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Purified Influenza Virus Hemagglutinin and Neuraminidase Are Equivalent in Stimulation of Antibody Response but Induce Contrasting Types of Immunity to Infection

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BALB/c mice immunized with graded doses of chromatographically purified hemagglutinin (HA) and neuraminidase (NA) antigens derived from A/Hong Kong/1/68 (H3N2) influenza virus demonstrated equivalent responses when HA-specific and NA-specific serum antibodies were measured by enzyme-linked immunosorbent assays (ELISAs). Antibody responses measured by hemagglutination inhibition or neuraminidase inhibition titrations showed similar kinetic patterns, except for more rapid decline in hemagglutination inhibition antibody. Injection of mice with either purified HA or NA resulted in immunity manifested by reduction in pulmonary virus following challenge with virus containing homologous antigens. However, the nature of the immunity induced by the two antigens differed markedly. While HA immunization with all but the lowest doses of antigen prevented manifest infection, immunization with NA was infection-permissive at all antigen doses, although reduction in pulmonary virus was proportional to the amount of antigen administered. The immunizing but infection-permissive effect of NA immunization over a wide range of doses is in accord with results of earlier studies with mice in which single doses of NA and antigenically hybrid viruses were used. The demonstrable immunogenicity of highly purified NA as a single glycoprotein without adjuvant offers a novel infection-permissive approach with potentially low toxicity for human immunization against influenza virus.

Influenza A viruses contain two major virus-coded surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA), which are the antigens primarily involved in induction of specific humoral immunity to influenza virus. Earlier studies in which NA and HA were segregated in antigenically hybrid reassortant viruses (21, 35, 37) or that employed chemically isolated NA antigen (24, 36) established the different roles of the two viral proteins in the induction of immunity to influenza virus infection. Anti-NA antibody is infection permissive in that antibody against NA does not prevent infection (25, 36). However, NA immunization can reduce viral replication below a pathogenic threshold so that infection can occur without apparent disease (2, 7, 25, 29). Antibody against HA is generally neutralizing, presumably preventing infection by interfering with virus attachment to host cells (14) or possibly by interfering with the fusion event subsequent to endocytosis (20, 41).

Vaccination with conventional, inactivated influenza virus vaccines, containing both HA and NA, stimulates immunity against both antigens (23), although the immunologic response to NA is severely suppressed in primed subjects through HA-NA antigenic competition (17, 21). Therefore, with biantigen vaccines, the effect of anti-HA immunity is overriding, and infection is usually prevented. However, the relative immunogenicity of NA can be increased in virus-primed humans (2, 5, 7, 21, 22) or mice (18) by administration of an antigenically hybrid reassortant virus vaccine containing an HA not previously experienced.

HA-conditioned immune response to intravirionic NA has been duplicated in a mouse model system. Studies with this system have identified the role of NA-specific T helper cells (16), NA-specific B cells, and the role of B- and T-cell collaboration in the intravirionic antigenic competition that results in immunodominance of one surface antigen over another (17).

We have shown that differential priming of HA- and NA-specific B and T cells is involved in the antigenic competition between HA and NA (17). This difference in priming ability could be due to the relatively greater molar amounts of HA than NA on the virion surface (9) or to intrinsic immunogenic differences between the two proteins. The present study compares the relative immunogenicity of influenza virus HA and NA in BALB/c mice injected with equivalent graded doses of purified H3 HA or N2 NA. Specific antibody response to each surface glycoprotein was evaluated by using reassortant viruses in hemagglutination inhibition (HI) and NA inhibition (NI) tests and purified antigens in enzyme-linked immunosorbent assays (ELISAs) and correlated with reduction of pulmonary virus levels in virus-infected mice.

MATERIALS AND METHODS

Viruses. The strains of influenza A viruses used in these studies are identified in Table 1. All viruses were grown in the allantoic sac of 10-day-old chicken embryos and stored in multiple aliquots at −70°C. Mouse-passaged wild-type H3N2 (A/Hong Kong/1/68) virus was used to infect mice. (Only a single egg passage was used following two sequential mouse lung passages at limiting dilution.)

Animals. Inbred BALB/c female mice (Jackson Laboratories, Bar Harbor, Maine) 8 weeks of age were used in this study.

Isolation of purified HA and NA from influenza A viruses. (i) Virus preparation. Reassortant influenza A virus strains H6N2 (A/Turkey/Mass/75-Aichi/2/68 [R]) and H3N1 (A/

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TABLE 1. Viruses used in this study

HA-NA subtype	Designation	Laboratory name	Specific use
H6N2	A/Turkey/Mass/75-Aichi/2/68 (R)	$H6N2_{HK}$	Source of purified N2 NA
H3N1	A/Hong Kong/1/68-PR/8/34 (R)	$H3_{HK}N1_{PR8}$	Source of purified H3 HA
H3N2	A/Aichi/2/68 (R)	$H3_{HK}N2_{HK}$	HI antigen
H1N2	A/PR/8/34-Hong Kong/1/68 (R)	$H1_{PR8}N2_{HK}$	NI antigen
H3N2	A/Hong Kong/1/68	$H3_{HK}N2_{HK}$	Mouse infection

Hong Kong/1/68-PR/8/34 [R]) were used as sources of N2 NA and H3 HA, respectively. Viruses were grown in the allantoic sac of 10-day-old chicken embryos at 37°C for 40 h. Eggs were chilled at 4°C for at least 18 h prior to harvesting. Cellular debris was removed by centrifugation at 11,000 rpm (Sorvall GSA rotor) for 10 min. Virus was pelleted from the clarified supernatant by a second centrifugation at 35,000 rpm (Beckman Ti45 rotor) for 45 min. The virus pellet was suspended in phosphate-buffered saline (PBS; 0.01 M sodium phosphate, 0.14 M NaCl [pH 7]), layered over a 30 to 60% sucrose gradient, and centrifuged at 25,000 rpm (Beckman SW27 rotor) for 90 min. The virus band was collected, diluted with PBS, and recentrifuged at 35,000 rpm (Beckman Ti45 rotor) for 45 min.

(ii) Extraction and purification of surface antigens. H3 HA and N2 NA were extracted and purified from influenza virus particles as described by Gallagher et al. (12) with several modifications. Virus pelleted from 200 eggs (yield, 20 to 40 mg) was suspended in 1 ml of sodium acetate buffer (0.05 M sodium acetate, 2 mM $CaCl_2$, 0.2 mM EDTA [pH 7.0]). The suspended virus preparation was refluxed through a 19-gauge needle to ensure a homogeneous suspension. An equal volume of 15% octylglucoside (octyl-β-D-thioglucoside; Calbiochem Co.) in sodium acetate buffer was added with vigorous vortexing. This suspension was centrifuged at 15,000 rpm (Beckman Ti50.1 rotor) for 60 min. The supernatant was carefully removed from the pellet and reserved as the HA-NA-rich fraction. Then, 2% aqueous cetyltrimethylammonium bromide (CTAB; Sigma Chemical Co.) was added to the HA-NA-rich fraction to a final concentration of 0.1% CTAB (100 μl of 2% CTAB per 2 ml of solution). This solution was applied to a DEAE-Sephadex (A-50; Pharmacia Fine Chemicals) ion-exchange column (bed, 0.7 by 6.0 cm) previously swollen and equilibrated with 0.05 M Tris-hydrochloride (pH 7.5) containing 0.1% octylglucoside (NA-eluting buffer). From 10 to 15 fractions (2 ml per fraction) were collected with the NA-eluting buffer, and then the elution buffer was changed to low-salt HA-eluting buffer (0.05 M Tris-hydrochloride [pH 7.5], 0.1 M NaCl, 0.1% Triton X-100). After 10 to 15 fractions (2 ml per fraction) were obtained, the elution buffer was changed to high-salt HA-eluting buffer (0.05 M Tris-hydrochloride [pH 7.5], 0.2 M NaCl, 0.1% Triton X-100). The addition of a high-salt HA-eluting buffer increased the overall yield of HA off the DEAE-Sephadex column (12). After chromatography, individual fractions were dialyzed against sodium acetate buffer with 2 mM $CaCl_2$ for 96 h to remove any residual detergent. Ca^{2+} in buffers stabilizes viral NA enzymatic activity (3, 8) and immunogenicity (E. D. Kilbourne, unpublished results). Each fraction was tested for neuraminidase activity with fetuin substrate (1) and for hemagglutinating activity with chicken erythrocytes (31). Fractions showing optimal activity were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis under nonreducing conditions by the method of O'Farrell (28). Gels were silver stained by the method of Ohsawa and Ibata (30).

NA isolated by this procedure coelutes with viral lipids and spontaneously forms liposomes after dialysis (12). Protein was quantitated by the method of Lowry et al. (26).

Serologic methods. Sera were obtained from mice by retroorbital bleeding. Mice were bled five times at 7-day intervals before boosting. Nonspecific inhibitors were destroyed with *Vibrio cholerae* NA before use of sera in HI tests. HI tests were performed by the microtiter method (38) with the reassortant viruses H3N2 (A/Aichi/2/68 [R] [X-31]) and H6N1 (A/Turkey/Mass 75-India/1/80 [R]). Tests for NI with the reassortant viruses H1N2 (A/PR/8/54-Hong Kong/1/68 [R]) and H1N1 (A/PR/8/34) were performed as described previously (21). ELISAs were done (19) on individual serum specimens from every animal and every bleeding. Purified N2 NA or purified H3, in carbonate buffer (0.15 M Na_2CO_3, 0.035 M $NaCO_3$ [pH 9.6]), was used to sensitize the plates. NA or HA was used at 400 ng/ml in a volume of 100 μl per well. Antibody binding was detected by alkaline phosphatase-conjugated sheep anti-mouse immunoglobulin G1 (IgG1), IgG2a, IgG2b, IgG3, and IgM used at the manufacturer's recommended dilution (ICN Biomedical, Inc., Costa Mesa, Calif.).

Immunization. The study design and immunization protocol are outlined in Table 2. A total of 180 BALB/c mice were randomly divided into two groups of 90 animals; each group received injections of either purified H3 HA or purified N2 NA. These two groups were further subdivided into nine subgroups of 10 animals, and each member of these subgroups received injections of antigen as indicated (Table 2). Injections were given either with Freund complete adjuvant (FCA) into the hind foot pads or intravenously (i.v.) without adjuvant. Day 42 boosting injections of 1 μg of HA or NA were given intraperitoneally (i.p.) without adjuvant.

Infection protocols. At 50 days after an i.p. booster injection of 1 μg of purified H3 HA or N2 NA, 10 mice from each primary dose group were inoculated intranasally under light ether anesthesia with 100 50% mouse infective doses of H3N2 virus. The surface glycoproteins contained in this

TABLE 2. Study design and immunization protocol

Group[a]	H3 HA or N2 NA used	FCA[b]	Day 42 boost (μg)[c]
A	0.2	−	1.0
B	1.0	−	1.0
C	5.0	−	1.0
D	25.0	−	1.0
1	0.2	+	1.0
2	1.0	+	1.0
3	5.0	+	1.0
4	25.0	+	1.0
X	0.0	−	1.0

[a] There were a total of 18 groups of 10 mice each; 9 groups received HA and 9 groups received NA at the indicated concentrations.
[b] Animals given antigen in FCA were injected in the hind footpads; animals given antigen alone were injected i.v. by the tail vein.
[c] All 1.0-μg booster injections were given i.p.

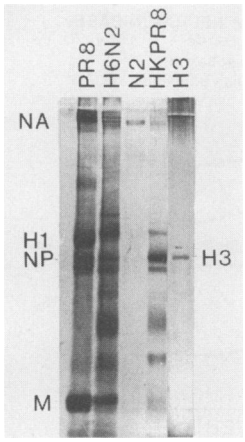

FIG. 1. A 1-μg amount of each detergent-disrupted whole virus was run in the lanes marked PR8, H6N2, and HKPR8, and 100 ng of purified antigens was run in the lanes marked N2 and H3. Proteins identified on the left indicate proteins from PR8 virus in this 10% SDS–polyacrylamide gel under nonreducing conditions. Additional bands appearing in whole-virus lanes are chicken embryo proteins that copurify with virus. N1 and N2 NAs migrate to approximately the same position under these conditions, whereas H3 HA migrates slightly faster than H1. M, 27-kilodalton protein; NP, 56-kilodalton protein.

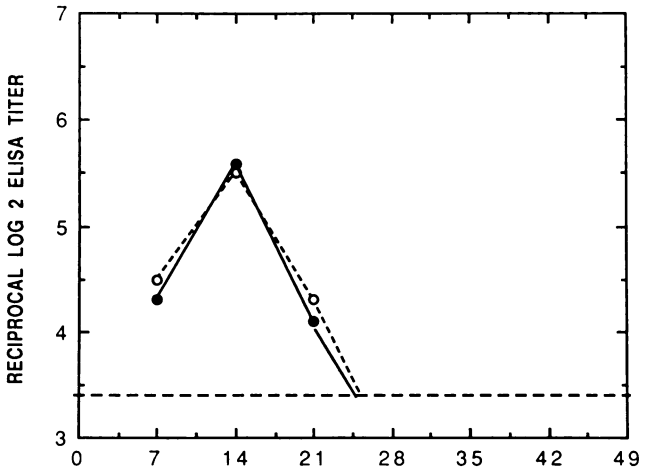

FIG. 2. Primary antibody response to purified H3 HA and N2 NA. Mice were injected with sterile PBS on day 0 and 42 days later were immunized with 1 μg of N2 NA (●) or 1 μg of H3 HA (○). Sera taken on the days specified were tested by ELISA. The dotted line represents the mean background level of binding for serum samples obtained from five uninoculated mice.

virus are identical to the purified H3 HA and N2 NA used to immunize the animals.

Titration of pulmonary virus. Three days after inoculation of virus, five mice from each group were killed, and 10^{-2} screening dilutions of homogenized lung suspensions were injected into 10-day-old chicken embryos. The presence of HA in harvested allantoic fluids identified virus-positive lungs. Individual virus-positive lungs were titrated for plaque-forming infective virus by inoculation of decimal dilutions (10^{-2} to 10^{-8}) of lung (lung homogenized at 10^{-1} [wt/vol] dilution) in Madin-Darby canine kidney (MDCK) cell cultures (three 60-mm plastic plates per dilution). After incubation at 37°C for 72 h (15), cell monolayers were stained with 0.1% crystal violet and plaques were counted.

RESULTS

Characterization of purified antigens. Fractions from the N2 NA purification from H6N2 virus that had high NA activity (≥3.5 μM *N*-acetyl neuraminic acid released/100 μl per h) and did not show any hemagglutination of chicken erythrocytes were pooled and analyzed by silver staining of polyacrylamide-SDS gels following electrophoresis. As shown in Fig. 1, no viral protein bands were detected except for a single band in the position of N2 NA. (Incomplete penetration of the band with the silver reagents superficially suggested a doublet.) Fractions eluted from chromatography of disrupted H3N1 virus were pooled based on high HA activity (>1,280 HA units [HAU] per 50 μl) and minimal NA activity (<0.032 μM *N*-acetyl neuraminic acid released/100 μl per h). Analysis on SDS gels revealed only a single band in the position corresponding to H3 HA (Fig. 1). Furthermore, at the highest dose of antigen, no antibody was detectable by ELISA (threshold of detection, 6.2 $\log_2$) against H6 HA in mice immunized with N2 NA purified from H6N2 virus or antibody specific for N1 NA in mice immunized with H3 HA purified from H3N1 virus (data not shown).

Primary immune response to purified HA and NA. In both the H3 HA and N2 NA immunization protocols, 1 μg of purified antigen without adjuvant was administered i.p. 42 days following a control i.v. injection of sterile PBS (Table 2, group X). The 1-μg doses of H3 HA and N2 NA induced equivalent but low levels of specific ELISA antibody, which peaked 14 days after injection (Fig. 2). There were no significant differences in HA and NA antigen-specific primary immune responses as measured by ELISA with respect to the maximum antibody titers induced or response kinetics. Measurements of antibody by HI or NI proved less sensitive. No NI antibody against N2 NA was found in any of the 10 mice injected with 1 μg of NA antigen (Fig. 3A). Of the 10 mice injected with purified H3 HA, only 1 mouse had detectable levels of HI antibody against H3 (Fig. 3C).

Secondary immune response to purified HA and NA. Data on secondary response are summarized graphically in Fig. 3 and 4. Purified N2 NA and purified H3 HA were immunogenic at all doses (0.2 to 25.0 μg) whether injected with or without FCA. As expected, there were significant differences in antibody response between groups receiving injections containing FCA and those that were not given FCA (analysis of variance, $P > 0.0001$). Antibody response to any given dose was always greater in the presence of adjuvant. Specific antibody titers in mice immunized with either purified H3 HA or N2 NA peaked 14 days after administration of a 1-μg boost. This 14-day peak was observed in HI tests (H3-primed mice), NI tests (N2-primed mice), and ELISA (both antigen groups) regardless of the antigen dose.

Overall, slightly higher antibody titers measured by ELISA were found in mice injected with N2 NA. This difference was not statistically significant. There were significant differences among most dose groups ($P > 0.0001$), but tests subsequent to analysis of variance found no statistically significant differences in NI or ELISA antibody titers in animals injected with 5 or 25 μg of N2 NA (Fig. 4). Both of these groups had a significantly greater antibody response than the groups given 0.2 or 1 μg. However, when purified N2 NA was given with FCA, significant differences were

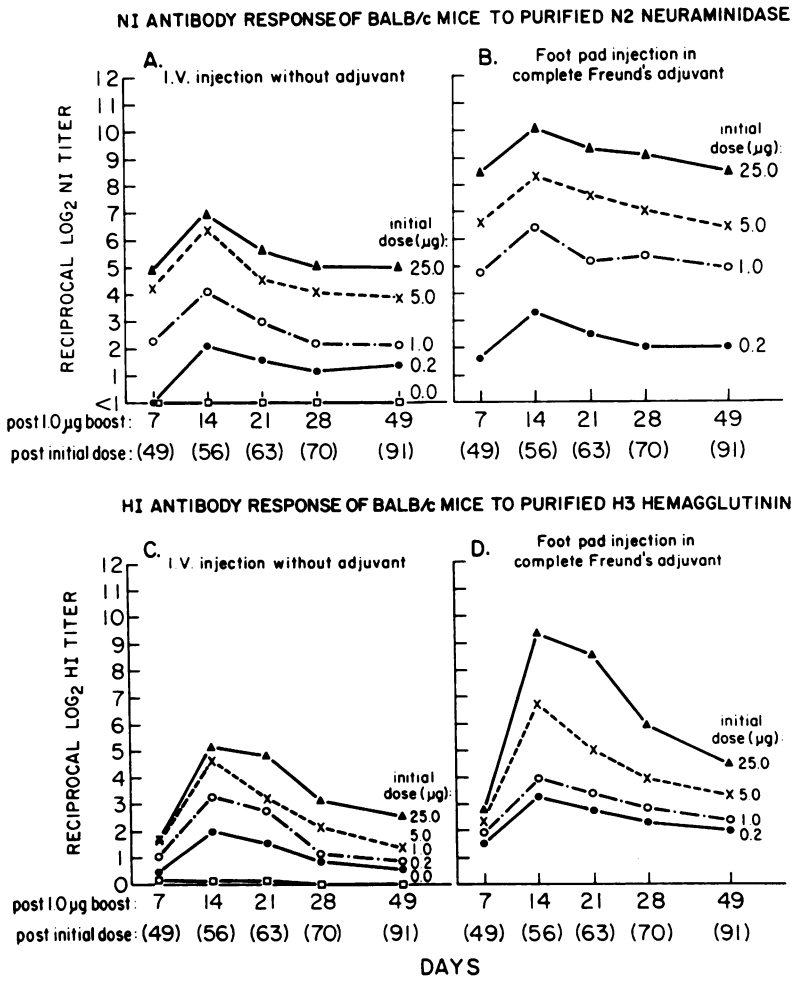

FIG. 3. NI and HI antibody responses. Antibody to N2 from mice injected with purified N2 alone (A) or N2-FCA (B) was measured by NI with whole H1N2 virus. Antibody to H3 from mice injected with purified H3 alone (C) or H3-FCA (D) was measured by HI with whole H3N2 virus.

found between 5-μg and 25-μg groups as well as among groups given smaller doses.

The pattern of differences in H3 antibody measured by ELISA and HI tests in animals that received purified H3 was similar to that found with N2-immunized animals. The results of injections of 5 or 25 μg of H3 antigen given without FCA were not significantly different from each other but were significantly different from those for the 1.0-μg and 0.2 μg groups. When FCA was included in the inoculum, significant differences ($P > 0.0003$) in ELISA (Fig. 4) and HI (Fig. 3) tests were found among all groups, with response proportional to antigen dose. The kinetics of HI and NI antibody responses differed. Although both glycoproteins induced significant rises in antibody titer from day 7 post-boost to maximum titer levels on day 14, mice injected with N2 NA experienced a 15 to 39% decline in antibody titer by day 49 (Fig. 3), whereas the decline from maximal HI titer in H3-immunized mice by that time ranged from 53 to 77%. However, these apparent differences in the rate of decline of HA and NA antibody were not as pronounced when antibodies were measured by ELISA. In N2-immunized mice, there was no significant difference between NA antibody measured by ELISA on days 14 and 49 (69 to 83% of the maximum titer was found on day 49). In comparison, H3 antibody titers were 53 to 87% of maximum titers by days 49, which represents a slightly significant difference ($P > 0.076$) between day 14 and day 49 ELISA antibody titers to HA. Furthermore, although NI antibody titers correlated highly with N2 antibody titers measured by ELISA ($r = 0.94$), the intensity of association between HI and H3 ELISA antibody was not as great ($r = 0.63$).

Infection in unimmunized mice. Infective virus was recovered from all 10 mice that had not been immunized with H3 HA or N2 NA antigens. Titration of pulmonary virus in each of these animals in MDCK cells gave a geometric mean endpoint titer of 1.53×10^{-8} PFU/ml of undiluted lung preparation.

H3N2 virus infection in N2-immunized mice. Consistent with past evidence that NA antibody is infection permissive (15, 25), we found that all mice immunized with purified N2 NA were infected when challenged with H3N2 virus containing homologous NA. The occurrence of infection in these N2-primed mice was independent of N2 antibody concentrations, i.e., all mice were infected over a broad range of antibody levels (Fig. 5). However, the concentration of infective virus decreased in direct proportion to preinfection levels of anti-N2 antibody (Fig. 6). At the lowest N2 antibody level, reduction in pulmonary virus less than

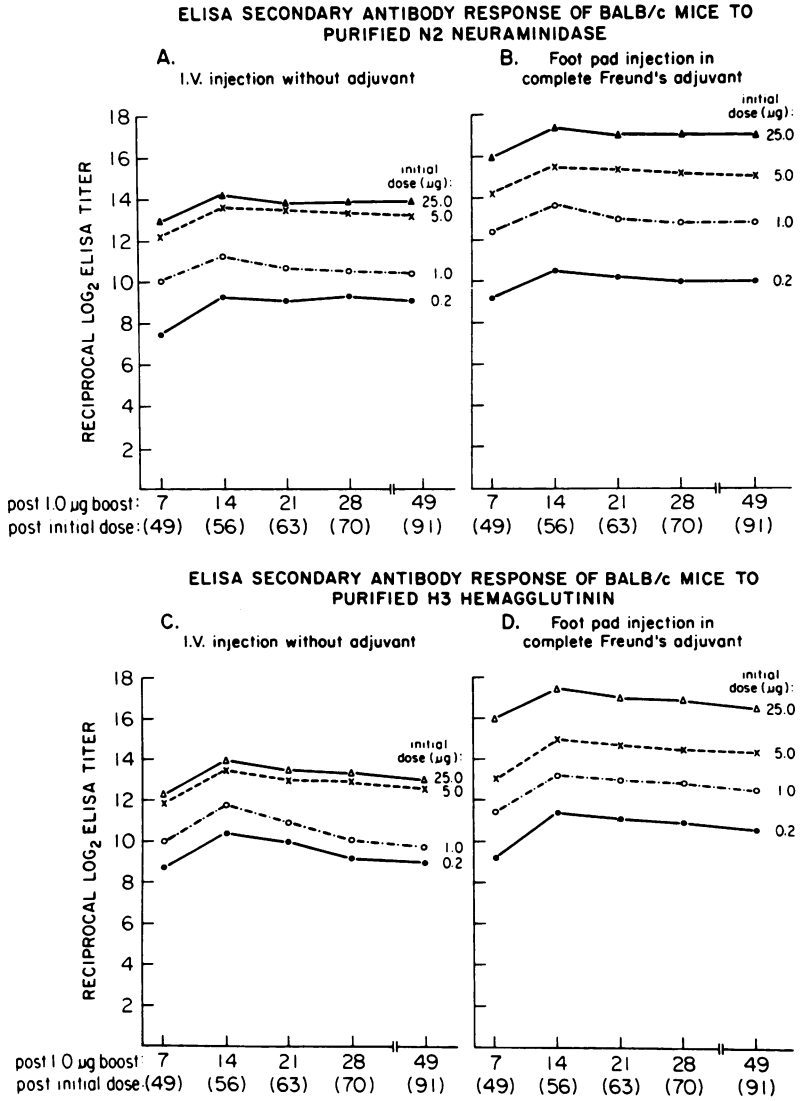

FIG. 4. Secondary antibody response. ELISA antibody to N2 from mice injected with purified N2 alone (A) or N2-FCA (B); ELISA antibody to H3 from mice injected with purified H3 alone (C) or H3-FCA (D). Purified N2 (A and B) or H3 (C and D) was used to coat ELISA plates. The time in days after the boost (after the initial dose) is indicated on the horizontal axis.

twofold (80%) was observed, but a further increase in N2 antibody level of approximately 200-fold was associated with a 10-fold reduction in virus.

H3N2 virus infection in H3-immunized mice. Mice immunized with purified H3 HA had antibody titers comparable to those of N2-immunized animals. The HA and NA antibody titers of animals on the day of challenge infection are summarized in Fig. 3 and 4. However, unlike N2 NA-primed animals, H3-immunized mice were susceptible to manifest infection only at relatively low HA antibody levels (HI, $\leq$1:4). Suppression of infection was evident at HI titers of only 1:8. Infection was not detected in animals with HI antibody titers greater than 1:16 (Fig. 5).

As in the case of NI antibody, different levels of HI antibody were associated with proportional and reciprocal changes of pulmonary virus concentration (Fig. 6). At the lowest HI antibody level, an approximately 2-fold reduction in pulmonary virus was observed, but a further 2-fold increase in HI titer was associated with an approximately 100-fold reduction in virus (Fig. 6). These curves (Fig. 6) demonstrate that HA immunization was infection permissive only in association with a low level of immunization, reflected in a very narrow antibody titer range. At most antibody levels, detectable infection was suppressed; in contrast, NA immunization, while associated with decreased virus levels, was infection permissive over a broad range of antibody concentrations.

DISCUSSION

In the present study we have shown that the influenza A virus surface glycoproteins HA and NA are equivalent immunogens in primary and secondary immune responses when equal amounts of these antigens are administered as purified proteins separated from other viral proteins. Injection of purified HA or NA induced identical maximum levels of specific antibody in ELISA tests (Fig. 4). Because there are apparently no intrinsic differences between HA and NA

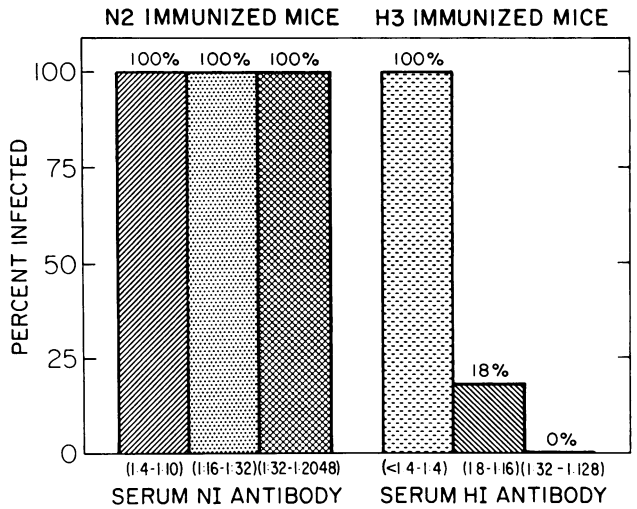

FIG. 5. Incidence of infection in mice immunized with N2 (left) or H3 antigen (right) and infected with H3N2 virus containing homologous antigen. Infection was defined by the detection of egg-infective virus in normal lungs in a 10^{-2} dilution of lung suspension.

in immunogenicity, the antigenic competition between HA and NA that results in greater B- and T-cell priming to HA antigen (32) is most likely a manifestation of the greater molar amount of HA found on the virion surface and hence its presentation in greater amount to the antigen recognition elements of the immune system. This is consistent with one of the features of antigenic competition between mixed antigens, i.e., a marked dependence on the relative proportions of the antigens in the mixture. The direction and extent of antigen competition are influenced by the dose of the antigens (4) and their relative molar ratios (39). Therefore, one way to avoid competition when mixed antigens are injected is to "balance" the mixture, i.e., adjust the proportions of the antigens so that competition does not occur. Such adjustment may assume particular importance when influenza virus vaccines are prepared for human use. Because there appear to be no intrinsic differences in the immunogenicity of HA and NA, balanced formulation of the two antigens might induce a more balanced anti-influenza

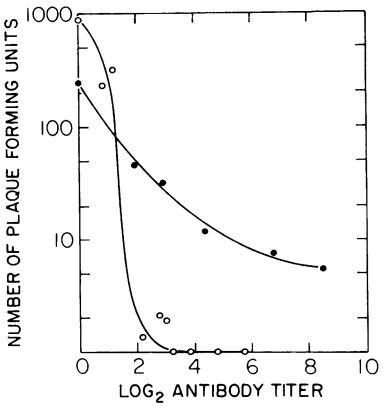

FIG. 6. Reduction in pulmonary virus levels. Plot of PFU versus amount of NI antibody in N2-immunized mice (●) and of HI antibody in H3-immunized mice (○) challenged with 100 50% mouse infective doses of H3N2 virus.

virus response, avoiding the HA-skewed response to the presently available inactivated influenza virus vaccines (22).

Was NA antibody response enhanced by small amounts of viral lipid in purified NA preparations? Earlier work has shown differences in the kinetics of primary response between antigen administered with adjuvant and antigen given without adjuvant (33). As evidenced by the identical kinetics of primary response to purified NA and HA (Fig. 2), the presence of some viral lipid in the NA preparation had no apparent effect on antibody response. This is consistent with observations suggesting that liposomal effects are not manifest on i.v. administration but depend on focal injection of antigen and lipid (11). Also, it was shown recently that lipids enhance specific T-cell subclasses rather than antibody formation per se (6, 13).

Although HA and NA induced equivalent levels of specific antibody and similar response kinetics in ELISA, some differences in antibody response curves were apparent when antibody responses to the two antigens were measured by NI or HI tests. However, using IgG and IgM isotype-specific reagents in the ELISA, similar patterns of response to HA and NA were found (data not shown). Measured by any method (ELISA, HI, or NI), antibody response to both HA and NA peaked 14 days after secondary antigenic stimulation. Thereafter, the degree of antibody decline varied in relationship to the immunizing antigen used (HA or NA) and the test used in the measurement of antibody. The trends observed for both increase and decline of antibody levels were essentially uninfluenced by the use of adjuvant except for the greater response in the adjuvant groups (Fig. 3).

The sustained levels of ELISA antibodies seen with both antigens (Fig. 4) probably reflect both the greater accessibility of epitopes in that assay system and its lack of dependence on the biologic activity of the antigens to which antibody is measured. Thus, changes in antibody avidity might go undetected in a system that scores multivalent antibody to multiple antigenic sites. In contrast, both HI and NI tests depend on competition between antibody and either erythrocyte receptors or enzyme substrate for combination with virion antigens and, understandably, might be more susceptible to decline of high-avidity antibodies. In fact, the rate of decline was greatest (down to 20% of the peak) for HI antibody, while the difference between NI and NA ELISA titers at 49 days after boost was minimal. Even though the NI test measures the blocking of biologic (i.e., enzymatic) function, it may be more comparable to the ELISA in that it may measure a broad range of antibodies to different sites, any of which can act by steric hindrance to block enzymatic activity (10, 40).

Challenge of mice immunized with isolated purified antigens confirms earlier evidence that NA-specific immunity is infection permissive over a broad range of antibody levels (2, 29, 35, 36) and that anti-HA immunity can prevent infection at relatively low antibody concentrations. That the effect of NA immunization may be mediated directly by circulating N2 antibody is suggested by previous evidence that passive administration of NA-specific antibody can ameliorate infection (36).

Precise measurement of infectious virus present in immunized animals revealed critical differences between immunity induced by HA and NA. Immunization with purified HA in a wide range of doses prevented infection by virus bearing an antigenically homologous HA, a situation analogous to the immunity established by vaccination with conventional influenza virus vaccines. Purified N2 NA induced immunity similar to that seen after vaccination by antigenically hybrid

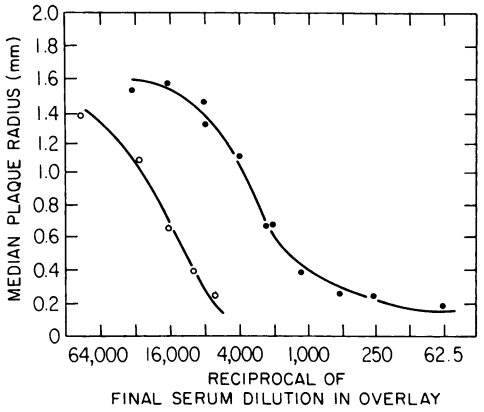

FIG. 7. Plot of median plaque radius of virus versus decreasing amount of specific antibody. Cells were infected with reassortant H1N2 virus; agar overlay contained either antibody to H1N1 virus (H1 specific) (○) or to H2N2 virus (N2 specific) (●). (Data replotted from Jahiel and Kilbourne [15]).

"NA-specific" vaccine viruses (2, 7, 21, 29). Although challenge virus contained N2 antigenically identical to the N2 NA used for priming, evidence of infection was found even in mice given the highest doses of NA.

Although immunity is seldom absolute, the nonpermissive, anti-infective immunity induced by the influenza virus HA appeared to be operative over a range of antibody concentrations, with manifest pulmonary infection occurring only at the lowest antibody levels. The wide window of permissiveness afforded by even the highest concentration of NA antibody was anticipated from earlier in vitro studies. Investigations by Jahiel and Kilbourne (15) on the influence of antiserum on H1N1 and H2N2 influenza viruses on the plaquing characteristics of an H1N2 antigenically hybrid virus showed kinetics in the reduction of virus replication remarkedly similar to those found in intact animals in the present study. A plot of virus plaque size against antibody concentration demonstrated that reduction in plaque size was principally a function of NA-specific antibody, while HA-specific antibody completely inhibited plaque formation except for a narrow zone of plaque size reduction near the titration endpoint (Fig. 7).

The contrasting effects both in vitro and in vivo of antibodies to HA and NA proteins most likely reflect the different biologic roles that HA and NA play in the virus replication cycle, HA being essential to virus attachment and initiation of infection and NA being inhibitory to virus release (25). The present study of purified surface antigens demonstrates that a cardinal element in establishing immunity to influenza virus is recognition of the surface glycoproteins of the virus. However, these findings do not negate the importance of cognate help in B- and T-cell interactions, in which humoral immune responses to HA or NA are supported by T cells that react with internal components of influenza virus (17, 32, 34). After sequential H3N2 virus infection of mice, T cells from these animals proliferated equally in response to heterosubtypic (H1N1) influenza virus internal proteins but varied in their response to HA and NA (17). The present study shows clearly that immunization with either antigen alone can alter the course of infection. Most important, we have shown unequivocally that NA is not merely a weaker HA in its immunizing effect on virus replication but that the partial immunity it induces permits subsequent (further immunizing) infection to occur even at the highest level of immunity induced. Thus, immunization with purified NA offers a unique approach to the prevention of influenza. It is likely that as a single viral protein NA will have less toxicity than whole-virus vaccines, live or inactivated. As isolated from detergent-disrupted virus, NA is highly antigenic, and adjuvants, although demonstrably enhancing, should not be required for its use in humans. Finally, the slower rate of antigenic drift of NA in both H1N1 (E. D. Kilbourne, unpublished data) and H3N2 (18) subtypes offers the prospect of longer-lasting immunity than that induced by the HA antigen or by conventional vaccines.

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