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Variation in the Divalent Cation Requirements of Influenza A Virus N2 Neuraminidases

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Influenza virus N2 neuraminidases were chromatographically purified from several vaccine candidate strains from 1957 to 1994. Enzymatic kinetic parameters and immunogenicity were tested for each strain. For each NA tested, with ionic strength held constant, Ca^{2+} or Mg^{2+} increased the initial rate of enzymatic activity. Earlier N2-NA strains had the highest initial velocity, V_{max}/K_m and V_{max}. There were significant differences among the influenza virus strains in enzymatic activity before and after addition of Ca^{2+} or Mg^{2+}: V_{max}/K_m varied from 0.54 M^{-1} s^{-1} to 0.88 M^{-1} s^{-1} and V_{max} varied from 2.45 s^{-1} to 4.3 s^{-1} before the addition of a divalent cation; and increased approximately 2-fold each of these kinetic parameters for each strain after the addition of exogenous Ca^{2+} or Mg^{2+}. Exhaustive dialysis with EDTA reduced the initial velocity of each strain with significant differences found among strains, with a range of 0.1% to 8% of original activity. Activity was partially restored by the addition of exogenous Ca^{2+} or Mg^{2+}, varying from 8% to 60% of pre-dialysis levels, but original rates were not achieved. This reduction in enzymatic activity for the tested strains (*i.e.*, A/Japan/57 and A/Johannesburg/94) was accompanied by a parallel decrease in NA-immunogenicity, with antibody response decreasing by as much as 76% as measured by NI titer, and ELISA titer decreasing by as much as 68%. The addition of Ca^{2+} or Mg^{2+} to the post-dialysis sample restored immunogenicity to as much as 80% of pre-dialysis NI titers and as much as 78% of pre-dialysis ELISA titers. Dialysis had the least effect on early strains as measured by enzymatic kinetic parameters and immunogenicity studies. Zn^{2+} had a slight inhibitory effect on the activity of all tested strains. Review of the nucleic acid sequence of each of these strains could not predict their enzymatic activity, immunogenicity or response to dialysis. If immunity against neuraminidase is desirable in vaccination against influenza, selection of vaccine candidate strains must include not only analysis of antigenic changes and sequence analysis but also enzymatic studies and determination of the requirement of divalent cations to maintain immunogenicity and activity during production.

Key words: influenza virus, neuraminidase.

Influenza A virus contains two membrane bound glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The NA (*N*-acetylneuraminyl glycohydrolase, EC 3.2.1.18) is a tetramer with a molecular mass of 240,000 daltons (*1*) that catalyses the cleavage of the terminal α-ketosidic linkage between sialic acid and adjacent sugar residues (*2*). This reaction allows transport of virions through mucin, elution of progeny virus particles from infected cells by destroying the HA receptor (*3*) and removal of sialic acid from the carbohydrate moiety of newly synthesized HA and NA, thereby preventing self-aggregation of virions (*4*).

Interest in the antigenicity and immunogenicity of NA has grown recently (*5–8*), while interest in NA as an enzyme has not been as great. Yet, with the possibility of regulation of the NA content in conventional trivalent inactivated influenza vaccines, understanding the enzymology and ion requirements of NA is imperative. Although the structure of NA has been extensively studied by X-ray crystallography (*1*, *9–10*) and nucleotide sequencing (*11*) and Ca^{2+} binding sites have been located in the crystal structure of N2-NA derived from the A/Tokyo/3/67 H3N2 strain (*1*) the roles of Ca^{2+} and other divalent cations in enzymatic activity are not well delineated. There have been several studies of NA activity and the requirement for divalent cations, especially Ca^{2+} (*12–16*), but none has examined the differences in ion requirement among strains within a subtype. Several groups have reported on the stimulatory effect of Ca^{2+} on influenza NA activity or stability (*12*, *14*, *15*, *17*). Wilson and Rafelson (*17*) reported that the addition of Ca^{2+} increased the V_{max} but had no effect on $k_{m,}$ and that the enzyme lacked an absolute requirement for Ca^{2+}. Others have shown that addition of Ca^{2+} did not alter the enzyme activity of N2-NA, but treatment with EDTA (*14*, *18*, *19*) inhibited the activity, which could be restored by the addition of Ca^{2+}, Mg^{2+}, or Mn^{2+}. Conversely, Baker and Gandhi (*20*) reported that N2-NAs were stable to dialysis

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against EDTA but that residual activity was strain-dependent. This report describes increased NA activity with all strains with increasing Ca^{2+} or Mg^{2+} concentrations, as well as a requirement for Ca^{2+} or Mg^{2+} in stabilizing the enzyme during EDTA treatment and a strain dependent restoration of activity with the addition of exogenous Ca^{2+} or Mg^{2+}. Enzymatic kinetic parameters varied among strains, with recent N2-strains requiring greater amounts of divalent cation for maximal activity and showing a greater decrease of activity after exhaustive EDTA dialysis. In contrast, Zn^{2+} had a slight inhibitory effect on NA activity for all strains tested. This report also demonstrates a dramatic decrease in the immunogenicity of the NA as a result of exhaustive dialysis, which was only partially restored by incubation with Ca^{2+}.

MATERIALS AND METHODS

Animals—Sixty inbred BALB/c female mice eight weeks old at the beginning of this experiment were used (Charles River Breeding Laboratories).

Viruses—The influenza A virus strains used in this study were: A/Japan/305/57 H2N2 [R]; A/Aichi/2/68 H3N2 [R]; A/Victoria/3/75 H3N2 [R]; A/Shanghai/11/87 H3N2 [R]; A/Beijing/32/92 H3N2 [R]; and A/Johannesburg/33/94 H3N2 [R] (supplied by E.D. Kilbourne, Valhalla, NY). All viruses selected for analysis were epidemiologically significant strains that were used in the vaccine for their respective year. These viruses are high-yield reassortants containing the genes coding for the surface antigens, HA and NA from their indicated strain and the genes coding for internal and nonstructural proteins are derived from A/PR/8/34 (*21*).

Purification of N2 Neuraminidase—All NAs used in this study were chromatographically purified from β-octylglucoside (Sigma Fine Chemical) disrupted influenza virions passed through a Sephadex-DEAE A-20 column (Pharmacia) as described (*21*). To remove metal ions, exhaustive dialysis against EDTA was used (*15*, *16*). Briefly, NA was dialyzed at 4°C against 1000 volumes of buffer containing 0.01 M Bes, 0.001 M EDTA, and 0.001 M β-mercaptoethanol (pH = 7.6) for 48 h with four changes, then against the same buffer without EDTA for an additional 48 h.

Fluorimetric Assay for Neuraminidase—NA activity was assayed by employing 2′-4′-methylumbelliferyl-α-D-*N*-acetylneuraminic acid substrate (Sigma Chemical, St Louis, Mo.) (*22*) in a modification of the method described by Myers *et al*. (*23*) To control for differences in specific activity among preparations, NA was incubated at 37°C at a concentration of 0.5 mU/100 μl in 40 mM sodium acetate (pH 5.9) with varying substrate concentrations between 50 and 500 μM in a final reaction volume of 100 μl. Enzyme activity (1 mU) is defined as the amount of neuraminidase required to liberate 1 nM of substrate per minute at 37°C in 50 mM $CaCl_2$ at pH 5.9. $CaCl_2$, $MgCl_2$, or $ZnCl_2$ were included in concentrations varying from 0 to 50 mM. Because increasing ionic strength also activates neuraminidase (*15*, *16*) buffers of constant ionic strength (I = 0.15 M with KCl) were designed and used as described by Ellis and Morrison (*24*). All reagents were chilled to 4°C prior to use. Reaction mixtures were incubated at 37°C for 8 min. The enzyme reaction was stopped with the addition of 200 μl of 250 mM glycine buffer adjusted to pH 10.4 with NaOH. Fluorescence was read on a Cambridge Technology Series 700 microplate fluorometer (λ_{excite} = 360 nm; $\lambda_{emission}$ = 430 nm). Reaction rates and velocities were linear with respect to time under the conditions described. Kinetic parameters were calculated from non-linear regression of initial velocities to the appropriate Michaelis-Menton equation.

Preparation of Laboratoryware—Prior to use, dialysis tubing was heated to a minimum of 75°C in 0.001 M EDTA in doubly deionized water for 4 h with four changes. All laboratoryware was pretreated to remove residual metal ions by soaking in 0.001 M EDTA for 30 min. then washing with 20% HNO_3. The labware was rinsed with doubly deionized water before use (*15*, *16*, *24*).

Serologic Methods—Neuraminidase inhibition (NI) assays were performed using purified $N2_{JAP}$-NA or purified $N2_{JH}$-NA in NI as described (*21*, *25*). NA-specific IgG subclass antibodies were measured using a previously described enzyme-linked immunoabsorbent assay (ELISA) utilizing purified $N2_{JAP}$-NA or purified $N2_{JH}$-NA as test antigens (*25*). All specimens were tested in triplicate. Serum specimens were obtained from each mouse by retro-orbital bleeding while under light Metofane (Mallinckrodt Veterinarian) anesthesia on the specified days. The sera were stored at –20°C.

Immunization Schedule—Sixty mice were randomly divided into six groups of ten animals. Mice were initially immunized *via* intraperitoneal (i.p.) injection as follows: 10 μg of purified non-dialyzed $N2_{JAP}$-NA (Group 1); 10 μg of purified dialyzed $N2_{JAP}$-NA (Group 2); 10 μg of dialyzed $N2_{JAP}$-NA incubated with 25 mM Ca^{2+} for 6 h prior to injection (Group 3); 10 μg of purified non-dialyzed $N2_{JH}$-NA (Group 4); 10 μg of purified dialyzed $N2_{JH}$-NA (Group 5); 10 μg of dialyzed $N2_{JH}$-NA incubated with 25 mM Ca^{2+} for 6 h prior to injection (Group 6). Animals received initial immunization on day 1, then a second i.p. injection of 1 μg of their respective vaccines 21 days after primary immunization. To assay the serologic response to immunization, all mice were bled 7 and 28 days after the boosting injection. All sera were then tested for NI antibodies and in ELISA.

Sequence Analysis—Protein sequences were downloaded from Genebank (*i.e.*, A/Japan/305/57; A/Aichi/2/68; A/Victoria/3/75; A/Shanghai/11/87; A/Beijing/32/92) or translated from unpublished nucleotide sequences (*i.e.*, A/Johannesburg/33/94) using Omiga 2.0 sequence analysis software (Oxford Molecular, Beaverton, OR). Protein sequences were aligned with ClustalW: amino acid (aa) positions determined by Varghese *et al*. (*1*) to correlate with Ca^{2+} binding, *N*-linked glycosylation, proton binding, sialic acid binding or cleavage were grouped for further analysis. The program NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc) utilizing the search formula Asn-Xaa-Ser/Thr was used to locate and predict potential N-glycosylation sites on the six NA strains used in this study. Additionally, regions of aa mutations were grouped by year, analyzed and correlation made between primary aa sequence changes and enzyme activity.

Statistical Analysis—Differences in NA-activity among strains and ion concentrations were analyzed by multivariate analysis of variance (mANOVA) and Tukey test

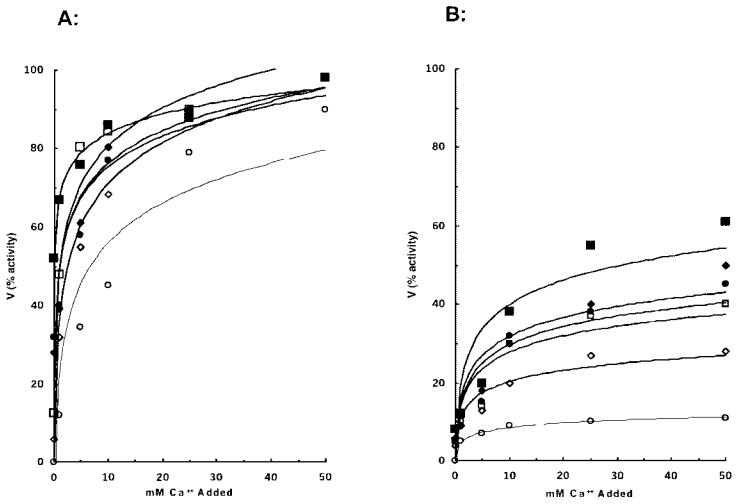

Fig. 1. **Effect of added Ca^{2+} on initial velocity of N2 neuraminidases.** Reactions were carried out with 100 μM 2′-4′-methylumbelliferyl-α-D-*N*-acetylneuraminic acid substrate and 0.5 mU of NA at pH 5.9. Initial velocities (v) are expressed as percent activity relative to that of non-dialyzed NA in the presence of 50 mM $CaCl_2$ (100% activity). (A): Non-dialyzed enzyme. (B): Dialyzed enzyme. Standard errors are reflected by the size of the symbol used: 1957 (solid circles), 1968 (solid squares), 1975 (solid triangles), 1987 (open circles), 1992 (open squares), and 1994 (open triangles). (C): Comparison of NA activity before and after exhaustive dialysis without added Ca^{2+}. Numbers are percent activity (SEM) as calculated above.

C:

Virus	‘57	‘68	‘75	‘87	‘92	‘94
Before	52 (4)	28 (5.6)	32 (3.2)	25 (6.7)	20 (2.3)	3 (0.7)
After	8 (2.1)	6 (0.8)	5 (1.1)	5 (0.8)	4 (1.0)	0.1 (0.1)

subsequent to ANOVA using the SAS statistical package for PCs (Cary, NC).

RESULTS

Effect of Ca^{2+} Concentration on Initial Velocity—Initial velocities of non-dialyzed N2-NA before the addition of exogenous Ca^{2+} varied significantly ($p < 0.001$) among strains (Fig. 1, A and C); the most recent strain tested, N2-NA 1994, had the lowest initial velocity of 3% of optimal activity, whereas the earliest strain examined, N2-NA 1957, had the greatest inital velocity, with 52% of optimal activity. The addition of up to 50 mM Ca^{2+} resulted in a 2- to 10-fold increase in initial velocity (Fig. 1A) with significant variation among strains ($p < 0.03$). The 1957 to 1992 N2 strains clustered together, and the 1994 strain had significantly the greatest requirement for exogenous Ca^{2+}.

Dialysis of the N2 neuraminidase with EDTA resulted in significant ($p < 0.001$) reduction of sialidase activity for all strains tested (Fig. 1, B and C). As expected, the degree of reduction varied with the strain tested. The residual enzymatic activity relative to optimal activity ranged from 8% (reduced from 52%) in the N2 1957 strain to 0.1% (reduced from 3%) in the N2 1994 strain before the addition of exogenous Ca^{2+} (Fig. 1C). The addition of up to 50 mM of Ca^{2+} to the reaction did not restore activity to pre-dialysis levels for any strain tested. Again there were strain dependent statistically significant differences ($p < 0.001$) in activity, with the peak increase in activity for each strain ranging from 60% of optimal activity for the N2 1957 strain to 8% of optimal activity for the N2 1994 strain. The clustering of the 1957 to 1992 strains non-dialyzed NA activity after the addition of exogenous Ca^{2+} was not observed after dialysis; indeed greater variation and separation of NA activity was demonstrated after exhaustive dialysis (Fig. 1B). The earliest N2 strain tested, N2 1957, required the least Ca^{2+} for restoration of activity, the 1968 and 1987 strains clustered together but were statistically significantly different ($p < 0.01$) from earlier strains, the 1992 strain ranked next and was significantly different ($p < 0.01$) from the N2 1994 strain which required the greatest amount of Ca^{2+} and had the smallest increase in activity.

Effect of Ca^{2+} Concentration on V and V/K—V and V/K were determined for each NA strain using non-dialyzed enzyme without exogenous Ca^{2+} and with 50 mM Ca^{2+} (Fig. 2). There were significant differences ($p < 0.001$) among the strains in V and V/K before and after addition of Ca^{2+}. Interestingly, there were no significant differences in k_m among strains or significant changes in k_m on

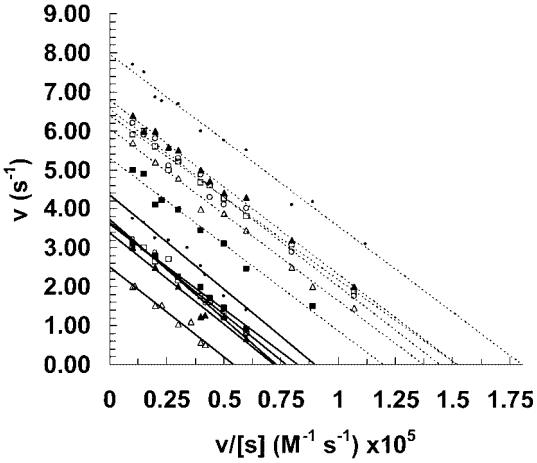

Fig. 2. **Effect of varying Ca^{2+} concentration on V and V/K.** Reactions were carried out at I = 0.15 M with non-dialyzed enzyme in the presence of no added Ca^{2+} (solid lines) or 50 mM Ca^{2+} (dashed lines). For each NA strain standard errors are reflected by the size of the symbol used: 1957 (solid circles), 1968 (solid squares), 1975 (solid triangles), 1987 (open circles), 1992 (open squares), and 1994 (open triangles).

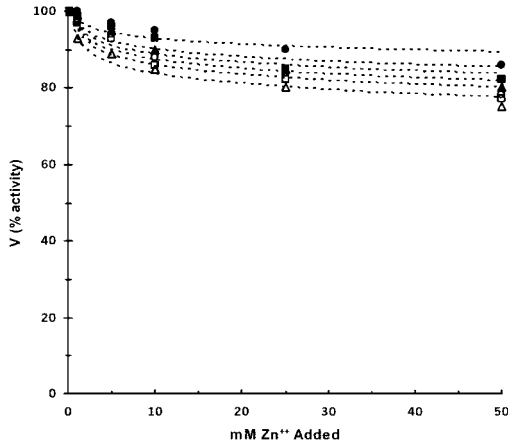

Fig. 3. **Effect of added Zn^{2+} on initial velocity of N2 neuraminidases.** Reactions were carried out with 100 μM 2′-4′-methylumbelliferyl-α-D-*N*-acetylneuraminic acid substrate and 0.5 mU of non-dialyzed NA at pH 5.9. Initial velocities (v) are expressed as percent activity relative to that of non-dialyzed NA in the absence of Zn^{2+} (100% activity). (A): Non-dialyzed enzyme. (B): Dialyzed enzyme. Standard errors are reflected by the size of the symbol used: 1957 (solid circles), 1968 (solid squares), 1975 (solid triangles), 1987 (open circles), 1992 (open squares), and 1994 (open triangles).

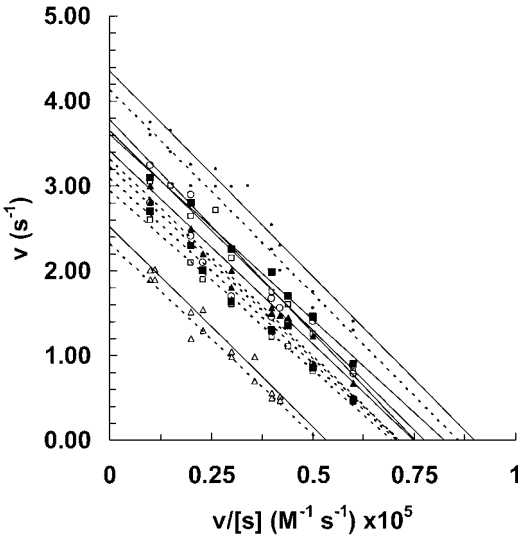

Fig. 4. **Effect of varying Zn^{2+} concentration on *V* and *V/K*.** Reactions were carried out at I = 0.15 M with non-dialyzed enzyme in the presence of no added Zn^{2+} (solid lines) or 25 mM Zn^{2+} (dashed lines). For each NA strain standard errors are reflected by the size of the symbol used: 1957 (solid circles), 1968 (solid squares), 1975 (solid triangles), 1987 (open circles), 1992 (open squares), and 1994 (open triangles).

addition of Ca^{2+}. V without Ca^{2+} was 2.45, 3.39, 3.55, 3.54, 3.67, and 4.3 s^{-1} for the 1994, 1992, 1987, 1975, 1968, and 1957 strains, respectively; and these values increased approximately 2-fold (range 1.8 to 2.2) to 5.2, 6.0, 6.51, 6.2, 6.52, and 8.0 s^{-1}, respectively, on addition of 50 mM Ca^{2+}. There were no statistically significant differences among strains in the relative increase of V with Ca^{2+}. In comparison, V/K without Ca^{2+} were: 0.54, 0.73, 0.74, 0.77, 0.79, and 0.88 M^{-1} s^{-1} for the 1994; 1992; 1987; 1975; 1968, and 1957 strains, respectively; and again these values increased approximately 2-fold (range 2.0 to 2.2) to 1.2 M, 1.37, 1.45 M, 1.56, 1.56, and 1.8 M^{-1} s^{-1}, respectively, on addition of 50 mM Ca^{2+}. There were no statistically significant differences among strains in the relative increase of V/K with Ca^{2+}.

Effects of Mg^{2+} Concentration on Initial Velocity—Identical studies were performed using Mg^{2+} in place of Ca^{2+} (data not shown). NA activities determined with Mg^{2+} in the reaction mixtures were statistically indistinguishable from comparable reactions containing Ca^{2+}. Initial velocities of non-dialyzed N2-NA before the addition of Mg^{2+} varied significantly among strains ($p < 0.003$), with the most recent strain, 1994 N2-NA, having the lowest activity with 1% of optimal activity, and the 1957 N2-NA strain having the greatest activity with 52% of optimal activity. The addition of up to 50 mM $MgCl_2$ resulted in 2- to 10-fold increase in initial velocity, and as observed with Ca^{2+} there were significant differences ($p < 0.01$) among strains. The N2 strains 1957 to 1992 clustered together, and the 1994 strain had significantly the smallest increase in initial velocity in response to exogenous Mg^{2+}. The 1957 to 1992 strains had the lowest requirement for additional Mg^{2+}, and the N2 1994 strain had the greatest requirement for exogenous Mg^{2+} (data not shown).

Dialysis with EDTA resulted in significant ($p < 0.001$) reduction of sialidase activity for all strains tested. The degree of reduction varied with the strain tested. The residual enzymatic activity relative to optimal activity ranged from 6% (reduced from 52%) in the N2 1957 strain to 0.1% (reduced from 1%) in the N2 1994 strain before the addition of exogenous Mg^{2+} (Fig. 1C, strains tested before addition of either cation). As observed with Ca^{2+}, the addition of up to 50 mM Mg^{2+} to the reaction did not restore activity to pre-dialysis levels for any strain tested. Again there were strain-dependent statistically significant differences ($p < 0.001$) in activity, with the peak increase in activity for each strain ranging from 63% of optimal activity for the N2 1957 strain to 7.8% of optimal activity for the N2 1994 strain.

Effect of Mg^{2+} Concentration on V and V/K—V and V/K were determined for each strain using non-dialyzed enzyme without exogenous Ca^{2+} or Mg^{2+} and with 50 mM Mg^{2+} (data not shown). NA activities determined with Mg^{2+} in the reaction mixture were statistically indistinguishable from comparable reactions containing Ca^{2+}. There were significant differences ($p < 0.001$) among the strains in V and V/K before and after addition of Mg^{2+}. Interestingly, as with Ca^{2+} there were no significant differences in k_m among strains or changes in k_m on addition of Mg^{2+}. V without Mg^{2+} was 2.45, 3.39, 3.55, 3.54, 3.67, and 4.3 s^{-1} for the 1994, 1992, 1987, 1975, 1968, and 1957 strains, respectively; and these values increased approximately 2-fold (range 1.6 to 2.3) to 5.2, 5.9, 6.51, 6.50, 6.52, and 7.8 s^{-1}, respectively, on addition of 50 mM Mg^{2+}. There were no statistically significant differences among strains in the relative increase of V with Mg^{2+}. In comparison, V/K without Mg^{2+} was 0.54 M, 0.73 M, 0.74 M, 0.77 M, 0.79, and 0.88 M^{-1} s^{-1} for the 1994, 1992, 1987, 1975, 1968, and 1957 strains, respectively; and these values again increased approximately 2-fold (range 2.0 to 2.1) to 1.21, 1.31 M, 1.44, 1.51 M, 1.53, and 1.75 M^{-1} s^{-1}, respectively, on addition of 50 mM Mg^{2+}. There were no statisti-

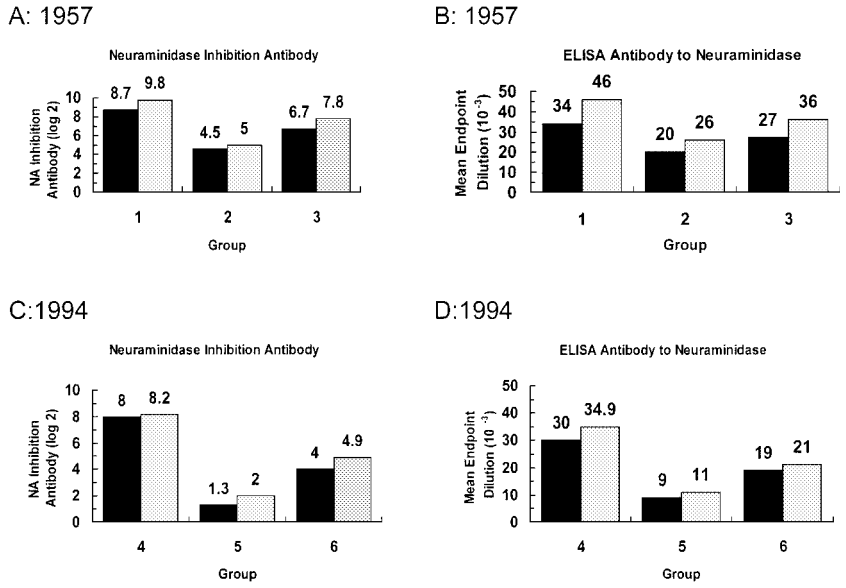

Fig. 5. **Effect of Dialysis on NA immunogenicity.** (A and C): Immune response measured by neuraminidase inhibition antibody. Numbers are $\log_2$ mean dilution endpoints of triplicate wells. (B and D): Immune response measured by ELISA antibody to the appropriate purified neuraminidase. Numbers represent the mean endpoint titers from triplicate wells. The endpoint was taken as the highest dilution of antibody producing an absorbance value threefold above background (absorbance value from an antibody free well). Significant differences were tested by ANOVA ($p < 0.001$) and Tukey test subsequent to ANOVA as described in the text. For all panels, mice were immunized as follows: Group 1: non-dialyzed A/Japan/1957 NA; Group 2: dialyzed A/Japan/1957 NA; Group 3: dialyzed A/Japan/1957 NA incubated with 25 mM Ca^{2+} before assay; Group 4: non-dialyzed A/Johannesburg/1994 NA; Group 5: dialyzed A/Johannesburg/1994 NA; Group 6: dialyzed A/Johannesburg/1994 NA incubated with 25 mM Ca^{2+} before assay. Black bars are Day 7 antisera, stippled bars are Day 28 antisera.

cally significant differences among strains in the relative increase of V/K with Mg^{2+}.

Effect of Zn^{2+} Concentration on Initial Velocity—The addition of Zn^{2+} to reaction mixtures of non-dialyzed NA was slightly inhibitory in contrast to the effect of both Ca^{2+} and Mg^{2+}. The initial velocity of all strains was decreased (Fig. 3), maximal inhibition of the NA (~80% of optimal activity) was observed at 10 mM of Zn^{2+}. There were, however, no statistically significant differences among strains in percent reduction of NA activity.

Effect of Zn^{2+} Concentration on V and V/K—The addition of exogenous Zn^{2+} to the reaction mixtures resulted in slight decreases in both V and V/K for all strains. Although as before there was significant variation in the initial V and V/K among strains, there were no statistically significant differences in percent decrease in activity (Fig. 4). V for each strain without Zn^{2+} was 2.45, 3.39, 3.55, 3.54, 3.67, and 4.3 s^{-1} for the 1994, 1992, 1987, 1975, 1968, and 1957 strains, respectively; and these values decreased approximately 20% to 30% for each strain tested to 2.15, 2.95, 3.12, 3.29, 3.30, and 4.09 s^{-1}, respectively, on addition of 25 mM Zn^{2+}. There were no statistically significant differences among strains in relative decrease of V with Zn^{2+}. In comparison, V/K without Zn^{2+} was 0.54, 0.73, 0.74, 0.77, 0.79, and 0.88 M^{-1} s^{-1} for the 1994, 1992, 1987, 1975, 1968, and 1957 strains, respectively; and again these values decreased to 0.51 M^{-1} s^{-1}, 0.68 M^{-1} s^{-1}, 0.70, 0.71, 0.712, and 0.80 M^{-1} s^{-1}, respectively, on addition of 25 mM Zn^{2+}.

Effect Ca^{2+} on NA Immunogenicity—Non-dialyzed NA, dialyzed NA and dialyzed NA incubated with Ca^{2+} from the A/Japan/1957 and A/Johannesburg/1994 strains were injected into immunologically naive mice. The immune response was measured by NI antibody assay and NA-specific ELISA (Fig. 5). Non-dialyzed NA from both strains was highly immunogenic. Dialysis resulted in a significant decrease ($p < 0.001$) in immunogenicity of both strains, and the decrease in antibody response was significantly greater for the A/Johannesburg/1994 NA in both assays: 76% (8.2 to 2 log2) and 49% (5 to 7.8 log2) decrease in NI titer and 68% (34.9 to 11 × 10–3) and 43% (46 to 26 × 10–3) decrease in ELISA titer, for the JH/94 and Japan/57 viral strains, respectively. Interestingly, incubation of dialyzed NA with Ca^{2+} resulted in a partial restoration of immunogenicity of both strains tested to 60% (2 to 4.9 log2) and 80% (5 to 7.8 log2) of non-dialyzed NI titers and 60% (11 to 21 × 10–3) and 78% (26 to 36 × 10–3) of non-dialyzed ELISA titers for the JH/94 and Japan/57 strains, respectively. The Ca^{2+}-restored A/Japan/1957 NA induced a statistically significant ($p < 0.01$) greater antibody response than the A/Johannesburg strain. The immunogenicity studies parallel the enzymatic kinetic parameters in that the earlier A/Japan/1957 N2-NA enzymatic activity was more resilient to Ca^{2+} removal by exhaustive dialysis than the more contemporary strain, A/Johannesberg/1994 N2-NA.

Sequence Analysis—NA amino acid sequences were analyzed (Table 1) for changes in previously defined amino acids and regions determined to correlate with Ca^{2+} binding (aa113, aa141), proton binding (aa153), sialic acid binding (aa118, aa119, aa152, aa178, aa222, aa292) or catalysis (aa279, aa276, aa277) (*1*). The only aa change in any of these regions was identified in the Ca^{2+} binding site, aa 141: at this position the 1957 and 1968 strains have an *asp*, the 1975 strain a *glu*, and the 1987 through 1994 have an *asn*. Not unexpectedly there were numerous aa changes in putative antigenic sites. Up to eight potential *N*-linked glycosylation sites were predicted for any given strain, with seven of those sites being conserved across all strains: (aa 61, aa70, aa86, aa146, aa200, aa234, aa402). The NA isolated from A/Aichi/2/68 had an additional site identified at aa 69, which was predicted to be *N*-glycosylated, while A/Beijing/32/92 and A/Johannesburg/33/94 had a potential site located at aa 329, which was predicted to not be *N*-glycosylated.

Table 1. **1957–1994 N2 neuraminidase amino acid changes.** Shown are all amino acid changes in the studied N2 neuraminidases from 1957–1994. Sequences are either unpublished or downloaded from GenBank. Highlighted are changes in residues that occur in previously defined sites of interest. See text for further detail.

AA	Japan/305/57	Aichi/2/68	Victoria/3/75	Shanghai/11/87	Beijing/32/92	Johannesburg/33/94	Comments
20	V	V	I	I	I	I	
30	V	A	V	V	V	V	
40	Y	H	Y	Y	Y	Y	
43	D	D	D	S	S	N	
46	A	A	A	P	P	P	
47	N	S	N	N	N	N	
51	M	M	M	M	I	M	
52	P	P	P	P	P	L	
54	E	E	E	E	Q	E	
56	I	I	I	T	T	T	
69	T	N	T	T	T	T	Potential N-gly Site
80	K	E	K	K	K	E	
81	L	V	L	L	L	L	
82	V	V	V	V	V	A	
93	K	Q	K	K	K	K	
127	G	G	G	G	S	G	
141	**D**	**D**	**E**	**N**	**N**	**N**	**Ca^{2+} Binding Site**
143	K	K	K	R	R	R	
147	D	G	D	D	D	D	
149	I	I	I	V	V	V	
153	**I**	**I**	**I**	**T**	**T**	**T**	**Antigenic Site VII**
155	H	H	H	Y	Y	Y	
172	R	K	R	K	K	K	
176	I	V	I	I	I	I	
183	**R**	**C**	**C**	**C**	**C**	**C**	**Disulfide Bonding**
197	**D**	**D**	**Y**	**H**	**H**	**H**	**Antigenic Site VII**
199	**K**	**R**	**K**	**K**	**E**	**E**	**Antigenic Site VII**
220	Q	Q	Q	K	K	K	
248	G	G	G	E	E	E	
258	E	K	E	E	E	E	
262	I	I	I	I	V	I	
275	V	I	V	V	V	V	
286	G	D	G	G	G	G	
302	V	V	V	I	I	I	
303	A	I	V	V	V	V	
307	M	M	V	V	V	V	
308	E	E	K	K	K	K	
313	D	D	D	V	V	V	
315	S	G	S	S	S	S	
328	**N**	**N**	**K**	**K**	**K**	**K**	**Antigenic Site I**
329	**D**	**D**	**N**	**N**	**N**	**N**	**Ag. Site I, Potential N-gly Site**
331	**R**	**S**	**R**	**R**	**S**	**S**	**Antigenic Site I**
334	**N**	**N**	**S**	**S**	**S**	**S**	**Antigenic Site I**
336	**N**	**N**	**Y**	**Y**	**Y**	**Y**	**Antigenic Site I**
338	R	R	R	R	R	W	
339	**N**	**D**	**N**	**N**	**N**	**N**	**Antigenic Site II**
344	**R**	**R**	**K**	**K**	**K**	**K**	**Antigenic Site II**
346	**N**	**N**	**N**	**S**	**S**	**G**	**Antigenic Site II**
347	**Q**	**P**	**H**	**H**	**H**	**H**	**Antigenic Site II**
356	N	N	D	D	D	D	
358	D	D	N	N	N	N	
367	**S**	**N**	**S**	**G**	**S**	**S**	**Antigenic Site III**
368	**K**	**K**	**E**	**E**	**E**	**E**	**Antigenic Site III**
369	**D**	**E**	**D**	**E**	**E**	**E**	**Antigenic Site III**
370	**L**	**S**	**S**	**L**	**L**	**L**	**Antigenic Site III**
385	T	T	T	T	T	K	
390	S	S	L	L	L	L	
392	I	V	I	I	I	I	
400	**S**	**N**	**S**	**S**	**S**	**R**	**Antigenic Site IV**
401	**D**	**N**	**A**	**G**	**G**	**G**	**Antigenic Site IV**
403	**R**	**W**	**R**	**R**	**R**	**R**	**Antigenic Site IV**
410	F	F	F	F	F	S	
431	**K**	**P**	**E**	**E**	**K**	**K**	**Antigenic Site V**
435	R	R	R	R	K	E	
462	A	A	A	A	G	A	
463	D	N	D	D	D	D	
466	F	F	L	L	L	L	

*Ag. = Antigenic, N-gly = *N*-glycosylation.

DISCUSSION

The N2-neuraminidase of influenza virus has long been of interest as a component of the conventional influenza vaccine. The activation and stabilization of the enzyme by divalent cations, Ca^{2+} in particular, has been examined by several investigators (*12*, *14–16*, *20*) with inconsistent findings. The findings of this report may help explain some of these disparate results, by demonstrating strain-dependent differences in V_{max}, V_{max}/K_m and divalent cation requirement for activity and immunogenic stabilization. For each strain, k_m was nearly identical, yet there were significant differences among strains prior to the addition of exogenous divalent cations in V (range: 2.45 to 4.35 s^{-1}) and V/K (range: 0.54 to 0.88 M^{-1} s^{-1}). Consistent with the findings of Chong *et al*. (*15*) with the single A/Tokyo/3/67 N2-NA strain, addition of Ca^{2+} or Mg^{2+} to the reaction mixtures increased the initial velocity of all the neuraminidase strains tested, but initial velocities varied significantly among strains, before and after the addition of Ca^{2+} or Mg^{2+}. Additionally, it was observed that the addition of Ca^{2+} or Mg^{2+} to the reaction resulted in an enhanced rate of substrate binding, as measured by increased V_{max}/K_m, again with significant strain-dependent differences; and both V and V/K were increased 2-fold for each strain. V and V/K were greater in the older strains tested (*i.e*., 1957) and declined steadily with more recent strains (*i.e.*, 1994). Our data indicate that in enhancing neuraminidase activity and stabilizing the enzyme, Ca^{2+} and Mg^{2+} are interchangeable under the conditions described here. In contrast, the addition of Zn^{2+} was slightly inhibitory to the N2-NA, but without differences among strains. Ca^{2+} and Mg^{2+} are both class IIA active metals and despite their difference in atomic mass and radius they have similar electron configurations (Ca = $[Ar]4s^2$; Mg = $[Ne]3s^2$). In contrast, Zn^{2+} is a class IIB transition metal with a very different electron structure (Zn = $[Ar]4s^23d^{10}$). Thus, it appears that enhancement of NA's activity is not a nonspecific divalent cation effect: the protein *requires* Ca^{2+} or Mg^{2+}. The differences in activity and cation ion requirement among viral strains probably reflect the results of strong evolutionary pressure on the viral protein. The NA must alter antigenic epitopes ringing the enzymatically active site (*10*) in response to host immune pressure yet maintain a functional enzymatic activity. Review and analysis of the amino acid sites implicated in Ca^{2+} binding (Table 1) for all the strains used in this study failed to reveal consistent amino acid sequence changes that could be used to predict the observed variation in enzymatic activity and cationic requirement. The only aa change among these NA strains in previously defined functional sites was in a putative Ca^{2+} binding site, aa 141. The change, a negatively charged *asp* residue in early viral strains (1957–1968) to positively charged *glu* (1975) or *asn* (1987–1994) may affect catalytic activity of the NA by changes in Ca^{2+} binding. This amino acid change is however, not consistent with the statistical grouping of kinetic activities of these strains. This posits two different conclusions: first, that this change at aa 141 is irrelevant; or second, that genetic haplotypes exist, and in that the change at aa 141 is necessary but not sufficient to explain the changes in kinetic activity, another mutation or mutations must occur elsewhere, and in this case in a site not previously known to affect enzyme activity. Similarly, Takahashi *et al*. (*26*) demonstrated that neuraminidase from duck and human pandemic influenza A strains maintain enzymatic activity under low pH (<4.5) conditions, whereas activity was lost in non-pandemic human and pig strains; sequence comparisons implicated several amino acid sites (153, 253, 307, 329, 344, 347, 356, 368, 390, and 431) that may be associated with low pH stability of the duck NA. The exact role that Ca^{2+} plays in the catalysis of sialic acid residues by NA is unknown. However, the nearly identical k_ms from NAs of viruses from widely spaced years suggest that Ca^{2+} does not play a role in either binding of sialic acid (*i.e.*, formation of enzyme-substrate complex) or hydrolysis of sialic acid (*i.e*., catalysis). Analysis of NA amino acid sequences through the years 1957 to 1994 demonstrate many amino acid changes (Table 1); however, there is no single mutation or set of mutations that can be correlated to the changes seen in NA enzymatic activity over the same period. What is it about the A/Johannesburg/33/94 NA that makes it sensitive to Ca^{2+} concentration and chelation? For each NA, there are multiple mutations that are the result of immunologic pressure and the requirement to preserve enzymatic structure and function. Do these same pressures contribute to the varying requirement for Ca^{2+} among strains? The data from this study indicate a general increasing dependence on Ca^{2+} for the enzymatic activity of NA, and by a mechanism not clearly understood using amino acid sequence data, Ca^{2+} appears to play an important role in stabilizing the NA's tertiary and quaternary structure.

The effects of removing divalent cations by dialysis are not limited to changes in enzymatic activity. Dialysis resulted in an 84% (52% to 8%) decrease in the 1957 strain's initial velocity and a 96% (3% to 0.1%) decrease in the 1994 strain's (Fig. 1C). Significant reduction in immunogenicity was observed after the removal of divalent cations by exhaustive dialysis; and the magnitude of this immunogenic reduction and subsequent recovery of immunogenicity after incubation with Ca^{2+}, albeit not a linear relationship, was strain dependent (Fig. 5). These differences in immunogenicity were measured by two distinct antibody assay systems: NI and ELISA. The NI test is a functional assay, dependent upon the enzymatic activity of the NA: it requires the binding of antibodies to antigenic sites near or in the catalytic site of intact NA tetramers, thereby preventing substrate binding. In contrast, the ELISA measures antibody binding to epitopes anywhere on the surface of the NA, including but obviously not limited to the enzymatically active site. The reduction in NA immunogenicity after dialysis and its partial restoration by the addition of Ca^{2+} was measured in parallel with NI and ELISA antibody titers. In view of the relationship of NA activity and immunogenicity, the reason for the concordance of ELISA and NI results is not clear. The most likely explanation would appear to posit a partially reversible conformational change in the entire NA tetramer influencing more than the epitopes surrounding the enzymatically active site accompanying removal and subsequent restoration of Ca^{2+}.

The strain dependent requirement of Ca^{2+} for optimum enzymatic activity and for the retention of NA immuno-

genicity is of potential importance to influenza vaccine manufacturers. Current vaccine preparation methods do not control for the presence or concentration of divalent cations during processing; indeed, this may in part account for the poor NA-immunogenicity of conventional influenza vaccines (*5*, *25*, *27*). Therefore, it is proposed that those responsible for strain selection and manufacturing determine the kinetic parameters of the neuraminidase for each potential vaccine candidate, perhaps simultaneously with the antigenic selection of strains, in order to select an antigenically appropriate but more enzymatically and more immunogenically robust NA. Furthermore, because of the possible emergence of a quasi-species variant within a given strain, manufacturers may wish to continually monitor the NA activity of the vaccine preparation.

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