Isolation of Immunogenic Neuraminidases of Human Influenza Viruses by a Combination of Genetic and Biochemical Procedures

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Neuraminidases were purified from reassortant viruses (H6N1 and H6N2) containing the two antigenic subtypes (N1 and N2) found in human influenza viruses. Surface glycoproteins were solubilized with octylglucoside, and the neuraminidase was isolated by chromatography on DEAE-Sephadex. Neuraminidase isolated by this technique coeluted with viral lipids and spontaneously formed liposomes on dialysis. The purified neuraminidase was immunogenic in rabbits, producing a significant antibody response at dose levels as low as 1 µg.

Hemagglutinin (HA) and neuraminidase (NA) are spike-like structures inserted in the envelope of the influenza virion (19, 22). Because both surface antigens are membrane-associated glycoproteins with similar biochemical properties, separation of the two components is difficult.

Many approaches have been used to purify the NA antigen (for review, see reference 5). Before purification, NA must be removed from the lipid bilayer by either proteolysis or detergent solubilization. Proteolytic techniques involve severing the hydrophobic region, which permanently eliminates the ability of the enzyme to incorporate into membranes or form micelles, properties which may be important for production of immunogenic structures. Detergent solubilization of NA can be achieved with maintenance of enzymatic activity and thus permits recovery of the intact NA molecule (4, 6, 14). Detergent-derived NA maintains the hydrophobic segment necessary for interaction with lipid bilayers or micelle formation.

HA and NA can be stripped from the viral particle with octylglucoside (1-*O*-*n*-octyl-β-D-glucopyranoside), a mild dialyzable detergent permitting recovery of both hemagglutinating and NA activities (10). This provides a logical first step for enrichment of the preparation for both surface antigens and elimination of nucleoprotein and M-protein contaminants. Purification of NA or HA or both is dependent on development of conditions to exploit biochemical differences between the two antigens.

The use of specifically designed influenza virus reassortants permits the production of virus with the combination of surface antigens most amenable to chromatographic separation. In the present study we found that use of an H6 HA best facilitated the separation of the N1 NAs; reassortants (H6N2) were also generated to provide ready purification of N2, the other antigenic NA subtype found in human influenza viruses. A previous report described the use of N1 purified by this technique as the adsorbent antigen in an

enzyme-linked immunosorbent assay for detecting NA antibodies in a clinical population (11).

NA prepared by the technique described above eluted with the viral lipids and spontaneously formed liposomes on dialysis. This NA was immunogenic, 1 µg producing an antibody response in rabbits.

MATERIALS AND METHODS

Preparation of virus. Recombinant virus strains X-76 (H6N1) and X-78 (H6N2) were used as the viral sources. X-76 is a recombinant containing HA (H6) derived from A/Turkey/Mass/3740/75 (H6N2) and NA (N1) derived from A/India/6263/80 (H1N1). Reassortment of X-76 with A/Bangkok/1/79 (H3N2) produced X-78 (H6N2). Virus was inoculated into 11-day-old embryonated chicken eggs, and the allantoic fluids were harvested 42 h later. Cellular debris was removed by centrifugation at 11,000 rpm (Sorvall GSA rotor) for 10 min. Virus was pelleted by a second centrifugation at 11,000 rpm (Sorvall GSA rotor) for 3 h. The virus pellet was resuspended in phosphate-buffered saline (PBS; 0.01 M sodium phosphate, 0.14 M NaCl [pH 7]) and centrifuged until the virus banded at the interface of 30% sucrose-PBS overlaid on 60% sucrose-PBS (25,000 rpm for 60 min; Beckman SW27 rotor). The virus band was collected, diluted with PBS, and recentrifuged under the same conditions to pellet the virus.

Extraction of surface antigens. Pelleted virus (20 to 40 mg of viral protein; virus yield from 200 eggs) was resuspended in 1 ml of sodium acetate buffer (0.05 M sodium acetate, 2 mM NaCl, 0.2 mM EDTA [pH 7.0]); resuspension was assisted by refluxing through a syringe equipped with a 19-gauge needle. An equal volume of 15% octylglucoside (Sigma Chemical Co.) in sodium acetate buffer was then added with mild vortexing. The resulting suspension was centrifuged at 15,000 rpm for 60 min (Beckman SW50.1 rotor), and the supernatant was reserved as the HA-NA-rich fraction.

Chromatographic separation of HA from NA. The HA-NA-rich fraction was adjusted to 0.1% cetyltrimethylammonium bromide (Sigma) with a 2% aqueous cetyltrimethylammonium bromide solution. The protein solution was then applied to a DEAE-Sephadex (A-50; Pharmacia Fine Chemicals) ion-exchange column (bed, 0.7 by 3 cm) previously swollen and equilibrated with 0.05 M Tris-hydrochloride (pH 7.5) buffer containing 0.1% octylglucoside. After 10 to 15 fractions (2 ml per fraction) were collected, the eluting buffer

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FIG. 1. DEAE-Sephadex chromatography of octylglucoside-solubilized HA-NA from H6N1 virus preparation (see the text for details). The arrow denotes an increase in the ionic strength of the eluting buffer to 0.5 M NaCl and substitution of 0.1% Triton X-100 for 0.1% octylglucoside. Fractions comprising pool A (NA rich) and pool B (HA rich) are denoted by lines at the bottom of the figure. NA activity is indicated by a solid line (—), HA activity is shown by a broken line (— —), and protein is indicated by a dashed line (-----). (A = 549 nm), Absorbance at 549 nm.

was changed to 0.05 M Tris-hydrochloride (pH 7.5) buffer containing 0.5 M NaCl and 0.1% Triton X-100.

NA, HA, and protein assays. NA activity was measured by the Warren assay as modified by Aminoff (1, 20). HA titers were determined in microtiter plates (Dynatech Laboratories, Inc.) by serial dilution of 25 μ l of column effluent in 25- μ l volumes of PBS, with 25 μ l of 0.5% human erythrocytes (type O) added after dilution. Protein was quantitated by the method of Lowry et al. (15).

SDS-gel electrophoresis. Sodium dodecyl sulfate (SDS) slab gels were run by the method of O'Farrell (16).

Electron microscopy. Purified NA was dialyzed against sodium acetate buffer (0.05 M sodium acetate, 2 mM NaCl, 0.2 mM EDTA [pH 7.0]) for 72 h. A drop was applied to a carbon-coated grid, and the specimen was stained with 2% phosphotungstic acid (pH 6.0). The specimen was examined at a magnification of 50,000 with a Philips 300 electron microscope.

Immunization protocols. After purification of the NA, the

preparation was dialyzed for 72 h to remove any residual detergent. Rabbits were immunized by two different regimens. The first group received an initial dose on day 0 and a booster dose on day 42; both doses were administered intravenously. The second group received an additional dose on day 0 in Freund adjuvant, administered intradermally. Doses of 1 to 10 μ g were used; a total of either 2 or 20 μ g for the first group and a total of either 3 or 30 μ g for the second group was used. NA was presented as dialyzed or with the addition of 1% octylglucoside to disperse the NA.

NI titers. Neuraminidase inhibition (NI) titers were obtained on heat-inactivated sera (56°C for 30 min) by the method of Aymard-Henry and associates (2) with fetuin as the substrate. The NI titer was expressed as the inverse of the titer at which 50% inhibition of control level enzymatic activity (absorbance at 549 nm) was observed. Titers were derived by plotting the absorbance at 549 nm versus dilutions of antiserum on semi-log paper.

HA inhibition assay. Antisera were titrated for HA inhibi-

TABLE 1. Recovery of NA activity and enhancement of specific activity during NA purification from H6N1 virus^a

Purification stage	Protein		NA activity ^b		Sp act (nm/min per mg)
	Total (mg)	Recovery (%)	Total (nm/min)	Recovery (%)	
Total virus preparation ^c	39.8	100	7,755	100	194
Supernatant ^d	22.3	56	6,179	80	277
Pellet ^d	ND	ND	790	10	ND
DEAE-Sephadex pool A ^e	3.6	9	3,889	50	1,080

^a ND, Not determined.

^b N-Acetyl neuraminic acid released at 37°C.

^c After the addition of octylglucoside.

^d After the addition of octylglucoside and centrifugation.

^e Activity was measured after overnight dialysis versus water.

FIG. 2. SDS-polyacrylamide gel electrophoresis analysis of H6N1 chromatographic fractions shown in Fig. 1. Electrophoresis was performed under reducing conditions. Isolated NA (N1) or pool A is shown in lane E. Molecular weight standards were run in lane A and include bovine serum albumin (BSA), ovalbumin (OVA), and chymotrypsinogen (CHY). Other samples include (lanes): B, total virus protein; C, octylglucoside supernatant; and D, pellet after octylglucoside extraction.

tion activity to H6 HA in the microtiter (Dynatech) system (9, 18). A reassortant virus (H6N4; A/duck/France/MB/42/76) with heterologous NA was used as the testing antigen (11).

RESULTS

Purification of N1 NA. The elution profile for the separation of N1 NA from the H6 HA on a DEAE-Sephadex column is shown in Fig. 1. NA eluted in the flow-through volume with no detectable hemagglutinating activity. HA was eluted by a stepwise increase in NaCl concentration and simultaneous substitution of detergents in the eluting buffer; Triton X-100 was substituted for octylglucoside. Neither salt nor Triton X-100 used individually was sufficient to elute HA. When gradient elution was performed, the minimum salt concentration required to elute HA was 0.1 M NaCl. N2 NA was isolated from the genetic reassortant H6N2 in a manner directly comparable to the procedure described for N1.

Recovery of enzymatic activity for this preparation is shown in Table 1. Initial activity measurements were made after the addition of octylglucoside. Detergent disruption of the viral particle with octylglucoside resulted in enhancement of the NA activity of 1.2- to 1.5-fold. A total of 80% of the NA activity could be recovered in the octylglucoside supernatant; 50% of the total activity was recovered in the NA peak obtained after DEAE-Sephadex chromatography. Protein recovery during the purification procedure is shown in Table 1; 56% of the total protein was solubilized by octylglucoside, and 9% of the total viral protein was associated with the NA peak obtained after DEAE-Sephadex chromatography. The specific activity of NA in pool A increased 5.6-fold during purification.

TABLE 2. Antibody titer to purified NA measured by NI

NA administration (total)	Rabbit no.	Final NI titer
Intravenous; no adjuvant		
1 µg per dose (2 µg)	129 ^a	64
	130 ^a	360
	125 ^b	24
	126 ^b	— ^c
10 µg per dose (20 µg)	127 ^a	— ^c
	128 ^a	130
	123 ^b	210
	124 ^b	74
Intravenous; additional intradermal dose with adjuvant		
1 µg per dose (3 µg)	137 ^a	600
	138 ^a	220
	133 ^b	56
	134 ^b	450
10 µg per dose (30 µg)	135 ^a	1,300 ^d
	136 ^a	600
	131 ^b	76
	132 ^b	240

^a Purified NA was dialyzed for 72 h to remove detergents (see the text for details).

^b Purified NA was dialyzed for 72 h; 1% octylglucoside was added to disperse NA before immunization (see the text for details).

^c —, No detectable NI.

^d Sole responder to HA antigen; HA inhibition titer of 1:40.

SDS-gel electrophoresis of the various fractions is shown in Fig. 2. Under reducing conditions (Fig. 2A through E), NA migrated with an apparent molecular weight of 63,500 and was shown to be the principal component of DEAE-Sephadex pool A (Fig. 2E). The octylglucoside supernatant (2C) was devoid of matrix protein and contained most of the HA and NA; a significant amount of nucleoprotein was also found in this experiment. The amount of nucleoprotein found in the octylglucoside supernatant varied from undetectable to high concentrations; variation of extraction conditions with respect to temperature, time, ionic strength, pH, detergent concentration, and velocity of trituration during pellet resuspension did not consistently enhance or reduce extracted nucleoprotein. Nucleoprotein was shown to elute with the HA pool. Use of gradient elution (0.1 to 0.5 M NaCl) permitted separation of HA from nucleoprotein on the DEAE-Sephadex column; NP was more firmly bound and eluted at a higher salt concentration.

NA prepared by this technique was immunogenic when administered to rabbits. Three of four rabbits responded to as little as 2 µg given in two divided 1-µg doses administered intravenously; NI titers ranged from 24 to 360 (Table 2). When a 1-µg dose was administered in Freund adjuvant in addition to the two intravenous doses (total of 3 µg), four of four rabbits responded with NI titers ranging from 56 to 600 (Table 2). Similar responses were seen at a 10-fold higher dose with or without adjuvant, but adjuvant enhanced NI titers. Responses were NA specific in all but one animal in which an HA inhibition titer of 1:40 was demonstrated in the day 47 postboost serum sample after immunization with 10 µg of an N1 dialyzed aqueous preparation in Freund adjuvant. This serum caused a 50% reduction of plaque number of H6N2 virus at a 1:40 dilution in Madin-Darby canine kidney cell monolayers infected with H6N2 virus. In this

FIG. 3. Electron micrograph of purified N1 NA or pool A fraction from DEAE-Sephadex chromatography of octylglucoside-solubilized HA-NA (Fig. 1). The sample was dialyzed to remove the detergent before electron microscopic analysis. Arrows denote some of the NA molecules forming the "fringe" on the periphery of the liposome. Instrument magnification was 50,000, with a final magnification of 250,000.

same rabbit (no. 135) the maximal NI titer (1:1,300) was demonstrated.

Electron microscopic examination of the purified NA showed that on dialysis NA spontaneously formed liposomes, with the virus-associated lipid coeluting with the enzyme (Fig. 3). The HA preparation consisted of star-shaped structures as eluted without dialysis; the size of the centers of the HA aggregates suggested that a certain amount of lipid might also be associated and coeluting with the HA (Fig. 4).

DISCUSSION

NAs of both N1 and N2 subtypes were purified by the chromatographic technique described above. Genetic reassortants containing H6 HA in combination with N1 or N2 were used as the viral source of NA. The H6 NA is immunologically irrelevant to antibody in the human population. Therefore, even when traces of HA persist in these preparations, their usefulness as potential NA-specific vaccines or testing reagents is not significantly diminished (11, 13).

Octylglucoside is a dialyzable, mild, nonionic detergent which is capable of stripping surface antigens from the lipid bilayer in which they are embedded (8). We found, however, that even at detergent concentrations as high as 7.5%, NA and HA were not fully dissociated from one another and the addition of small amounts of cetyltrimethylammonium bromide was required for complete dissociation of the antigens.

FIG. 4. Electron micrograph of H6 HA-rich fraction or pool B from DEAE-Sephadex chromatography of octylglucoside-solubilized HA-NA (Fig. 1). The sample was analyzed directly from the column with no dialysis performed. Arrows denote some of the clusters of HA molecules. Instrument magnification was 40,000, with a final magnification of 200,000.

Cetyltrimethylammonium bromide bears a positive charge and as such does not interact with DEAE-Sephadex, preventing mediation of protein-ion exchanger interactions. Conditions of chromatography were established to maximize the absorption of non-NA polypeptides, permitting a high recovery of NA. The use of a commercially available chromatographic material, DEAE-Sephadex, eliminated the difficulties inherent in the preparation of specific adsorbents for isolation of NA (4, 6).

Chromatographic behavior and subsequent isolation of purified NA was dependent on the HA with which the NA was associated. Earlier, the use of the X-7 (14) or X-7 (F1) (21) H1N2 influenza virus reassortants had been shown to facilitate isolation of N2 NA by cellulose acetate electrophoresis. The HA derived from the NWS (H1) strain was SDS sensitive and readily denatured on exposure to SDS, with alteration of its electrophoretic properties.

The method as described above was developed to isolate the NAs (N1 and N2) currently prevalent in human influenza viruses. Because the primary structures of both HA and NA vary among viral strains, it is possible that accumulated amino acid changes during mutation of these proteins could become sufficient to alter the elution profile, which is nominally related to net charge. The most striking example of this chromatographic behavior was observed with regard to the genetic reassortant H1N2 (X-7) which incorporates the N2 of 1957 strains. The surface antigens of this virus eluted reciprocally as compared with their counterparts in the reassortant viruses H6N1 and H6N2. Thus, H1 eluted first and N2 was eluted upon change of the buffer. Other

strains have shown intermediate results, with HA and NA coeluting in both pools. Explanations for these observations may include enduring protein-protein interactions, vesicle formation, protein-charge heterogeneity, and changes in net charge which render the ion-exchange adsorption suboptimal.

Conventional whole virus vaccines deliver both surface antigens (NA and HA) to the vaccinee, and although the vaccine protects against natural infection, the immunity that results is of short duration. By viral genetic reassortment, whole virus vaccines have been prepared which incorporate into an antigenically hybrid virus a currently prevalent NA antigen along with an HA that is not normally encountered in human infection. Such vaccines allow infection to occur, but antibodies to NA attenuate the response so that clinical manifestations of disease are limited (3, 7, 17). Low-level heterotypic reactions among HA antigens even of different subtypes may confound the induction of NA-specific immunity with whole virus antigenic hybrids (12). Therefore, definitive testing of the concept of NA-specific vaccine will require the development of purified antigen vaccine. Elimination of other viral proteins should not only facilitate the presentation of NA antigen but reduce the probability of adverse vaccine reactions.

The present rapid, simple, and efficient method for preparation of NA from influenza virus should be useful not only in producing an influenza vaccine based upon purified NA but also for the preparation of NA for study of structure-activity relationships and in providing diagnostic reagents for serological testing of vaccine responses (11).

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