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Variation in the Divalent Cation Requirements of Influenza A Virus N1 Neuraminidases

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Enzymatic kinetic parameters of influenza A virus N1 neuraminidases (NA) chromatographically purified from several vaccine candidate strains were tested. With ionic strength held constant, Ca^{2+} or Mg^{2+} increased the initial rate of enzymatic activity. The 1934 and 1943 strains had statistically significant highest initial velocities, V_{max}/K_m and V_{max}. There were no significant differences among the influenza virus strains from 1947 to 1991. Measured K_m for the 1943 strain (6.2×10^{-5} M) was significantly higher than other strains ($3.1–4.7 \times 10^{-5}$ M). V_{max}/K_m varied from 0.78 M^{-1} s^{-1} to 0.91 M^{-1} s^{-1} and V_{max} varied from 3.0 s^{-1} to 5.5 s^{-1} before the addition of a divalent cation and increased approximately 2-fold for each of these kinetic parameters for each strain after the addition of exogenous Ca^{2+} or Mg^{2+}. Dialysis reduced the initial velocity and immunogenicity of each strain with significant differences found among strains. Enzymatic activity and immunogenicity were partially restored by the addition of exogenous Ca^{2+}. Nucleic acid sequence analysis could not predict these differences. Selection of vaccine strains must include analysis of antigenic changes, but also enzymatic studies and determination of the requirement of divalent cations to maintain immunogenicity and activity during production.

Key words: influenza virus, N1, neuraminidase.

Neuraminidase (EC 3.2.1.18, *N*-acetylneuraminyl glycohydrolase) (NA) is one of two membrane-bound glycoproteins on the surface of influenza virus particles (*1*). NA catalyzes the cleavage of the terminal α-keto group of *N*-neuraminic acid and adjacent sugar residues (*2–4*). Physiologically the NA works in concert with hemagglutinin (HA), which binds terminal sialic acid residues ubiquitous in the mucus of the respiratory tract, or present on the surface glycoproteins of other virions. NA disrupts these interactions, prevents aggregation of emerging virions and facilitates infection by clearing paths through respiratory mucus (*1–4*). There are nine NA antigenic subtypes (N1–N9); all are found in birds, only two in humans (N1, N2), swine and horses (*1*, *2*). Antigenicity and immunogenicity of the N1 and N2 subtypes have been examined over the past three decades (*4–8*); similarly, NA structure has been extensively studied with nucleotide sequence data from a large number of antigenic variants (*9*, *10*) and to a lesser extent X-ray crystallography (*1*, *9–11*). However, many aspects of NA biochemistry and enzymology, such as the role of divalent cations in catalysis, stabilizing the tetrameric structure and preserving immunogenicity, remain less well studied (*12–21*).

Our laboratory has reported previously on the variation in enzymatic activity and divalent cation requirement among N2 influenza strains (*21*). Enzymatic parameters V_{max} and V_{max}/K_m varied among N2-strains, with a trend toward recent strains requiring greater amounts of divalent cation for maximal activity, a greater decrease of activity after exhaustive EDTA dialysis or incubation with Zn^{2+} and 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+} (*21*). Baker and Gandhi (*20*) showed that early N1-strains were unstable at 37°C in the absence of Ca^{2+}, whereas N2-neuraminidases were stable. Furthermore, the amount of Ca^{2+} required for maximal activity varied among these early N1-strains more than 10-fold (*14*). Many 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+} to N1 or N2-NA 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 NA, but treatment with EDTA inhibited the activity, which could be restored by the addition of Ca^{2+}, Mg^{2+}, or Mn^{2+} (*14*, *18*, *19*). Conversely, Baker and Gandhi (*20*) reported that N2-NAs were stable to dialysis against EDTA, but that residual activity was strain-dependent. None of these studies has examined the differences in ion requirement among recent strains within the N1 subtype or more than two N1-NA strains.

As part of an ongoing effort to study the biology of influenza NA (*21*), this report describes increased NA activity with the 1934 to 1991 strains with increasing Ca^{2+} or Mg^{2+} concentrations. There was 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

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of exogenous Ca^{2+} or Mg^{2+}. Enzymatic kinetic parameters varied among strains, with recent N1-strains requiring greater amounts of divalent cation for maximal activity and showing a greater decrease of activity after exhaustive EDTA dialysis. The 1943 NA strain was an exception in that its K_m was significantly higher than other strains tested and the 1943 strain had only a modest increase in activity with the addition of Ca^{2+}. This report also demonstrated a dramatic decrease in the immunogenicity of the N1-NA after exhaustive EDTA 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/Puerto Rico/8/34 H1N1; A/Weiss/43 H1N1; A/Fort Monmouth/1/47 H1N1; A/USSR/90/77 H1N1 [R]; A/Taiwan/1/86 H1N1 [R]; and A/Texas/36/91 H1N1 [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. A/Puerto Rico/8/34 and A/Weiss/43 viruses were initially isolated in ferrets (the principal experimental host at that time); all other viral strains were initially isolated from clinical specimens in embryonated chicken eggs. The 1977, 1986 and 1991 virus strains are high-yield reassortants containing the genes coding for the surface antigens, hemagglutinin (HA) and neuraminidase (NA) from their indicated strain and the genes coding for internal and nonstructural proteins are derived from A/Puerto Rico/8/34 (*22*). All viruses were propagated in eggs and stored at –70°C until used.

Purification of N1 Neuraminidase—All NAs used in this study were chromatographically purified from α-octylglucoside (Sigma Fine Chemical)–disrupted influenza virions passed through a Sephadex-DEAE A-20 column (Pharmacia) as described (*22*). To remove metal ions, exhaustive dialysis against EDTA was used (*15*, *16*). Briefly, NA was dialyzed at 4°C against 1,000 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.) (*23*) in a modification of the method described by Myers *et al*. (*24*) 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. $CaCl_2$ or $MgCl_2$ was 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 (*25*). 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-Menten equation. Graphs were plotted using Curvefit (Graphpad Software, Melbourne, Australia)

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*, *25*).

Serologic Methods—Neuraminidase inhibition (NI) assays were performed using purified $N1_{PR8}$-NA or purified $N1_{Texas}$-NA in NI as described (*22*, *26*). NA-specific IgG subclass antibodies were measured using a previously described enzyme-linked immunoabsorbent assay (ELISA) utilizing purified $N1_{PR8}$-NA or purified $N1_{Texas}$-NA as test antigens (*26*). 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 $N1_{PR8}$-NA (Group 1); 10 μg of purified dialyzed $N1_{PR8}$-NA (Group 2); 10 μg of dialyzed $N1_{PR8}$-NA incubated with 25 mM Ca^{2+} for 6 h prior to injection (Group 3); 10 μg of purified non-dialyzed $N1_{Texas}$-NA (Group 4); 10 μg of purified dialyzed $N1_{Texas}$-NA (Group 5); 10 μg of dialyzed $N1_{Texas}$-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 NA-specific ELISA.

Sequence Analysis—Protein sequences were downloaded from GenBank or translated from unpublished nucleotide sequences obtained in our laboratory using Omiga 2.0 sequence analysis software (Oxford Molecular, Beaverton, OR). Protein sequences were aligned using ClustalW alongside and numbered with respect to A/Tokyo/3/67 (H3N2). Amino acid (aa) positions determined by Varghese *et al*. (*1*) and Burmeister *et al.* (*27*) 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 and correlations between primary aa sequence changes and enzyme activity were analyzed.

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

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

RESULTS

Effect of Ca^{2+} Concentration on Initial Velocity—Initial velocities of non-dialyzed N1-NA before the addition of exogenous Ca^{2+} had no significant differences among strains (Fig. 1, A and C); the A/Weiss/43 strain, N1-NA, had the greatest requirement to achieve maximum velocity. 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 ($p < 0.01$). The 1947 to 1991 N1 strains clustered together, and the 1943 strain had significantly the greatest and the 1934 strain the least requirement for exogenous Ca^{2+}. Dialysis of the N1 neuraminidase with EDTA resulted in significant ($p < 0.001$) reduction of sialidase activity for all strains tested (Fig. 1B, Table 4). The degree of reduction varied with the strain tested. The residual enzymatic activity relative to optimal activity ranged from 7% (reduced from 52%) in the N1 1934 strain to 2% (reduced from 20%) in the N1 1991 strain before the addition of exogenous Ca^{2+} (Table 4). The addition of up to 50 mM 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 40% of optimal activity for the N1 1934 strain to an average of 18% of optimal activity for the other N1 strains tested. The N1-NA of the 1934 strain did not cluster with the 1943 to 1991 strains non-dialyzed NA activity after the addition of exogenous Ca^{2+}; indeed greater variation and separation of NA activity was demonstrated after exhaustive dialysis (Fig. 1B). The earliest N1 strain tested, A/PR/8/34, required the least Ca^{2+} for restoration of activity; and the 1943 to 1991 strains clustered together.

Effect of Ca^{2+} Concentration on V and V/K—V_{max} and V_{max}/K_m were determined for each NA strain using non-dialyzed enzyme without exogenous Ca^{2+} and with 50 mM Ca^{2+} (Fig. 2). These data are summarized in Table 1. There were significant differences ($p < 0.01$) among the strains in V_{max} and V_{max}/K_m before and after addition of Ca^{2+}. Interestingly, there was a single N1-NA, from the 1943 strain that had a significantly different K_m from other tested strains (6.3 *vs.* 4.0 s^{-1}). There were no significant changes in a given N1-NA strain's K_m after the addition of Ca^{2+} (Table 1). As summarized in Table 1, V_{max} without Ca^{2+} were 4.37, 5.50, 3.38, 3.63, 3.70 and 3.10 s^{-1} for the 1934, 1943, 1947, 1977, 1986 and 1991 strains, respectively; and with the exception of the 1943 strain these values increased approximately 2-fold (range 1.7 to 2.0) on addition of 50 mM Ca^{2+}. The 1943 strain had only a 1.1-fold increase, which is statistically significantly different from other strains tested ($p < 0.01$). Interestingly, there was no statistically significant difference between V_{max} values obtained for the 1943 strain with or without the addition of Ca^{2+}. In comparison, V_{max}/K_m without Ca^{2+} were: 0.91, 0.88, 0.72, 0.72, 0.79 and 0.78 M^{-1} s^{-1} for the 1934, 1943, 1947, 1977, 1986 and 1991 strains,

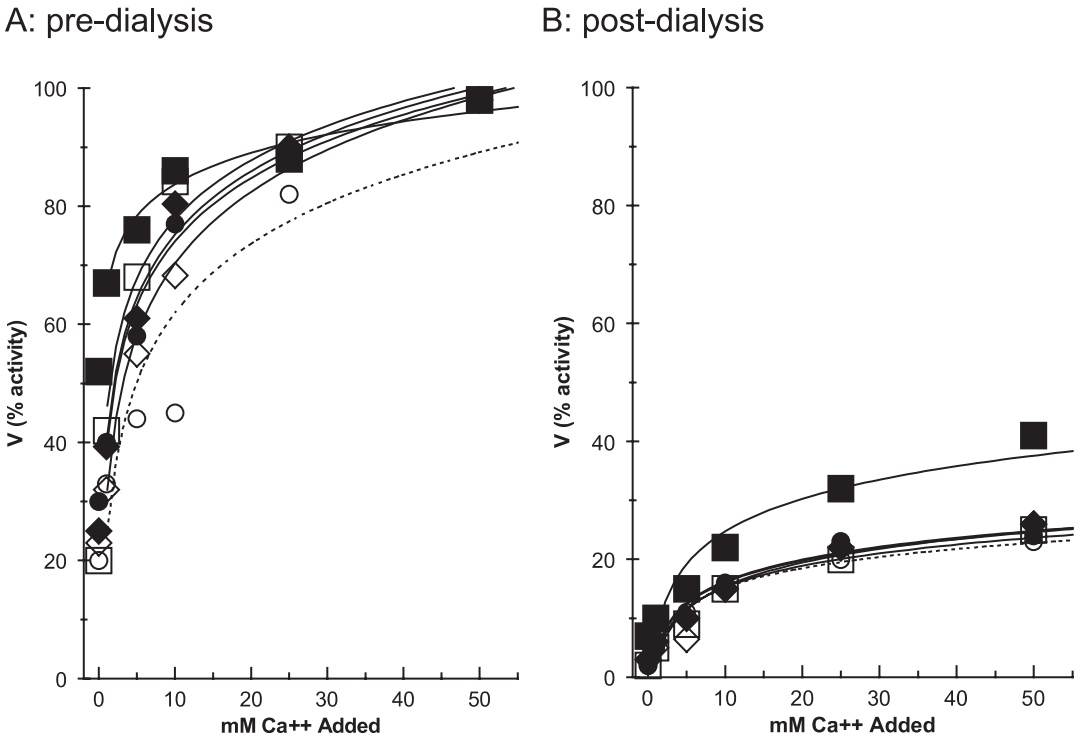

Fig. 1 **Effect of added Ca^{2+} on initial velocity of N1 neuraminidases.** Reactions were carried out with 100 μM 2′-4′-methylumbelliferyl-α-D-*N*-acetylneuraminic acid substrate and 0.5 mU of NA at pH 5.9. Initial velocities (v) are expressed as percent activity relative to that of non-dialyzed NA 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: 1934 (solid squares), 1943 (open circles), 1947 (open triangles), 1977 (solid triangles), 1986 (solid circles), and 1991 (open squares).

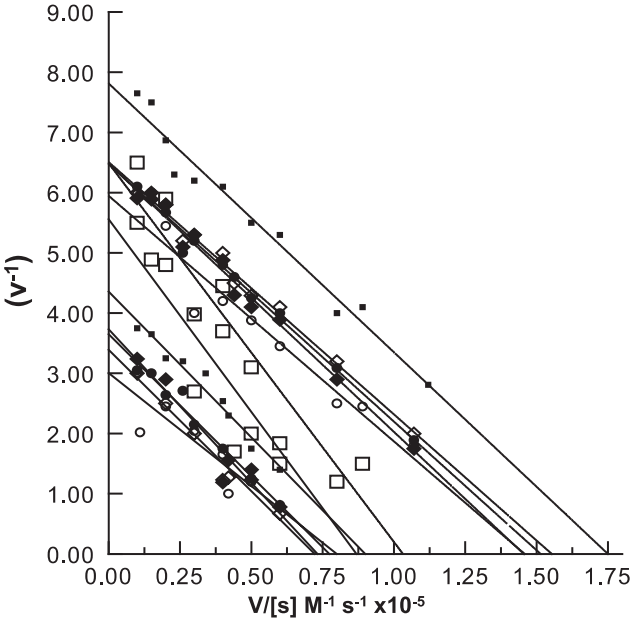

Fig. 2. **Effect of varying Ca^{2+} concentration on *V* and *V/K*.** Reactions were carried out at $I = 0.15$ M with non-dialyzed enzyme in the presence of no added Ca^{2+} (solid lines) or 50 mM Ca^{2+} (dashed lines). For each NA strain standard errors are reflected by the size.

Table 1. **Summary of N1 neuraminidase kinetic parameters.** Kinetic parameters were calculated from non-linear regression of initial velocities to the appropriate Michaelis-Menten equation. Woolf-Augustinsson-Hofstee graphs were plotted, the appropriate intercept or slope determined and summarized here. See text for further detail.

Viruses	Without Ca^{2+}			With Ca^{2+}		
	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m
PR/8/34	4.37	0.91	4.7	7.78	1.75	4.5
Weiss/43	5.50	0.88	6.2	6.50	1.03	6.3
FM/1/47	3.38	0.72	4.5	6.51	1.56	4.1
USSR/90/77	3.63	0.72	4.9	6.50	1.47	4.3
Taiwan/1/86	3.70	0.79	4.6	6.50	1.51	4.4
Texas/36/91	3.10	0.78	4.1	6.11	1.47	4.0

respectively; the V_{max}/K_m values obtained from the 1934 and 1943 N1-NA strains were statistically significantly different from the other tested strains ($p < 0.01$). After the addition of 50 mM Ca^{2+}, V_{max}/K_m increased approximately 2-fold (range 1.65 to 2.2) for all strains tested except the 1943 strain which had an increase of only 1.29-fold, statistically significantly different from other strains ($p < 0.01$).

Effects of Mg^{2+} Concentration on Initial Velocity and on V *and V/K*—Identical studies were performed using Mg^{2+} instead 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 N1-NA before the addition of exogenous Mg^{2+} had no significant differences among strains; the A/Weiss/43 strain, N1-NA, had the greatest requirement to achieve maximum velocity. The addition of up to 50 mM Mg^{2+} resulted in a 2- to 10-fold increase in initial velocity with significant variation among strains ($p < 0.01$). The 1947 to 1991 N1 strains clustered together, and the 1943 strain had significantly the greatest and the 1934 strain the least requirement for exogenous Mg^{2+}. Dialysis of the N1 neuraminidase 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 7% (reduced from 52%) in the N1 1934 strain to 2% (reduced from 20%) in the N1 1991 strain before the addition of exogenous Mg^{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 45% of optimal activity for the N1 1934 strain to an average of 15% of optimal activity for the other N1 strains tested. The N1-NA of the 1934 strain did not cluster with the 1943 to 1991 strains non-dialyzed NA activity after the addition of exogenous Mg^{2+}; indeed greater variation and separation of NA activity was demonstrated after exhaustive dialysis. The earliest N1 strain tested, A/PR/8/34 required the least Mg^{2+} for restoration of activity, the 1943 to 1991 strains clustered together.

Effect of Mg^{2+} Concentration on V *and V/K*—V_{max} and V_{max}/K_m were determined for each NA strain using non-dialyzed enzyme without exogenous Mg^{2+} and with 50 mM Mg^{2+} (data not shown). There were significant differences ($p < 0.01$) among the strains in V_{max} and V_{max}/K_m before and after addition of Mg^{2+}. The 1943 N1-NA strain had a significantly different K_m from other tested strains (6.3 *vs.* 4.0 s^{-1}). There were no significant changes in a given N1-NA strain's K_m after the addition of Mg^{2+}. V_{max} without Mg^{2+} were 4.37, 5.50, 3.38, 3.63, 3.70 and 3.10 s^{-1} for the 1934, 1943, 1947, 1977, 1986 and 1991 strains, respectively; and with the exception of the 1943 strain these values increased approximately 2-fold (range 1.5 to 2.1) on addition of 50 mM Mg^{2+}. The 1943 strain had only a 1.1-fold increase, which is statistically significantly different from other strains tested ($p < 0.01$). There was no statistically significant difference between V_{max} values obtained for the 1943 strain with or without the addition of Mg^{2+}. In comparison, V_{max}/K_m without Mg^{2+} were 0.91, 0.88, 0.72, 0.72, 0.79, and 0.78 M^{-1} s^{-1} for the 1934, 1943, 1947, 1977, 1986 and 1991 strains, respectively. The V_{max}/K_m values obtained from the 1934 and 1943 N1-NA strains were statistically significantly different from the other tested strains ($p < 0.01$). After the addition of 50 mM Mg^{2+}, V_{max}/K_m increased approximately 2-fold (range 1.51 to 2.0) for all strains tested except the 1943 strain, which had an increase of only 1.1-fold, statistically significantly different from other strains ($p < 0.01$).

Effects of Zn^{2+} Concentration on Initial Velocity and on V *and V/K*—The addition of up to 50 mM $ZnCl_2$ had no effect on any measured enzymatic kinetic parameters (data not shown).

Effect Ca^{2+} on NA Immunogenicity—Non-dialyzed NA, dialyzed NA and dialyzed NA incubated with Ca^{2+} from the A/Puerto Rico/8/34 and A/Texas/36/91 strains were injected into immunologically naive mice. The antibody response was measured by NI and NA-specific ELISA antibody assays (Fig. 3). Non-dialyzed NA from both strains was highly immunogenic. Dialysis resulted in a significant decrease ($p < 0.001$) in immunogenicity of both strains, and

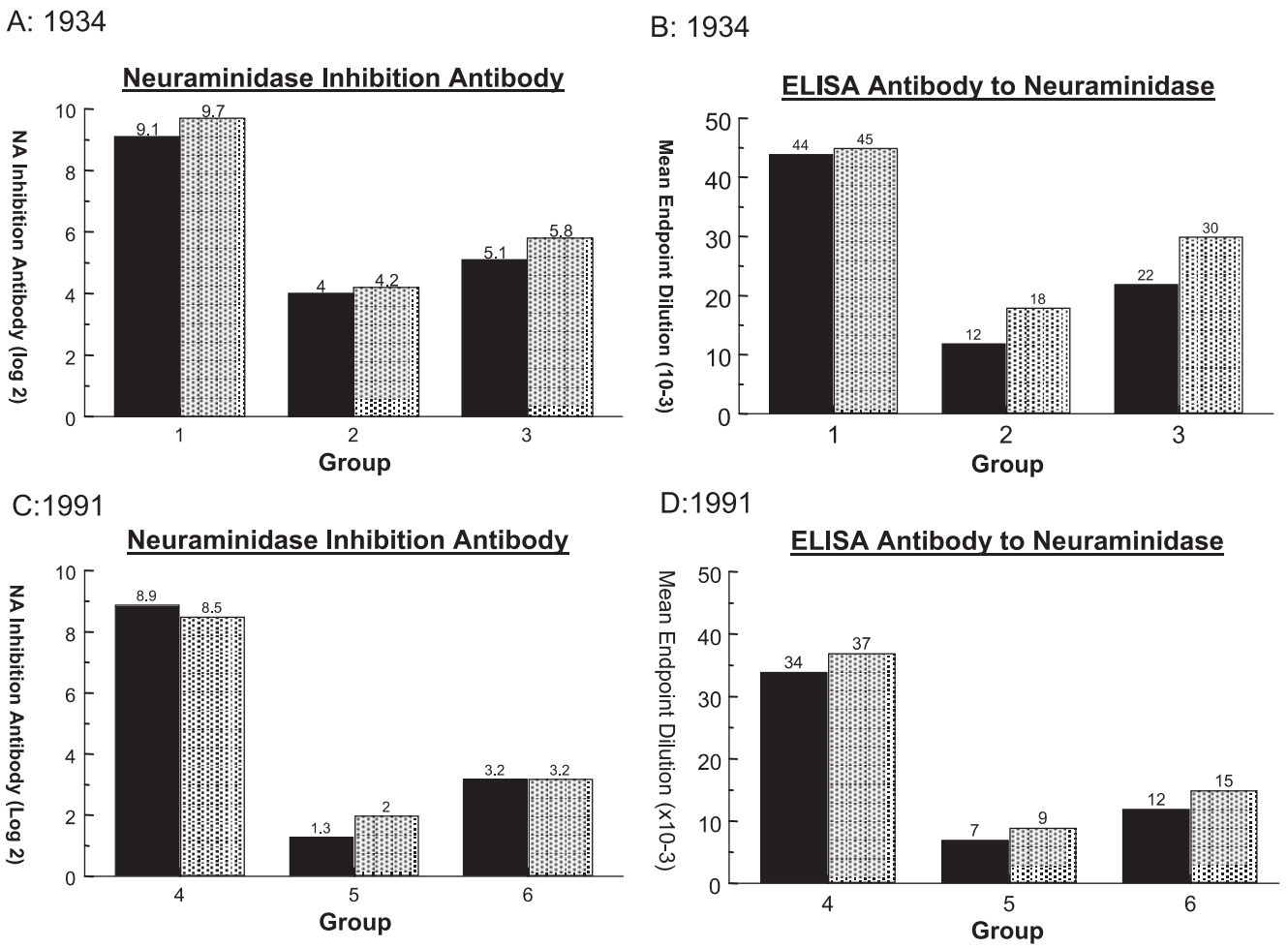

Fig. 3. **Effect of Dialysis on NA immunogenicity.** (A and C) Immune response measured by neuraminidase inhibition antibody. Numbers are $\log_2$ mean dilution endpoints of triplicate wells. (B and D) Immune response measured by ELISA antibody to the appropriate purified neuraminidase. Numbers represent 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/Puerto Rico/8/1934 NA; Group 2: dialyzed A/Puerto Rico/8/1934 NA; Group 3: dialyzed A/Puerto Rico/8/1934 NA incubated with 25 mM Ca^{2+} before assay; Group 4: non-dialyzed A/Texas/36/1991 NA; Group 5: dialyzed A/Texas/36/1991 NA; Group 6: dialyzed A/Texas/36/1991 NA incubated with 25 mM Ca^{2+} before assay. Black bars are Day 7 antisera, stippled bars are Day 28 antisera.

the decrease in antibody response was significantly greater for the A/Texas/36/91 NA in both assays: 78% (9.7 to 4.2 $\log_2$) and 99% (8.5 to 2.0 $\log_2$) decrease in NI titer and 70% (45 to 18×10^3) and 75% (37 to 9×10^3) decrease in ELISA titer, for the A/Puerto Rico/8/34 and A/Texas/36/91 viral strains, respectively. Interestingly, incubation of dialyzed NA with Ca^{+2} resulted in a partial restoration of immunogenicity of both strains tested to 21% (4.2 to 5.8 $\log_2$) and 13% (2 to 3.2 $\log_2$) of non-dialyzed NI titers and 67% (18 to 30×10^3) and 41% (9 to 15×10^3) of non-dialyzed ELISA titers for the 1934 and 1991 strains, respectively. Incubation with Ca^{2+} restored A/Puerto Rico/8/34 NA to a statistically significant ($p < 0.01$) greater antibody response than the A/Texas/36/91 NA. The immunogenicity studies parallel the enzymatic kinetic parameters in that the earlier A/Puerto Rico/8/34 N1-NA enzymatic activity was relatively more resilient to exhaustive EDTA dialysis than the more contemporary strain, A/Texas/36/91 NA.

Sequence Analysis—NA amino acid sequences were analyzed (Table 2) for changes in previously defined amino acids and regions determined to correlate with Ca^{2+} binding based on previous structural studies on neuraminidases from influenza A and B. The low affinity binding site includes (Asp113 and, Asn141/Asp142), and the high affinity binding site includes Asp293, Gly297, Asp324, Pro326, Gly345 and Asp/Asn347. According to Burmeister (*27*) and Varghese *et al.* (*1*), position 141 is an Asp in both N2 and B neuraminidases. However, our research has shown that both Asn141 and Asp142 are conserved in N1 neuraminidases, and it is unclear which is important for Ca^{2+} binding along the 4-fold axis. Also analyzed were residues known for proton binding (aa153), sialic acid binding (aa118, aa119, aa152, aa178, aa222, or catalysis site (aa279, aa276, aa277) (*1*, *27*). All catalytic and sialic acid binding residues were conserved throughout all 6 N1 NA sequences. Only the Weiss/43 strain had a change (Ser153

Table 2. **1934–1991 N1 neuraminidase amino acid changes.** Shown are all amino acid changes in the studied N1 neuraminidases from 1934–1991. 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	PR/ 8/34	Weiss/ 43	FM/1/ 47	USSR/ 90/77	Taiwan/ 1/86	Texas /36/91	Comments
8	T	I	I	I	I	I	
14	C	C	C	C	S	C	
15	L	M	M	M	M	M	
16	V	V	V	A	A	A	
17	V	V	V	I	I	I	
19	L	I	I	I	I	I	
30	I	I	V	I	I	V	
34	I	I	I	V	A	I	
42	S	S	N	S	S	S	
49	H	H	H	H	H	Y	
52	I	I	T	I	I	I	
54	N	N	D	N	N	N	Potential N-Gly site
56	N	S	S	R	R	R	
61	K	K	K	E	E	E	
67	-	N	N	N	N	N	Potential N-Gly site
68	-	Q	Q	Q	Q	Q	
69	-	T	T	T	T	T	
70	-	Y	Y	Y	Y	Y	
71	-	V	V	V	V	V	
72	-	N	–	N	N	N	Potential N-Gly site
73	-	I	–	I	I	I	
74	-	S	–	S	N	N	
75	-	N	–	N	N	N	
76	-	T	–	T	T	T	
77	-	N	N	N	N	N	
78	-	V	V	V	V	V	
79	-	V	V	V	V	V	
80	-	A	A	A	A	A	
81	-	G	G	G	G	G	
82	K	K	K	K	K	Q	
83	D	G	D	D	D	D	
84	T	T	T	T	K	T	
87	V	V	V	M	V	V	
88	I	I	I	T	T	E	
90	T	A	A	A	A	A	
95	R	R	R	R	R	S	
101	S	S	S	S	T	S	
105	S	G	G	S	S	S	
106	I	I	V	I	I	I	
111	K	K	K	K	K	T	
153	S	T	S	S	S	S	Antigenic site VII
163	V	V	V	I	L	I	
167	S	S	S	C	S	S	
181	S	C	S	S	S	S	
199	N	D	D	D	N	D	Antigenic site VI
219	R	R	R	R	K	R	
221	K	K	E	Q	R	R	
231	A	V	V	V	V	V	
246	S	S	S	S	S	N	
247	D	D	G	D	N	N	
253	K	K	K	R	R	K	
262	V	V	V	I	I	I	
269	N	D	D	D	D	D	
273	S	S	S	S	Y	S	
284	T	T	T	T	T	A	
285	G	G	S	G	G	G	
286	K	K	K	T	T	T	
306	D	D	D	N	N	N	
314	I	I	M	I	I	I	
329	E	K	K	K	K	K	Antigenic site I
332	T	K	K	K	E	K	Antigenic site I
339	G	G	G	D	N	G	Anitgenic site II
342	Y	Y	Y	N	T	T	Antigenic site II
347	N	N	N	D	D	D	Antigenic site II, high affinity Ca^{2+} binding site
349	V	V	V	I	V	V	
352	F	I	F	F	F	F	
355	R	R	K	K	R	R	
367	S	S	S	T	S	S	Antigenic site III
368	H	D	N	N	N	N	Antigenic site III, potential N-Gly site
372	H	Q	Q	K	K	R	
385	E	E	E	D	D	D	Antigenic site VIII
390	K	N	N	N	D	K	Antigenic site VIII
392	S	F	F	L	S	L	Antigenic site VIII
393	V	V	T	V	V	V	Antigenic site VIII
394	R	K	K	K	K	K	Antigenic site VIII
397	V	I	V	V	V	V	Antigenic site VIII
399	A	A	A	A	P	A	Antigenic site VIII
400	M	M	V	M	M	M	Antigenic site IV
432	K	K	K	R	R	R	Antigenic site V
434	K	K	N	K	N	N	Antigenic site V
435	–	–	–	T	T	T	
441	A	G	G	A	G	G	
451	S	S	S	S	S	G	
453	T	T	T	T	A	T	
454	V	V	V	V	A	A	
455	D	D	D	N	N	N	Potential N-Gly site
461	G	D	G	G	D	D	
466	F	L	F	F	L	L	
467	S	T	T	T	T	T	

to Thr) at the proton-binding residue. Analysis of potential glycosylation sites yielded an interesting trend. More recent viruses tended to have more glycosylation sites than earlier viruses, and those sites were likely to be glycosylated (Table 3). Both residues involved in the low affinity Ca^{2+} binding site were conserved across all 6 N1 NA sequences, and 5/6 residues considered to be Ca^{2+} ligands (*27*) were conserved. The only change, Asn347 to Asp, first appeared in the 1977 strain and remained an aspartic acid in the two more recent viruses.

DISCUSSION

The N1-neuraminidase of influenza virus is a component of the conventional influenza vaccine (CIV) and

Table 3. **Predicted glycosylation sites in tested strains.** Shown are predicted glycosylation sites in all strains using the Asn-Xaa-Ser/Thr motif. The probability of glycosylation at a particular site is predicted by a jury of 9 glycosylation prediction mechanisms described by NetNGlyc 1.0. Sites that are predicted to be not glycosylated are in italics.

Strain	# probable/ predicted sites	% Glycosylated	Predicted sites
PR/8/34	4/5	80	44, *58*, 91a, 146, 234
Weiss/43	7/8	88	44, 50, *58*, 63, 68, 91a, 146, 234
FM/1/47	5/7	71	44, *58*, 63, 91a, 146, 234, *368*
USSR/ 90/77	7/9	78	44, 58, 63, 70, 91a, 146, 234, *368*, 455
Taiwan/ 1/86	8/10	80	44, 58, 63, 70, 91a, 146, 234, *368*, 434, 455
Texas/ 36/91	9/10	90	44, 58, 63, 70, 91a, 146, 234, *368*, 434, 455

Table 4. **NA activity before and after dialysis.** Comparison of NA activity before and after exhaustive dialysis without added Ca^{2+}. Numbers are percent activity (SEM) as calculated above.

Virus	'34	'43	'47	'77	'86	'91
Before	52 (8)	25 (5)	28 (6)	20 (6.2)	23 (4.5)	20 (6.1)
After	7 (4.1)	2 (0.7)	2 (0.4)	3 (1.4)	2 (0.8)	2 (0.9)

live-attenuated vaccine (LAV). But, currently, there are no guidelines governing the NA content or NA immunogenicity of influenza vaccines. The administration of CIV and LAV to immunologically naïve or primed subjects has repeatedly been shown to induce better HI than NI responses to the corresponding HA and NA antigens of the component strain(s) (*6*, *7*, *8*, *22*, *26*). This observation has been attributed to a manifestation of intermolecular HA-NA antigenic competition that occurs when the two glycoproteins are presented as structurally associated antigens on intact or disrupted viral particles (*28*, *29*, *30*). However, the lack of immunogenic NA antigen in the final vaccine preparation may play an additional role in the lack of a vigorous antibody response to NA (*5*, *21*, *26*, *31*, *32*). The activation and stabilization of the NA enzyme by divalent cations, Ca^{2+} in particular, has been examined by several investigators (*12*, *14–16*, *20*, *21*) with variable results. Our laboratory and others have shown previously that removal of divalent cations by exhaustive EDTA dialysis of N1 and N2-NAs results in a marked decrease in enzymatic activity and immunogenicity (*17*, *19*, *20*, *21*). The activity cannot be restored to original pre-dialysis levels by adding exogenous Ca^{2+}. This finding strongly suggests that with dialysis there is an irreversible enzyme conformational change. This is in accord with current structural theory about tetrameric NA. Burmeister *et al.* (*27*) postulate the existence of two calcium binding sites, a low affinity site along the 4-fold axis that likely stabilizes tetrameric NA and a high affinity site near antigenic site II that is thought to be involved with structure of the monomer and, more specifically, the structure of the enzyme's active site. During dialysis to remove Ca^{2+}, it is likely that two thresholds are reached: the first which facilitates the tetramer's dissociation into dimers and possibly monomers, which is responsible for loss of immunogenicity; and a second that correlates with the removal of the Ca^{2+} ion from its octahedral coordination in the high affinity binding site and with near-complete loss of enzymatic activity. Based on these data, speculations on the functional structure of neuraminidase can be made. The loss of enzymatic, antigenic and immunogenic properties during dialysis suggested that the Ca^{2+} cation is involved in the conformational integrity of the tetrameric molecule or at least that region of the NA molecule bearing sites responsible for these properties. The loss of all three of these properties concurrently indicates that these sites are the same or very closely approximated on the surface of the NA molecule. The difference in NI and ELISA titers after exhaustive dialysis and restoration with Ca^{2+} is consistent with this conclusion. The NI assay is a functional test, measuring antibody binding in or in close proximity to the catalytic site such that binding with substrate is physically blocked; and ELISA measures antibody binding anywhere on the NA molecule. Differences in titers measured by these two methods suggest that Ca^{2+} may exert a greater influence on the structure of the NA molecule in and around the catalytic site than the stalk regions. Detailed dialysis studies are needed to determine the relative affinities of the two Ca^{2+} binding sites and their relative importance on structure and enzymatic activity.

For each strain, K_m was nearly identical with the exception of the A/Weiss/43 strain, yet there were significant differences among strains prior to the addition of exogenous divalent cations in V_{max} (range: 3.1 to 5.5 s^{-1}) and V_{max}/K_m (range: 0.72 to 0.91 M^{-1} s^{-1}). Consistent with earlier findings with N2-NA (*15*, *21*), 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+}. Addition of Ca^{2+} or Mg^{2+} to the reaction resulted in an enhanced rate of substrate binding, as noted by increased V_{max} and V_{max}/K_m. These were increased approximately 2-fold for most strains, with the exception of the A/Weiss/43 strain, which had a minimal increase (Table 1). V_{max} and V_{max}/K_m were greater for the older strains tested (*i.e.*, 1934) and declined steadily with more recent strains (*i.e.*, 1991). 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+}, which was slightly inhibitory to the N2-NA (*15*, *21*), had no measurable effect on the enzymatic activity of the N1-NAs tested. Despite significant overlap of kinetic parameters for N1 and N2-NAs, N1-NA demonstrated a greater sensitivity to exhaustive dialysis and a lesser recovery of activity when exogenous Ca^{2+} was returned (*21*). The A/Weiss/43 N1-NA's K_m of 6.2 s^{-1} was significantly different from all other N1 strains tested (average K_m = 4.4 s^{-1}) and, unlike other tested strains, had only a minimal increase in V_{max} after the addition of exogenous Ca^{2+}. What molecular and evolutionary forces explain this finding? The three earliest N1 strains used in this study, A/PR/8/34, A/Weiss/43 and A/FM/47 were initially isolated in ferrets then passaged in embryonated chicken eggs. However, the A/PR/8/34 and A/FM/47 strains have had over a hundred passages in chick eggs whereas the A/Weiss/43 strain used in this

study had less than ten passages in eggs. This posits that if sialic acid levels in chick eggs are very low, an NA strain with a relatively low K_m would be at a selective advantage there. Conversely, it is possible that the sialic acid concentration in the respiratory tract of a ferret is much higher than the chick egg and an NA strain with a larger K_m would have a selective advantage there. This suggests that adaptation to growth in eggs imposes a selective pressure that selects not only cell tropism but also enzymatic activity. Although the viruses used in this study have had no selective pressure from antibody since they were isolated in the wild, virions are under additional selective pressure from host immune systems. The most effective immune responses might be those that target the regions of a viral protein where escape mutation inflicts the largest fitness cost to the virus, which may manifest, among other things as changes in enzymatic activity. Comparison of the A/Weiss/43 sequence with other N1-NAs shows several residues unique to this strain: Gly83, Thr153, Cys181, Glu200, Ile352, Asp368 and Ile397. It is also the only one of the three earlier strains to possess a complete stalk region. Additionally, the stalk region contains a potential glycosylation site unique to this strain (aa50, Table 3), presumably made possible because of the Ser at position 52. Could this additional glycosylation site help stabilize tetrameric structure, or maintain enzymatic function against Ca^{2+} chelation? Is it one of these unique mutations that allow an elevated V_{max} in the absence of Ca^{2+}, or is it a combination of them? Or is it a combination of one or more of these mutations in conjunction with some of the more seemingly random mutations? It seems more plausible that residues closer to the Ca^{2+} binding site (Ile352, Asp368) have a higher chance of affecting these characteristics, yet without further study it is impossible to conclusively prove whether this is the case. As was previously seen with N2 neuraminidase (*21*), simple sequence analysis alone cannot explain the increasing dependence on Ca^{2+} seen in the activity and immunogenicity data. The change in the Ca^{2+} binding residue at aa347 from Asn to Asp is interesting, but its significance remains unclear. Also unclear is how the changes in the aa's surrounding the Ca^{2+} binding residues affect Ca^{2+} requirements. Because no structural studies on N1 neuraminidase have been conducted, it is difficult to speculate on the effects of this change. Different isolates of influenza virus show a high degree of amino acid sequence variation in their surface glycoproteins. Conserved residues located in the substrate-binding pocket of the influenza virus neuraminidase are likely to be involved in substrate binding or enzyme catalysis (*33*, *34*). Similarly, to study the structure and function of the active site of this protein Lentz *et al.* (*33*) cloned a full-length cDNA of the neuraminidase gene from influenza A/Tokyo/3/67 and amino acid substitutions were made in selected residues. The mutant neuraminidase genes were expressed and the proteins were analyzed for synthesis, transport to the cell surface, and proper three-dimensional folding. Twelve of the 14 mutant proteins were correctly folded and were transported to the cell surface in a manner identical with that of the wild type. Two of these have full enzyme activity, but seven mutants, despite correct three-dimensional structure, completely lost neuraminidase activity, similar to our observation that removal of Ca^{2+} has a greater effect on NI than ELISA. Two mutants were active at low pH. Similarly, Takahashi *et al.* (*34*) 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. In the final analysis, it is likely that multiple mutations work together to allow for a functional enzyme and antigen, and more work needs to be done to elucidate how this occurs. The larger question, however, still looms: what governs this trend of selection for viral strains whose NA has an increasing reliance on calcium? Is it host system dependent, or is Ca^{2+} independence a consequence of repeated passage?

Previous work has shown that stimulating immunity to influenza NA as well as HA results in a broadened, more balanced immune response to influenza (*35*) and affords greater protection from challenge, especially with antigenically hetereogenous strains (*35–37*). If immunity to influenza NA is desirable, and immunogenicity of influenza neuraminidase is indeed linked to calcium, then it follows that three things should happen. First, vaccine manufacturers should control for calcium content in the production process. This may help to preserve the immunogenicity of the NA recovered in the influenza vaccine production process. Second, as an additional criterion for selection, vaccine strain selection should take into account enzymatic kinetics of the NA antigen, in order to select the most antigenically and immunogenically appropriate strain. Lastly, more research needs to be done on the structure of and enzymatic mechanism of NA, and how calcium influences these aspects of this important protein.

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