tip of the three-dimensional structure described for H3 [10, 71–73]), which overlaps with the majority of *H-2*^{d}-restricted helper T-cell epitopes (5, 6). How the immune system is able to distinguish between these HA variants, which are essentially structurally alike, is unknown. To address this issue, we compared the HA-specific B-cell activities in lymphoid tissues elicited by single amino acid variants and correlated these with the relative efficacy in protecting mice from challenge by virus expressing the native HA. Several parameters of B-cell responsiveness were examined, including the kinetics, the immunoglobulin (Ig) isotype profile and tissue distribution, memory, and the frequency of antibody producers, as well as the levels of hemagglutination inhibition (HI) and neutralizing antibodies in serum.

Our findings demonstrate the capacity for a single amino acid change in the HA (Glu to Lys at position 156) to markedly influence the immune recognition of influenza virus.

MATERIALS AND METHODS

Viruses. The A/Memphis/7/90 (Mem/7/90) (H3N2) virus strain was isolated from an original human throat sample in MDCK cells and in the amniotic cavities of 10-day-old embryonated eggs. After initial isolation in the amnion, egg-grown virus was passaged in the allantoic cavity and biologically cloned. Cloning was performed at limiting dilution in the allantoic cavities of embryonated eggs or in MDCK cells by using threefold dilutions of virus. Clones generated at the highest dilutions were harvested, recloned, and used to make high-yield reassortants with the A/PR/8/34 (PR8) (H1N1) virus. Virus titers were determined by hemagglutination with 0.5% chicken erythrocytes (70).

Reassortment. To limit the possibility that differences in the immunogenicity of vaccines were due to variable ratios of HA in the virions, to changes in the NA or other gene products, or to differences in infectivity, as well to derive high-yield vaccines, we made reassortants between Mem/7/90 (H3N2) and the high-growth PR8 virus (52). Reassortment was done in eggs for H3N2 variants with one mutation (Ile-186 or Lys-156) and in MDCK cells for MDCK cell-grown Mem/7/90. Eggs were coinfected with both parental viruses, and the progeny was treated with goat anti-PR8 serum and repassaged. Progeny from the second passage (in the presence of antiserum) was passaged twice more in eggs at limiting dilution. The fourth egg passage was analyzed to determine the genotype of the reassortants and passaged further to produce sufficient virus for vaccine studies with animals. The reassortment of the MDCK cell-grown variant of Mem/7/90 with PR8 was performed with MDCK cells by the same procedure used for eggs, except that protein A-purified goat IgG antibody was utilized. Genotyping of reassortants was based on PCR amplification: appropriate primers specific for the H3, H1, N2, and N1 genes were designed to correspond to unique target regions, and primer pairs were tested for the ability to amplify the correct gene. Samples were analyzed by agarose gel electrophoresis with ethidium bromide staining. For determination of the source of the other genes (PB1, PB2, PA, NP, M, and NS), primers corresponding to conserved regions of these genes were used for PCR amplification and the products were purified by the MagicPrep (Promega, Madison, Wis.) procedure. Purified samples were examined by partial sequencing (fmol; Promega), and the genotype was determined by comparison with the parental gene sequence.

Virus reassortants to be used for vaccination and the Mem/7/90 challenge virus were purified by equilibrium density centrifugation through 25 to 75% sucrose gradients (39). Purified virus at a concentration of 40,000 HA units/ml was inactivated with 0.025% formalin at 4°C for 3 days (32); this resulted in complete loss of infectivity.

Extraction and purification of surface antigens. The glycoproteins from each of the three reassortant viruses were used to coat plates for the B-cell assay (see below). They were removed and purified by the method of Johansson et al. (27, 29). Briefly, surface glycoproteins HA and NA were extracted from influenza virus particles by treatment with the nonionic detergent *n*-octyl β-thioglucopyranoside (15%) in sodium acetate buffer (pH 7.0). This suspension was centrifuged at 15,000 rpm (Beckman 50Ti rotor) for 60 min, and the supernatant was carefully removed from the pellet and reserved as the HA-NA-rich fraction. The HA-NA-rich fraction was assessed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The amounts of HA per total virus protein of MDCK cell-grown and egg-grown viruses were compared under reducing conditions by polyacrylamide gel electrophoresis. Density scans of the HA bands on Coomassie blue-stained gels were performed on a Sparc station 2 densitometer (Sun Systems, Mountain View, Calif.) with Bioimaging Visage 110 software (Millipore, Bedford, Mass.) and showed that HA made up 55% of the total virus protein. The relative HA content was also evaluated by determining the ratio of HA units to total viral protein. The protein content was estimated by a modification of the Bradford method (Repligen, Cambridge, Mass.).

Sequence analysis. The RNA from tissue culture and allantoic fluid was sequenced as described earlier (7). Briefly, RNA was precipitated, resuspended for reverse transcription in 3.5 μl of water with 1 μl of DNA U12 primer (1 μg/μl; 5′AGCAAAAGCAGG3′, corresponding to viral nucleotides 1 to 12), and the cDNA was generated with 20 U of reverse transcriptase, 2 μl of 2.5 mM deoxynucleoside triphosphates (Pharmacia, Piscataway, N.J.), and 20 U of RNAsin (Promega) in 0.25 M Tris-HCl (pH 8.0)–50 mM MgCl–50 mM dithiothreitol–0.35 M KCl. One hour after incubation at 42°C, the reaction was stopped by heating at 100°C for 5 min. The cDNA corresponding to the HA1 region was amplified by PCR amplification (35) with primers 5′TTCGCCCAAAAACTTCCCGG3′ and 5′CCGTCTACCATTCCCTCCCA3′, which are complementary to RNA nucleotides 72 to 91 and 1183 to 1164, respectively. Amplified products were purified with the Geneclean II kit (Bio 101, La Jolla, Calif.) as previously described (35). Analysis of the cloned virus HA1 sequence was done by fmol DNA sequencing (Promega) with end-labeled primers.

Mouse immunizations. Six- to eight-week-old female BALB/c mice (*H-2*^{d}) were used since the HA recognition specificity and the immunogenetic mechanisms that define it have been well characterized (12, 14, 36, 63, 67), better than in other mouse strains (65). Mice were immunized subcutaneously with inactivated reassortant vaccines containing 5 μg of HA in the presence of the adjuvant aluminum hydroxide [$Al(OH)_3$; Rehydragel LV; Reheis Inc., Berkeley Heights, N.J.] (2, 22). Four weeks later, mice received an intraperitoneal (i.p.) booster inoculation of the same dose of vaccine without the adjuvant. One week later, mice were anesthetized with Metofane (Pitman-Moore, Mundelein, Ill.) and challenged intranasally (i.n.) with 200 50% mouse infectious doses (MID_{50}) of MDCK cell-grown Mem/7/90 virus.

Determination of virus titers in lungs. Three days postchallenge, mice were sacrificed and lungs were collected for virus titration. Lungs were processed for virus titration by the method described by Liang et al. (40). Briefly, the mice were anesthetized and sacrificed, the thorax was opened, the trachea was cut below the glottis, and the lobes of the lungs were lifted and removed. The lungs were stored in 1 ml of sterile phosphate-buffered saline (PBS) at −20°C until used for virus titration. Lung tissue was ground and centrifuged for 10 min at 400 × *g* at 4°C. The supernatant was tested for the concentration of infectious virus in MDCK cells. Titers are expressed as 50% tissue culture infectious doses ($TCID_{50}$) per lung.

Assay of B-cell response. Immunized mice were anesthetized and exsanguinated by heart puncture. An assortment of lymphoid tissues (cervical lymph nodes [CLN], mediastinal lymph nodes [MLN], inguinofemoral lymph nodes [IFLN], and spleens) were removed and processed individually (1). Single-cell suspensions prepared freshly were sampled from individual mice on the same day; tissues from one or three mice per group were analyzed individually.

B-cell responses were measured by a modification of the enzyme-linked immunospot assay (15, 25, 26, 56, 59). Briefly, 96-well nitrocellulose microtiter plates (MultiScreen filtration plates; Millipore) were coated with 0.5 μg of HA of HA-NA glycoprotein preparations in 100 μl of PBS and incubated overnight at 4°C. Control plates were coated with 5 μg of protein from normal allantoic fluid per ml. Plates were washed three times with PBS and blocked with 2% fetal calf serum for 90 min at room temperature. To washed plates, 10^5 cells were added and incubated for 4 h at 37°C in a humid 5% CO_2 atmosphere. Plates were then washed once with PBS, once with PBS–0.1% Tween 20, and three times with PBS. Antibody-forming cells (AFCs) were detected as spots following incubation with alkaline phosphatase-conjugated anti-mouse Ig isotype-specific reagents diluted in 5% bovine serum albumin and development with the peroxidase substrate 5-bromo-4-chloro-3-indolylphosphate. The results shown are the means normalized to the number of AFCs per 10^5 total cells for each tissue per vaccine group at each time point.

Monoclonal antibodies (MAbs). Mouse ascitic fluids were used as the source of MAbs to the receptor-binding site of the H3 HA of egg-grown A/Bangkok/1/79 (MAbs 45/5, 49/4, and 85/1) and MDCK cell-grown A/Memphis/2/85 (MAbs 2/1 and 2/5) and A/Memphis/12/85 (MAb 12/3) viruses. These MAbs have been shown to detect differences between host cell variants of H3N2 viruses (21) and were used to study antigenic relatedness between H3 variants by the HI test (70).

Serologic tests. Neutralization assays were done by mixing 100 $TCID_{50}$ of virus and antisera for 1 h at 23°C; this was followed by titration of the mixtures for residual virus infectivity on MDCK cell monolayers in 96-well plates. After 3 days of incubation at 37°C in 5% CO_2, neutralization titers were assessed for the presence of a cytopathic effect in the cultures and for HA activity in the supernatant. Neutralization titers are expressed as the reciprocal of the antibody dilution that completely inhibited virus infectivity in 50% of triplicate cultures.

TABLE 1. Antigenic relatedness of MDCK cell-grown and egg-grown Mem/7/90 HA Ile-186 or HA Lys-156 H3N1 reassortants as defined by HI analysis with MAbs

Host cells used (virus)	HI titer in response to H3 receptor-binding site-differentiating MAb:					
	2/1	2/5	12/3	45/5	49/4	85/1
Eggs (HA Lys-156)	<100	<100	<100	1,600	<100	100
Eggs (HA Ile-186)	6,400	6,400	3,200	800	6,400	6,400
MDCK	6,400	6,400	6,400	800	6,400	6,400

RESULTS

Serologic reactivity of Mem/7/90 MDCK cell-grown and egg-grown variants. We have previously reported that the HAs of MDCK cell-grown and egg-grown viruses differ by a single amino acid change at residue 186 or 156 (21). MDCK cell-grown and egg-grown reassortants were tested in HI assays against a panel of MAbs directed against the receptor-binding site of H3N2 viruses to determine their antigenic relationship. The MDCK cell-grown and egg-grown HA Ile-186 variant reacted with all of the MAbs, but the egg-grown HA Lys-156 variant was recognized only by MAb 45/5 (Table 1). It is clear, then, that the egg-grown HA Ile-186 variant does not differ antigenically from the MDCK cell-grown variant, whereas the egg-grown HA Lys-156 variant is antigenically different from each of the other two.

Sequencing of MDCK cell-grown and egg-grown variants and genotyping of reassortants. The three variants of Mem/7/90 (H3N2) virus—one MDCK cell grown and two egg grown—were sequenced. Sequence analysis of the HA1 region of the MDCK cell-grown variant showed it to be identical to that of the virus from the original clinical gargle. Egg-derived variants have a single mutation within HA1, at position 186 (Ser→Ile) or 156 (Glu→Lys) (data not shown).

Because newly isolated strains of influenza virus do not usually grow to a high titer, the yield of the candidate vaccines was enhanced by reassortment with the high-growth PR8 (H1N1) laboratory strain. Preparation of the H3N1 reassortants was also important for analysis of the effect of a single mutation within the HA on the immune response. Reassortment of the MDCK cell-grown variant was performed with MDCK cells under the selective pressure of purified anti-PR8 IgG to avoid the possible introduction of new mutations with the use of whole goat antiserum (55). Genotyping confirmed the identity of the HA gene from the original variant: the three high-growth reassortants had the same 7/1 gene constellation but differed by a single mutation in the HA1 region of the HA gene (i.e., Lys-156 and Ile-186 [data not shown]).

Specificity of serum antibodies produced by immunized mice. The ability of formalin-inactivated reassortant vaccine prepared from an MDCK cell-grown or egg-grown virus to induce serum antibodies was examined. Mice were inoculated subcutaneously with adjuvanted-inactivated reassortant vaccines containing 5 μg of HA, and the antibody titers in serum were determined by HI (Table 2) and neutralization (Table 3) analyses 14, 42, and 47 days later. Within the first 2 weeks postvaccination, no marked difference was detectable in HI antibody titer or specificity in all three vaccine groups (Table 2). In contrast, booster vaccination (day 35) generated a marked difference in the increased HI and neutralizing antibody titers in the sera of all three groups within 7 days: neutralizing antibodies induced by MDCK cell-grown and egg-grown HA Ile-186 variant reassortants were cross-reactive with the two vaccines. However, the egg-grown HA Lys-156 variant induced HI antibodies that were strain specific and capable of neutralizing homologous HA 100-fold better than the heterologous HAs. Therefore, the antigenic difference between the egg-grown HA Lys-156 variant and the other two HA analogs is reflected by the biological functions of the serum antibodies generated.

B-cell responsiveness to immunization. Since the MDCK cell-grown and egg-grown variant reassortant vaccines induce serum antibodies that exhibit differential recognition of the HA analogs, we next examined their effect on B-cell responsiveness. B-cell activity—in particular, AFC frequency, Ig isotype profile determination, and lymphoid tissue localization—against glycoprotein preparations from the MDCK cell-grown and egg-grown reassortants (H3N1) and the PR8 (H1N1) virus was estimated by the enzyme-linked immunospot assay in various lymphoid tissues (CLN, MLN, IFLN, and spleen). Lymphoid tissues were sampled 14 days postvaccination and 5 days postboost (day 40 overall).

All vaccine groups showed low (average of fewer than 10 AFCs per 10^5 total cells) or undetectable numbers of HA-specific AFCs 14 days postvaccination, and AFCs were present only in the spleen (data not shown).

MDCK cell-grown reassortant vaccine group. Within 5 days of the boost, higher numbers of IgG AFCs were seen in the MLN and spleen (Fig. 1a to c). The highest response measured was in the MLN (75 AFCs per 10^5 total cells) by assay against glycoproteins from the egg-grown HA Ile-186 reassortant, and the AFCs were of the IgG isotype (Fig. 1b); minor populations of IgM AFCs against the same reassortant were also detected in the MLN and spleen (up to $20/10^5$ cells). AFCs producing antibodies to homologous HA were essentially restricted to the IgG isotype in the MLN ($27/10^5$ cells; Fig. 1a), whereas responsiveness to the egg-grown HA Lys-156 reassortant was low and equivalently distributed among IgM and IgG producers in

TABLE 2. Antibody titers in sera of mice immunized with inactivated MDCK cell-grown or egg-grown Mem/7/90 × PR8 (H3N1) reassortants

Reassortant vaccine group[a]	Primary HI antibody[b] $\log_{10}$ titer in response to reassortants (14 days postvaccination)			Secondary HI antibody[c] $\log_{10}$ titer in response to reassortants (42 days postvaccination)		
	MDCK cell-grown virus	Egg-grown HA Ile-186	Egg-grown HA Lys-156	MDCK cell-grown virus	Egg-grown HA Ile-186	Egg-grown HA Lys-156
MDCK cell-grown virus	2.7	2.3	2.3	3.0	2.7	2.3
Egg-grown HA Ile-186	2.6	2.5	2.3	3.2	3.1	2.0
Egg-grown HA Lys-156	2.1	2.4	2.5	2.8	2.9	2.7

[a] Mice were immunized subcutaneously with formalin-inactivated reassortants (5 μg of viral HA) in $Al(OH)_3$. Five weeks later, animals were boosted i.p. with the same dose of vaccine without the adjuvant.
[b] Mice were bled 14 days postvaccination with vaccine and the adjuvant.
[c] Mice were bled 7 days postboosting with vaccine but no adjuvant.

TABLE 3. Neutralizing antibody responses in sera of mice in response to immunization with inactivated MDCK cell-grown or egg-grown Mem/7/90 × PR8 (H3N1) reassortants

Reassortant vaccine group[a]	Postvaccination[b] titer[c] (log_{10}) of neutralizing antibody to reassortant virus		
	MDCK cell-grown virus	Egg-grown HA Ile-186	Egg-grown HA Lys-156
MDCK cell-grown virus	3.6	3.2	3.6
Egg-grown HA Ile-186	3.4	3.4	3.5
Egg-grown HA Lys-156	2.2	2.5	4.5

[a] Mice were immunized subcutaneously with formalin-inactivated reassortants [5 μg of viral HA in $Al(OH)_3$]. Five weeks later, mice were boosted i.p. with the same HA dose without the adjuvant.

[b] Mice were bled 7 days postboosting with vaccine but no adjuvant.

[c] The mean titer is expressed as the reciprocal of the highest dilution of serum that neutralized 100 $TCID_{50}$ in 50% of virus-infected MDCK cell cultures. Neutralizing antibody titers of serum samples from unvaccinated mice were below detectable levels.

the MLN and spleen (range of 6 to 21 AFCs per 10^5 cells; Fig. 1c).

Cross-reactivity between subtypes was estimated by an assay with PR8 glycoproteins. Responsiveness varied between 4 (spleen) and 16 (MLN) AFCs per 10^5 total cells (Fig. 1d), which, interestingly, was equivalent to the responsiveness to the egg-grown HA Lys-156 reassortant. This suggests that the AFCs within this vaccine group produced mostly subtype-specific antibodies whose fine specificity was restricted to the MDCK cell-grown and egg-grown HA Ile-186 variants. Assay of anti-H1N1 glycoprotein AFCs further indicated that B-cell responsiveness to N1 was minor.

Egg-grown HA Ile-186 reassortant vaccine group. No AFC responsiveness was observed in the MLN following boosting when assayed against each of the four glycoprotein preparations (Fig. 1e to h). Very low numbers of both IgG and IgM AFCs (0 to $6/10^5$ cells in the two mice sampled) were detected in the spleen (Fig. 1f).

Egg-grown HA Lys-156 reassortant vaccine group. Mice primed and boosted with the egg-grown HA Lys-156 reassortant vaccine (Fig. 1i to l) also showed low numbers of HA-specific IgM AFCs (0 to $10/10^5$ cells) in the spleen and none elsewhere.

Overall, AFC responsiveness to boosting was detected only in the MLN and spleens of mice primed with the MDCK cell-grown reassortant vaccine.

Memory B-cell responsiveness to lethal challenge. We next determined the antigenic repertoire of memory B cells of each reassortant vaccine group 5 and 13 days following i.n. challenge (days 47 and 55 overall) with 10^3 $TCID_{50}$ (equivalent to 200 MID_{50}) of infectious MDCK cell-grown reassortant virus (H3N1).

MDCK cell-grown reassortant vaccine group. Near predominance of the IgG isotype was evident in the MLN response in an assay against homologous HA, but there was less activity (two- to eightfold) in the CLN (Fig. 1a). Surprisingly, the MLN responded to all three H3 variants with remarkable AFC frequencies, ranging from 235 to 427 IgG AFCs per 10^5 cells (Fig. 1a to c) on day 5 postchallenge. Responsiveness all but disappeared by the end of the second week postinfection.

There were considerable IgA AFC frequencies (average, $40/10^5$ cells) in the MLN on day 5, directed primarily to the MDCK cell-grown and egg-grown HA Ile-186 variants, with less activity (14 AFCs per 10^5 cells) against the egg-grown HA Lys-156 variant. Surprisingly, IgA AFC responsiveness persisted for 2 weeks (Fig. 1a). On day 5, IgA AFC numbers were much lower in the CLN (2- to 5-fold) than in the MLN and were substantially reduced (3- to 10-fold) from the IgG AFC frequencies. In comparison with the MLN numbers, anti-H3 variant IgG AFCs in the CLN (41 to $102/10^5$ cells) and spleen (5 to $29/10^5$ cells) were substantially lower.

Similarly, in contrast to the IgG AFC numbers, the IgM AFC frequencies in the MLN and CLN were much reduced (over 200-fold); only increased numbers of IgM AFCs (9 to $25/10^5$ cells) were found in the spleen.

Assay of AFCs on PR8 glycoproteins (Fig. 1d) indicated limited subtype cross-reactivity, mediated primarily by IgG antibodies produced in the MLN ($112/10^5$ cells) and by IgM- and IgG-producing populations in the spleen (about $30/10^5$ cells).

Egg-grown HA Ile-186 reassortant vaccine group. The Ile-186 substitution had an appreciable effect on the memory AFC responsiveness to challenge (Fig. 1e to g). There was a shift in the major responding tissue against all three H3 variants. The CLN predominated (328 to 405 AFCs per 10^5 cells), with two to three times the activity in the MLN when assayed against the egg-grown H3 variants on day 5 (Fig. 1f and g). This and the low CLN and MLN responsiveness against the MDCK cell-grown H3 (59 IgG AFCs per 10^5 cells; Fig. 1e) suggest that the challenge predominantly stimulated memory AFCs specific for the egg-grown virus. The fact that AFCs in the CLN showed poor specificity for PR8 (Fig. 1h) indicates that H3 recognition specificity was retained.

In addition, the considerable numbers of IgM AFCs in the spleen, especially those producing antibodies to homologous H3 ($80/10^5$ cells), supplanted the very poor IgA AFC responsiveness and were the second largest population in this group. Twice as many splenic AFCs were IgM producers as were IgG producers. The frequency of splenic IgM AFCs was reduced by day 13.

Egg-grown HA Lys-156 reassortant vaccine group. There was yet another shift in the responding tissue. The predominant AFC responsiveness to challenge on day 5 was localized in the spleen, was of the IgM isotype, and was directed equally against all three H3 variants, as well as H1 (Fig. 1i to l). The extent of the splenic IgM AFC response (100 to $180/10^5$ cells) was approximately three times less than the average IgG AFC maxima of the MDCK cell-grown and egg-grown HA Ile-186 vaccine groups in the MLN and CLN, respectively. The spleen also contributed the largest number of IgG AFCs in the host (average of $100/10^5$ cells). In fact, IgG AFC responsiveness was diminished six- to ninefold below that observed in the other two vaccine groups.

In comparison with the other two vaccine groups, the CLN and MLN contribution was much reduced (about sevenfold) and was largely targeted to the egg-selected H3 variants (Fig. 1j and k). IgA AFC responsiveness (0 to $10/10^5$ cells) was the poorest of the three vaccine groups. Reactivity against control plates coated with allantoic fluid was on a similar order of scale in each of the vaccine groups.

Levels of protection against challenge are influenced by the Glu→Lys substitution at position 156. Vaccine efficacy was monitored by clearance of virus from the lungs following a lethal challenge (200 MID_{50}) with the MDCK cell-grown reassortant virus (Table 4). The MDCK cell-grown and egg-grown HA Ile-186 reassortant vaccine groups were completely (100%) protected. This was associated with complete clearance of the virus from the lungs. In contrast, 50% of mice immunized with the egg-grown HA Lys-156 reassortant vaccine shed infectious virus, as much as control animals. The data for the egg-grown HA Lys-156 reassortant vaccine support the findings reported by Wood et al. (74) and Katz and Webster

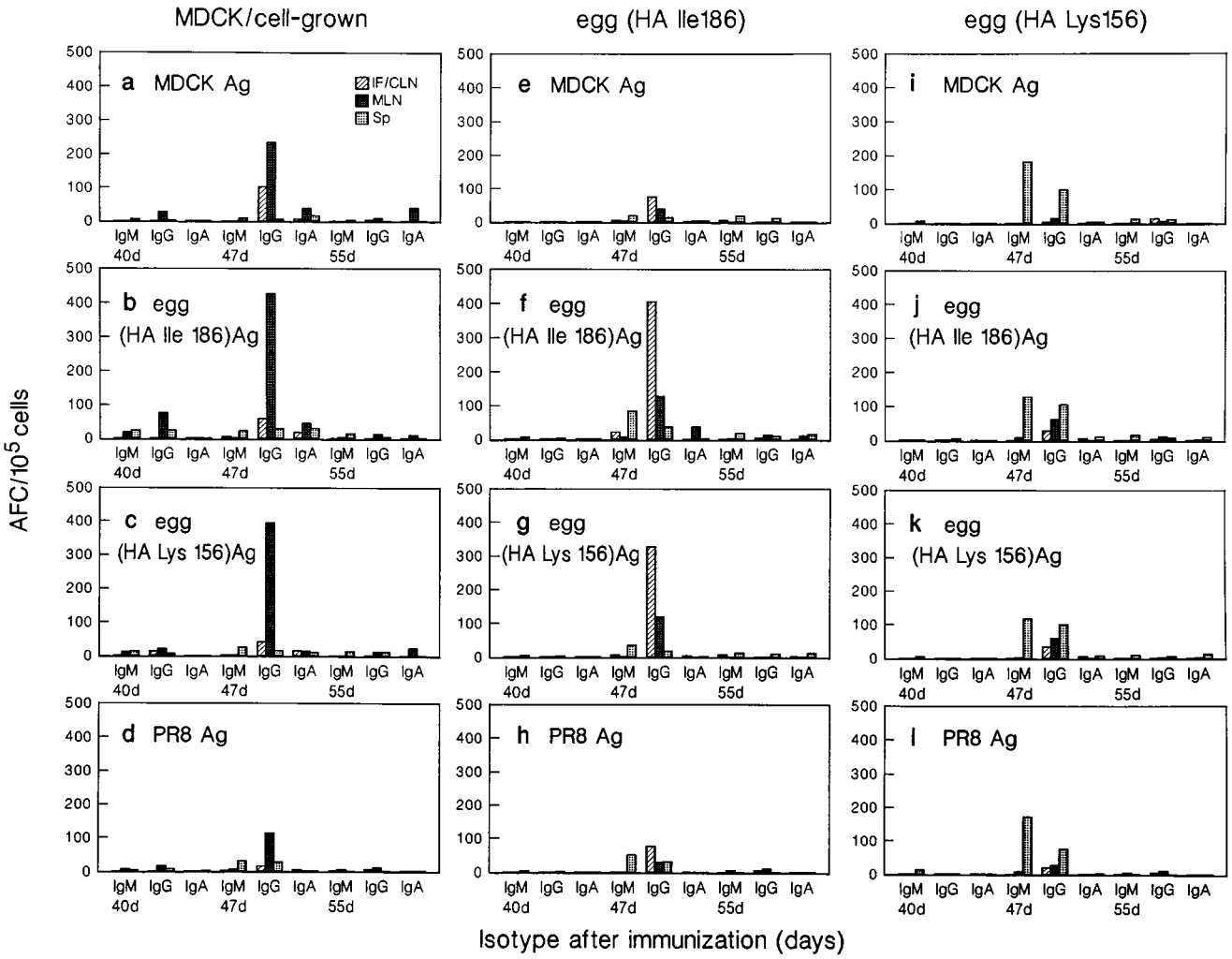

FIG. 1. B-cell responsiveness induced in mice by inactivated vaccines of the A/Mem/7/90 × PR8 (H3N1) reassortant viruses representative of the original clinical isolate or egg-grown variants and in response to a lethal challenge. BALB/c mice were immunized with 5 μg of HA of one of three reassortant vaccines (MDCK cell-grown and egg-grown HA Ile-186 and HA Lys-156) in combination with an adjuvant on day 0 and alone 35 days later and challenged within 5 days by i.n. inoculation with 200 MID_{50} of the live MDCK cell-grown (H3N1) reassortant. Lymphoid tissues of immunized mice were removed 5 days following boosting (day 40) with homologous vaccine and 7 and 15 days after challenge (days 47 and 55, respectively). Freshly isolated cells from each tissue (IFLN, MLN, and spleen [Sp]) were sampled for Ig isotype determination at each time point in the enzyme-linked immunospot assay against glycoprotein preparations derived from the MDCK cell-grown (a, e, and i) and egg-grown HA Ile-186 (b, f, and j) and HA Lys-156 (c, g, and k) reassortants, as well as PR8 (d, h, and l). Following challenge, B-cell response in the CLN was substituted for the earlier IFLN. Ig-specific AFC frequencies in one to three individual mice per sampling time are shown. The data shown are from one mouse or are means of two or three mice per group; the maxima on day 7 postchallenge for each tissue in each vaccine group were significantly different, and there was little variability in the number of AFCs between mice in any group. Ag, antigen; d, days.

(31) on the ineffectiveness of egg-grown vaccine against an MDCK cell-grown virus challenge.

Correlation of AFC numbers with HI antibody titers in serum. To further evaluate the efficacy of the three vaccine reassortants, we compared the total AFC number per mouse in each vaccine group with the corresponding HI titer in serum.

TABLE 4. Response of immunized mice to challenge with MDCK cell-grown Mem/7/90 × PR8 (H3N1) reassortant virus

Reassortant vaccine group	Mean virus titer (log_{10} $TCID_{50}$)[a]	No. of mice shedding/total	Protection (%)
MDCK cell-grown virus	$<10^1$	0/4	100
Egg-grown HA Ile-186	$<10^1$	0/4	100
Egg-grown HA Lys-156	$10^{2.16}$	2/4	50
Control	$10^{2.25}$	4/4	0

[a] The mean virus titer in lungs of four mice per group is expressed as log_{10} $TCID_{50}$ per lung measured 3 days following challenge with 10^3 $TCID_{50}$ of the MDCK cell-grown Mem/7/90 × PR8 (H3N1) reassortant.

First, we calculated the median AFC totals against the three vaccine HAs for each lymphoid tissue on days 40 (prechallenge) and 47 (postchallenge). The MDCK cell-grown reassortant group showed the greatest median AFC total of all three groups within 1 week of boosting (day 40 overall), and 70% of the AFCs were localized in the spleen (Fig. 2a). Half of these AFCs produced IgG, unlike those in the egg-grown HA Lys-156 reassortant group, which mostly (70%) secreted IgM (data not shown). Almost 10-fold greater AFC totals per mouse were found in the MDCK group than in the other two (see Table 6). By the fifth day after challenge, median AFC totals had increased 10 to 100 times, to about 2.0×10^4 in each vaccine group (see Table 6). Distribution of median AFC totals per organ reflected the frequencies seen earlier (Fig. 1): approximately 60% of the total AFCs in the MDCK group were

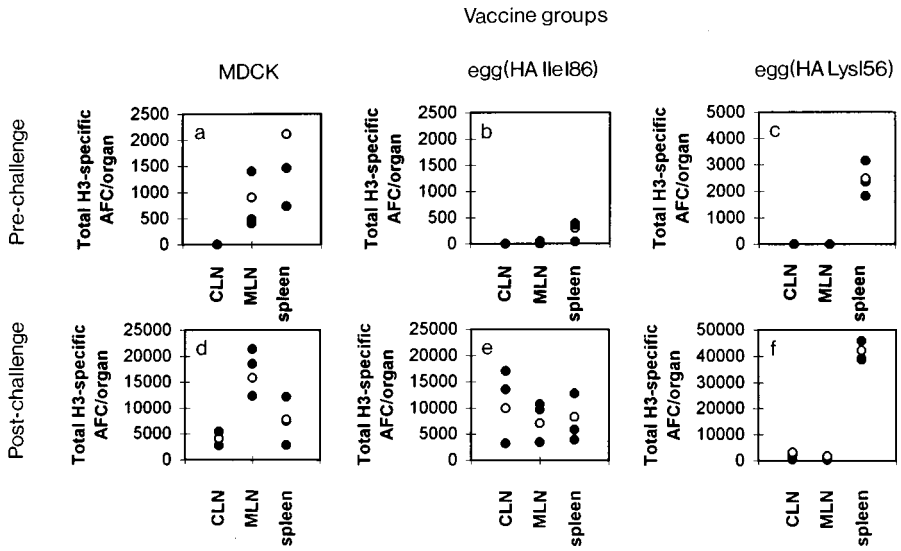

FIG. 2. Localization of H3-specific AFCs in vaccinated mice. The sums of the total numbers of AFCs in the CLN, MLN, and spleens in each vaccine group (MDCK cell-grown [a and d] and egg-grown HA Ile-186 [b and e] and HA Lys-156 [c and f]) are shown for day 5 postvaccination (day 40 overall) and day 7 postchallenge (day 47 overall). These AFCs produced antibodies against glycoprotein preparations from each H3N1 reassortant vaccine (closed circles). Total numbers of AFCs that recognized PR8 were not included. Prechallenge, the median numbers of AFCs producing antibodies against the three vaccine glycoproteins (open circles) were as follows: a, 0 (CLN and IFLN), 900 (MLN), and 2,117 (spleen); b, 0 (CLN and IFLN), 23 (MLN), and 301 (spleen); c, 0 (CLN and IFLN), 0 (MLN), and 2,600 (spleen). The postchallenge numbers were as follows: d, 4,105 (CLN), 15,815 (MLN), and 7,479 (spleen); e, 10,180 (CLN), 7,160 (MLN), and 8,399 (spleen); f, 1,880 (CLN), 1,050 (MLN), and 42,240 (spleen). Individual symbols indicate AFC numbers determined in organs from the same mice (Fig. 1). For calculations of total numbers of AFCs, AFC frequencies for each tissue in a given mouse were multiplied by the number of lymphocytes in the CLN, MLN (assuming that 50% are B cells), or spleen (assuming that 30% are B cells).

localized in the MLN (Fig. 2d), whereas AFCs in the CLN predominated (40%) in the egg-grown HA Ile-186 group (Fig. 2e) and there was a further shift in localization to the spleen (93%) in the egg-grown HA Lys-156 group (Fig. 2f). Despite the difference in tissue localization, the Ig isotype profile in the MDCK cell-grown and egg-grown HA Ile-186 groups was dominated by IgG production (about 80%), with the remainder shared equally by IgM and IgA secretors (data not shown). The total number of IgG AFCs per mouse in each vaccine group was the same (see Table 6). As expected from the data in Fig. 1, the share of IgG AFCs was reduced by half of the total in the egg-grown HA Lys-156 vaccine group, and about two-thirds of these were localized in the spleen. The proportions of IgG AFCs (versus IgM producers) in the spleen and MLN among vaccine groups were equivalent at 32 and 90%, respectively. In comparison with the MDCK cell-grown and egg-grown HA Ile-186 vaccine groups, the 27,102-cell (40%; see Table 6) increase in the total number of AFCs in the egg-grown HA Lys-156 group was made up entirely of splenic IgM AFCs, which contributed to half of the total number of AFCs per mouse.

Second, HI titers in serum before and after challenge (days 5 and 13) were determined. In the MDCK cell-grown and egg-grown HA Ile-186 reassortant vaccine groups, only antibodies that recognized the HAs from the two vaccines exhibited 10-fold increased titers (Table 5). In contrast, a challenge of the egg-grown HA Lys-156 reassortant vaccine group generated HI antibodies that exhibited broader reactivity to all three HAs yet were not protective.

To correlate the total number of HA-specific AFCs with serum HI titers in the vaccine groups, we compared the relative avidity of the IgG antibodies that can inhibit hemagglutination. The 8-fold increase in the median HI titer in the MDCK cell-grown reassortant vaccine group postchallenge (day 47 overall) was associated with a nearly 10-fold greater number of IgG AFCs (Table 6). In contrast, almost 100 times more IgG AFCs were needed to raise the median HI titers in serum three- to eightfold in the egg-grown HA Ile-186 and HA Lys-156 reassortant groups, respectively. The relative avidity for IgG in these two vaccine groups was, on average, less than 1/10 of that of the MDCK cell-grown reassortant vaccine group. It is likely, nevertheless, that the avidity of the anti-HA

TABLE 5. Antibody titers in sera of mice immunized with the MDCK cell-grown Mem/7/90 × PR8 (H3N1) reassortant

Reassortant vaccine group[a]	HI antibody $\log_{10}$ titer to reassortant at postchallenge day:					
	5			13		
	MDCK cell-grown virus	Egg-grown HA Ile-186	Egg-grown HA Lys-156	MDCK cell-grown virus	Egg-grown HA Ile-186	Egg-grown HA Lys-156
MDCK cell-grown virus	3.7	3.2	2.4	3.7	3.1	2.2
Egg-grown HA Ile-186	3.4	3.5	2.2	3.4	3.7	1.9
Egg-grown HA Lys-156	3.2	3.1	3.5	3.4	3.1	3.7

[a] Mice were challenged with 200 MID_{50} of live MDCK cell-grown Mem/7/90 × PR8 (H3N1) reassortant virus at 42 days postvaccination.

TABLE 6. H3-specific IgG responses[a]

Sampling time	HI titer			Total no. of IgG AFCs/mouse[b]			IgG AFC/HI titer ratio		
	MDCK cell-grown virus	Egg-grown HA Ile-186	Egg-grown HA Lys-156	MDCK cell-grown virus	Egg-grown HA Ile-186	Egg-grown HA Lys-156	MDCK cell-grown virus	Egg-grown HA Ile-186	Egg-grown HA Lys-156
Prechallenge	327	457	282	2.2×10^3	1.8×10^2	2.6×10^2	6.7	0.4	0.9
Postchallenge	2,691	1,820	1,820	2.1×10^4	2.0×10^4	1.9×10^4	7.0	11.4	9.9

[a] Mice were immunized with MDCK cell-grown and egg-grown HA Ile-186 and HA Lys-156 reassortant vaccines, boosted with homologous vaccines on day 35, and killed 5 (prechallenge; day 40 overall) and 13 (7 days postchallenge; day 47 overall) days later. HI titers were determined from the sera of one or two mice per group at each time point.

[b] Total IgG AFC numbers were determined from the same one or two mice bled for HI titration and represent the median of the total number of IgG AFCs found per mouse which recognized the glycoprotein preparations derived from each MDCK cell-grown and egg-grown HA Ile-186 or HA Lys-156 H3N1 reassortant. Total IgG AFC numbers were calculated for the same mice whose tissue AFC totals are given in Fig. 2 and whose AFC frequencies are shown in Fig. 1.

IgG in the egg-grown HA Ile-186 reassortant vaccine group was greater than indicated, a large part of the initially produced HI antibody having been bound by the challenge virus because of its antigenic relatedness. This is supported by the absence of virus in the lungs of the egg-grown HA Ile-186 vaccine-treated group, whereas virus persisted in the egg-grown HA Lys-156 vaccine-treated group. These data indicate that the MDCK cell-grown reassortant vaccine was the most effective in generating HI antibodies at the challenge site and that the egg-grown HA Ile-186 vaccine was a close second; however, substitution with Lys at position 156 drastically altered the immunogenicity of the HA, which failed to generate a B-cell memory that was protective against a lethal challenge.

DISCUSSION

The goal of the present study was to understand the immunological basis of the differential vaccine efficacy between influenza virus vaccine candidates derived from passage of the same clinical isolate (Mem/7/90 [H3N2]) in MDCK cells or embryonated chicken eggs. Inactivated H3N1 reassortant vaccines containing single amino acid changes in the HA1 subunit selected by growth in eggs were compared with the MDCK cell-derived counterpart for the capacity to generate primary and memory B-cell responsiveness. By using the single-cell enzyme-linked immunospot assay, we could directly monitor and compare the Ig isotype profile, tissue localization, and kinetics of the B-cell responses to the variant HAs. Our findings indicate that the MDCK cell-grown virus is immunogenic and induces HI and neutralizing antibodies against homologous HA and the egg-grown HA Ile-186 variant, which is like the MDCK cell-grown variant; i.e., these antibodies are strain specific and are generated primarily in the lymph nodes that drain the upper and lower respiratory tracts. These antibodies provide protective immunity from challenge infection with the MDCK cell-grown high-growth reassortant virus. Unlike the MDCK cell-grown variant, the egg-selected Lys-156 substitution renders the vaccine poorly immunogenic, inducing antibodies that are cross-reactive prechallenge but predominantly egg-grown HA Lys-156 variant specific postchallenge, of the IgM isotype, and produced in the spleen. In contrast, the Ser-to-Ile change at residue 186 does not significantly alter immunogenicity and protective efficacy, the dominant IgG response associated with protection, nor its antigenicity but does restrict memory B-cell responsiveness to challenge to the MLN draining the upper respiratory tract. Our results support the view that within the codominant mixture of virus populations that arise during egg adaptation (21, 50), the minor HA antigenic variation of some, but not all, egg isolates may make these isolates sufficiently different from viruses that cause clinical disease to generate postvaccination B-cell responses that are clinically irrelevant.

The failure to observe equivalent immunogenicity with all of the HA variants when they were formulated as inactivated vaccine was not unexpected. Previous studies have investigated the significance of minor HA antigenic variation with regard to cross-protection in animal models (28, 32, 33, 38, 54, 74) and human volunteers (46). By and large, these studies have shown that vaccines prepared from MDCK cell-grown and egg-grown viruses that differ by as little as two amino acids in their HAs also differ in the ability to protect a host from infection. MDCK cell-grown vaccine is more broadly cross-reactive with related prevalent viruses and is more immunogenic and protective than egg-grown viruses. Kilbourne et al. (38) have shown that BALB/c mice could not distinguish between two egg-grown reassortant (H3N2) vaccines that differed by three amino acid substitutions in the HA1 subunit (including Ile-186). HI analysis of antibody levels in serum following challenge with an uncloned preparation of an egg-grown wild-type parental H3N2 virus demonstrated complete antigenic relatedness between the variant HAs. We have shown that a single amino acid change (HA Lys-156) could be responsible for generating a postchallenge antibody incapable of distinguishing antigenic differences, even between egg-grown and MDCK cell-grown variants. In contrast, the egg-grown HA Ile-186 variant behaves differently and is remarkably like the MDCK cell-grown variant in character, although Ile-186 may be insufficient to counter the negative effects of other substitutions that result from egg adaptation (38). Several possible explanations for the differences in immunogenicity and protective efficacy generated by the HA Lys-156 and HA Ile-186 egg-grown variants can be reasoned.

First, it is possible that B cells, in response to Lys-156 or Ile-186, modify the HA Glu-156- and HA Ser-186-specific V regions that are encoded by characteristic VH-Vk gene segment combinations. Stark and Caton (62) have shown that in response to a single Asp-to-Gly change at amino acid 225, which induces only a subtle local conformational change in the PR8 HA, entirely new VH-Vk combinations are used by PR8 Gly-225-specific splenic hybridomas. It is interesting that an identical change has been reported between an MDCK cell-derived virus and an egg-adapted A/Taiwan/86 (H1N1)-like virus (50). It is likely that in comparison with the Ser-186→Ile change, the Glu-156→Lys substitution on the exterior face of the position 187 to 196 α helix on the three-dimensional structure of the H3 HA (72) is not only larger but charged and may thus be more severe.

Second, the difference in immunogenicity between egg-grown variants could be anticipated in view of the proximity of the changes to antigenic site B (70, 71) and helper T-cell

determinants (5, 6, 11, 16). It is important to note that the majority of major histocompatibility complex I^d class II-restricted T-cell sites overlap antibody-binding regions of HA1 and are sensitive to the amino acid substitutions that have occurred in natural variant viruses (6, 7, 10). In fact, site B (positions 174 to 209) (5, 6, 16) is immunodominant in BALB/c mice for both antibody (10, 61) and helper T cells (6). The extensive reciprocity in B-cell and helper T-cell recognition of influenza virus HA (5, 6, 9) suggests that B cells can selectively process and present HA1 peptides to major histocompatibility complex class II-restricted T cells, thereby defining both B-cell memory and T-cell memory. The fine specificity of surface Ig receptors of HA-specific memory B cells (used for antigen capture) may determine the spectrum of peptides generated by antigen processing and delivered to HA-specific $CD4^+$ T cells (5, 8, 17, 64, 68). In most instances, antibodies would be expected to enhance the efficacy with which antigenic peptides are presented; however, some antibody specificities may result in differential enhancement (47), if not complete abrogation, of T-cell activation (69). It is interesting to speculate that the HA Lys-156 variant has potentially suppressive effects on the presentation of T-cell epitopes and, ultimately, on the B-cell response itself. Further description of the effects of the Lys-156 change on T-cell recognition awaits further determination, but even subtle conformational changes induced by sequences flanking epitopes have been shown to be sufficient to alter immunogenicity (68).

Third, it is possible that because of the restricted IgM composition of AFCs from HA Lys-156 variant-vaccinated mice following challenge, the failure is due to limited cognate help provided by virus-specific helper T cells (13) that promote virus clearance and recovery (18). Scherle and Gerhard (57) have shown that only adoptive transfer of HA-specific and matrix protein-specific $CD4^+$ T cells to nude mice supports enhanced antibody production and the Ig isotype switch necessary to generate a normal antibody (IgM, IgG, and IgA isotypes) response. Transfer of NP-specific clones fails to generate anti-HA antibodies other than those of the IgM isotype. Reduced isotype switching and memory B-cell responsiveness could additionally indicate an inability to generate germinal centers in lymphoid tissues (41, 42) as a result of skewed cytokine profiles (48, 60). Because these processes are dependent on the initial CD40-driven costimulatory signal to B cells delivered by activated helper T cells (reviewed in references 4, 20, and 66), lessening of this major contact-mediated signal may have diminished T-B cognate interaction in response to HA Lys-156 without affecting IgM secretion, which is independent of CD40 ligation (20, 37). Experiments that compare the differential cytokine secretion profile of cellular subsets of the spleen (e.g., B cells, helper T cells, and macrophages [66]) of HA Lys-156 variant-vaccinated mice with that in the CLN and MLN should allow this possibility to be addressed.

It is intriguing that in the HA Lys-156 vaccine group, memory AFC responsiveness to the MDCK cell-grown high-growth reassortant virus is largely (>90%) restricted to the spleen and only half of memory B cells are IgG^+. This indicates that with vaccination, memory B cells were not disseminated to secondary lymphoid tissues to reside within the recirculating lymphocyte pool (61), in readiness for subsequent reexposure to antigen (3). These memory B cells may represent long-term antigen-specific memory (19, 23, 44) and/or the subset of sessile splenic memory B cells described by MacLennan et al. (43). Furthermore, it is surprising that HA Lys-156 variant-specific memory B cells are committed to IgM production although similar splenic localization of memory IgM AFCs is seen following i.n. live-virus challenge of CD4-deficient mice primed i.p. with HA alone (31). Jones and Ada (30), in an early report on B-cell responsiveness, showed that AFCs elicited by inactivated egg-grown influenza vaccine given either i.p. or i.n. were also localized in the spleen. Whether restriction to the spleen is influenced by the unavailability of T help following priming with some egg-grown vaccines awaits to be determined in experiments with CD4-deficient mice, especially since class II major histocompatibility complex knockout mice fail to make virus-specific IgG (24) and are devoid of germinal centers (14).

It is not surprising that immunization induced only poor mucosal IgA memory, especially since mucosal immunity is not targeted by subcutaneous or i.p. inoculation (67). Previously, it was shown that i.p. or oral vaccination with inactivated influenza virus generated an overwhelmingly IgM AFC response in the spleen (30). Mucosal adjuvant does not help to provide protection with a single vaccine dose, although an immunization protocol which includes oral adjuvant-assisted vaccination followed by i.p. or oral boosting is 80 to 100% protective, by enhancing the mucosal IgA response (67). Failure to detect measurable AFC numbers following priming and boosting is evidence that B-cell responsiveness was limited to the development of memory, possibly as a result of adjuvant action (67). We are presently studying the mechanisms that determine mucosal immunity against challenge.

The present study elicits an interesting question. Would challenge of each vaccine group with a homologous virus generate memory AFC responsiveness equivalent to that seen with the MDCK cell-grown virus? Our earlier study (32) indirectly addressed this issue and showed that MDCK cell-grown vaccine provided better protection than did an egg-grown counterpart to challenges with both the homologous MDCK cell-grown and egg-grown viruses. On the basis of the close antigenic relatedness between the MDCK cell-isolated variant and the egg-grown HA Ile-186 variant that resembles the MDCK cell-grown variant and the B-cell data here, we would expect the memory responsiveness of the HA Ile-186 vaccine group to challenge with a homologous virus to be equivalent to that observed with the MDCK cell-grown virus. Challenge of the HA Lys-156 vaccine group with a homologous virus would be similarly poorly effective. A similar situation may exist in the human population. Newman et al. (46) have reported the existence, in unvaccinated human sera from volunteers with previous natural infections, of a strain-specific antibody to viruses that resemble the MDCK cell-grown virus but not to the egg-grown counterpart. These results suggested that the close antigenic relatedness with prevalent natural viruses may be responsible for the greater immunogenicity of MDCK cell-grown virus-like candidate vaccines, a conclusion supported by our findings.

Caution against the selection of some egg-adapted strains for use in vaccine has been much emphasized (33, 38, 46, 50, 74). We have previously recommended a number of steps to minimize the selection of minor variants of influenza viruses which may rapidly overgrow the desired virus population (21). This includes virological cloning of vaccine virus at a very early passage and preparation of high-growth reassortants. Sequencing and antigenic analysis assured us that the egg-grown HA Ile-186 and HA Lys-156 variants were homogeneous populations. Our results suggest that it is therefore practical to recommend removal of the egg-grown HA Lys-156 variant or similar isolates as vaccine candidates and instead to select isolates which minimally have Ile at residue 186 and which most closely resemble the clinical specimen or viruses that resemble the MDCK cell-grown virus. This is particularly important, since protection against the prevalent natural virus

that may differ from a vaccine virus by only one egg-derived amino acid is needed.

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