ANTI-NEURAMINIDASE ANTIBODY AFTER FIVE ANNUAL INFLUENZA VACCINATIONS IN THE AGED AND CHRONICALLY ILL.

By

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ABSTRACT

Increasing evidence suggests that neuraminidase-inhibition antibodies (NIAB) contribute to immunity against influenza. The serum NIAB response to N1 and N2 neuraminidase antigens was measured after each of five annual vaccinations (1976 - 1980) in 69 aged and chronically ill patients (mean birth date 1902±2). Of the 69 patients selected for this clinical study, 47 were vaccinated whereas 22 served as non-vaccinated controls. Sera were collected before, two months, and one year after each vaccination.

Test antigens used were recombinant viruses A/Prague/1/56 - A/USSR/92/77 (H7N1) and A/Prague/1/56 - A/Port Chalmers/73 (H7N2).

An economical mini neuraminidase-inhibition (MNI) test, based on the same principles as the standard WHO method, was developed in our laboratory. The standardized and reproducible MNI test was used to measure NIAB in test sera.

The data obtained from testing patient sera for NIAB demonstrated the following:

1. The most significant increase in NIAB response to the N1 component of the vaccines occurred following the first N1 immunization with the bivalent A/New Jersey/8/76 - A/Victoria/3/75 (NJ-VIC) vaccine. A smaller increase occurred after the second N1 vaccination by the

trivalent A/USSR/92/77 - A/Texas/1/77 - B/Hong Kong/2/73 (USSR-TEX-BHK) in vaccine 3. Of the 39 patients vaccinated with the NJ-VIC vaccine, the number (%) with NIAB titres $\geqslant$ 1:10 increased from 25(64%) pre-vaccination to 33(85%) after, and included 21(54%) with a $\geqslant$ 2-fold increase. The geometric mean titre (GMT) demonstrated a highly significant increase from 10.5 to 18.3 ($p<0.001$). Corresponding values in the 22 non-vaccinated controls remained unchanged with a prevalence of NIAB titre $\geqslant$ 1:10 in 9(41%) and a GMT of 8.8. Following the second N1 challenge, the prevalence in vaccinees of NIAB titre $\geqslant$ 1:10 increased from 32/47(68%) pre-vaccination to 35/47(75%) after, and included 11/47(23%) with $\geqslant$ 2-fold increase; the GMT rose insignificantly from 11.8 to 13.6 ($p>0.05$). The controls remained relatively stable, the prevalence of titres $\geqslant$ 1:10 in 8/20(40%) and the GMT at 9.0.

2. In the same group of patients, the only significant increase in NIAB response to the N2 component of the vaccines occurred following vaccine 2; bivalent A/Victoria/3/75 - B/Hong Kong/72; (VIC-BHK). The NIAB response also increased, but insignificantly, after vaccine 3(USSR-TEX-BHK). Following vaccine 2; patients with NIAB titre $\geqslant$ 1:10 increased from 27/47(57%) before to 36/46(77%) after vaccination, including 18/47(38%) with a $\geqslant$ 2-fold increase, and a highly signficant rise in GMT from 11.6 to 17.3 ($p<0.001$). Correspondingly, in the controls the prevalence increased slightly from 11/21(52%) to 14/21(67%), including 2/21(10%) with a $\geqslant$ 2-fold increase, whereas the GMT was maintained at 11.0. After vaccine 3,

the prevalence of vaccinees with NIAB titre $\geq$1:10 increased from 31/47(66%) before vaccination to 34/47(72%) after and the GMT rose insignificantly from 14.0 to 16.3 ($p > 0.05$). The controls remained relatively stable, the prevalence of titres $\geq$1:10 in 13/20(65%) and the GMT at 10.7.

3. Continuing immunization with either N1 or N2 vaccine components did not elicit further significant NIAB response.

4. The GMT usually declined within one year after each vaccination (N1 or N2). Re-immunization, however, boosted the GMT which was maintained at levels equivalent to, or slightly higher than, that of the initial pre-immunization GMT.

5. During the five year period, usually the majority of the same patients responded well to N1 and N2 vaccine challenges. Of the vaccinees 27/47(57%) responded to at least one or more vaccines, whereas 20/47 (43%) did not respond at all to any of the vaccines.

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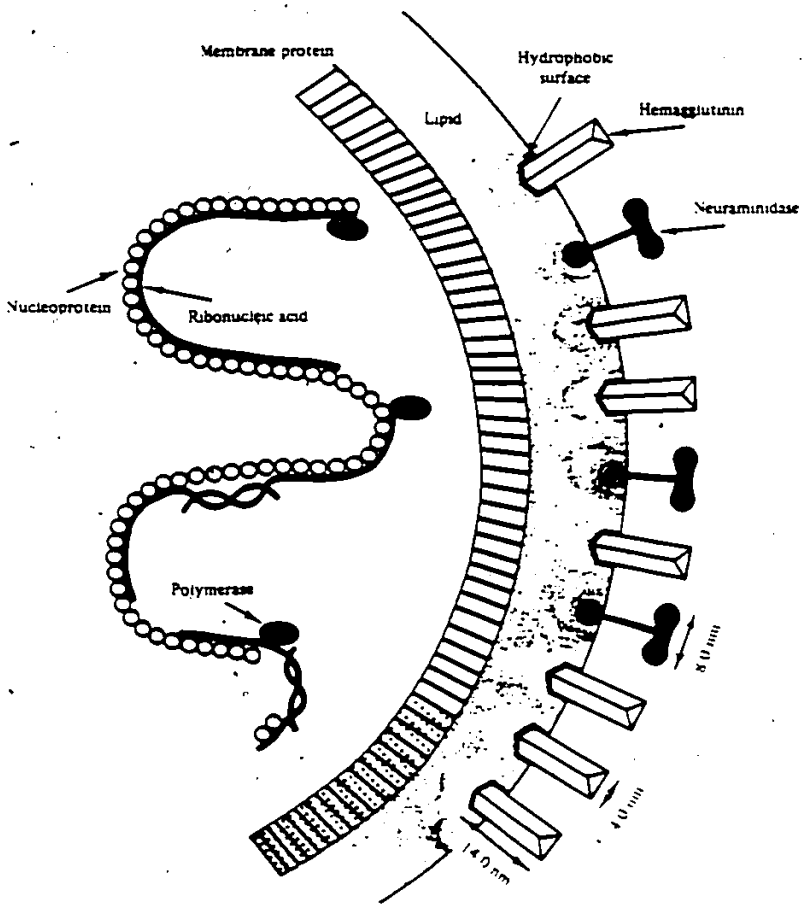
INTRODUCTION

Influenza viruses are well known etiologic agents of acute respiratory diseases causing sporadic, epidemic and pandemic infections not only in human population but also in a wide range of animal and avian species. Influenza viruses, particularly type A , have been shown to give rise to infectious disease characterized by a high rate of morbidity which can result in excess mortality in high risk groups such as the aged and chronically ill.

Since the first isolation of influenza virus in ferrets by Smith et al (1933), in England, it has become one of the most extensively studied viruses. Due to their affinity for certain mucins, influenza viruses, were named as Myxoviruses and ultimately grouped in the family Orthomyxoviridae (Fenner, 1975). Orthomyxoviruses are characterized by a single-stranded segmented ribonucleic acid (RNA) in a helical nucleocapsid surrounded by a matrix and an ether-sensitive, lipid-bilayer envelope through which the morphologically and antigenically different surface glycoproteins, hemagglutinin (H) and neuraminidase (N) protrude (Fig 1). Influenza viruses were shown to adsorb to, agglutinate, and elute from fowl erythrocytes and other animal species (Hirst, 1942). Based on antigenically distinct ribonucleo- protein (RNP) and matrix (M) protein antigens, three different types of influenza viruses have been characterized and designated as A, B, and C (Chanock et al, 1971). Such antigenic distinctions were documented using the complement fixation test (Lief and Henle, 1959), immunodouble

Figure 1: Schematic illustration of the structure of influenza virus particle. (From Laver W.G.. In "Medical Virology" - Orthomyxoviruses -, Fenner F.J. and White D.O., eds. (1976), p. 378. Academic Press, New York, San Francisco, London.



diffusion (Schild and Pereira, 1969: Schild, 1970), and hybridization techniques (Scholtissek et al, 1976). The new recommendation by Assaad et al, (1980) proposes different designations for both human and avian hemagglutinin and neuraminidase antigens of influenza viruses (Table 1).

Antigenic variation in influenza viruses is well documented in its continual occurrence within group A influenza (Pereira, 1979; Laver et al, 1980; Schild et al, 1980; Van Wyke et al, 1980) and to a lesser extent in group B (Webster and Laver, 1975; Briedis et al, 1981) whereas in group C it appears to be very minor to stable (Meier-Ewert et al, 1981; Chakraverty, 1978: Compans et al, 1977).

Influenza Virus Structure

Influenza are relatively large viruses with molecular weights ranging from 151 x 10^6 to 360 x 10^6 daltons (Scholtissek, 1978). The pleomorphic particles are usually spherical, with diameters of approximately 80-120 nM, established by ultrafiltration and by negatively stained electron micrographs (Horne et al, 1960; Hoyle et al, 1961). Filamentous forms usually occur on fresh isolation from human clinical specimens (Choppin et al, 1960). The specific infectivity of the filamentous forms was found to be higher than that of spherical virions, and they contain more RNA per particle than do the spheres (Ada et al, 1958). Electron-dense micrographs and biochemical studies reveal that the virus core nucleocapsid contains nucleoprotein in association with single-stranded RNA genome of a negative (non-message) polarity (Baltimore, 1972) and is

Table 1:*Proposed subtypes of hemagglutinin and neuraminidase antigens of influenza A viruses:

Proposed subtypes of hemaglutinin antigens	Previous subtypes (1971 system)
H1	H0, H1, Hsw1
H2	H2
H3	H3, Heq2, Hav7
H4	Hav4
H5	Hav5
H6	Hav6
H7	Heq1, Hav1
H8	Hav8
H9	Hav9
H10	Hav2
H11	Hav3
H12	Hav10
Proposed subtypes of neuraminidase antigens	
N1	N1
N2	N2
N3	Nav2, Nav3
N4	Nav4
N5	Nav5
N6	Nav1
N7	Neq1
N8	Neq2
N9	Nav6

*From: Assaad, F.A., Brés, P., Chi-Ming, C., and Dowdle, W.R. (1980). A revision of the system of nomenclature for influenza viruses: a WHO memorandum. Bull. W.H.O. 58: 585 - 591.

associated with an RNA-dependent RNA transcriptase. The RNA genome is segmented and currently eight genomic segments are identified (Barry & Mahy, 1979, Pons, 1976; Palese & Schulman, 1976; McGeoch et al, 1976), which code for seven viral structural proteins and three non-structural proteins (Dimmock et al, 1980; Baez et al, 1981). The total molecular weight (M.W.) of the RNA genome is approximately $4-5 \times 10^6$ daltons (Choppin and Compans, 1975a). The molecular weights and characteristics of each of the proteins coded for by the eight segments are listed in Table 2. It is evident that due to the presence of a segmented genome, influenza virus undergoes a high frequency recombination or gene reassortment resulting in the production of novel viral strains of different antigenic composition (Bean et al, 1980; Webster and Laver, 1975; Webster & Laver, 1980; Moss et al 1980; Schild et al, 1980) to which the human population lacks previous experience.

The nucleoprotein (NP) which has a M.W. ranging between 55-65,000 daltons (Choppin and Compans, 1975b; Pons, 1976; Inglis et al, 1976, Rohde et al, 1977) is closely associated with the RNA genome in the form of RNA-protein complexes. This type specific antigen forms the basis for the classification of influenza viruses into the designated types A, B, and C (Chanock et al, 1971; Assaad et al, 1980). The nucleocapsid is overlaid by the matrix (M) protein antigen which is the most abundant component of the virus particle (Compans et al, 1970; Schulze, 1970). The M protein is in turn encapsulated by an outer lipid bilayer, which is of host origin, and in which the hydrophobic ends of the two surface glycoprotein projections, hemagglutinin (H) and neuraminidase (N), are

Table 2: Virus specific polypeptides and antigens of influenza virus particle and infected cells[a].

Polypeptide	Polypeptide M.W.	Function	Approximate no. of molecules per virus particle	Assignment to structural and antigenic components of virus
P1 P2 P3	84,000-94,000	unknown	50	minor, internal non-glycosylated proteins, associated with polymerase activity. Unknown antigenicity.
N	60,000	Neuramin-idase	100-200	surface glycoprotein, antigenically variable, strain specific
NP	53,000	Nucleo-capsid	1000	Internal, non-glycosylated protein, associated with RNA. Type specific. Antigenically stable.
HA1 HA2	58,000 28,000	Hemag-glutinin	1000	Surface glycoprotein. Heavy (HA1) and light components(HA2) chains linked by disulphide bonds. Anti-genically variable & strain specific.
M1 M2	27,000 11,000[b]	Matrix Protein	3000	Major internal non-glyco-sylated proteins. Antigenically stable. Type specific associated with lipid bilayer.
NS1 NS2 NS3	23,000 11,000 unknown	unknown		Non-structural virus-coded proteins. Synthesized in cytoplasm and migrate to nucleus of infected cells.

*Modified from Stuart-Harris and Schild (1976); Scholtissek (1978); Schild (1979).
From: Lamb and Choppin (1981); and Allen et al (1980).
M.W. : Molecular Weight.

embedded (Laver and Valentine, 1969).

The structure of influenza viruses has been extensively reviewed by many authors (Kilbourne, 1975, Stuart-Harris and Schild, 1976; Scholtissek, 1978; Laver & Air, 1980). In this thesis we will focus upon current data available regarding the structure and function of influenza viruses.

Nucleic Acid

Influenza A virus genome ranges in size from 890-2500 nucleotides (Palese & Schulman, 1976) and contains eight single stranded RNA segments (McGeoch et al, 1976; Palese & Schulman, 1976). Each strand encodes at least one monocistronic polypeptide (Ritchey & Palese, 1976; Scholtissek et al, 1976; Lamb and Choppin, 1979) hence, the genome contains information for seven structural polypetides and two non- structural NS1 and NS2 polypeptides and possibly a third NS3 polypeptide (Baez et al, 1981). The genome undergoes continuous variations of which two are:

a) reassortment between human influenza viruses and animal influenza viruses (Young and Palese, 1979; Desselberger et al, 1978) and

b) sequential mutation in individual genes of viruses of the same type particularly type A, (Young et al, 1979). Gradual mutations can cause changes in antigenic properties known as "drifts". More abrupt changes in antigen known as "shifts" result from recombination or reassortment involving exchange of one or both of the RNA segments 4 and 6 which code for hemagglutinin (H) and neuraminidase (N), respectively (Laver & Webster 1979; Porter et al, 1980). Each segment codes for a single viral polypeptide protein (Scholtissek, 1978), with the exception of segment

8 which codes for the two nonstructural proteins, NS1 and NS2, (Lamb and Choppin, 1979; Inglis et al, 1979). Little is known concerning the functions of the nonstructural polypeptides in the virus replication cycle. Early in the infection NS1 (M.W. 23,000) is synthesized in the cytoplasm (Skehel, 1973) and migrates into the host cell nucleus (Krug & Soeiro, 1975) but later accumulates in the cytoplasm (Shaw & Compans, 1978). Synthesis of NS2 (M.W. 11,000) occurs only late in infection and remains in the cytoplasm (Lamb et al, 1978). The coding regions for NS1 and NS2 polypeptides on the RNA segment overlap by about 144-159 nucleotides (Lamb et al, 1980). Hall and Air (1981) utilizing complementary DNA techniques, examined gene segment 8 from a number of human influenza A viruses isolated over a period of 43 years and representing H2N2 and H3N2 subtypes. They suggested that unlike segments 4 and 6 which code for H and N, respectively, segment 8 was far more conserved during the reassortment events which were responsible for the antigenic shifts H1N1 to H2N2 to H3N2. Segment 7 was recently found to code for two polypeptides of 27,000 (matrix protein) and an additional protein of approximately 11,000 daltons (Allen et al, 1980). The other polypeptides coded for by viral RNA include nucleocapsid protein (NP) coded for by segment 5, and three polypeptides (P1, P2, P3) associated with polymerase activity, coded for by segments 2,3, and 1, respectively (Scholtissek, 1978).

Nucleoprotein (NP)

The NP has a molecular weight of 55,000 to 65,000 daltons (Pons, 1976; Ritchey and Palese, 1976; Rohde et al, 1977, Winter & Fields, 1981). It binds several molecules of the P polypeptides (Bishop et al, 1972) and

is also specifically associated with matrix (M) protein (Rees & Dimmock, 1981). The NP is believed to play an important role in the replication of influenza virus. Hudson et al (1978) have shown that during infection at 4°C, which allows events to proceed up to viral transcription, influenza virus NP accumulates in the nucleus, but when cells are warmed to 37°C the majority of the NP migrates from the nucleus to the cytoplasm. The NP is sequestered within the virion (Compans & Dimmock, 1969) and, therefore, is not subject to the antigenic selection pressure exerted on the externally located H and N. At least five different antigenic sites can be resolved in nucleoproteins from different human influenza A strains (Oxford et al, 1978; VanWyke et al, 1980). The first direct evidence for antigenic variation in the NP was reported by Schild et al (1979) who demonstrated that NP of PR/8/34 (HON1) strain differs antigenically from the NP of H3N2 strains. This variation was confirmed by Van Wyke et al (1980) who also suggested that antigenic variation in the NP of influenza A viruses proceeds independently of changes in the viral surface antigens and that point mutations and genetic reassortment may account for NP variability./ The complete structure of the gene encoding the NP of human influenza virus A/PR/8/34 has been determined. It was found that RNA segment 5 contains 1565 nucleotides and encodes a protein of 498 amino acid residues of 56,106 dalton molecular weight (Winter & Fields, 1981).

Matrix (M) Protein

The Matrix (M) protein which is the most abundant component of the

influenza virus particle, is situated beneath the outer lipid bilayer envelope and surrounds the nucleoprotein (Schulze, 1970, 1972). It has been shown that purified M protein has a marked affinity for lipids and is easily incorporated into lipid vesicles (Bucher et al, 1980; Gregoriades, 1980; Gregoriades & Frangione, 1981). The M protein appears to be anchored to lipid vesicles by a fragment of approximately 5,000 daltons which remains in association with the lipid vesicle. This may indicate that a specific portion of the M molecule has a high affinity for lipid bilayer in situ (Gregoriades & Frangione, 1981) and planar lipid bilayer membranes (Kharitonenkov et al., 1980). Matrix protein is found on smooth and rough endoplasmic reticulum and never free in the cytoplasm (Hay, 1974). It is also present in high concentrations in the nucleus even early after infection (Gregoriades, 1977) and on the surface of infected cells (Yewdell et al, 1981). The recently obtained nucleotide sequence of RNA segment 7 of PR/8/34 strain has been found to code for two M proteins: M1 containing 252 amino acids which is of 27,000 daltons; and M2 containing 97 amino acids which is of 11,000 daltons (Allen et al, 1980; Winter & Fields 1980; Lamb & Choppin, 1981). Antibody to M protein is not apparently protective against influenza as was shown in infected mice (Virelizier et al, 1976).

Hemagglutinin (H)

Structure and Biochemical Properties

Of the two surface antigens, hemagglutinin is considered quantitatively the major surface glycoprotein (White, 1974) and the antigen against

which neutralizing antibodies are directed (Drezniek et al, 1966; Laver and Kilbourne, 1966). Structurally, H is an elongated cylindrical trimer, 135A long, with a triangular cross-section varying in radius from 15A to 40A (Wilson et al, 1981). The H is initially synthesized as a single polypeptide of about 580 amino acids with an apparent molecular weight of 65,000 on SDS polyacrylamide gels (Skehel, 1972; Klenk and Rott, 1973; Nakamura and Compans, 1978). Subsequently, the polypeptide is glycosylated in the rough endoplasmic reticulum and the molecular weight increases to approximately 75,000 (Elder et al, 1979; Waterfield et al, 1979). During biosynthesis disulphide bonds are formed and three identical H molecules, through non-covalent interactions, form a trimer of a molecular weight of 210,000 (Wiley et al, 1977). Each H monomer is found to be cleaved by host plasma protease to form two disulphide bonded polypeptides HA1 and HA2, respectively (Laver, 1971; Brown et al, 1980; Garten et al, 1981), a heavier HA1 chain of M.W. 47,000, and a lighter chain HA2 M.W. 30,000 (Dopheide and Ward, 1981). The cleavage has been found to be essential for virus infectivity (Klenk et al, 1975; Lazarowitz and Choppin, 1975), and a precondition for the fusion capacity of the H (Huang et al, 1980 b, 1981; Rott et al, 1981). Helenius et al (1980), Huange et al (1980; 1981), Meada et al (1981) and White et al (1981: 1982) demonstrated that the hemagglutinin (H) plays a key role in the fusion of influenza viruses with liposomes or tissue culture target cells. It was shown that fusion occurred only when 1) The precursor H was proteolytically cleaved into HA1 and HA2, respectively where the sialic acid-receptor binding site(s) and the fusogenic activity were located 2) The pH as acidic (low) ranging from

5.2 - 5.5 (as in the lysosomal compartment). The acidity causes the protonation of the exposed N-terminal of HA2 rendering it hydrophobic, thus enabling it to insert into the cell membrane.

In addition to H, Huang et al (1980) demonstrated that the presence of Neuraminidase (N) was also required for fusion. They found out that although H caused the adsorption of the virus to the liposome, however, fusion occurred only when soluble N was added to the medium concluding that both H and N were required and play a complementary role in fusion. Therefore, antibodies against both H and N would be important in preventing the fusion capaicty of these antigens with the cell membrane and consequently may prevent penetration and infection.

Strain specific differences in the susceptibility of the H to cleavage account for differences in host range and pathogenicity (Rott et al,(1980; Bosch et al, 1979). Two major types of carbohydrate side chains exist on the H polypeptide, a) complex type I that contains mannose, glucosamine, galactose, and fucose; b) mannose-rich type II which contains only mannose and glucosamine (Schwarz et al, 1977; Nakamura and Compans, 1978; Collins and Knight, 1978). Schwarz and

Klenk (1981), found distinct quantitative and qualitative differences between the carbohydrates of 21 influenza A strains in the distribution of type I and type II side chains on HA1 and HA2 fragments; the majority being located on HA1. Schwarz and Klenk (1981) suggested that the primary structure of the polypeptide is an important determinant for the carbohydrate moiety of the H. The structure of the H trimer consists of two distinct regions, the first being a long fibrous region containing residues from both HA2 and HA1 and is centered on a 76Å triple-stranded α-helical coiled-coil extending from the membrane surface. The second region consisting of a globular region which contains residues entirely from HA1, sits on top of the fibrous stem, and includes an 8-stranded β-structure which provides the framework for the sialic acid receptor binding site (Wilson et al, 1981). The amino acid sequence analysis of several strains has recently been completed, and it was shown that depending on the subtype, each monomer of the trimeric glycoprotein consists of 318-328 amino acid residues on the heavy (HA1) chain and 221-222 residues on the light (HA2) chain (Ward and Dophiede, 1980; Dopheide and Ward; 1980, Min-Jou et al, 1980; Gething et al, 1980; Sleigh et al, 1980),and that both HA1 and HA2 are connected by a disulphide bond. The HA1, besides containing most if not all of the antigenic activity of hemagglutinin (Jackson et al, 1979; Sleigh et al, 1981), contains most of the carbohydrate whereas HA2 has hydrophobic properties. Antigenic drift in influenza A viruses occurs by selection of variants with altered hemagglutinin and neuraminidase molecules that are imperfectly neutralized by the host's immune system (Dowdle et al, 1974). Webster & Laver (1980) suggested that changes in certain amino

acid sequence residues of the HA1 are more important than others in producing a new variant with an epidemiological potential.

Determinant Sites of Hemagglutinin

The most highly conserved sequence in the H is the amino terminus of HA2. This region is associated with the activity by which the virus penetrates the host-cell membrane (Wilson et al, 1981). Further analysis of the antigenic structure of H revealed that there are 3-4 defined antigenic sites or determinants on the hemagglutinin molecule, as revealed by highly specific hybridoma (monoclonal) antibodies (Gerhard et al, 1981; Breschkin et al, 1981; Webster and Laver, 1980). One of the four regions is completely distinct and contains epitopes recognized by several monoclones. The two other regions are apparently distinct, but not completely remote, from the first major region. The fourth region is physically unrelated to the others, and unlike the first three regions which show neutralizing activity, it has no such activity (Breschkin et al, 1981). Lubeck and Gerhard (1981) suggested that two of the four antigenic sites are predominantly cross-reactive, which suggests that parts of these sites are either structurally overlapping or in close proximity.

Functions of Hemagglutinin

Hemagglutinin antigen is recognized as the major antigenic component playing a significant role in infection as well as immunity (Kilbourne, 1978). Hemagglutinin is the means by which influenza virus adheres to susceptible cells. It was demonstrated <u>in vitro</u> using liposomes

containing both the H and N, that such liposomes would adhere to cell membranes if H was in the cleaved form. If uncleaved H was used instead, liposomes only adsorbed to cells without causing fusion of membranes (Huang et al, 1979; Rott, 1980).

Following natural infection or immunization, antibody response to the hemagglutinin was found to exhibit extensive cross-reactivity with related influenza viruses which is a reflection of the cross reactivity of the antigenic determinants of the H antigen of the virus subtype (Schild et al, 1974). However, strain-specific (SS) antibodies to the H were also found to appear, particularly after a second influenza infection or immunization by virus of the same type (Laver et al, 1976; Oxford and Schild, 1979; Tyrrell and Smith, 1979; Virelizier et al, 1979). It was demonstrated by Haaheim and Schild (1980) that the strain-specific antibodies not only were considerably more efficient than the cross- reactive (CR) antibodies in direct virus neutralization test, but were also approximately four-fold more effective than the cross-reactive antibodies in protection against fatal infection in mice. Similarly, humans (primed adults in particular) responded to initial infection or immunization by producing CR and SS antibodies directed against the first encountered subtype (Oxford et al, 1979). Furthermore, the SS antibodies had a higher capacity to neutralize the virus (Schild et al, 1977) and to inhibit hemagglutination (Haahein, 1980). Potter et al (1977) and Larson et al (1978) indicated that infection or immunization with heterologous virus vaccine may induce protective heterotypic hemagglutination inhibiting antibodies against challenge virus infection. However, homologous virus induces the

highest degree of protection.

Neuraminidase (N)

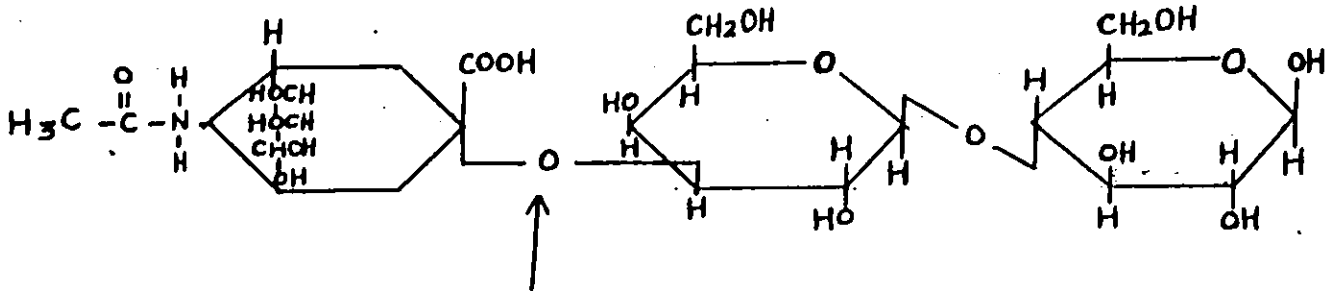
Hirst (1942) was the first to suggest the presence of a functioning enzyme on the surface of influenza virus. Gottschalk (1957, 1958) and Blix et al (1957) characterized the enzyme and established that it specifically cleaves the α,2-3 glycosidic linkage (Fig. 2) joining the keto group of N-acetyl-neuraminic acid (NANA), also known as sialic acid, to carbohydrate moieties such as D-galactose or D-galactosamine, hence the enzyme was named neuraminidase. The enzyme was later systematically designated as mucopolysaccharide-N-acetyl neuraminyl-hydrolase, E.C. 3.2.1.18 according to Enzyme Nomenclature (1965).

Several researchers and authors have discussed and reviewed what is known of the structure, genetics, chemistry, biological function and the immunological role of neuraminidase (Stuart-Harris and Schild, 1976; Bucher and Palese, 1975).

Structure

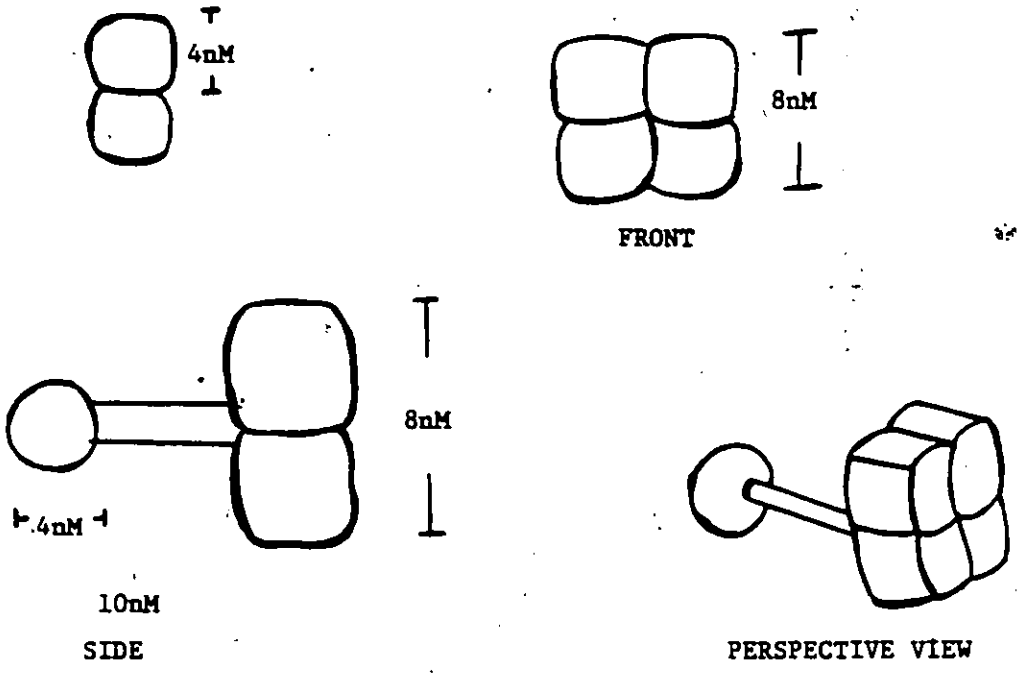
The N exists on the viral membrane as a tetrameric head, 8 x 8 nM in dimension, which is attached to a "fibre" or "tail" of 10 nM in length with a "foot" of about 4 nM in diameter (Fig. 3) (Laver & Valentine, 1969; Wrigley et al, 1973). The "heads" have a total molecular weight of approximately 207,000 daltons while N subunits with fibers have a M.W. of approximately 240,000 daltons (Wrigley et al, 1973). More

Fig. 2 : Action of neuraminidase enzyme on substrate. Cleavage of α, 2 - 3 linkage.



N-Acetylneuraminyl (α, 2 - 3) lactose which is a low molecular weight substrate for influenza virus neuraminidase (Bucher and Palese, 1975. In "The influenza virus and influenza". E.D. Kilbourne, ed. p. 88. Academic Press.

Figure 3: Schematic illustration of the structure of neuraminidase.



From: Kilbourne, E.D. (1975). Influenza Viruses and Influenza. Academic Press., N.Y. p. 82.

recently Cabezas et al (1980) using filtration through sephadex G-200 suggested a M.W. of 270,000. Proteolytically derived heads contain polypeptides of 48,000 dalton M.W. whereas detergent-derived N has a M.W. of 60,000 to 63,000 daltons which indicates that the fibre is of approximately 12,000 daltons M.W. (Wrigley et al, 1973; Fields et al, 1981). The N must be in a tetrameric form to possess enzymatic activity (Kendal & Eckert, 1972). It is not known yet whether the tetramer exists as four equivalent monomers or two types of non-equivalent monomers. The sedimentation coefficient ranges between 8-9S (Drezniek et al, 1966; Bucher & Kilbourne, 1972).

The complete structure of the neuraminidase gene in influenza A/PR/8/34 has been determined. Fields et al (1981) cloning N gene into bacteriophage M13 and sequencing with dideoxynucleotide chain terminator, found that the gene is 1,413 nucleotides long, codes for a protein of 454 amino acids, has a molecular weight of 50,087 daltons, excluding carbohydrates, and has five potential glycosylation sites. In addition, the amino acid composition of the N in A/PR/8/34 correlates reasonably well with the amino acid composition of A/Bel/42 (N1) which was sequenced by Laver & Baker (1972). The amino acid composition indicated that the N is rich in cysteine (19 residues), whose position is still unknown. Fields et al (1981) suggested the presence of one major hydrophobic region located near the N-terminus and that the attachment of protein to the virus membrane occurs through this region. The RNA segment 6 (which codes for neuraminidase) obtained from six N1 and five N2 influenza A strains have been sequenced from the 3' end of the viral RNA using the dideoxy method. The above comparative

nucleotide sequencing revealed that in the first 200 nucleotides, 20 single base changes occurred within the N1 subtype and 26 among the strains of the N2 subtype. However, there is a region at the N-terminus of the predicted amino acid sequence which is conserved between the N1 and the N2 strains (Blok & Air, 1980a; Fields et al, 1981).

The antigenic variation of neuraminidase has been examined by several workers using methods such as RNA-RNA hybridization (Scholtissek et al, 1976), oligonucleotide mapping techniques (Nakajima et al, 1978) and neuraminidase-inhibition test (Aymard-Henry et al, 1973). Antigenic "drifts" (Paniker, 1968; Schulman & Kilbourne, 1969) and "antigenic shifts" (Paniker, 1968) have been documented. Antigenic shift in human influenza viruses occurred in 1957 when the N2 subtype replaced the N1. A comparison of the N-terminal amino acid sequences predicted from RNA sequencing (Blok & Air, 1980b) show that within a subtype there is a high degree of amino acid conservation, but between the different subtypes there is little homology after the first 12 amino acids. Scholtissek (1979) using the hybridization technique, and Nakajima et al (1978) utilizing oligonucleotide mapping, demonstrated that the recent influenza virus isolate A/USSR/77 (H1N1) is very similar to the strain which was present in the 1950's. Only 2 of the 200 nucleotides were different between the H1N1 subtypes A/FM/4/50 and A/Memphis/10/78 (an A/USSR/77 type strain).

Biological Properties

Although N enzymatic activity has a wide range in cleaving the

α-glycosidic bond, it was found that N activity was most efficient and specific in cleaving α, 2-3 linkages (Drezniek, 1972). Neuraminidase acts on several natural substrates such as fetuin, bovine submaxillary mucin and porcine submaxillary mucin, or synthetic substrates such as 2-phenyl-N-acetylneuraminic acid, 2,P-nitrophenyl-N-acetylneuraminic acid, 2-(3 -methoxyphenyl) -N- acetylneuraminic acid, and 4 -methyl umbelliferyl -N- acetylneuraminic acid (Bucher and Palese, 1975). The pH optimum for N activity is 5.9-6.0 (Aymard-Henry et al, 1973; A.P. Kendal, Personal Communication via R.E. Fyson). The temperature optimum varied with different strains (Bucher and Palese, 1975), although 37°C was found satisfactory in most instances. Dimmock (1971) suggested that 0.3 mM Ca^{++} was required for N2 neuraminidse to achieve its maximal activity, whereas a 10-fold greater concentration (3mM Ca^{++}) was required for N1 neuraminidase for its maximal acivity. Carroll and Paulson (1982) also suggested that the addition of 5 mM $CaCl_2$ stimulated N activity 20-100 fold and prevented the loss of such activity in the N1 serotype. However, such requirements are still controversial and may largely depend on the strain of the virus and techniques used.

The amount of N incorporated into the virion can be quite variable, depending on the host cell in which the virus is grown, as well as on the strain of the virus, ranging from 6% to 14% of the total protein of the influenza virion (Bucher and Palese, 1975). Kendal et al (1968) suggested that the number of N molecules on the virion can be estimated if the following were known: 1) N content of the envelope; 2) viral protein content; 3) molecular weight of the viral particle; 4) The

incorporation of Avogadro's number. It was estimated that between 100-200 molecules of N exist on the surface of influenza virus compared to approximately 1000 molecules of H.

Solubilization of N

Neuraminidase can be solubilized by either of two major methods: 1) proteolysis, 2) lysis by detergent. Proteolytic agents utilized included trypsin (Noll et al, 1962), chymotrypsin (Rafelson et al, 1966) and protease (Biddle, 1968). Proteolytically-derived N did not aggregate, although the 7000-12,000 dalton fiber peptide was lost during the process (Wrigley et al, 1973). Detergent agents used included Sodium dodecyl sulfate (SDS) (Laver, 1963), Tween 20 and Tween 80 (Webster and Darlington, 1969), NP40 (Gregoriades, 1972), Triton x-100 (Bucher, 1975), Sarkosyl (Schild et al, 1971).

Function and Role of Neuraminidase

Neuraminidase exhibits both enzymatic and antigenic activities. This has lead many investigators to devise methods for the assessment of N, particularly as an antigen and its relation to immunity. Several techniques have been devised to measure the interaction of N with antibodies:

1. Inhibition of enzymatic activity (Aymard-Henry et al, 1973); Siekmann et al, 1975)
2. Immunoprecipitation (Easterday et al, 1969)
3. Reduction in plaque size (Jahiel and Kilbourne, 1966)

4. Mouse titration test (Schulman et al, 1968)
5. Improved hemagglutination - inhibition procedure for measurement of NI antibodies (Kendal et al, 1971)
6. Single-radial hemolysis in agarose gels (Callow and Beare, 1976; Farrohi et al, 1977)
7. Photometric antibody concentration unit (ACU) for titration of neuraminidase antibodies (Drescher and Desselberger, 1976) .
8. Enzyme - linked immunosorbent assay (Murphy et al, 1980).
9. Lectin-neuraminidase test (Luther et al, 1980).

Currently, the neuraminidase-inhibition test (Aymard-Henry et al, 1973-WHO recommendation; CDC 1975) is the best standardized and most widely used method to identify N antigens. It was adapted for the assessment of N antibodies (Details of the method on page 41).

The function of N is still under investigation. Kilbourne et al (1968), and Compans et al (1969) demonstrated that neuraminidase-inhibition antibodies (NIAB) greatly reduced or inhibited viral release from infected cells but did not affect the infectivity of influenza virus. Therefore they concluded that N aids in the release of mature influenza virions from receptors at the surface of infected cells. Kilbourne (1974) demonstrated that monovalent antineuraminidase antibodies inhibited viral replication in a plaque reduction test. Dowdle et al (1974), and Palese et al (1974) demonstrated that antineuraminidase antibodies prevented virus aggregation. They suggested that N removed sialic acid from the virion, thus eliminating receptors on the surface

which could cause aggregation of the virus to form conglomerates of up to 500 particles.

Recently, Rott (1980), and Huang et al (1980a) suggested that the presence of neuraminidase is essential for fusion between the virion and the plasma membrane of the susceptible cells. They found that the hemagglutinin alone even in the cleaved form is not able to induce fusion. When liposomes coated with cleaved H without N are strongly adsorbed to cell membranes; fusion occurred only when soluble N, viral or even V. cholerae enzyme (RDE) is introduced into the liposome - host cell mixture. This suggests that both H and N glycoproteins are required for penetration and infectivity of influenza virus.

Neuraminidase and Immunity

Currently there is general agreement about the significance and protective value of antibodies to hemagglutinin (Potter et al, 1977; Potter and Oxford, 1979) but some doubt still exists as to whether antibodies specific for neuraminidase are of significance in protection against influenza. Evidence presented by Schulman et al (1968) has demonstrated that antineuraminidase antibodies do offer protection from further influenza infection by limiting the spread of the virus from already infected cells and the subsequent spread of infection. Thus antineuraminidase antibodies do complement the protective effect of antibodies to hemagglutinin. Monto and Kendall (1973) observed that during the 1968-1969 outbreak of influenza due to A/Hong Kong/68 (A/HK) (H3N2) in a population lacking antibodies to H3 of the new virus, elevated titres of antibody to N were associated with a lower incidence

of serologically proven infection. Those lacking pre-existing serum anti-N2 antibodies demonstrated double the rate of infection by A/HK virus compared with those possessing higher anti-N2 titres. Murphy et al (1972) observed that, in artificially infected volunteers, the clinical response, duration of virus shedding and maximal level of virus shedding correlated inversely with the level of anti-neuraminidase antibodies in serum. Kilbourne et al (1972) proposed a new approach to the vaccination against influenza in which recombinant viruses with relevant or homotypic N to the anticipated challenge virus were used. This was based on the concept that such an immunization will result in the induction of monospecific antineuraminidase antibodies which would not prevent the infection with the challenge virus but would prevent its spread. However, a superior immunity should develop on subsequent natural exposure to the virus and consequently the individual would possess a biphasic immunity (anti-H and anti-N) and thus will be resistant to both infection and illness. Employing the above system Couch et al (1974) immunized mice and humans with inactivated vaccine prepared from recombinant viruses with irrelevant H from A/Equi 1 (H7) but a relevant N from A/Hong Kong/68. They demonstrated that the vaccine induced protection against illness caused by infection with an influenza virus containing identical N and that the infection induced resistance to subsequent challenge with an antigenically related virus. Kilbourne (1976) vaccinated student volunteers with either the conventional biphasic A/England/42/72 (H3N2) vaccine or an experimental antigenic hybrid (X-38) [(Heq1N2Eng)=(H7N2Eng)] vaccine containing comparable amounts of the same N. His comparative studies indicated that the hybrid vaccine was of a superior potency in inducing a response

to N2, whereas a significant antibody response to N2 ($\geq$4-fold increase) was observed in 25% of those vaccinated with the conventional vaccine and in 69% of those who received the hybrid vaccine. Mean antibody response to N2 was two fold greater in those vaccinated with hybrid vaccine. Ogra et al (1977) immunized young school children (mean age 14 years) with an inactivated recombinant influenza virus vaccine specific for N antigen of A/Port Chalmers/73 [(Heq1N2Ch)=(H7N2Ch)] and with conventional biphasic Port Chalmers vaccine (H3Ch N2Ch). Specific antibody response to the N2Ch antigen of Heq1N2Ch was observed in 90% of vaccines. The investigators indicated that vaccinees with serologically proven infection but without clinical illness were found to have titres of antibody to N before infection that were four- to eight-fold higher than the titres in vaccinees who were infected, and who became clinically ill. However, during a subsequent natural outbreak of influenza only 51.4% of H7N2Ch recipients in contrast to 74.3% of the H3Ch N2Ch recipients were protected against infection. This finding suggests that high levels of anti-neuraminidase antibody offer a certain degree of protection, and that protection against illness correlates with the level of pre-existing HI and NI antibodies rather than with HI antibody alone. Kendal et al (1977) proposed that the antibody response to N can be suppressed if subjects receiving the vaccine have been primed with its HA component. The results support the hypothesis that the immunogenicity of N is greater in populations primed with N but lacking antibodies to H specific for the immunizing virus. This was demonstrated by studies from 1963-1968, when H2N2 vaccine never induced NIAB response in more than one-quarter to one-third of the vaccinees,

whereas H3N2 vaccine (containing novel H) induced NIAB response in 66%-96% of the vaccinees (Mostow et al, 1975). Utilizing ts-vaccines in volunteers, Murphy et al (1978) indicated that the failure of A/HK/68, Udorn/72 and Georgia/74 ts-1(E) vaccinees to develop vaccine induced systemic reactions may reflect the presence of pre-inoculation NIAB before infection. However the Victoria/75-ts-1(E) vaccinees who lacked neuraminidase inhibition antibodies, or possessed a low titre of this antibody, developed symptoms and shed more virus, suggesting that NI immunity can decrease the infectivity of a virus in addition to modulating the clinical response.

Little work has been done on the response of anti-neuraminidase antibodies in the aged. Kendal et al (1973) studied the influenza neuraminidase antibody patterns in a population whose birthdates fell between 1880 and 1953. Sera tested were pooled by two-year age groups. The number of pools was 39 and each contained sera from 25 individuals. The results of the study demonstrated a) distinctive distribution by age of anti-neuraminidase antibodies, and b) that the antigens which characterize the enzymes of strains of the first infections in childhood permanently orient the antibody forming mechanisms. Therefore, on subsequent exposure to antigenically related enzymes, the level of primary anti-neuraminidase antibody is reinforced. Besides the humoral immunity represented by antibodies specific for either H or N, there is a role played by cell mediated immunity (CMI) although this role is yet not very clear. Cytotoxic T lymphocyte response has been demonstrated in mice to both primary and secondary stimulation and can be strain specific (Ennis et al, 1977), or cross-reactive (Doherty et al, 1977) or

both (Effros et al, 1977) depending upon the target-cell system or viruses employed in cytotoxicity assays. Cambridge et al (1976) indicated a lack of response in cytotoxic assays to homologous neuraminidase antigen. Chow et al (1979) demonstrated that influenza immunization induced CMI response to the H and N surface antigens and suggested that these responses may be important in immunity and recovery from influenza infection. Greenberg et al (1977) indicated that lymphocyte cytotoxicity to influenza virus-infected cells may be mediated by small quantities of antibody. Armerding & Rossiter (1980) suggested that inoculation of mice with live influenza virus resulted in the induction of cytotoxic thymus-derived (T) lymphocytes and of bone marrow-derived (B) cells producing antiviral antibodies. Lazer & Wright (1980) suggested that influenza virus H1N1 which circulated before 1957 induced long-term CMI for periods of at least 20 years as was demonstrated by the use of A/USSR/77 (H1N1) virus as test antigen.

Background

In our present study we have utilized the two types of distinct human related neuraminidases currently known, the N1 of A/USSR/92/73 (H1N1) and the N2 of A/Port Chalmers/73 (H3N2). They represent respectively the neuraminidase type prevalent before 1957 (N1) which reappeared in autumn of 1977, and the N2 type which emerged after 1957 and is still the prevalent type (MMWR, 1982). The choice of A/USSR/92/77 (H1N1) and A/Port Chalmers/73 (H3N2) is justified because of the following facts.

The study population consists of elderly and chronically ill patients of mean age 80±2 years (see Materials and Methods, p 46) who were previously exposed to the N1 type neuraminidse of influenza H1N1 (=Hsw1N1) which was thought to circulate in 1918-1919, to the N1 of A/PR/8/34 (H1N1) circulating in 1934-1946, and the N1 of A/FM/1/47 (H1N1) circulating between 1947-1957. With the reappearance of H1N1 of A/USSR/92/77 in 1977 this population could demonstrate reinforced resistance to H1N1 infection by possession of pre-existing anti-neuraminidase antibodies in the serum and the restimulation of such antibodies upon a second exposure in accordance with the concept of serological recapitulation (Kendal et al, 1973). If that holds true, and this defined population shows considerable anti- neuraminidase antibody (NIAB) in their serum, then this may reflect the fact that such NIAB contribute to protection.

In September and October 1973, A/Port Chalmers/73 (H3N2) a new variant from the parent A/Hong Kong/68 (H3N2) emerged. In the new variant, antigenic "drift" occurred particularly in the neuraminidase antigen (Schild et al, 1974). Thus this variant was subsequently accepted as the prototype for proceeding (H3N2) strains (Schild et al, 1974; Jennings and Miles, 1978). Although the defined elderly population had had previous exposure to (H3N2) type, nevertheless, the new variant A/Port Chalmers/73 showed a neuraminidase which "drifted" from A/England/41/72 as demonstrated by a 32-fold reduction in neuraminidase inhibition antibodies (Schild et al, 1974). Thus it could be possible that the elderly population is more susceptible to infection by A/Port Chalmers/73 (H3N2) due to the lack of pre-existing neuraminidase

antibody to A/Port Chalmers strain in their sera (Jennings and Miles, 1978).

The elderly and chronically ill population selected for this pilot study has continually participated in a five-year influenza vaccination program initiated by E. Perry and R.E. Fyson in July 1976 (see Materials and Methods). This study is unique because the anti-neuraminidase antibodies in a defined elderly population have been monitored and evaluated over a five-year period of annual influenza immunization. The results of this study were utilized to investigate a) the neuraminidase inhibition antibody response in defined aged and chronically ill patients to repeated annual influenza immunizations, and b) monitoring the persistance and duration of these antibodies.

MATERIALS AND METHODS

Viruses

Variants

Recombinant influenza viruses were used bearing relevant N but irrelevant H antigen. To minimize the possibility of "steric hindrance" by HI antibody, recombinant viruses were used.

Influenza virus recombinants were:
A/Equi/Prague/1/56 (H7) - USSR/92/77 (N1) (H7N1), and A/Equi/Prague/1/56 (H7) - Port Chalmers/73 (N2) (H7N2). The designation of H7 for A/Prague/1/56 is in accordance with the new system of nomenclature for influenza viruses (Assaad et al, 1980). The H7 in the new system (Assaad et al, 1980) corresponds to Heq1 in the old system (Chanock et al, 1971). Designations for N1 and N2 remain as in the old system. Each recombinant virus used contains an irrelevant hemagglutinin (H7), but relevant neuraminidases (N1 and N2). Recombinant virus reference strains were kindly provided by D.A. McLeod, Influenza Reference Centre, Bureau of Microbiology, LCDC, Health and Welfare Canada.

Virus Antigens

Virus Propagation and Antigen Preparation

Recombinant viruses were grown in 10-11 day old chick embryo (CE) as follows. Ten-fold virus dilutions were made in phosphate-buffered saline (PBS), pH 7.2, containing 1000 units of pencillin and 1000 ug of

streptomycin sulfate per ml (Appendix A). Volumes of 0.1 ml of virus dilutions were aseptically inoculated by the allantoic route using a tuberculin syringe. Each recombinant strain was passaged and harvested on different days to avoid any cross-contamination. A minimum of four eggs per each dilution were inoculated. Virus dilutions ranged from 10^{-1} to 10^{-8}. Eggs were incubated at 33.5°C for 48 hours, chilled overnight at 4°C and the allantoic fluid (ALF) harvested. The allantoic fluid from each egg infected was tested for its viral hemagglutinating activity by spot hemagglutination test (Appendix B). Viruses were repassaged in eggs 11 times for H7N1 and 10 times for H7N2, until maximum hemagglutinating activities of 1:640 and 1:320, respectively, were obtained. Parallel to HA tests, the neuraminidase activity in virus-infected allantoic fluid (V-ALF) of the last few passages was also measured by the neuraminidase activity test (Aymard-Henry, 1973; CDC, 1975) to determine the virus dilution required to produce the antigen with the maximum neuraminidase activity. Seed virus was obtained by inoculating eggs with the V-ALF dilution demonstrating the highest neuraminidase activity. Volumes of 0.1 ml of 10^{-5} of H7N1 and 10^{-3} of H7N2 were inoculated in eggs (on separate days to avoid cross-contamination), incubated for 24 hours at 33.5°C and then chilled overnight at 4°C. The V-ALF of each recombinant virus was harvested separately and clarified by centrifugation at 3500 rpm (2000xg), 4°C, for 30-45 minutes to remove cell debris.

The clarified V-ALF was then dispensed in 1 and 2 ml volumes in clean, sterile glass vials, labelled as virus seed, and stored at -80°C. All harvested V-ALF were tested for bacterial contamination by inoculation to the media, blood agar plate (BA), cooked meat, thioglycolate and trypticase soy agar plate. The seed virus was then used to prepare a uniform and homogenous bulk antigen, and the same procedure for egg inoculation as described above was followed but incubation was for 48 hours. The H7N1 and H7N2 bulk antigens were prepared on separate days to avoid any possibility of cross-contamination. The identity of the bulk V-ALF antigen was checked by cross-reaction with reference ferret specific antisera to H7N1 and H7N2 in neuraminidase inhibition tests (Appendix B). Bulk antigens prepared were dispensed in 2, 5 and 10 ml volumes in clean and sterile glass vials, and stored at -80°C.

Micro hemagglutination (HA) test. Virus titration was done as outlined by the Centre for Disease Control (CDC) Manual series No. 6 (1975), incorporating a slight modification. Viruses were titrated in Linbro disposable U-type microtitration plates using 0.05 ml microdroppers, and 0.05 ml microdilutors. Doubling dilutions of virus in 0.05 mL volumes were made in PBS (pH 7.2) from 1:10 to 1:5120. Rooster RBC (0.5%) at 0.05 mL were added to each well, plates were agitated then incubated on ice for 30 minutes. Controls consisted of 0.05 ml of 0.5% fowl RBC (FRBC) plus 0.05 ml of PBS (pH 7.2) only. HA titres were read at a 50% end point, a modification from the 100% endpoint used by CDC (1975), and expressed as the reciprocal of the dilution.

Serological Techniques

Hemagglutination-Inhibition (HI) test

The method followed was as described in detail by the CDC Manual (1975). In brief, serial doubling dilutions of 0.025 ml volumes of receptor destroying enzyme (RDE)-treated sera (Appendix B) were made in 0.025 ml PBS (pH 7.2) at dilutions ranging from 1:10 to 1:5120. Four hemagglutinating units (HAU) (Appendix B) of V-ALF test antigen 0.025 ml were added to each well. Contents were mixed and allowed to stand at room temperature (RT) for 30 minutes. A volume of 0.05 ml of 0.5% FRBC was added to all wells including RBC controls (0.05 ml PBS and 0.05 ml of 0.5% RBC) and serum controls (0.025 ml PBS, 0.025 ml RDE treated and serially diluted reference antiserum and 0.05 ml of 0.5% fowl RBC). The mixed contents were held at room temperature and read after 30 minutes at a 100% end point.

Neuraminidase Activity (NAC) and Neuraminidase- Inhibition (NI) Tests

These tests which are usually used for the identification of viral strains, have been applied to assess the effect of anti-neuraminidase antibodies (NIAB) in inhibiting neuraminidase activity (Aymard-Henry et al, 1973). Both the NAC and NI tests used followed the procedure outlined by Aymard-Henry et al (1973) and CDC (1975) modified from Warren (1959) and Aminoff (1961).

The NI test consists of two essential parts:

1. The assay of neuraminidase activity (Flow Chart No. 1).

Principal steps are as follows:

a. Release of free N- acetylneuraminic acid (5- acelamido -3, 5 - dideoxy - N - acetyl - D - glycerol - D - glycerol - nonulosonic acid) from the fetuin substrate by the action of neuraminidase.

b. Conversion of the free N - acetylneuraminic acid (NANA) to B - formyl pyruvic acid by periodate oxidation.

c. Extraction of the chromophore in an organic solvent for spectrophotometric analysis.

This method assesses the potency or activity of neuraminidase from which the standard dose for use in the NI test is determined.

2. Neuraminidase-Inhibition test (Flow Chart No. 2).

The principal steps are:

a. Incubation of standard neuraminidase dose with serial dilutions of normal and test sera

b. Determination of the inhibitory action of serum on neuraminidase activity.

c. Calculation of NI titres.

Flow Chart 1 : Step-like procedure of the Mini- and Standard (WHO)- neuraminidase activity (NAC) tests.

0.05 ml virus -ALF antigen dilutions (1:3 to 1:96)

+

0.05 ml fetuin mixture

↓

shake & incubate: 18 hours/37°C (waterbath)

+

add 0.05 ml periodate reagent

↓

shake & incubate: 20 minutes/20°C (waterbath)

+

add 0.05 ml arsenite reagent

↓

shake

+

add 0.05 ml thiobarbituric acid reagent

↓

shake & place in boiling waterbath/15 minutes

↓

place in ice-water for 5 minutes

+

add 1.25 ml butanol reagent

↓

shake tubes on mechanical vortex

↓

centrifuge 2-3 minutes 500 xg

↓

read in spectrophotometer at 549 nM

Flow Chart 2 : Step-like procedure of the Mini- and Standard (WHO)-neuraminidase inhibition (NI) tests.

0.05 ml test serum of doubling dilutions (1:10 to 1:320)

+

0.05 ml standardized virus antigen dilution

↓

shake & incubate: 1 hour/37°C (waterbath)

+

add 0.05 ml fetuin mixture

↓

shake & incubate: 18 hours/[illegible]°C (waterbath)

+

add 0.05 ml periodate reagent

↓

shake & incubate 20 minutes/20°C (waterbath)

+

add 0.05 ml arsenite reagent

↓

shake

+

add 0.05 ml thiobarbituric acid reagent

↓

shake & place in boiling waterbath for 15 minutes

↓

place in ice-water for 5 minutes

+

add 1.25 ml butanol reagent

↓

shake tubes on mechanical vortex

↓

centrifuge 2-3 minutes 500 xg

↓

read in spectrophotometer at 549 nM

Although NAC and NI tests were used as described by Aymard-Henry et al (1973) and CDC (1975), certain modifications were made: 0.4 M phosphate buffer (PB) was used at pH 6.0 and without the addition of $CaCl_2 . 6H_2O$ (R.E. Fyson from A.P. Kendal - personal communication). After the addition of arsenite reagent, test tubes were held at room temperature for 5 minutes before the addition of thiobarbituric acid reagent (TAR), which was used at pH 2.0 instead of pH 2.6 (our own experimental trials). After the addition of butanol reagent, test tubes were incubated for 3 minutes at 37°C before being shaken for color extraction and then replaced in ice-water. After centrifugation, test tubes were incubated at 37°C for 3 minutes before reading the optical density (O.D.) at 549 nM. All initial dilutions in the neuraminidase tests were done on ice. Incubation temperatures of 31°, 33°, 35° and 37°C were tested for best results. We found that 37°C was the optimal temperature in our own laboratory in contrast to 31°C (Results) as suggested by A.P. Kendal (personal communication via R.E. Fyson), and Kendal et al, (1980).

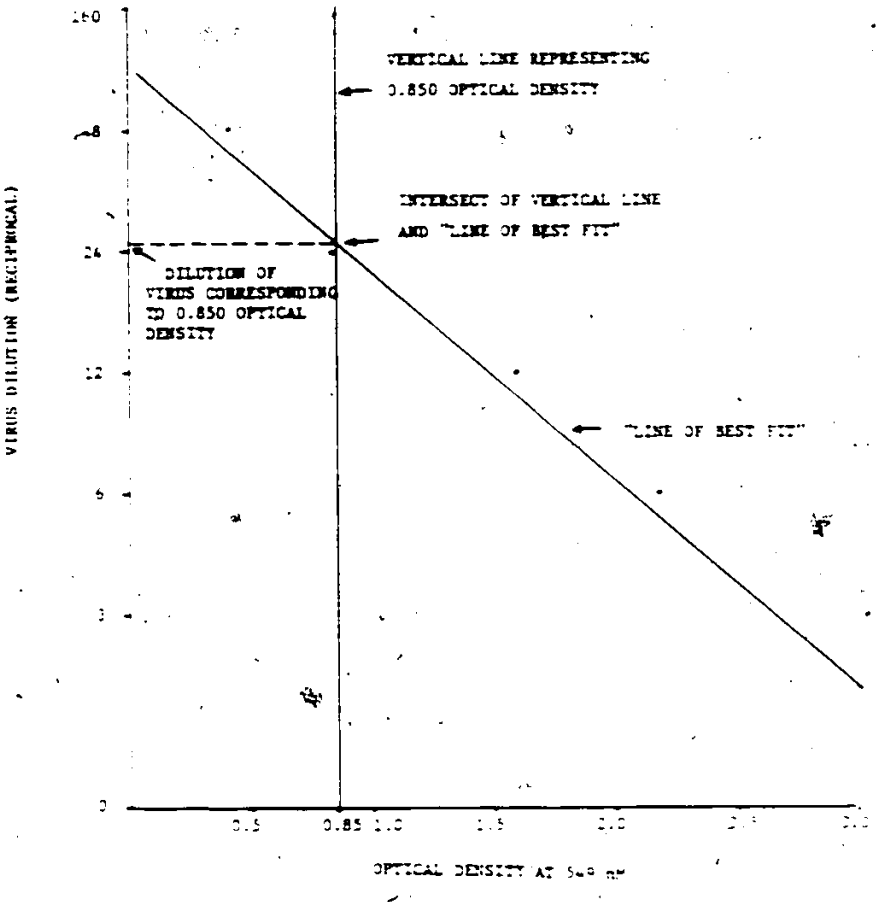
The Standard Neuraminidase Activity Test (CDC, 1975). Initially, doubling dilutions of virus -ALF antigen in 0.5 ml volumes were prepared in 12 x 75 mm test tubes using 0.85% NaCl as diluent. The initial dilutions ranged from 1:2 to 1:128 and were later modified to range from 1:3 to 1:96 in 0.6 ml volumes. To 0.05 ml of each dilution which was transferred into corresponding 16 x 100 mm test tubes using 0.05 ml micropipet (SMI type D), was added 0.15 ml of fetuin (Spiro method) mixture (freshly reconstituted fetuin mixed with equal volumes of 0.85% NaCl and PB), pH 6.0. Controls consisted of a) virus control: 0.05 ml

virus and 0.1 ml of 0.85% NaCl and 0.05 ml PB at pH 6.0. b) fetuin control: 0.05 ml of 0.85% NaCl and 0.15 ml of fetuin mixture. After shaking, tubes were incubated at 37°C (waterbath) for 18 hours, followed by 20°C (waterbath) for a few minutes, then 0.1 ml of periodate ($NaIO_4$) reagent (Appendix A) was rapidly added to all tubes, the contents thoroughly mixed and incubated at 20°C (waterbath) for exactly 20 minutes. With a Cornwall pipettor, 0.1 ml of arsenite ($NaAsO_2$) reagent (Appendix A) was added to all tubes, mixed thoroughly until the brown color disappeared, followed by addition of 2.5 ml of freshly prepared thiobarbituric acid reagent (Appendix A). After thorough mixing, tubes were placed in a boiling water bath for 15 minutes. The appearance of a pink color indicated the presence of neuraminidase activity. Tubes were rapidly cooled in an ice water bath, transferred into a water bath at 37°C, and held for approximately 5 minutes. Immediately 4.0 ml of butanol reagent (Appendix A) was added, the tubes rubber-stoppered and mixed well with a mechanical vortex, and centrifuged at 4°C for 3 minutes at 500Xg. The upper butanol-extracted pink layer was examined in a spectrophotometer (Beckman Model DB-25) at 549nM using a sipper flow cell. Virus and fetuin controls are color-free. The fetuin control was used to calibrate the spectrophotometer to 100% transmission (zero O.D.). on a 3-cycle semilogarithmic graph paper, the vertical (logarithmic) axis is labelled as the "virus dilution factor" and the horizontal (arithmetic) axis as the "Optical Density". From a specific "virus dilution factor" on the vertical axis an imaginary line was drawn until reaching the level on the horizontal axis corresponding to the O.D. reading obtained for that specific dilution factor and a mark was made at that point. This step is repeated for the other specific

"dilution factors" and their corresponding O.D. readings and marks were made. A "line of best fit" was then drawn through the points (marks). Then a vertical line from the point on the horizontal axis corresponding to 0.85 O.D. was drawn upwards intersecting with the "line of best fit". From the point of intersection of the "line of best fit" and the vertical line, a horizontal line was constructed towards the vertical line bearing the "virus dilution factor", and the point of intersection of these two lines represent the dilution of the virus at O.D. 0.85 which was used in the neuraminidase-inhibition test (Fig. 4). Therefore, the dilution of the virus obtained at O.D. 549 nM contains neuraminidase enzymatic activity approximately equivalent to one unit. One unit of enzymatic activity is defined as the amount of the enzyme that liberates 1 μmol of N-acetylneuraminic acid (NANA)/minute at pH 5.9-6.0 and 37°C with fetuin as a substrate (CDC, 1980).

The Standard Neuraminidase-Inhibition (NI) test. The same equipment and reagents used for the NACtest were utilized for the NI test. In performing the test, serial doubling dilutions of test sera were made in 0.85% NaCl in 12 x 75 mm test tubes. Doubling dilutions ranged from 1:10 - 1:320. With a 0.05 ml micropipet, 0.05 ml of each serum dilution was transferred to corresponding 16 x 100 mm tubes, and 0.05 ml of virus antigen, previously standardized by NACtest, was then added to each test tube. Controls for each test included: 1) reference ferret specific antiserum (0.05 ml reference serum & 0.05 ml virus antigen), 2) normal ferret serum (NFtS) (0.05 ml of NFtS & 0.05 ml virus antigen), and 3) fetuin control (0.1 ml of 0.85% NaCl solution).

Fig. 4 : Example showing plot of data for estimating dilution of virus to be used in the NI test. (From Centre for Disease Control. Immunology Series No. 6. Advanced laboratory techniques for influenza diagnosis (1975) p. 116.)



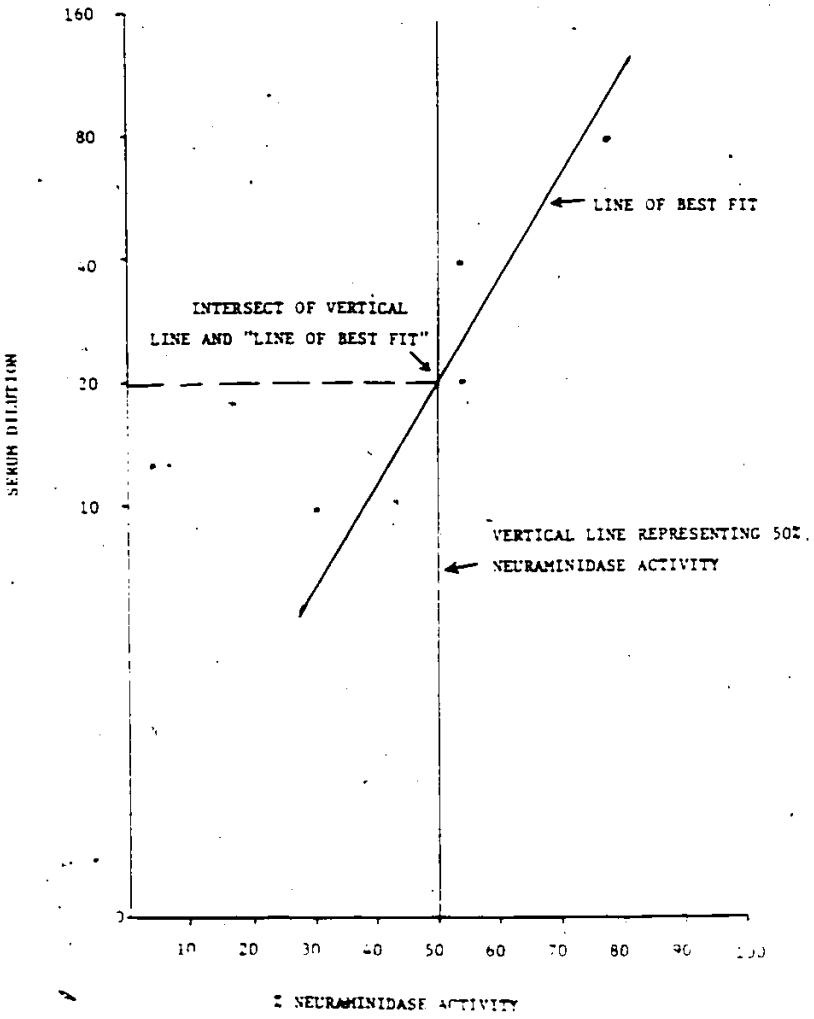
All tubes were incubated at 37°C (waterbath) 1 hour, then 0.1 ml of freshly prepared fetuin mixture (reconstituted fetuin in an equal volume of PB, pH 6.0), was added to all test and control tubes which were further incubated at 37°C (waterbath) for 18 hours. The inhibition effect of the test run was assessed by utilizing the same procedure as used for neuraminidase activity described above. Optimal O.D. for test and control sera were measured in a spectrophotometer at 549 nM. To obtain the percentage of remaining neuraminidase activity the following formula was applied:

$$\frac{\text{O.D. of 1:10 test serum/virus mixture}}{\text{O.D. of 1:10 normal serum/virus mixture}} \times 100 = \% \text{ neuraminidase activity}$$

On 3-cycle semilogarithmic paper, the vertical (logarithmic) axis was marked with "serum dilution factors" and the horizontal (arithmetic) axis was percentage (%) "neuraminidase activity" from 0% to 100%. Construction of the "line of best fit" was done as follows:

From the 1:10 dilution of serum on the vertical axis (Fig. 5), an imaginary line was traced moving to the right until reaching the level on the horizontal axis corresponding to the computed neuraminidase activity for the 1:10 dilution of serum and a mark was made. This process is repeated for the other dilutions of serum. A vertical line is drawn upward from the horizontal axis corresponding to 50% neuraminidase activity. A "line of best fit" is drawn through the points which are linearly arrayed on both sides of the vertical line representing 50% neuraminidase activity. From the point of intersection

Fig. 5 : Example showing plot of data for estimating NI titres of antiserum.
(From Centre for Disease Control. Immunology Series No. 6. Advanced laboratory techniques for influenza diagnosis (1975) p. 116.)



between the "line of best fit" and the line representing 50% neuraminidase activity, a horizontal line is drawn towards the vertical axis bearing the dilution factors and record the dilution factor for the serum which results in 50% neuraminidase activity with the antigen tested. This is the neuraminidase inhibition titre of the antiserum with this antigen.

Mini Neuraminidase-Inhibition (MNI) Test

A mini neuraminidase inhibition test was developed in our laboratory for the purpose of economy. The test was based on the same principle as the standard test, except that volumes used were modified (Table 3). The volumes utilized in the mini test were obtained by a proportional increase of volumes used in the micro (Essen) test (Thraenhart and Kuwert, 1976). The volumes incorporated were as follows: 0.05 ml serum, 0.05 ml standardized virus antigen, 0.05 ml fetuin mixture, 0.05 ml periodate reagent, 0.05 ml arsenite reagent, 0.5 ml thiobarbituric acid reagent, and 1.25 ml butanol reagent. The complete mini test (NAC or NI) from start to end was carried out in 12 x 75 mm test tubes unlike the standard procedure which required the tranfer of 0.05 ml of the initial dilutions made in 12 x 75 mm test tubes to 16 x 100 mm test tubes to accomodate the complete test. Therefore, this was another valuable advantage in the mini test by avoiding any error in the tranfer of dilutions from one set of test tubes to another. Incubation temperature, pH, and methodology of reagent preparation were carried out as described for the standard method (Flow Chart No. 2).

The micro neuraminidase-inhibition test (Thraenhart and Kuwert, 1976) was also tested, but was abandoned because it could not be adapted to the conditions in our laboratory. The micro test exhibited lower O.D. readings (Table 3 and Fig. 14 in Results page 74, 124) than the minimum required O.D. 0.85 which contains one enzymatic unit of neuraminidase. The comparison of volumes used in standard (macro) -, mini-, and micro-NI tests, is detailed in Table 3.

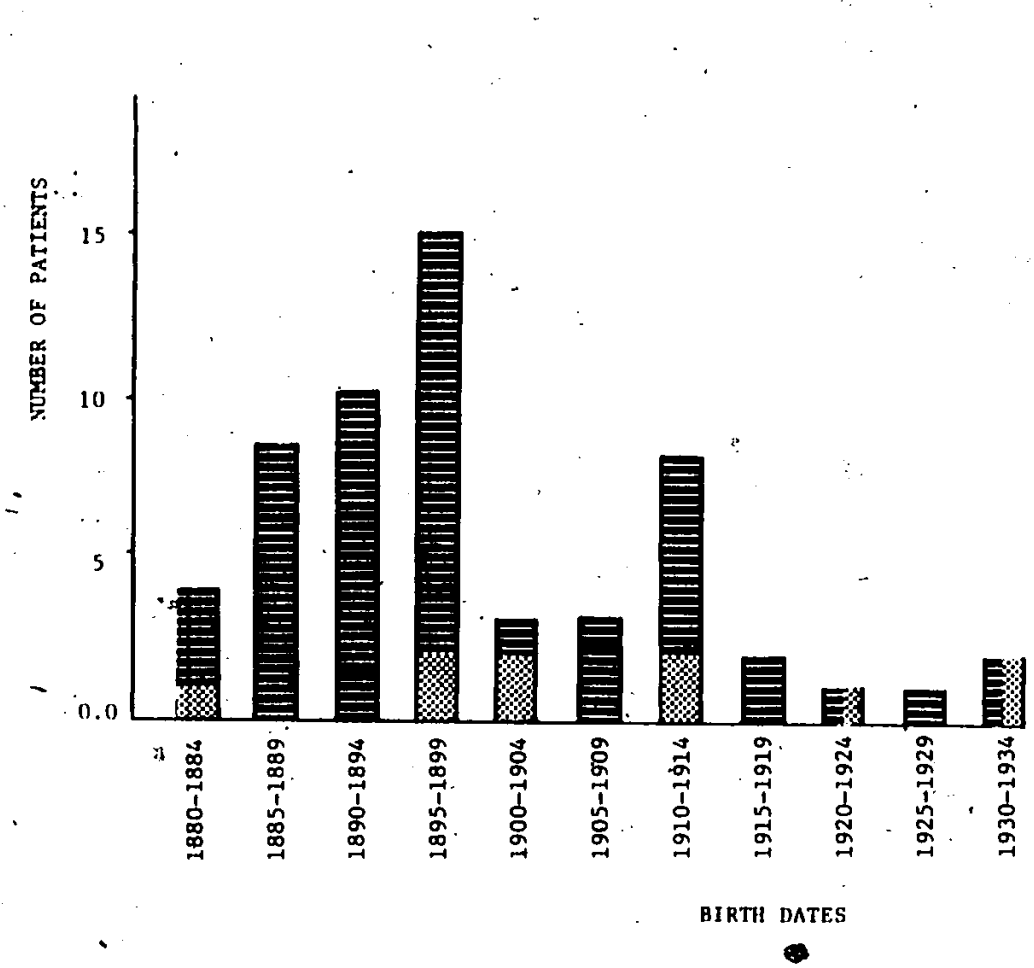
Human Sera

The participants selected from the total program for the neuraminidase antibody study included 69 consenting aged and chronically ill patients (mean B.D. 1902 $\pm$12). Selection of patients was restricted to those who had participated continually for five years in the influenza vaccination program and from whom complete serum sampling had been possible to obtain. Of the 69 patients 59 were females and 10 were males (Fig. 6). In this project 47 vaccinees and 22 non-vaccinated controls were included. Of the vaccinees 40 were females and 7 were males. The controls consisted of 19 females and 3 males (Fig. 6).

Vaccination Program

An influenza vaccination program in an Ottawa hospital for the aged and chronically ill was initiated in July 1976 by hospital authorities in collaboration with E. Petry and R.E. Pyson and is being continued on an annual basis. The study is clinical and observational. The neuraminidase antibody aspect of the continuing study covers a period in which five annual influenza vaccines have been administered, the last in

Fig. 6 : Age distribution of patients at 5-year intervals.



MEAN BIRTH DATE

VACCINEES: 47 (39 females and 8 males)

CONTROLS: 22 (19 females and 3 males)

November 1980. Vaccines employed were in accordance with the recommendations of the National Advisory Committee on Immunization (Canada), U.S. Surgeon General (CDC Atlanta, Ga.), and the World Health Organization (WHO). Vaccines were subcutaneously administered. All vaccines were the inactivated whole-virus type and consisted of the following formulations (according to the data of R.E. Fyson and E. Perry, with permission 1982-1983):

Vaccine 1. Administered December, 1976. Bivalent Influenza A/New Jersey/76 (H1N1) and A/Victoria/3/75 (H3N2) (NJ-VIC) at 200 chick cell agglutinating (CCA) units per 0.5 ml dose.

Vaccine 2. Administered February, 1978. Bivalent influenza A/Victoria/3/75 (H3N2) and B/Hong Kong/72 (VIC-BHK) at 200 CCA units per 0.5 ml dose.

Vaccine 3. Administered December, 1978. Trivalent influenza A/USSR/92/77 (H1N1), A/Texas/1/77 (H3N2), and B/Hong Kong/2/73 (USSR-TEX-BHK) at 7 µg hemagglutinin (HA) per 0.5 ml dose.

Vaccine 4. Administered November, 1979. Trivalent influenza A/Brazil/11/78(H1N1), A/Texas/1/77 (H3N2) and B/Hong Kong/2/73 (BRZ-TEX-BHK) at 7 µg HA per 0.5 ml dose.

Vaccine 5. Administered November, 1980. Trivalent influenza A/Brazil/11/78 (H1N1), A/Bangkok/1/79 (H3N2) and B/Singapore/222/79 (BRZ-BGK-BSP) at 7 µg HA per 0.5 ml dose.

Sera

Sera for this aspect of the total study numbered 691 collected from vaccinated and non-vaccinated patients over the five-year period. Sera for testing were kindly provided by R.E. Fyson and E. Perry, Department of Microbiology and Immunology, School of Medicine, University of Ottawa. Collection of sera was made before, 4-8 weeks following, and approximately one year after each immunization according to the following schedule:

Pre-vaccine 1 bleed : July, 1976
Post-vaccine 1 bleed: January, 1977
Post-vaccine 1 and pre-vaccine 2 bleed: December, 1977
Post-vaccine 2 bleed: March-April, 1978
Post-vaccine 2 and pre-vaccine 3 bleed: November-December, 1978
Post-vaccine 3 bleed: February, 1979
Post-vaccine 3 and pre-vaccine 4 bleed: November, 1979
Post-vaccine 4 bleed: January 1980
Post-vaccine 4 and pre-vaccine 5 bleed: November-December, 1980
Post-vaccine 5 bleed: December 1980 to January 1981.

All sera allocated for this study were tested by the mini NI test. At intervals, a number of patient sera were selected at random and tested by the standard NI test in parallel with the MNI test. All sera collected from each patient over the five year period were tested on the same day and in the same test. With every new batch of sera to be tested, 3-4 previously tested sera were also included in the new batch to serve as controls for the reproducibility of the test. All sera were tested against recombinant viruses H7N1 and H7N2. Reference normal and

specific ferret antisera for H7N1 and H7N2 were always included as controls. Antibody dilutions of test sera from 1:10 to 1:320 were made in 0.85% NaCl.

Statistical Methods

The statistical methods used were those outlined by Colton (1974) and Taylor et al (1979) are as follows:

Mean $(\bar{x}) = \frac{x}{n}$ where: x= total of observations
n= number of observations

Geometric Mean titre (GMT) = $\sqrt[n]{(x1)\ (x2)\ \ldots\ (xn)}$

where: $(x1)(x2)\ \ldots\ (xn)$ = titre of observations

n = number of observations

Standard Deviation, Arithmetic (SD) = $\sqrt{\frac{\Sigma x^2 - (\Sigma x)^2 / n}{n-1}}$

where: Σx^2 = sum of squared observations

$(\Sigma x)^2$ = sum of observations, squared

n = number of observations.

Standard Deviation, Geometric (SDG) = $\frac{\Sigma(\log x - \log \bar{x})^2}{n-1}$

where: $\Sigma \log x$ = logarithmic base 10 of titre observations

$\Sigma \log x$ = logarithmic base 10 of the geometric mean titre

n = number of observations

$$\text{Standard Error (SE)} = \frac{SD}{\sqrt{n}}$$

where SD = Standard deviation

n = number of observations

Standard Error (SE) for percent responders was done by binomial distribution:

$$\text{Percent (\%) SE} = \frac{P \cdot q}{n}$$

where: P = Percent with positive antibody

q = Percent with negative antibody

n = number of observations

$$\text{Chi Square } (x^2) = \frac{[(b-c)-1]^2}{b + c} \quad \text{(with one degree of freedom)}$$

where b = number of patients demonstrating antibody change from low to high

c = number of patients demonstrating antibody change from high to low.

RESULTS

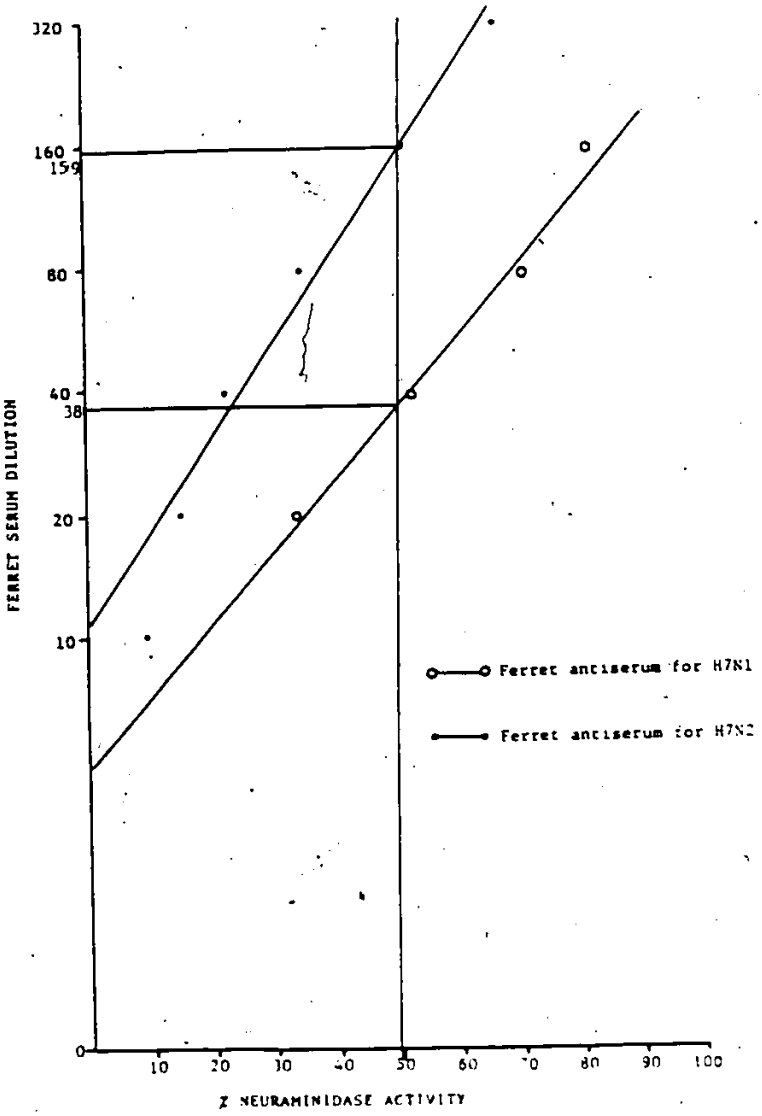
Virus-Infected Allantoic Fluid (V-ALF)

After 11 passages, recombinant virus H7N1 reached its highest HA titre (1:640) whereas recombinant H7N2 had its highest HA titre (1:320) at the end of 10 passages. The last few passaged V-ALFs were tested by the standard WHO neuraminidase test (Aymard-Henry, 1973; CDC, 1975) for N activity. The neuraminidase activity unit can be defined as the highest dilution of the virus which still contains enzymatic activity approximately equivalent to one enzymatic unit measured at O.D. 549 nM (Materials and Methods). The ALF infected with the recombinant H7N1 at optimal dilution of 10^{-5} consistently yielded the optimal N activity of one unit of enzyme (corresponding to 0.85 at O.D. 549 nM) when the V-ALF was diluted 1:18 in the NAC test. In contrast, the desired N activity of ALF infected with recombinant H7N2 at optimal dilution of 10^{-3} was obtained when the V-ALF was diluted 1:25 in the NAC test. This bulk antigen was used as a source of antigen (neuraminidase) throughout this study.

Reference Specific Antisera for H7N1 and H7N2

Reference normal and specific ferret sera (kindly provided by D. McLeod, Influenza Reference Centre, Bureau of Virology, LCDC, Health and Welfare Canada) were used throughout the testing. Specific ferret sera maintained reproducible NIAB titres of 1:34 to 1:40 for H7N1 and 1:151 to 1:160 for H7N2 (Fig. 7).

Figure 7 : The dilution factor of reference ferret antisera specific for influenza H7N1 and H7N2 resulting in 50% inhibition of neuraminidase activity.



Neuraminidase Inhibition Test

The WHO standard NI test was used as described by Aymard-Henry et al (1973) and CDC (1975) with certain modifications. Several experimental trials revealed that using 0.4M PB without CaCl2.6H20 produced equivalent and reproducible N activities at dilutions of 1:18 and 1:25 for H7N1 and H7N2, respectively, (Fig. 8) to the PB used with the addition of $CaCl_2.6H_2O$ (Fig. 9). We adopted the use of PB at pH 6.0 (Kendal et al, 1980; A. P. Kendal via R.E. Fyson - personal communication) instead of pH 5.9 (CDC, 1975). Thiobarbituric acid (TAR) reagent plays the key role in color extraction. The TBA was found to have a pH 2.6 when prepared according to the method outlined by Aymard-Henry et al (1973) and CDC (1975). Usually somewhat cloudy or turbid TAR was obtained when prepared according to the standard method. This cloudiness may have contributed to the formation of a white precipitate in the test after the process of adding TAR and placing test tubes in a boiling water bath for 15 minutes. The precipitation was almost completely prevented by lowering the pH of TAR to 2.0 - 2.1. This gave a reproducibly sharp, clear pink color without the interference of precipitate in O.D. readings (Figs. 10,11). Although CDC (1975) suggested that TBA could be stored in a glass-stoppered bottle at room temperature indefinitely, we found that TAR became rather turbid and developed some precipitates within 48 hours whether it was used at pH 2.0 or 2.6. Therefore, a fresh TAR reagent was prepared for each test to substantially reduce the presence of interferring

Fig. 8 : The dilution of influenza H7N1 and H7N2 (containing one unit of neuraminidase activity) obtained using 0.4 M phosphate buffer with the addition of $CaCl_2.6H_2O$.

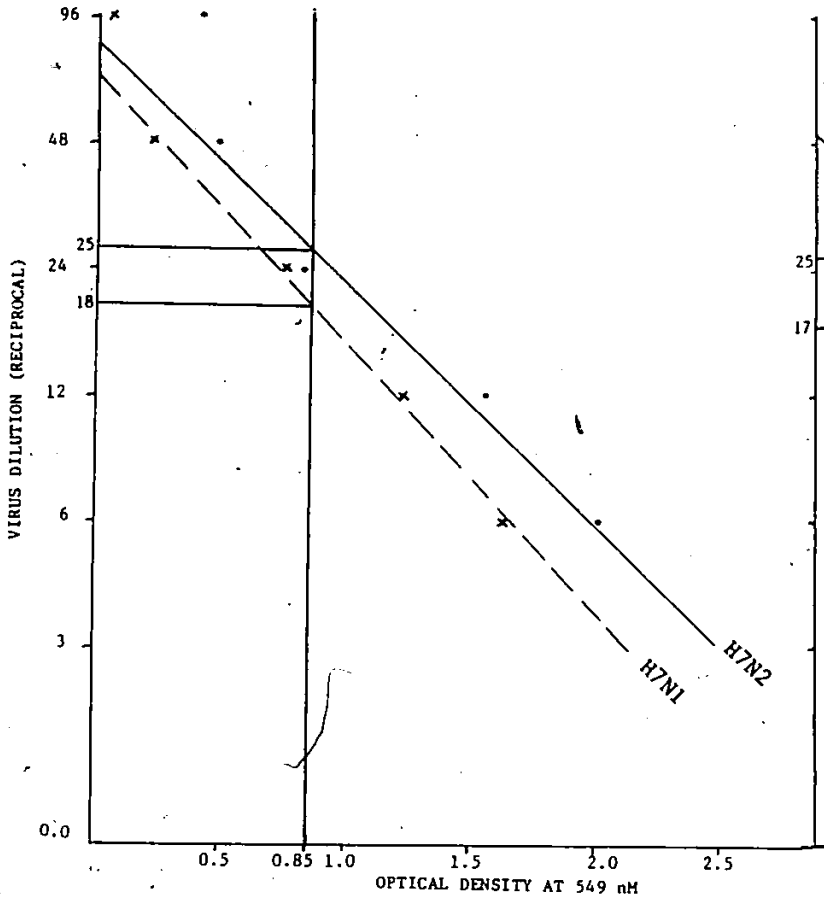


Fig. 9 : The dilution of influenza H7N1 and H7N2 (containing one unit of neuraminidase activity) obtained in the standard (WHO) neuraminidase test using 0.4 M phosphate buffer without the addition of $CaCl_2.6H_2O$.

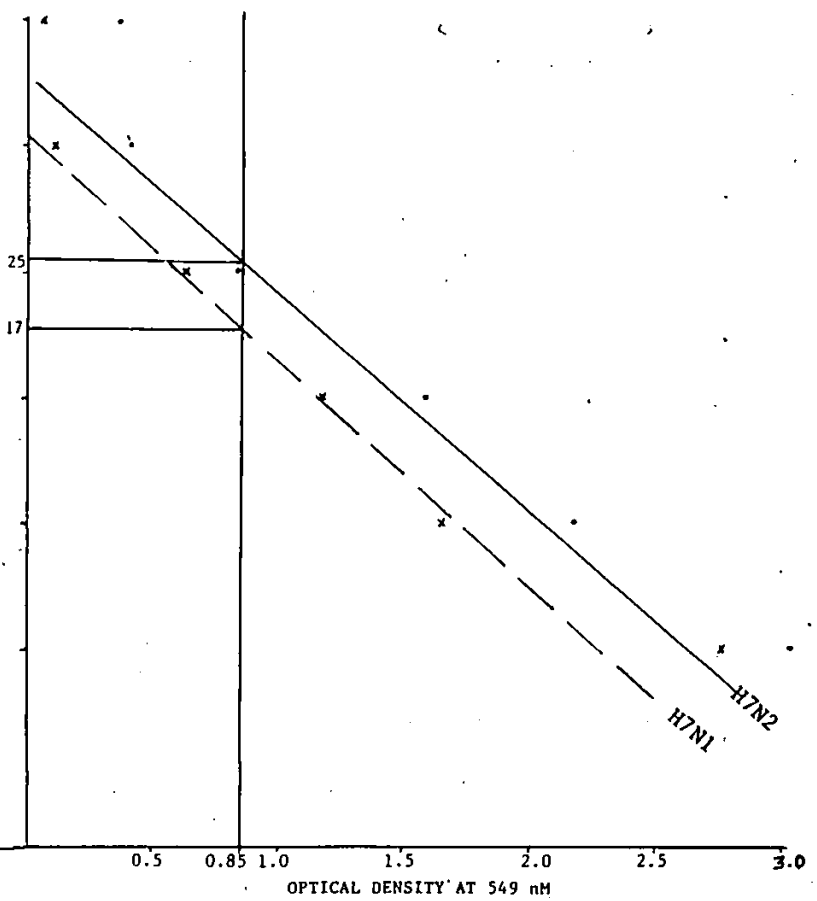


Fig. 10 The dilution of influenza H7N1 (containing one unit of neuraminidase activity) obtained in the Standard (WHO) neuraminidase test using thiobarbituric acid (TAR) reageht at pH 2.0 and pH 2.6.

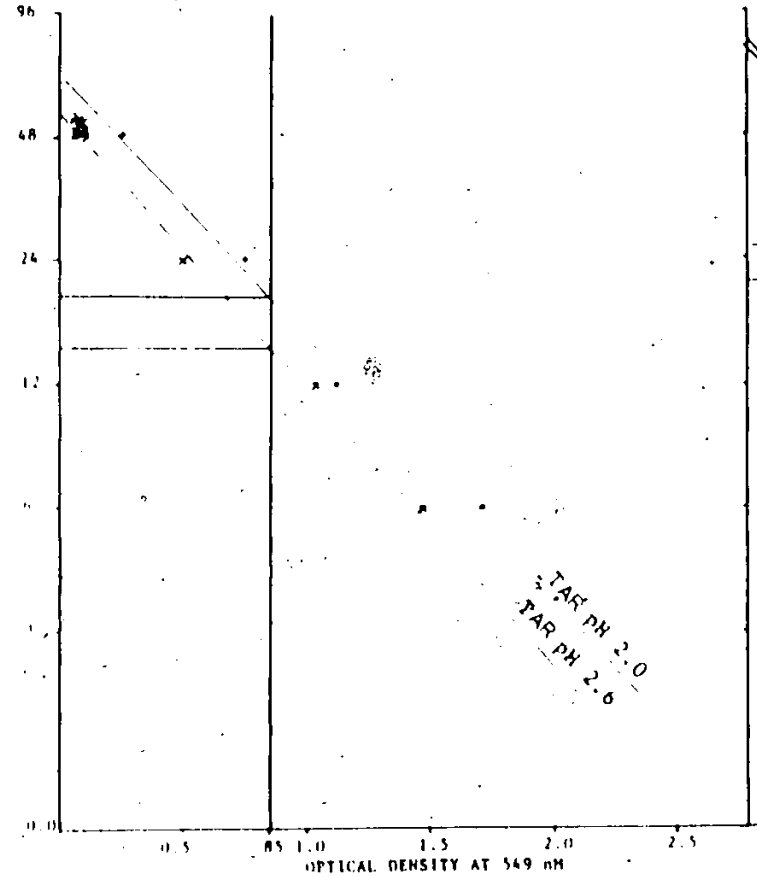
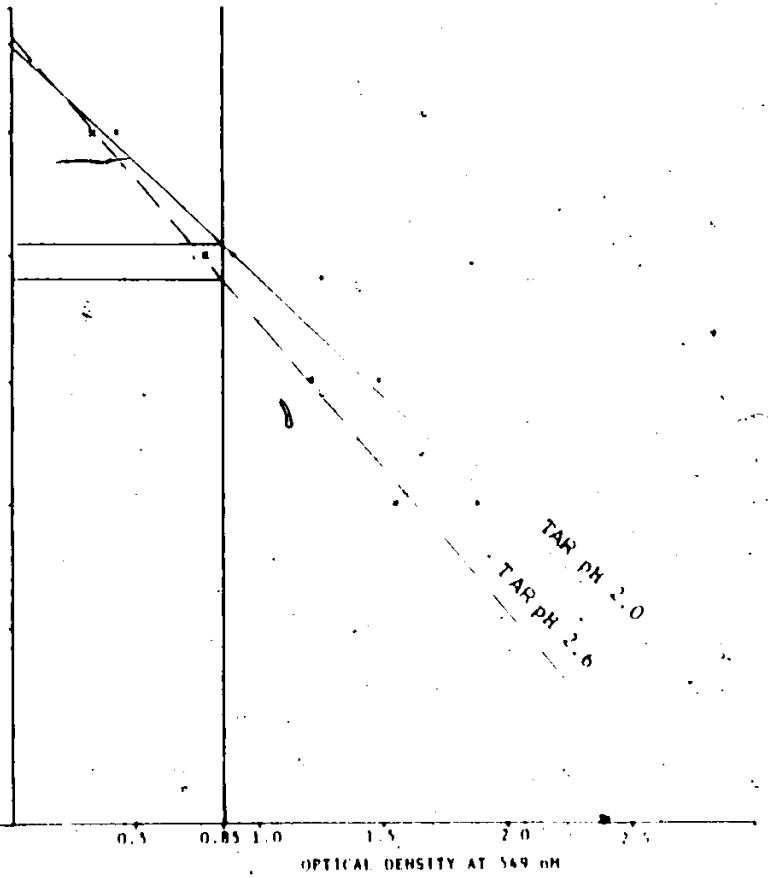


Fig. 11 : The dilution of influenza H7N2 (containing one unit of neuraminidase activity) obtained in the standard (WHO) neuraminidase test using thiobarbituric acid (TAR) reagent at pH 2.0 and pH 2.6.



precipitates in O.D. readings. After centrifugation for color extraction in preparation for spectrophotometer readings, test tubes were incubated at 37°C (waterbath) for 3 minutes to stabilize O.D. readings which fluctuated when the tubes were held at 4°C as recommended in the standard method. Incubation at 37°C (waterbath) for 3 minutes may have rendered the temperature of the material tested closer to that of the spectrophotometer, thus adding a stabilizing factor to the readings. Incubation temperatures of 31°, 33°, 35°, and 37°C were tested for optimal neuraminidase activity. Our findings suggest that 37°C was most satisfactory and resulted in the production of sharp and intense pink color with consequent high O.D. readings (Figs. 12, 13).

Mini Neuraminidase-Inhibition Test.

The mini test was based on principles similar to those of the standard test (Table 3). The volumes used in the mini test were obtained by a proportional increase of 2.5 fold the volumes suggested for the micro NI test developed by Seikmann et al (1975) and standardized by Thraenhart and Kuwert (1976). The micro test was tested, using fetuin as a substrate, but could not be adopted in our laboratory because it produced less than one unit of enzymatic activity at 0.85 O.D. reading (Table 4 and Fig. 14). The acid- α1-glycoprotein, which was utilized in the micro test, was not used in our laboratory because it was not cost effective. According to the requirements of a suitable NI test, approximately one unit of enzyme activity is required. A 0.85 O.D.

Fig. 12 : The dilution of influenza H7N1 (containing one unit of neuraminidase activity) obtained in the standard (WHO) neuraminidase test at incubation temperatures of 31 , 33 , 35 , and 37°C.

Fig. 13 : The dilution of influenza H7N2 (containing one unit of neuraminidase activity) obtained in the standard (WHO) neuraminidase test at incubation temperatures of 31 , 33 , 35 , and 37°C.

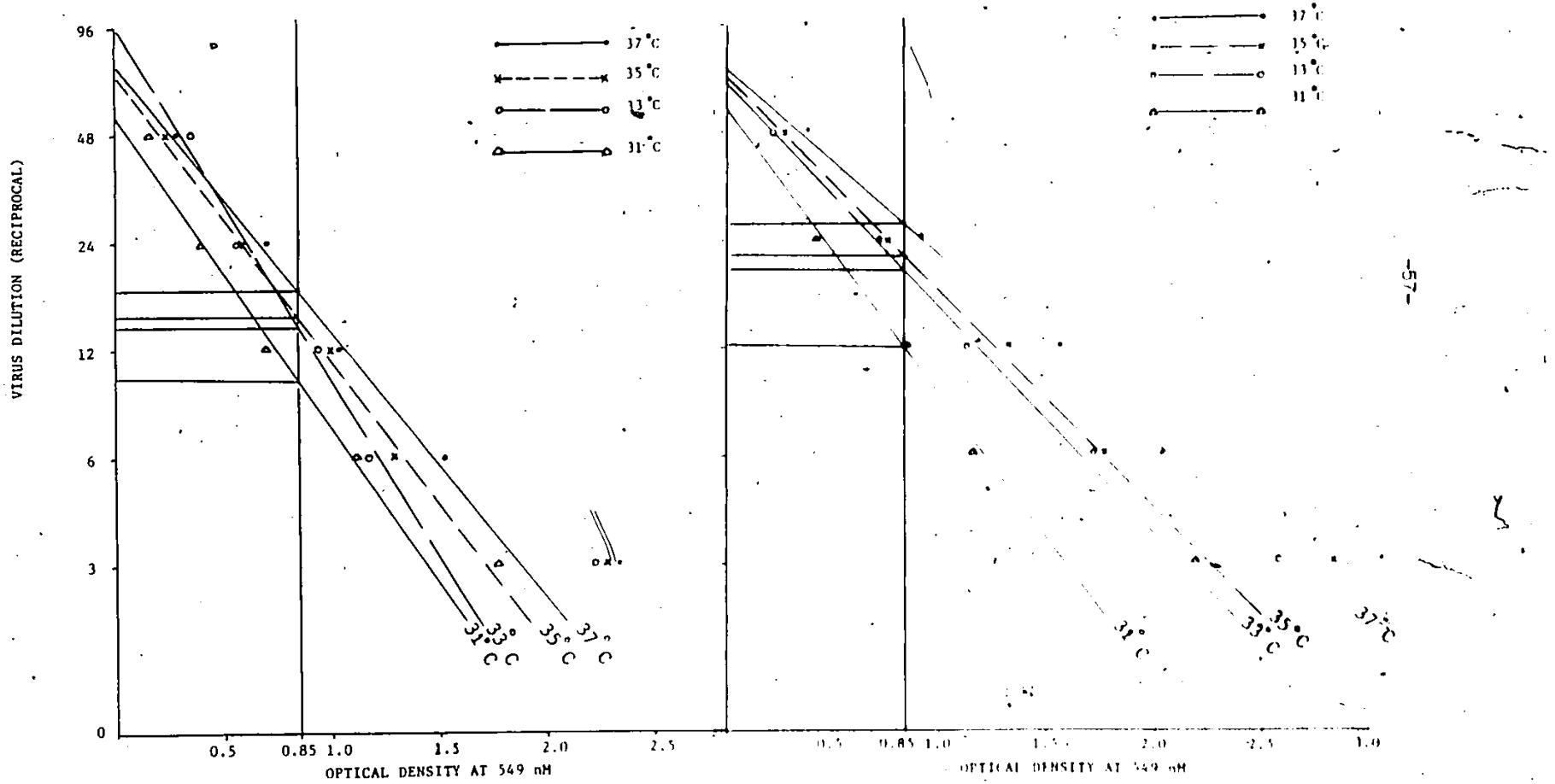


Table 3: Comparison of volumes used in the Standard -, Mini-, and (Essen) Micro - neuraminidase inhibition (NI) tests.

Material/Reagent	* Standard (ml)	Mini (ml)	**(Essen) Micro (ml)
Virus (antigen)	0.05	0.05	0.02
Serum	0.05	0.05	0.02
Substrate	0.15	0.05	0.02
Periodate reagent	0.10	0.05	0.02
Arsenite reagent	1.00	0.05	0.02
Thiobarbituric acid-reagent	2.50	0.50	0.20
butanol reagent	4.00	1.25	0.50

* Centre for Disease Control (1975). Advanced laboratory tecniques for influenza virus. Immunology series No. 6. Procedural Guide. pp. 107-129.

** Thraenhart, O., and Kuwert, E.K. (1976). Standardization of a rapid modified micro-neuraminidase inhibition test (Essen-NIT) for influenza virus - neuraminidase antibody assay and comparison with the W.H.O. method. J. Biol. Stand. 4:225-241.

Table 4: Comparison of the O.D. readings (neuraminidase activities at optical density 549 nM) of H7N1 and H7N2 obtained by the standard, mini, and Essen (micro) neuraminidase activity tests.

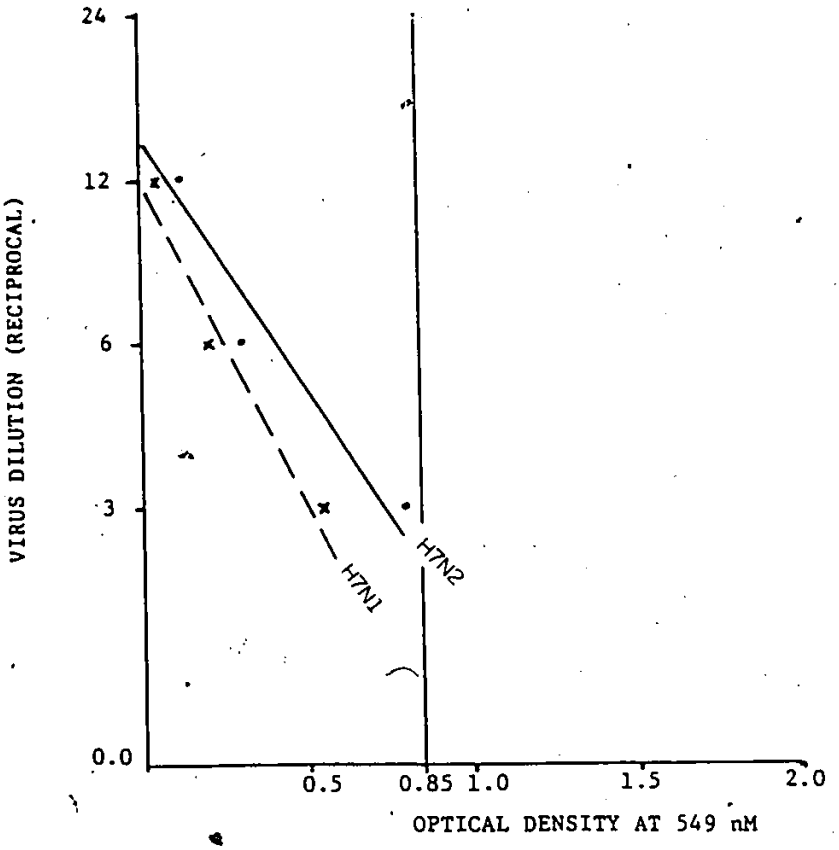
Virus dilution (reciprocal)	Standard				Mini				Essen (Micro)			
	H7N1 *	SE **	H7N2	SE	H7N1	SE	H7N2	SE	H7N1	SE	H7N2	SE
96	0.05	0.024	0.33	0.09	0.25	0.03	0.46	0.06	nil	--	nil	--
48	0.13	0.035	0.43	0.10	0.5	0.05	0.66	0.08	nil	--	nil	--
24	0.69	0.13	0.78	0.08	1.03	0.10	1.45	0.08	nil	--	nil	--
12	1.37	0.2	1.64	0.13	1.62	0.08	1.97	0.12	0.05	0.01	0.11	0.01
6	1.76	0.16	2.24	0.10	2.28	0.11	2.7	0.11	0.17	0.04	0.28	0.04
3	2.8	0.11	3.06	0.15	3.00	0.14	3.15	0.29	0.51	0.08	0.75	0.06

* the O.D. reading representing the neuraminidase activity of the virus at the corresponding dilution. The O.D. reading is averaged from 32 consecutive trials of each of the standard, mini, and Essen (micro) neuraminidase activity tests.

** standard error of the mean at 95% confidence.

O.D. = Optical Density.

Fig. 14 : The dilution of influenza H7N1 and H7N2 (containing one unit of neuraminidase activity) obtained in the Essen (micro) neuraminidase test using 0.4 M phosphate buffer without the addition of $CaCl_2.6H_2O$.



reading represents approximately one unit of enzyme activity contained in the corresponding particular virus dilution (Aymard-Henry et al., 1973; CDC, 1975).

A total of 63 experimental trials were carried out to standardize the mini test for reproducibility. The smaller volumes used, compared to the standard test, produced a sharp and usually more intense color (neuraminidase activity) without the presence of interfering precipitates (Table 4 and Fig. 15).

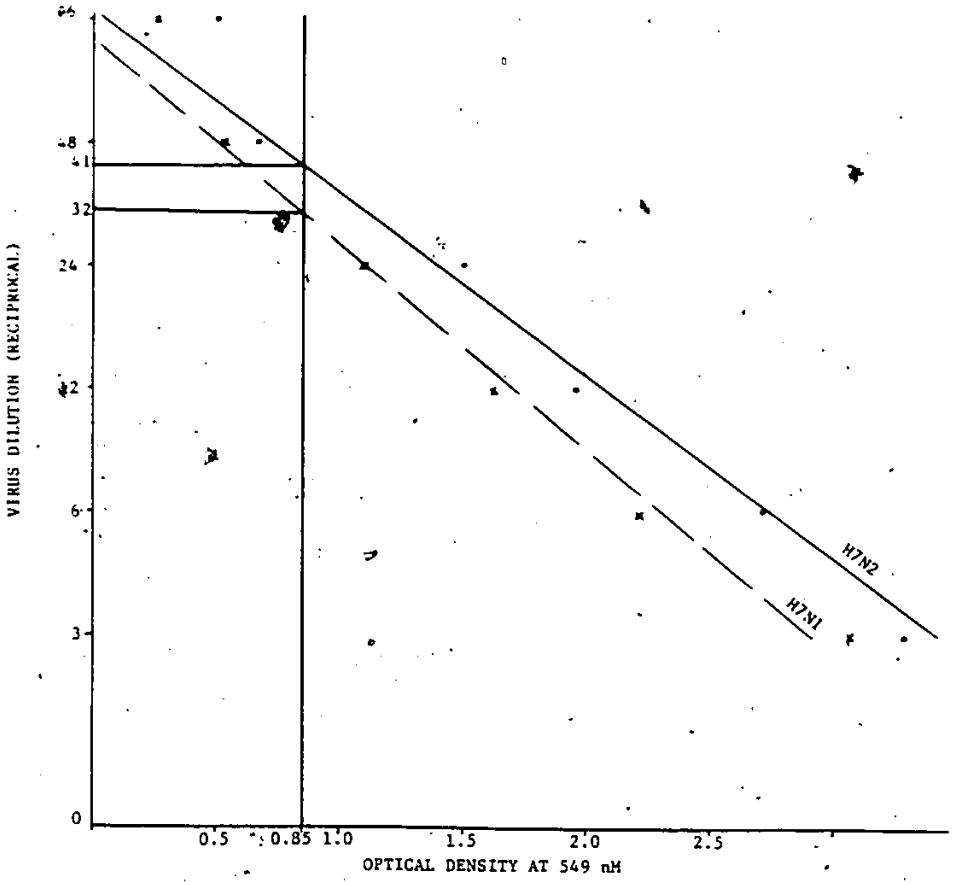
The advantages gained by using smaller volumes in the mini test included: 1) considerable economy on reduction by one-half to two-thirds the amount of fetuin substrate required 2) increasing the number of sera tested by the same batch of certain reagents, thus minimizing factors of variation in the test, 3) the complete mini NAC or NI tests were accommodated in one set of test tubes, unlike the Standard (WHO) procedure wherein the initial dilutions were made in one set of test tubes and the required volumes transferred into another set of tubes, 4) increasing the sensitivity of the test (Table 4).

The mini test produced almost 2-fold higher O.D. readings (NAC) (1:32 and 1:41 for H7N1 and H7N2, respectively) (Table 4 and Fig. 15) than that obtained by the standard test (Fig. 9).

Human Participants (Vaccinees and Controls)

A five-year annual influenza immunization program was carried out in an

Fig. 15 : The dilution of influenza H7N1 and H7N2 (containing one unit of neuraminidase activity) obtained utilizing the mini neuraminidase activity test.



Ottawa hospital for the aged and chronically ill housing over 500 patients. The total study involved 250-330 vaccinees and over 50 controls each year.

For the NIAB aspect of the study, 69 resident patients (mean B.D. 1902 ±12) were selected. The population chosen represents a well defined group of elderly and chronically ill patients most of whom were born before 1900 or shortly after. The probability that they have been exposed to both N1 and N2 antigens is high. Distribution by age and sex is illustrated in Fig. 6. However, because of attrition due to death or transfer out of hospital, continual participation in the program involved a limited number of patients. Therefore, this thesis is confined to a core group of patients referred to as group I consisting of up to 47 vaccinees who participated continually in the program, receiving all five annual influenza vaccines (V 1-5) and from whom complete serum sampling was obtained and up to 22 non-vaccinated controls during the same five-year period (CT 5X) from whom, also, complete serum sampling was obtained. Vaccination and serum sampling were subject to ethical limitation.

Neuraminidase-Inhibition Antibodies (NIAB) to H7N1 in Patients Sera:

Group I (Table 5)

This group consisted of up to 47 vaccinees who in five annual vaccinations received four N1-vaccines. The controls included up to

LEGEND

Pre V1 - V5 : Pre-vaccination serum.
Post V1 - V5 : Post-vaccination serum.

2M : Two months.
Y : Approximately one year (10-12 months).

No. Patients Tested: The variation in the number of patients is due to death by attrition or transfer out of hospital.

SE of GMT : Standard error of the geometric mean titre (GMT).

% SE : Percent standard error of the binomial distribution.

- : Antibody activity not demonstrated.

NJ-VIC : A/New Jersey/76(H1N1)-A/Victoria/3/75(H3N2).

VIC-BHK : A/Victoria/3/75(H3N2)-B/Hong Kong/72.

USSR-TEX-BHK : A/USSR/92/77(H1N1)-A/Texas/1/77(H3N2)-B/Hong Kong/2/73.

BRAZ-TEX-BHK : A/Brazil/11/78(H1N1)-A/Texas/1/77(H3N2)-B/Hong Kong/2/73

BRAZ-BGK-BSP : A/Brazil/11/78(H1N1)-A/Bangkok/1/79(H3N2)-B/Singapore/222/79.

Table 5 : Prevalence and geometric mean titres (GMT) of neuraminidase-inhibition antibody (NIAB) to influenza H7N1 in vaccinated and non-vaccinated controls of group I during administration of vaccines 1-5.

Serum Sample	Sampling Interval	Serum Sample Date	No. Patients Tested	No. (%) with Reciprocal NIAB Titre <10	10	20	40	80	≥160	Total No.(%) with Reciprocal Titre ≥10	GMT	SE of GMT	P	No.(%) Sero-conversion <10 - ≥10	% SE	No.(%) Responders with NIAB Titre Increase ≥2-fold	≥4-fold
Vaccinees																	
Pre-V 1	-	Jly 76	39	14(36)	13(33)	8(20)	3(8)	1(3)	-	25(64)	10.5	0.34	-	-	7.7	-	-
Post-V 1	2M	Jan 77	39	6(15)	10(26)	10(26)	10(26)	2(5)	1(2)	33(85)	18.3	0.39	<0.001	10(26)	5.4	24(62)	12(31)
Post-V 1 =Pre-V 2	1Y	Jan 78	47	12(25)	15(32)	10(21)	7(15)	3(6)	-	35(75)	13.6	0.34	>0.05	5(11)	7.7	9(19)	6(13)
Post-V 2	2M	Mar 78	47	14(30)	12(26)	11(23)	8(17)	2(4)	-	33(70)	13.2	0.34	>0.05	1(2)	6.7	5(11)	2(4)
Post-V 2 =Pre-V 3	1Y	Nov 78	47	15(32)	16(34)	8(17)	6(13)	2(4)	-	32(68)	11.8	0.33	>0.05	2(4)	6.8	4(9)	3(6)
Post-V 3	2M	Feb 79	47	12(25)	17(36)	5(11)	11(23)	2(4)	-	35(75)	13.6	0.34	>0.05	5(11)	6.3	11(23)	8(17)
Post-V 3 =Pre-V 4	1Y	Nov 79	44	15(34)	11(25)	7(16)	10(23)	1(2)	-	29(66)	12.7	0.36	>0.05	2(4)	7.2	9(20)	3(7)
Post-V 4	2M	Jan 80	44	14(32)	15(34)	6(14)	7(16)	2(4)	-	30(68)	12.1	0.35	>0.05	2(4)	7.0	3(7)	2(4)
Post-V 4 =Pre-V 5	1Y	Nov 80	42	13(31)	16(38)	4(10)	8(19)	1(2)	-	29(69)	11.8	0.35	>0.05	3(7)	7.0	3(7)	3(7)
Post-V 5	1M	Dec 80	42	11(26)	18(43)	6(14)	6(14)	1(2)	-	31(74)	11.8	0.32	>0.05	3(7)	6.5	4(10)	3(7)
Controls																	
Pre-V 1	-	Jly 76	22	13(60)	3(13)	3(13)	3(13)	-	-	9(41)	8.8	0.49	-	-	10.5	-	-
Post-V 1	2M	Jan 77	22	13(60)	3(13)	3(13)	3(13)	-	-	9(41)	8.8	0.49	>0.05	1(5)	10.5	1(5)	1(5)
Post-V 1 =Pre-V 2	1Y	Jan 78	21	13(62)	3(14)	1(5)	4(19)	-	-	8(38)	8.8	0.50	>0.05	2(9)	10.6	3(14)	3(14)
Post-V 2	2M	Mar 78	21	13(62)	3(14)	1(5)	4(19)	-	-	8(38)	8.8	0.50	>0.05	2(9)	10.6	2(9)	2(9)
Post-V 2 =Pre-V 3	1Y	Nov 78	20	11(55)	4(20)	1(5)	4(20)	-	-	9(45)	9.3	0.52	>0.05	3(15)	11.1	3(15)	3(15)
Post-V 3	2M	Feb 79	20	12(60)	3(15)	1(5)	4(20)	-	-	8(40)	9.0	0.52	>0.05	0(0)	11.0	0(0)	0(0)
Post-V 3 =Pre-V 4	1Y	Nov 79	16	11(69)	2(12)	-	3(19)	-	-	5(31)	8.1	0.57	>0.05	0(0)	11.6	0(0)	0(0)
Post-V 4	2M	Jan 80	16	10(62)	3(19)	-	3(19)	-	-	6(38)	8.4	0.57	>0.05	1(6)	12.1	1(6)	1(6)
Post-V 4 =Pre-V 5	1Y	Nov 80	15	11(73)	1(7)	-	3(20)	-	-	4(27)	8.0	0.61	>0.05	0(0)	11.5	1(6)	1(6)
Post-V 5	1M	Dec 80	15	11(73)	1(7)	1(7)	2(13)	-	-	4(27)	7.6	0.61	>0.05	0(0)	9.5	0(0)	0(0)

22 non-vaccinated patients.

The highest NI antibody response occurred within 2 months after the administration of the first N1 vaccine (NJ-VIC). Of the 39 vaccinees those with NIAB titre $\geq$1:10 rose in number (%) from 25(64%) before vaccination to 33(85%) after, and those with titres between 1:20 to 1:160 increased from 12(31%) to 23(59%) before and after vaccination, respectively (Table 5, Fig. 16). This was paralleled by a highly significant rise in GMT from 10.5 before vaccination to 18.3 ($p < 0.001$) after (Table 5, Fig. 18). Of the 39 vaccinees, 24(62%) demonstrated a $\geq$2-fold increase in titre including 12(31%) with $\geq$4-fold change, and 10(26%) who seroconverted to titre $\geq$1:10 (Table 5, Fig. 19). By contrast, in the 22 patients of the control group, the corresponding values remained unchanged with NIAB titres $\geq$1:10 in 9(41%) and between 1:20 and 1:160 in 6(27%) patients (Table 5, Fig. 17), and the GMT remained stable at 8.8 (Table 5, Fig. 18), less than one-half the GMT (18.3) of the vaccinees.

Following administration of the second vaccine (VIC-BHK), which contained no N1 antigen, the prevalence in 47 patients of NIAB titre of $\geq$1:10 remained relatively unchanged dropping slightly from 35(75%) to 33(70%) (Table 5, Fig. 16) paralleled by an insignificant decline in GMT from 13.6 to 13.2 (Table 5, Fig. 18). This clearly illustrated that

Figure 16 : Prevalence of neuraminidase-inhibition antibody (NIAB) titres to influenza H7N1 in vaccinees of group I before and after administration of vaccines 1-5.

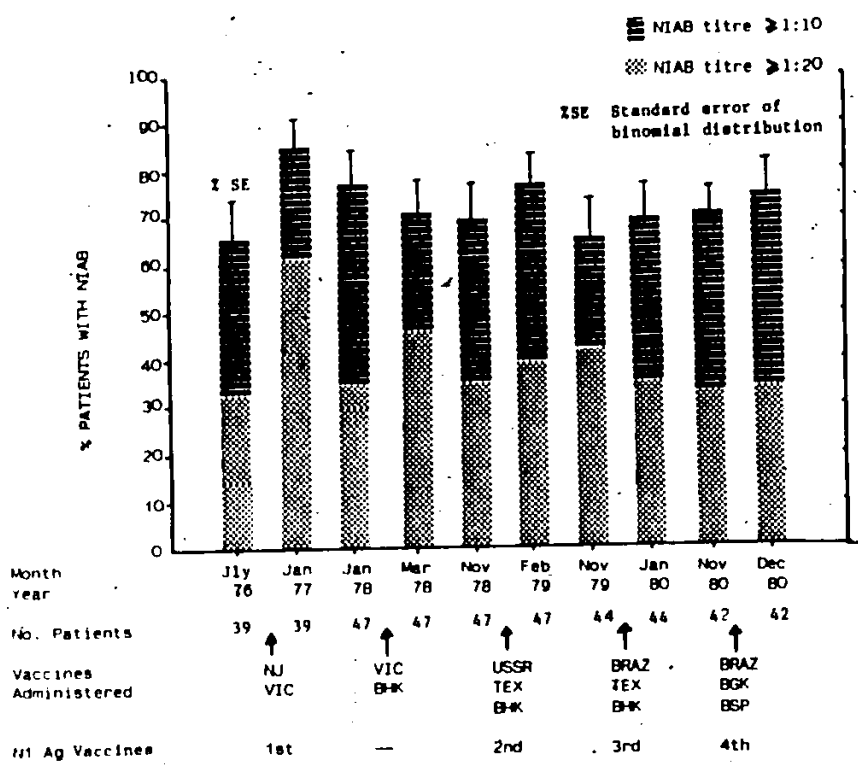


Figure 17 : Prevalence of neuraminidase-inhibition antibody (NIAB) titres to influenza H7N1 in non-vaccinated controls of group I before and after administration of vaccines 1-5.

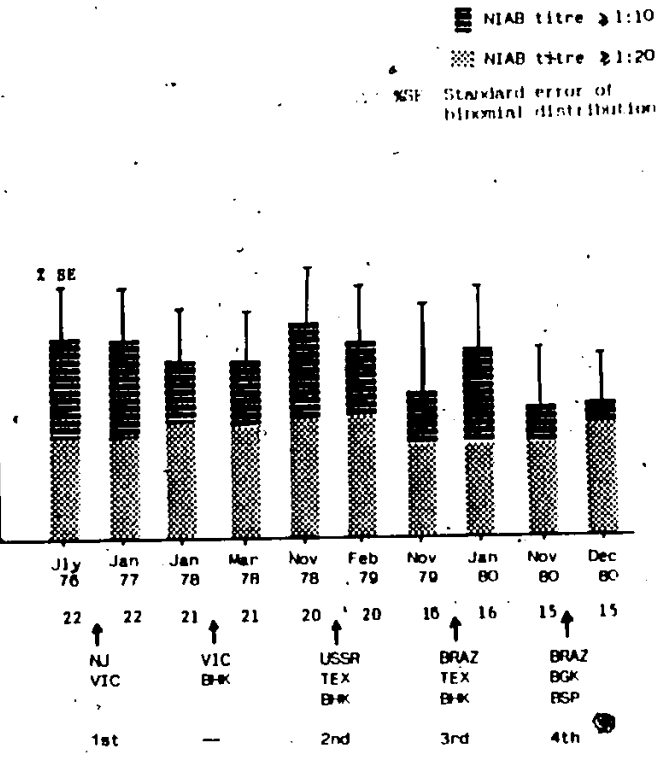
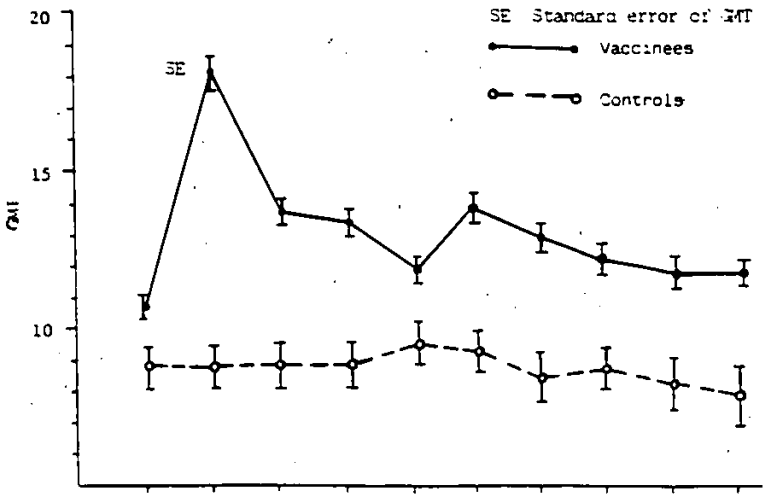


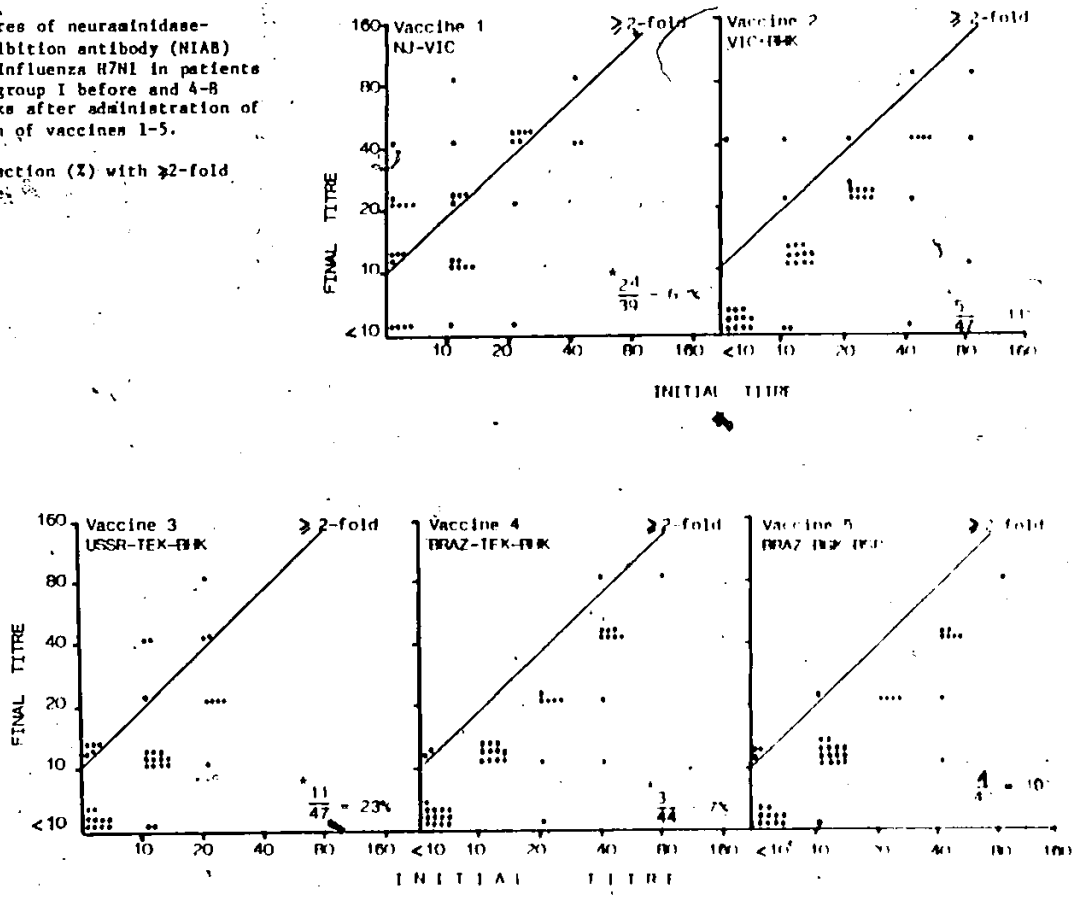
Figure 18 : Geometric mean titres (GMT) of neuraminidase-inhibition antibody (NIAB) to influenza H7N1 in vaccinees and non-vaccinated controls of group I before and after administration of each of vaccines 1-5.



Month	Jly	Jan	Jan	Mar	Nov	Feb	Nov	Jan	Nov	Dec
Year	76	77	78	78	78	79	79	80	80	80
No. Vaccinees	39	39	47	47	47	47	44	44	42	42
No. Controls	22	22	21	21	20	20	16	16	15	15
Vaccines Administered		NJ VIC		VIC BHK		USSR TEX BHK		BRAZ TEX BHK		BRAZ BGK BSP
N1 Ag Vaccines		1st		-		2nd		3rd		4th

Figure 19 Titres of neuraminidase-inhibition antibody (NIAB) to influenza H7N1 in patients of group I before and 4-8 weeks after administration of each of vaccines 1-5.

*Fraction (%) with ≥2-fold rise.



the lack of NIAB response in patients can be attributed to the absence of a second N1 annual stimulation by the second vaccine (VIC-BHK), and the lack of a natural H1N1 infection. Similarly, the NIAB prevalence and GMT of the control group of 22 patients remained unchanged.

Administration of the third vaccine (USSR-TEX-BHK), the second N1 stimulus, resulted in a slight rise in the prevalence of NIAB titres at $\geqslant$1:10 from 32/47(68%) before vaccination to 35/47 (75%) after and those with titres 1:20 to 1:80 increased from 16/47 (34%) to 18/47 (38%), respectively (Table 5, Fig. 16). This was accompanied by a statistically insignficant rise in the GMT from 11.8 before vaccination to 13.6 after ($p > 0.05$) (Table 5, Fig. 18). Of the 47 vaccinees, 11(23%) demonstrated a $\geqslant$ 2-fold, 8(17%) with a $\geqslant$ 4-fold increase in NIAB titre and 5(11%) serconverted from titre $<$ 1:10 to $\geqslant$ 1:10 (Table 5, Fig. 19). In contrast, the 20 controls remained relatively unchanged exhibiting a trend of decline in the prevalence of NIAB titre at $\geqslant$ 1:10 from 9(45%) to 8(40%) (Table 5, Fig. 17) and in the GMT from 9.3 to 9.0 ($p > 0.05$) (Table 5, Fig. 18).

Following the fourth vaccine (BRAZ-TEX-BHK), the third N1 stimulus, and the fifth vaccine (BRAZ-BGK-BSP), the fourth N1 stimulus, little change was elicited. The prevalence in patients of NIAB titre at $\geqslant$ 1:10 ranged between 30/44 - 31/42 (68% - 74%) (Table 5, Fig. 16) and the GMT ranged between 12.1 - 11.8 ($p > 0.05$) (Table 5, Fig. 18), respectively. In the

controls, the prevalence of NIAB titre at $\geqslant$1:10 declined steadily during the five year period reaching 4/15(27%) (Table 5, Fig. 17). In parallel the GMT drifted down to 7.6 ($p > 0.05$) which was approximately one-third less than that of the vaccinees at 11.8 (Table 5, Fig. 18).

The GMT dropped within a year after each vaccination, however, annual re-immunization boosted the levels of NIAB GMT and maintained them at higher levels ($\geqslant$11.8) than that of the initial GMT 10.5. In comparison, the GMT of the control group remained relatively unchanged dropping to as low as 7.6 (Fig. 18) by the end of the five-year period.

Neuraminidase-Inhibition Antibody Titres (NIAB) to H7N2 in Patients' Sera.

Group I (Table 6)

This group included up to 47 vaccinees who received five N2-vaccines and 22 non-vaccinated controls.

Administration of the first vaccine (NJ-VIC) did not elicit a significant increase in NIAB titre. The prevalence in 39 patients of NIAB titre at $\geqslant$1:10 only marginally increased from 25 (64%) before vaccination to 27(69%) after, but those with titres between 1:20 and

Table 6 : Prevalence and geometric mean titres (GMT) of neuraminidase-inhibition antibody (NIAB) to influenza H3N2 in vaccinated and non-vaccinated controls of group 1 during administration of vaccines 1-5.

Serum Sample	Sampling Interval	Serum Sample Date	No. Patients Tested	No. (%) with Reciprocal NIAB Titre <10	10	20	40	80	≥160	Total No.(%) with Reciprocal Titre ≥10	GMT	SE of GMT	P	No.(%) Sero-conversion <10 - ≥10	% SR	No.(%) Responders with NIAB Titre Increase ≥2-fold	≥4-fold
Vaccinees																	
Pre-V 1	-	Jly 76	39	14(36)	3(8)	8(21)	11(28)	2(5)	1(2)	25(64)	15.8	0.45	-	-	7.7	-	-
Post-V 1	2M	Jan 77	39	12(31)	6(15)	7(18)	14(36)	-	-	27(69)	15.1	0.39	>0.05	8(21)	6.5	10(26)	8(21)
Post-V 1 =Pre-V 2	1Y	Jan 78	47	20(43)	9(19)	7(15)	10(21)	1(2)	-	27(57)	11.6	0.35	>0.05	1(2)	7.2	1(2)	1(2)
Post-V 2	2M	Mar 78	47	11(23)	9(19)	8(17)	17(36)	2(4)	-	36(77)	17.3	0.36	<0.001	9(19)	6.1	18(38)	12(26)
Post-V 2 =Pre-V 3	1Y	Nov 78	47	16(34)	9(19)	5(11)	16(34)	1(2)	-	31(66)	14.2	0.37	>0.05	3(6)	6.9	8(17)	3(6)
Post-V 3	2M	Feb 79	47	13(28)	9(19)	5(11)	19(40)	1(2)	-	34(72)	16.3	0.36	>0.05	3(6)	6.5	8(17)	5(11)
Post-V 3 =Pre-V 4	1Y	Nov 79	44	11(25)	12(27)	8(18)	13(30)	-	-	34(77)	14.4	0.34	>0.05	5(11)	6.4	7(16)	5(11)
Post-V 4	2M	Jan 80	44	10(22)	13(30)	7(16)	14(32)	-	-	34(77)	14.8	0.34	>0.05	2(5)	6.3	5(11)	3(7)
Post-V 4 =Pre-V 5	1Y	Nov 80	42	10(24)	13(31)	11(26)	8(19)	-	-	32(76)	13.2	0.32	>0.05	1(2)	6.5	3(7)	2(5)
Post-V 5	1M	Dec 80	42	9(21)	13(31)	9(21)	11(26)	-	-	33(79)	14.4	0.33	0.05	2(5)	6.3	5(12)	3(7)
Controls																	
Pre-V 1	-	Jly 76	22	7(32)	6(27)	2(9)	7(32)	-		15(68)	11.3	0.51	-	-	9.9	-	-
Post-V 1	2M	Jan 77	22	9(41)	6(27)	1(5)	6(27)	-		13(59)	11.3	0.51	>0.05	1(5)	10.5	1(5)	1(5)
Post-V 1 =Pre-V 2	1Y	Jan 78	21	10(47)	5(24)	1(5)	5(24)	-		11(52)	10.3	0.52	>0.05	0(0)	10.9	0(0)	0(0)
Post-V 2	2M	Mar 78	21	8(38)	6(29)	2(10)	5(23)	-		14(67)	11.4	0.50	>0.05	2(10)	10.2	2(10)	2(10)
Post-V 2 =Pre-V 3	1Y	Nov 78	20	8(40)	5(25)	3(15)	4(20)	-		12(60)	11.1	0.51	>0.05	2(10)	10.9	2(10)	2(10)
Post-V 3	2M	Feb 79	20	7(35)	7(35)	3(15)	3(15)	-		13(65)	10.7	0.47	>0.05	1(5)	10.7	1(5)	1(5)
Post-V 3 =Pre-V 4	1Y	Nov 79	16	5(31)	6(38)	3(19)	2(13)	-		11(69)	10.9	0.51	>0.05	0(0)	11.0	0(0)	0(0)
Post-V 4	2M	Jan 80	16	6(38)	5(31)	2(12)	3(19)	-		10(63)	10.9	0.55	>0.05	0(0)	12.0	1(6)	0(0)
Post-V 4 =Pre-V 5	1Y	Nov 80	15	7(46)	6(40)	1(7)	1(7)	-		8(53)	8.3	0.48	>0.05	1(7)	12.9	1(7)	1(7)
Post-V 5	1M	Dec 80	15	7(47)	5(33)	1(7)	2(13)	-		9(60)	9.1	0.54	>0.05	0(0)	10.5	0(0)	0(0)

1:160 hardly changed from 22(56%) before to 21(54%) after vaccination (Table 6, Fig. 20). In parallel the GMT marginally dropped from 15.8 to 15.1 (Table 6, Fig. 22). The 22 controls exhibited a slight decline in the prevalence of NIAB titre at $\geq$ 1:10 and those with titres between 1:20 and 1:80, respectively from 15(68%) to 13(59%) and from 9(41%) to 7(32%) (Table 6, Fig. 21). The GMT of the controls dropped from 13.3 to 11.3 (Table 6, Fig. 22). Of the 39 vaccinees, 8(21%) seroconverted from $<$1:10 to $\geq$1:10, and 10(26%) demonstrated a $\geq$2-fold rise in titre including 8(21%) with a $\geq$4-fold increase (Table 6, Fig. 23). One (5%) of the controls seroconverted (Table 6).

The highest NIAB response occurred within 2 months after administration of the second vaccine (VIC-BHK). Patients with NIAB titres $\geq$1:10 and those between 1:20 and 1:80 increased in number (%) from 27/47(57%) before vaccination to 36/47(77%) after, and from 18/47(38%) before to 27/47(57%) after vaccination, respectively (Table 6, Fig. 20). This change in NIAB was accompanied by a highly significant rise in GMT from 11.6 to 17.3 ($p<0.001$) (Table 6, Fig. 22). Eighteen (38%) of the 47 vaccinees exhibited a $\geq$2-fold increase in titre including 12(26%) with a $\geq$4-fold rise, and 9(19%) who seroconverted to a titre $\geq$1:10 (Fig. 23). In contrast, pre and post vaccination controls with NIAB titres $\geq$1:10 and those between 1:20 and 1:80 slightly increased from 11/21(52%) to 14/21(67%) and from 6/21(29%) to 7/21(33%), respectively (Table 6, Fig. 21).

Figure 20: Prevalence of neuraminidase-inhibition antibody (NIAB) titres to influenza H7N2 in vaccinees of group I before and after administration of each of vaccines 1-5.

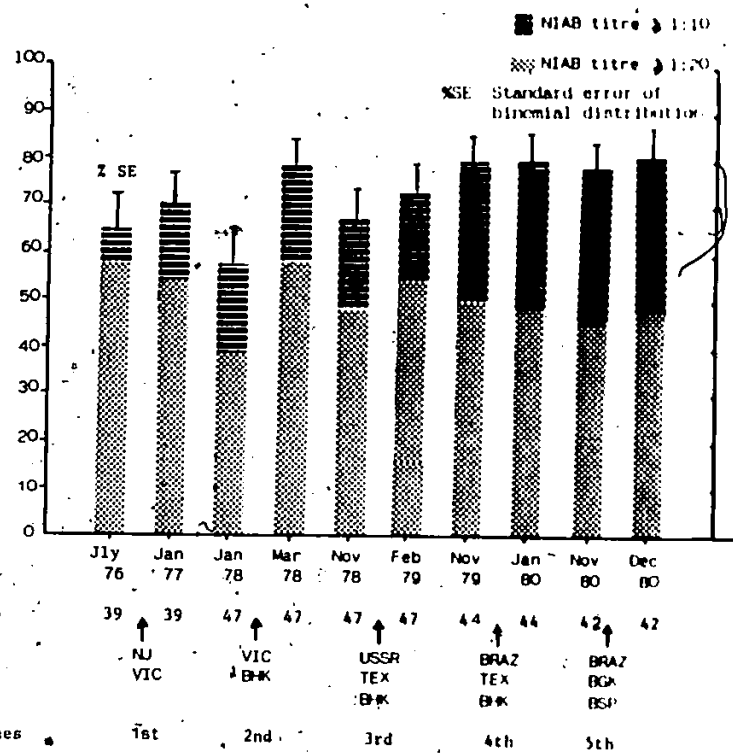


Figure 21: Prevalence of neuraminidase-inhibition antibody (NIAB) titres to influenza H7N2 in non-vaccinated controls of group I before and after administration of vaccines 1-5.

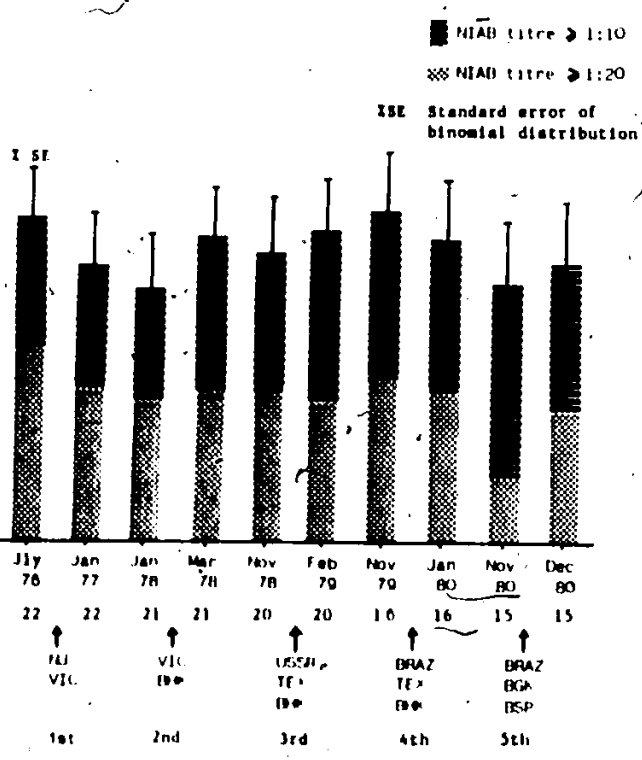
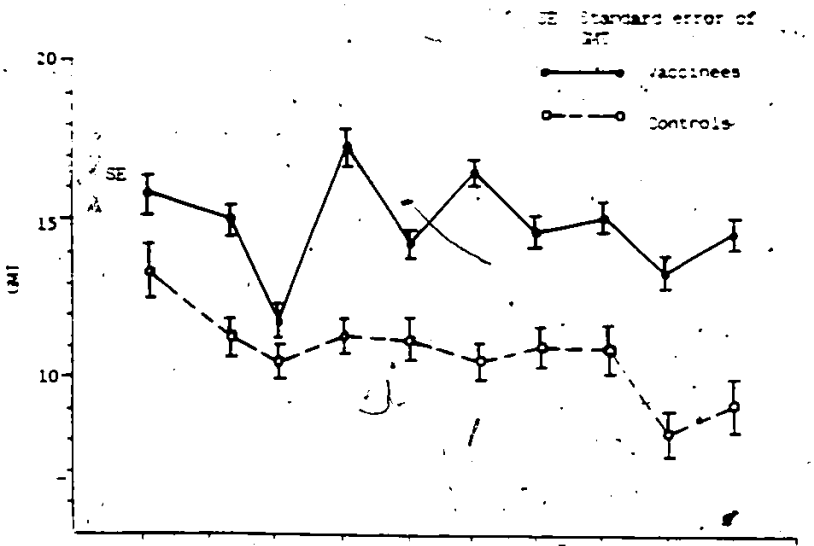


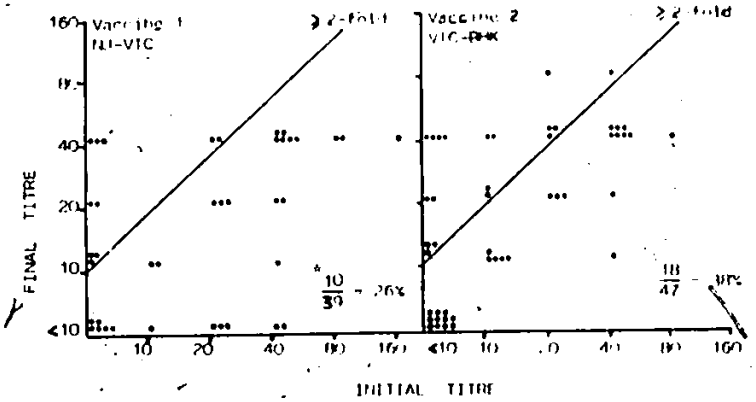
Figure 12 : Geometric mean titres (GMT) of neuraminidase-inhibition antibody (NIAB) to influenza H7N2 in vaccinees and non-vaccinated controls of group I before and after administration of each of vaccines 1-5.

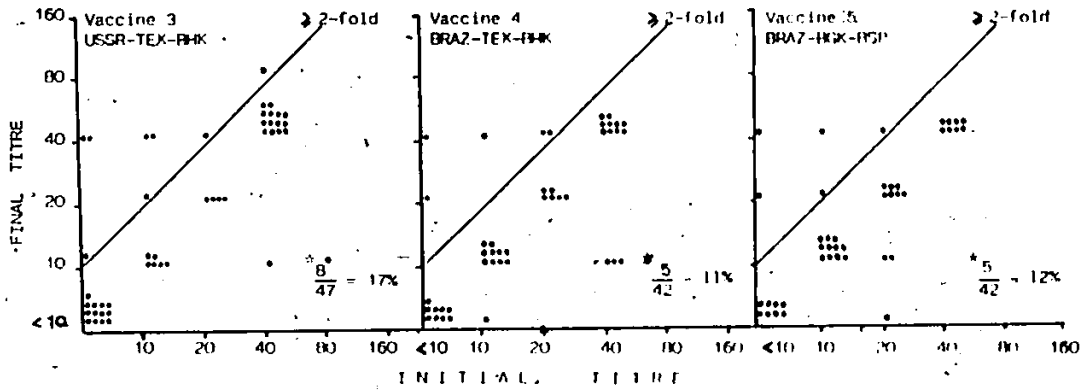


Month	Jly	Jan	Jan	Mar	Nov	Feb	Nov	Jan	Nov	Dec	Jan
Year	76	77	78	78	78	79	79	80	80	80	81
No. Vaccinees	39	39	47	47	47	47	44	44	42	42	
No. Controls	22	22	21	21	20	20	16	16	15	15	
Vaccines Administered	NJ VIC		VIC BHK		USSR TEX BHK		BRAZ TEX BHK		BRAZ BHK BSP		
N2 Ag Vaccines	1st		2nd		3rd		4th		5th		

Figure 23: Titres of neuraminidase-inhibition antibody (NIAB) to influenza H7N2 in patients of group I before and 4-8 weeks after administration of each of vaccines 1-5.

*Fraction (%) with ≥2-fold rise.





Two (10%) of the controls did seroconvert demonstrating a ⩾4-fold rise in titre (Table 6). The GMT slightly, but insignificantly increased from 10.3 before vaccination to 11.4 after ($p>0.05$) (Table 6, Fig. 22) which was almost one-third less than the GMT (17.3) of the vaccinees. The seroconversion exhibited by the controls may be attributed to either a subclinical infection with influenza virus or to experimental variations. Kendal et al (1980) indicated that despite full standardization, the NI test remains subject to an uncontrollable number of factors.

Following administration of the third N2 vaccine (USSR-TEX-BHK), the 47 patients exhibited a slight increase in prevalence of NIAB titres at ⩾1:10 and titres between 1:20 and 1:80 from 31(66%) before vaccination to 34(72%) after and from 22(47%) before to 25(53%) after vaccination, respectively (Table 6, Fig. 20). This increase was paralleled by a statistically insignificant rise in GMT from 14.2 to 16.3 ($p > 0.05$) (Table 6, Fig. 22). Of the vaccinees 8(17%) demonstrated a ⩾2-fold rise in titre including 5(11%) with ⩾4-fold increase and 3(6%) who seroconverted from < 1:10 to ⩾1:10 (Fig. 23). The controls remained relatively unchanged with a NIAB prevalence at titre ⩾1:10 of 13(65%) (Table 6, Fig. 21) and the GMT at 10.7 (Table 6, Fig. 22).

Administration of the fourth (BRAX-TEX-BHK) and fifth (BRAZ-BGK-BSP) N2 vaccines caused no significant changes in NIAB titre. Of the vaccinees 34/44 (77%) and 33/42 (79%) exhibited a titre of ⩾1:10 (Table 6, Fig.

20) with respective GMT of 14.8 and 14.4 (Table 6, Fig. 22) following the fourth and fifth vaccinations, respectively. Similarly 21/44 (48%) and 20/42 (48%) of the vaccinees exhibited a NIAB distribution ranging between titres 1:20 and 1:40 following the fourth and fifth vaccines, respectively (Table 6, Fig. 20). Correspondingly, 5(11%) of the vaccinees after the fourth, and 12% after the fifth vaccine demonstrated a $\geqslant$2-fold rise in titre including 3(7%) with a $\geqslant$4-fold increase and 2(5%) who seroconverted in both instances (Table 6, Fig. 23). The controls remained relatively unchanged when, after five years 9/15 (60%) had NIAB titre at $\geqslant$1:10 (Table 6, Fig. 21) and the GMT was 9.1 (Table 6, Fig. 22), one-third less than the GMT 14.4 of the vaccinees. The GMT in the vaccinees generally dropped within one year after each vaccination. Annual boosting slightly elevated the GMT, but after five years it still remained lower (14.4) than the initial GMT (15.8) but approximately one-third higher than that of the non-vaccinated controls (9.1).

DISCUSSION

For the purpose of assessing anti-neuraminidase antibody (NIAB) levels, recombinant influenza A viruses H7N1 and H7N2 were used carrying irrelevant H but relevant N. This enabled us to measure the levels of antibodies specific for N with minimal interference from H antibodies by what is known as "steric hindrance" (Kilbourne, 1968 and Hsia et al, 1980).

Normal and specific ferret antisera for N7N1 and H7N2 were utilized as controls throughout the testing. Our finding that the ferret serum was more suitable than rabbit serum is in agreement with the findings of Kendal et al (1973) who used ferret serum instead of rabbit serum in the neuraminidase-inhibition test. Therefore, rabbit serum was not used in our experimentation. The N inhibitory titres of ferret sera were consistently reproducible, thus proving to be accurate controls.

The Essen (micro) N test developed by Seikman et al (1975) was tested for possible use as an alternative for the uneconomical standard WHO method. However, in our Laboratory, little viral N activity (less than 0.85 O.D. reading which is the minimum required to contain one enzymatic unit) could be detected by this test, even when 3 and 18 hour incubation periods were tried. The low N activity detected by the Essen (micro) N test could be related to either or all of the following: a) the use of very small volumes, b) characteristics of different influenza virus strains, and c) use

of fetuin substrate rather than the acid α1-glycoprotein substrate utilized by Seikman et al (1975) in the Essen (micro) test. Therefore, a mini test based on the standard WHO test, was developed. The volumes used in the mini test were a proportional increase by a factor of 2.5 the volumes utilized in the micro test. Sixty-three experimental trials were carried out to ensure the reproducibility of the test and its suitability for testing NIAB in human sera. Approximately 2-fold higher N activity of the same virus antigens was obtained by using the mini test rather than by the standard test. This may be due to the greater sensitivity of the test because of the volumes used. Based on the 63 trials, the suitability of the mini test was demonstrated by the constant reproducibility of NIAB titres in normal and specific ferret antisera controls as well as in the fetuin and virus controls which were always incorporated with each set of human sera tested.

Although great care was taken in stabilizing and standardizing the accuracy and reproducibility of the test, nevertheless, the NI test remains subject to a few variables. This was confirmed by the CDC (1980) who indicated that the accuracy of the test relied on many variables such as buffer, temperature, pH, quality of substrate, and the possible irregularity in the quality of supplied reagents and chemicals. In our experimentation, we have found that even the variation in the batch of fetuin used resulted in extreme variation in the test which in some cases failed to produce any reaction. This was also true with respect to the thiobarbituric acid. Only

the product specified in this thesis was successful in extracting a sharp pink color whereas thiobarbituric acid from other manufacturers failed to produce the desired sharpness in color.

In this pilot clinical and observational study, the human participants represented an aged and chronically ill population (mean birth date 1902 ±12). The uniqueness of this project resides in the fact that the NIAB response following five annual influenza vaccines was monitored in a well defined elderly population during a five year period. Studies carried out by other investigators were limited to approximately two years and included human participants of a wide range of ages from less than one year to 60 years of age (Slepushkin et al, 1971; Murphy et al, 1972; Kendal et al, 1973; Couch et al, 1974; Kilbourne, 1976; Kendal et al, 1977; Ogra et al, 1977; Jennings and Miles, 1978; Drescher and Desselberger, 1980; Hsia et al, 1980; Sarateanu et al, 1980). Results were difficult to compare with those of others because: 1) little work has been done on the value and significance of NIAB, 2) much of the previous research on NIAB response was measured after one or two vaccinations utilizing different test antigens, and 3) the limited concrete data available such as individual NIAB titre values rather than titres obtained from pooled sera and the NIAB response in aged population (80 years of age) to both N1 and N2 antigens.

The elderly participants selected in our study reflect a past experience and exposure to both types of influenza neuraminidase (N1 and N2). Therefore, the majority were assumed to have had contact with the N1 antigen of the swine influenza which circulated in 1918-1919, the N1 of H1N1 (A/FM1/47) prevalent from 1947 to 1957 until replaced by H2N2 (Asian) in 1957, and finally contact with the N1 of influenza A/USSR/92/77 (H1N1) virus (Layde et al, 1980). These participants probably were also subject to previous contact with N2 antigen in 1957 with A/Singapore/57 (H2N2) and reinforced by the appearance of A/Hong Kong/68 (H3N2).

As in other clinical and observational studies involving human participants and our study was also to ethical constraints. In addition, the study was also limited by uncontrollable factors due to attrition by death, transfer out of hospital or lack of consent to continual participation on the part of the patients.

When investigating patients' sera for N1 antibody, our data indicated that the greatest response in vaccinees occurred after administration of the NJ-VIC vaccine when 62% showed a $\geq$ 2-fold rise in NIAB titre and a highly significant increase in the GMT from 10.5 before vaccination to 18.3 ($p < 0.001$) after. This is expected since the vaccine contained the N1 antigen of NJ/76. Our result is in close agreement with that of Kendal et al (1977) who demonstrated that after a single dose of HswlN1 (H1N1) vaccine, 31/57 (54%) of adults between 25-54 years old responded to the

vaccine demonstrating a $\geq$ 3-fold rise in NIAB titre. Kendal et al (1977) suggested that the reason for this high response is attributed to the priming effect as those adults were presumed to have been infected at sometime between 1930 and 1951 with viruses containing N1 neuraminidase.

Our data indicated that 25/39 (64%) had a $\geq$ 1:10 titre before immunization and increased by approximately 20% to 33/39 (85%) after vaccination with NJ-VIC. In comparison, Fletcher et al (1977) demonstrated that of the 20 adults > 65 years of age, 80% had a $\geq$ 1:10 NIAB titre before vaccination and 90% after administration of the monovalent NJ/76 vaccine. Thus, the response in our vaccinees seems to be close to those studied by Fletcher et al (1977). Kendal et al (1977) had suggested that the N of NJ/76 had a low activity and consequently a low immunogenicity. Nevertheless, at present the correlation between the activity and immunogenicity remains unestablished. Fazekas de St. Groth (1963) had demonstrated that the N enzymatic activity and antigenicity were located on different genomic sites and thus little or no correlation existed between the two.

The second vaccine (VIC-BHK) did not contain any N1 antigen. In the abscence of an artificial N1 stimulation, or a natural challenge by a wild virus, and over a period of two years, patients did not demonstrate any NIAB response. Therefore, administration of this vaccine and the response recorded clearly confirms that N1 antigen of NJ/76 administered in the first vaccine was solely

responsible for stimulating the N1 antibody. Vaccinees responded moderately following the third vaccine, containing the N1 of USSR/77, when 23% showed a $\geq$2-fold increase in titre and the GMT rose from 11.8 to 13.6 ($p>0.05$). The moderate response in vaccinees was expected since the vaccine contained a low concentration of N1 antigen of USSR/77 due to the use of a smaller dose (7µg H) of the vaccine as compared to the 200 CCA concentration used for the NJ/76 vaccine. Kendal et al (1980) had also shown that the N1 of USSR/77 in vaccines was of a low potency, thus lower immunogenicity. It may also be suggested that the N1 antigen of USSR/77 had reinforced the antibody directed against older antigens encountered such as the N1 of NJ/76-like thus contributing to the higher level of NIAB to NJ/76 rather than to the USSR/77 in accordance with the hypothesis of serological recapitulation (Kendal et al, 1973). The aged population in our study was presumed to have been primed by N1 antigen through exposure to H1N1 of swine virus in 1918 and restimulation by H1N1 of A/PR/8/34 from 1934-1946 followed by exposure to H1N1 (A/FM/1/47) from 1947-1957 and therefore immunization with the vaccine containing the USSR/77 reinforced the NIAB to earlier strains with related N1 (Kendal et al, 1977). Kendal et al (1980) demonstrated that of the 21 vaccinees, (>50 years of age) who received whole vaccine of USSR/77, 19% after the first vaccine and 24% following the second vaccine exhibited a $\geq$3-fold rise in NIAB titre. In our study, of the 47 vaccinees who received the USSR/77 containing vaccine, 23% exhibited a $\geq$2-fold rise in titre which could be considered close to the findings of Kendal et al (1980).

Further vaccination with the fourth and fifth vaccines did not stimulate any significant response in the vaccinees even though those vaccines contained N1 antigen of BRAZ/78. One possible explanation for the lack of response could be attributed to a saturation point with the N1 antigen in the antibody forming mechanism in the vaccinees or to the lower potency of the N1 antigen in the vaccines where lower concentration (7 µg H) was used in the last three vaccines as compared to 200 CCA units used in the first two vaccines. Thus the use of repeated annual vaccination should be re-evaluated in light of the results obtained.

It was observed that NIAB titre usually dropped within one year following each vaccination. However, annual re-vaccination was found to either slightly boost or maintain the GMT at levels higher or closely equivalent to that of the initial GMT. Re-vaccination probably stimulates the antibody forming memory cells (B-cells) to produce more antibody. After five years of the program, the GMT of the vaccinees was maintained at 11.8 which was higher than the initial pre-immunization GMT of 10.5. In comparison, the GMT of the controls remained unchanged at 8.8 and slightly dropped after five years to 7.6 which was less than the initial 8.8 and approximately one-third less than that of the vaccinees (11.8). Therefore, although the fourth and fifth vaccines did not boost the NIAB titre, they maintained the acquired higher value of GMT at that level.

The same set of patients' sera was also tested for N2 antibodies. The results obtained show that the vaccinees had a high initial pre-immunization NIAB titre at a GMT of 15.8. The high GMT is attributed to a severe in-hospital natural outbreak of influenza A/Victoria/3/75 (H3N2) in the spring of 1976 when 123 patients were afflicted in a three week period (R.E. Fyson - personal communication). Similarly, the high initial GMT demonstrated by the controls resulted from natural VIC/75 infection.

Administration of the first vaccine (NJ-VIC) did not elicit any significant response in the vaccinees when the GMT demonstrated a slight drop from 15.8 to 15.1. The lack of response can be explained by the fact that the natural infection with A/Victoria/3/75 (H3N2) had already triggered the antibody forming mechanism boosting the NIAB to a certain high level. Introduction of the first vaccine containing the N2 of VIC/75 not only was probably too soon (approximately 9 months) following the natural infection, but also, as has been established, the antigenic concentration and potency of a vaccine did not match that of the natural (wild) virus (Tyrrell and Smith, 1979). Consequently the vaccine did not stimulate a significant response in the vaccinees. Kendal et al (1977) demonstrated that the NIAB response to a second dose of VIC/75 vaccine was low in children (<17 years of age) presumed to have been primed with the N2 antigen as indicated by the presence of antibody to A/Victoria/3/75 neuraminidase in some

prevaccination sera and the finding of antibodies to A/Port Chalmers/1/73 neuraminidase.

In our study, the first vaccine administered containing the N2 of VIC/75 could be considered as a second dose following the natural infection with VIC/75. Therefore, based on that fact our results fall in agreement with the findings of Kendal et al (1977).

It was observed that the GMT of the vaccinees after the first vaccine dropped considerably within a year to 11.6. However, administration of the second vaccine (VIC-BHK) boosted the NIAB response to its maximum and the GMT significantly rose to 17.3 ($p<0.001$). Thus, the NIAB attained its highest level in the five year period. From this finding one can easily conclude that the NIAB usually drops to lower levels within one year of each vaccination and that annual re-immunization for at least two years was necessary to boost the NIAB antibody to a higher levels.

Although a higher GMT (17.3) was obtained upon vaccination with VIC-BHK than the GMT of 15.8 exhibited by the patients following the natural infection with VIC/75, nevertheless, the increase from 15.8 to 17.3 was not significant ($p>0.05$).

It was observed, however, that the drop of the NIAB GMT resulting from the natural infection from 15.8 to 15.1 was not as great as, after vaccine 2, from 17.3 to 14.2. It has been demonstrated that a natural infection produces a stronger stimulation for the

antibody-forming memory cells than that produced through artificial vaccination (Tyrell et al, 1979). Whether this indicates a better immunity against re-infection can only be evaluated upon artificial, or preferably, natural influenza challenge. The GMT of 17.3 dropped within one year of the second vaccination to 14.2, but was boosted insignificantly to 16.3 ($p > 0.05$) by the third vaccine containing the N2 of TEX/77. The lower response after the third vaccine could be attributed to the administration of the second vaccine (VIC-BHK) which had already triggered the antibody forming mechanism to its fullest capacity saturating the antibody-forming mechanism of the vaccinees with N2 antigen. Thus the third vaccine merely served in re-stimulating the process of antibody formation and maintaining its continuity.

Introduction of the fourth and fifth vaccines did not elicit any response in vaccinees and the GMT remained unchanged at almost 14.4. This finding is comparable to that of N1 antibody response and indirectly confirms the suggestion of a saturation point (Fyson and Perry, 1981) in vaccinees with the N2 antigen beyond which further vaccination has no or little effect in eliciting a significant NIAB response.

On comparison of the vaccinees with the controls, it was obvious that the GMT of the controls fluctuated insignificantly, exhibiting a declining trend reaching 9.1 after five years, approximately one-third less than that of the vaccinees (14.4) at the same time.

Therefore, it can be concluded that vaccinations played an important role in stimulating NIAB.

Similar to the trend exhibited by N1 antibody, the N2 antibody GMT declined within one year after each vaccination. However, re-vaccination had clearly boosted or maintained the titres at higher levels. It could be concluded from this observation that although continually repeated vaccinations may not significantly boost the antibody, nevertheless they seem to serve in maintaining the continuity of the antibody-forming mechanism.

It was suggested by Kendal et al (1977) that NIAB response to vaccination can be suppressed if subjects receiving the vaccine were immunologically primed to its hemagglutinin component, thus directing most of the antibody formation towards the H. This observation may also be applied to our data where the presence of the H antigen in the vaccine strain may have suppressed the magnitude (capacity) for the formation of NIAB, particularly in the later period of the program. A similarity in the response of vaccinees to N1 and N2 antigens was observed. In both cases, it was shown that one particular vaccine had significantly boosted the NIAB response to its maximum capacity and to almost comparable levels, whereas, the second consecutive vaccine slightly boosted the antibody, thus maintaining the continuity of the process. However, unlike the GMT of the N1 antibody which at the end of the program was raised to a higher level than that of the initial, the N2 antibody GMT seemed to remain slightly lower than the

initial GMT. This trend could be due to a specific behaviour of N2 antibody response, as an indirect result of the natural VIC/75 outbreak (Kendal et al, 1977) or may be due to the antigenic concentration of N2 in the vaccine which was insufficient to boost the NIAB beyond that produced by the natural wild type virus challenge.

Both N1 and N2 antibodies were detectable in patients' sera throughout the duration of the program. This is well confirmed by the detection of NIAB not only in the vaccinees but also in the controls. Therefore, it could be suggested that NIAB had persisted during the specified period. Kilbourne (1976) had suggested that N antibody persisted for a longer period than H antibody. Whether this holds true in our study will only be known when comparison is made of the persistance of anti-H antibody and NIAB in the same sera for the same period, which is presently underway (R.E. Pyson and E. Perry - personal communication).

Many investigators (Couch et al, 1974; Kendal et al, 1977; 1980; Kilbourne, 1976; Murphy et al, 1972; Ogra et al, 1977; and Slepushkin et al, 1971) have demonstrated that NIAB are of a protective value in artificial challenge or natural outbreaks. Influenza A/Port Chalmers/73 (H3N2) because of its chronological precedence was not included in the vaccine formulas in this program. The A/PC/73 was considered a prototype for succeeding (H3N2) viruses since it had undergone a considerable drift in its N from that of the A/Hong Kong/68 (Schild et al, 1974). Kilbourne

(1976) demonstrated that more NIAB to earlier or older N2 antigens were produced upon vaccination with N2 vaccines, thus providing additional evidence for the hypothesis of serological recapitulation. In our case, VIC-, TEX-, and BGK- containing vaccines could have reinforced NIAB to earlier N2 antigens such as A/PC/73 in accordance with the hypothesis of serological recapitulation. Thus it was appropriate to use A/PC/73 N2 as an antigen in our study where higher NIAB titre was detectable than if VIC or TEX viruses were used.

In considering the range of NIAB titres (<1:10 to 1:160) of both pre- and post- vaccination, our results agree with the findings of Jennings and Miles (1978). They investigated the prevalence of NIAB titres in families before the outbreak of influenza A/PC/73 and found that 7/91(8%) of the population had NIAB titres to PC/73 ranging between 1:10 to 1:100.

The role of cell-mediated immunity (CMI), although not investigated by us at this time, should not be ignored. There is increasing evidence that CMI plays an important role in immunity to influenza infection. In vitro testing by Lazar and Wright (1980) demonstrated that lymphocyte cultures obtained from adults who had their last contact with the H1N1 (A/FM/1/47 prototype) virus circulating between 1947-1957 were able to transform when incubated with A/USSR/77 (H1N1) virus. Therefore, it was suggested that influenza virus can induce long-term CMI for periods of at least 20 years. Investigating the correlation between antibody and CMI, Greenberg

et al (1977) showed that small quantities of HI antibody and killer (K) lymphocytes were required to mediate lymphocyte cytotoxicity to influenza virus-infected cells. Whether NIAB plays a role similar to that of HIAB in mediating lymphocyte cytotoxicity remains as yet unknown.

Further investigations have revealed a link between influenza virus infection, CMI, and genetic factors. Daisy et al (1980) indicated that infection with live influenza virus in humans had induced an HLA-restricted cytotoxic T lymphocyte (CTL) response. Ennis et al (1982) demonstrated that most of the human volunteers, who received either live attenuated or inactivated influenza vaccines, exhibited a rise in their HLA-restricted influenza-specific memory CTL response. They also noted, however, that some of the live vaccine recipients had a positive CTL response but not an antibody response. The study of Ennis et al (1982) left a few questions unanswered, for example why some individuals develop CTL response but have no antibody response and vice versa, and whether the presence of enhanced HLA-restricted influenza-specific CTL is associated with protection of the vaccinated individuals when exposed to virus. Biddison and Shaw (1979) had suggested that virus-immune T cells preferentially lyse virus-infected target cells with which they share and recognize self antigens coded by genes that are both linked to the HLA-A and -B loci. They also demonstrated that in some donors the lack of CTL responses to influenza virus in conjunction with HLA-A1 or B7 may be attributed

to the control by certain regulatory genes that act at the level of the responder cell.

Of the 47 vaccinees who participated continually in the five year vaccination program 27(57%) responded to one or more of the vaccines administered exhibiting a rise in NIAB titre. The response to various vaccines differed when some vaccinees responded to only one vaccine whereas others responded to more than one vaccine. In contrast, 20(43%) did not respond at all to any of the vaccines administered.

The good or poor response of NIAB exhibited by certain patients in our study could have been influenced by certain regulatory genes at the level of the major histocompatibility complex. Whether this possibility is true or not can only be clarified by designing a special study to investigate that aspect of immunity to influenza.

CONCLUSION:

A mini neuraminidase inhibition (MNI) test was developed in our laboratory for detecting the neuraminidase inhibition antibody (NIAB) in sera from aged and chronically ill patients. The MNI test was standardized and, besides its economical advantage, was found reproducible and suitable for detecting NIAB.

Recombinant influenza viruses H7N1 and H7N2 were used as test antigens, thus covering both N1 and N2 type neuraminidase known at present, with minimum steric hindrance by HI antibody.

The data obtained clearly indicate that at least one influenza vaccination is necessary to boost NIAB titre to a maximal magnitude. When testing sera against N1 antigen, it was obvious that the first vaccine (NJ-VIC) induced the highest NIAB response. The same sera, when tested against N2 antigen, demonstrated that the second vaccine (VIC-BHK) elicited the highest NIAB response.

A second consecutive annual vaccination may be required to maintain the higher levels of NIAB attained and thus the continuity of the antibody-forming mechanism.

Although further annual vaccinations did not significantly enhance the NI antibody, nevertheless, they seemed to consequently re-inforce the antibody titre which usually declined within one year following each annual vaccination.

Of the 47 vaccinees, 27(57%) responded to at least one or more vaccines whereas 20(43%) were non-responders.

Due to the absence of a major influenza epidemic, the protective significance of the NI antibody could not be assessed.

FUTURE WORK

Our pilot clinical and observational study raised a number of questions requiring further investigation:

1. Development of a new more sensitive, accurate and less variable-dependent neuraminidase-inhibition test.

2. The protective value of NIAB. This can only be accurately assessed in the face of a natural outbreak of influenza.

3. The correlation between NIAB and HIAB upon vaccination. This study is currently underway by R.E. Fyson and E. Perry.

4. The role of cell-mediated immunity against influenza infection and the HLA-histocompatibility link to good and non-responders upon vaccination.

5. The correlation between the enzymatic and antigenic sites of neuraminidase gene.

REFERENCES

Ada G.L., Perry, B.T., and Abbot, A. (1958). Biological and physical properties of the Ryan strain of filamentous influenza virus. J. Gen. Microbiol. 19:23.

Allen H., McCanley J., Waterfield M., and Gething M-J (1980). Influenza virus RNA segment 7 has the coding capacity for two polypetides. Virology 107:548-551.

Aminoff D. (1961). Methods for the quantitative estimation of N-acetyl-neuraminic acid and their application to hydrolysates of sialomucoids. Biochem. J. 81: 384-392.

Armerding D. and Rossiter H. (1980). Induction of cytolytic T- and B-cell responses against influenza virus infections. Infect. Immun. 28: 799-811.

Assaad F.A., Brés P., Chi-Ming C. and Dowdle W.R. (1980). A revision of the system of nomenclature for influenza viruses: a WHO memorandum. Bull. W.H.O. 58:585-591.

Aymard-Henry M., Colman M.T., Dowdle W.R., Laver W.G., Schild G.C., and Webster R.G. (1973). Influenza virus neuraminidase-inhibition test procedures. Bull. World Health Organization 48: 199-205.

Baez M., Zazra J.J., Elliott R.M., Young J.F., and Palese P. (1981). Nucleotide sequence of the influenza A/duck/Alberta/60/76 virus RNA: Conservation of the NS1/NS2 overlapping gene structure in a divergent influenza virus RNA segment. Virology 113: 397-402.

Baltimore D. (1972). Expression of animal virus genomes. Bacteriol. Rev. 35:235-241.

Barry R.D. and Mahy B.W.J. (1979). The influenza virus genome and its replication. Br. Med. Bull. 35:39-46.

Bean W.J., Jr., Cox N.J., and Kendal A.P. (1980). Naturally occurring recombinants of human influenza A viruses. In "Structure and Variation in Influenza Virus". G. Laver and G. Air (eds). pp. 105-114. Elseviers North Holland.

Biddison W.E. and Shaw S. (1979). Differences in HLA antigen recognition by human influenza virus-immune cytotoxic T cells. J. Immunol. 122: 1705-1709.

Biddle, F. (1968). The action of protease on influenza A2 virus. J. Gen. Virol. 2: 19-28.

Bishop D.H.L., Roy P., Bean W.J., and Simpson R.W. (1972). Transcription of the influenza ribonucleic acid genome by a virion polymerase. III. Completeness of the transcription process. J. Virol. 10: 689-697.

Blix G., Gottschalk A., and Klenk E. (1957). Proposed nomenclature in the field of neuraminic and sialic acids. Nature (London) 179:1088.

Blok J., and Air G.M. (1980a). Comparative nucleotide sequences at the 3' end of the neuraminidase gene from eleven influenza type A viruses. Virology 107: 50-60.

Blok J., and Air G.M. (1980b). Sequence variations observed in the neuraminidase of several strains of influenza virus. In: Structure and Variation of Influenza Virus. W.G. Laver and G.M. Air (eds.) pp. 125-133. Elsevier/North-Holland, New York.

Bosch G.X., Orlich M., Klenk H-D, and Rott R. (1979). The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. Virology 95: 197-207.

Breschkin A.M., Ahern J., and White D.O. (1981). Antigenic determinants of influenza virus hemagglutinin. Virology 113: 130-140.

Briedis D.J., Lamb R.A., and Choppin P.W. (1981). Influenza B virus RNA segment 8 codes for two nonstructural proteins. Virology 112: 417-425.

Brown L.E., Dopheide T.A.A., Ward C.W., White D.O., and Jackson D.C. (1980). Antigenic determinants of influenza virus hemagglutinin. I. Antigenicity of the HA2 chain. J. Immunol. 125: 1583-1588.

Bucher D.J. (1975). In "Negative Strand Viruses." (B.W.J. Mahy and R.D. Barry, eds.) pp. 611 - 620. Academic Press, New York.

Bucher D.J., Kharitonenkow L.G., Zakomiridin J.A., Grigoriev V.B., Klimenko S.M., and Davis J.F. (1980). Incorporation of influenza M-protein into liposomes. J. Virol. 36:586-590.

Bucher D.J., and Kilbourne E.D. (1972). A2(N2) neuraminidase of the X-7 influenza virus recombinant: Determination of molecular size and subunit composition of the active unit. J. Virol. 10: 60-66.

Bucher D.J., and Palese P. (1975). In "Influenza Viruses and Influenza". (E.D. Kilbourne ed.) pp. 83-123. Academic Press, New York.

Cabezas J.A., Calvo P., Eid P., Martin J.A., Perez N., Reglero A., and Hannoun C. (1980). Neuraminidase from influenza virus A (H3N2). Specificity towards several substrates and procedure of activity determination. Biochim. Biophys. Acta 616: 228-238.

Callow K.A. and Beare A.S. (1976). Measurement of antibody to influenza virus neuraminidase by single radial hemolysis in agarose gels. Infect. Immun. 13: 1-8.

Cambridge G., Mackenzie J.S., and Keast D. (1976). Cell-mediated immune response to influenza virus infection in mice. Infect. Immun. 13: 36-43.

Carroll S.M. and Paulson J.C. (1982). Complete metal ion requirement of influenza virus N1 neuraminidase. Arch. Virol. 71: 273-277.

CDC (Centre for Disease Control) (1975). Immunology Series No. 6. Advanced Laboratory Techniques For Influenza Diagnosis.

CDC (Centre for Disease Control) (1980). Class Session on "Measurement of Neuraminidase in Influenza Vaccines".

Chakraverty P. (1978). Antigenic relationship between influenza C viruses. Arch. Virol. 58: 341-348.

Chanock R.M., Cockburn W.C., Davenport F.M., Dowdle W.R., Fazekas de St Groth S., Fukumi H., Kilbourne E.D., Schild G.C., Schulman J.L., Sohier R., Soloview V.D., Tumora B., Webster R.G., Zakstel L.Ja., and Zdanov V.M. (1971). A revised system of nomenclature for influenza viruses. Bull. W.H.O. 45: 119-124.

Choppin P.W., and Compans R.W. (1975a). The structure of influenza virus. In: The Influenza Viruses and Influenza. Kilbourne E.D. (Ed.). New York-San Francisco. London. Academic Press. pp. 15-51.

Choppin P.W., and Compans R.W. (1975b). Reproduction of Paramyxoviruses. Rockefeller University, N.Y., New York. Comprehensive virology 4: 95-178.

Choppin P.W., Murphy J.S., and Tamm I. (1960). Studies of two kinds of virus particles which comprise influenza A2 virus strains. III- Morphological characteristics: Independence of morphological and functional traits. J. Exp. Med. 112: 945-952.

Chow T.C., Beutner K.R., and Ogra P.L. (1979). Cell-mediated immune response to the hemagglutinin and neuraminidase antigens of influenza A virus after immunization in humans. Infect. Immun. 25: 103-109.

Collins J.K., and Knight C.A. (1978). Purification of the influenza hemagglutinin glycoprotein and characteristics of its carbohydrate components. J. Virol. 26: 457-467.

Colton T. (1974). In "Statistics in Medicine". Little, Brown & Company. Boston.

Compans R.W., Bishop D.H.L., and Meier-Ewert H. (1977). Structural components of influenza C virions. J. Virol. 21: 658-665.

Compans R.W., and Dimmock N.J. (1969). An electron microscopic study of single-cycle infection of chick embryo fibroblasts by influenza virus. Virology 39 : 499-515.

Compans R.W., Dimmock N.J. and Meien-Ewert H. (1969). Effect of antibody to neuraminidase on the maturation and hemagglutinating activity of an influenza A2 virus. J. Virol. 4: 528-534.

Compans R.W., Klenk H-D., Caliguiri L.A., and Choppin P.W. (1970). Influenza virus proteins. 1. Analysis of polypeptides of the virion and identification of spike proteins. Virology 42: 880-889.

Couch R.B., Kasal J.A., Gerin J.L., Schulman L.J., and Kilbourne E.D. (1974). Induction of partial immunity to influenza by a neuraminidase-specific vaccine. J. Infect. Dis. 129: 411-420.

Daisy J., Tolpin M.D., Quinnan G.V., Rook A.H., Murphy B.R., Mittal K., Clements M.L., Mullinix M.G., Kiley S.C., and Ennis F.A. (1981). Cytotoxic cellular immune response during influenza A infection in human volunteers. In the Replication of Negative Strand Viruses, pp. 443-448. D.H.L. Bishop and R.W. Compans (eds.). Amsterdam: Elsevier/North Holland.

Desselberger U.K., Nakajama K., Alfino P., Pederson F.S., Haseltine W.A., Hannoun C., and Palese P. (1978). Biochemical evidence that "new" influenza virus strains in nature may arise by recombination (reassortment). Proc. Natl. Acad. Sci. U.S.A. 75: 3341-3345.

Dimmock N.J. (1971). Dependence of activity of an influenza virus neuraminidase upon Ca^{2+}. J. Gen. Virol. 13: 481-483.

Dimmock N.J., Carver A.S., and Webster R.G. (1980). Categorization of nucleoproteins and matrix proteins from type A influenza viruses by peptide mapping. Virology 103: 350-356.

Doherty P.C., Effros R.B., and Bennink J. (1977). Heterogeneity of the cytotoxic response of thymus-derived lymphocytes after immunization with influenza viruses. Proc. Natl. Acad. Sci. USA 74: 1209 - 1213.

Dophiede T.A., and Ward C.W. (1980). Structural studies on A Hong Kong influenza hemagglutinin. The structure of the light chain and the arrangement of the disulphide bonds. In "Structure and variation of influenza virus". G. Laver and G. Air (Eds.). pp. 21-26. Elsevier North Holland.

Dopheide T.A. and Ward C.W. (1981). The location of the bromelain cleavage site in a Hong Kong influenza hemagglutinin. J. Gen. Virol. 52: 367-370.

Dowdle W.R., Downie J.C., and Laver W.G. (1974). Inhibition of virus release by antibodies to surface antigen of influenza viruses. J. Virol. 13: 269-275.

Drescher J., and Desselberger U. (1980?). Comparison of the antineuraminidase antibody response to vaccination with intact virus and subunit preparations obtained by different techniques of the influenza virus strains A/Sing/1/57 (H2N2), A/Hong Kong/1/68 (H3N2), A/Port Chalmers/1/73 (H3N2). Comp. Immunol. Microbiol. Infect. Dis. 3: 219-223.

Drzeniek R. (1972). Viral and bacterial neuraminidases. Curr. Top. Microbiol. Immunol. 59: 35-74.

Drzeniek R., Seto J.T., and Rott R. (1966). Characterization of neuraminidase from myxovirus. Biochim. Biophys. Acta 128: 547-558.

Easterday B., Laver W.G., Pereira H.G., and Schild G.C. (1969). Antigenic composition of recombinant virus strains produced from human and avian influenza A viruses. J. Gen. Virol. 5: 83 - 91.

Effros R.B., Doherty P.C., Gerhard W., and Bennink J. (1977). Generation of both cross-reactive and virus-specific T-cells populations after immunization with serologically distinct influenza A viruses. J. Exp. Med. 145:557.

Elder K.T., Bye J.M., Skehel J.J., Waterfield M.D., and Smith A.E. (1979). In Vitro synthesis, glycosylation, and membrane insertion of influenza virus hemagglutinin. Virology 95: 343-350.

Ennis F.A., Martin W.J., and Verbonitz M.W. (1977). Hemagglutinin-specific cytotoxic T-cell response during influenza infection. J. Exp. Med. 146: 893-898.

Ennis F.A., Yi-Hua Q.I. and Schild G.C. (1982). Antibody and cytotoxic T- lymphocyte response of humans to live and inactivated influenza vaccines. J. Gen. Virol. 58: 273-281.

Enzyme Nomenclature (1965). List of the nomenclature of enzymes. Compr. Biochem. 13: 138.

Farrohi K., Farrohi F.K., Noble G.R., Kay H.S., and Kendal A.P. (1977). Evaluation of the single radial hemolysis test for measuring hemagglutinin-and neuraminidase-specific antibodies to H3N2 influenza strains and antibodies to influenza B. J. Clin. Microbiol. 5: 353-360.

Fazekas de St. Growth S. (1963). Steric inhibition: neutralization of a virus-born enzyme. Ann. N.Y. Acad. Sci. 103: 674.

Fenner F. (1975). Classification and nomenclature of viruses. Intervirology 6: 1-12.

Fenner F. and White D.O. (1976). Orthomyxoviruses. In "Medical Virology". Academic Press, New York, San Francisco, London.

Fields S., Winter G., and Brownlee G.C. (1981). Structure of the neuraminidase gene in human influenza virus A/PR/8/34. Nature 290: 213-217.

Fletcher W.B., Larson H.E., Murphy F.W., Sims P., Smith H., Wood J.M., and Yetts R. (1977). Antibody responses and reactogenicity of graded doses of inactivated influenza A/New Jersey/76 whole virus vaccine in humans. J. Infect. Dis. 136: (supplement) S 475- S 483.

Fyson R.E. and Perry E. (1981). Immune response to influenza vaccines in the aged. A five year study. Presentation to the Surgeon General's meeting on influenza. Atl., GA., USA Jan 27, 1981 (Abstract).

Garten W., Bosch F.X., Linder D., Rott R., and Klenk H-D. (1981). Proteolytic activation of the influenza virus hemagglutinin: The structure of the cleavage site and the enzymes involved in cleavage. Virology 115: 361-374.

Gerhard W., Yewdell J., Frankel E.M. and Webster R. (1981). Antigenic structure of influenza virus hemagglutinin defined by hybridoma antibodies. Nature (Lond.) 290: 713 - 717.

Gething M.J., Bye J., Skehel J.J., and Waterfield M.D. (1980). Cloning and DNA sequence of double stranded copies of hemagglutinin genes from H2 and H3 strains of influenza virus. In: Structure and variation in influenza virus. pp. 1-10. Ed. by Laver W.G. and Air G. New York : Elsevier/North Holland.

Gottschalk A. (1957). Neuraminidase: The specific enzyme of influenza virus and Vibrio cholerae. Biochim. Biophys. Acta 23: 645-646.

Gottschalk A. (1958). The influenza virus neuraminidase. Nature (London). 181: 377-378.

Greenberg S.B., Criswell B.S., Six H.R., and Couch R.B. (1977). Lymphocyte cytotoxicity to influenza virus-infected cells. II. Requirements for antibody and non-T Lymphocyte. J. Immunol. 119: 2100 - 2106.

Gregoriades A. (1972). Isolation of neuraminidase from the WSN strain of influenza virus. Virology 49: 333-336.

Gregoriades A. (1977). Influenza virus-induced proteins in nuclei and cytoplasm of infected cells. Virology 79: 449-454.

Gregoriades A. (1980). Interaction of influenza M protein with viral lipid phosphatidylcholine vesicles. J. Virol. 36: 470-479.

Gregoriades A., and Frangion B. (1981). Insertion of influenza M protein into the viral lipid bilayer and localization of site insertion. J. Virol. 40: 323-328.

Haaheim L.R. (1980). Antibodies to the strain-specific and cross-reactive determinants of the hemagglutinin of influenza H3N2 viruses. Acta Path. Microbiol. Scand. Sec. B. 88: 341-345.

Haaheim L.R., and Schild G.C. (1980). Antibodies to the strain-specific and cross-reactive determinants of the hemagglutinin of influenza H3N2 viruses. Pathol. Microbiol. Scand. Sect. B 88: 335-340.

Hall R.M., and Air G.M. (1981). Variation in nucleotide sequences coding for the N-terminal region of the matrix and non-structural proteins of influenza A viruses. J. Virol. 38: 1-7.

Hay, A.T. (1974). Studies on the formation of the influenza virus envelope. Virology 60: 398-418.

Helenius A., Kartenbeck J., Simons K. and Fries E. (1980). On the entry of semliki forest virus into BHK-21 cells. J. Cell Biology 84: 404-420.

Hirst G.K. (1942). The quantitative determination of influenza virus and antibodies by means of red cell agglutination. J. Exp. Med. 75: 47-64.

Horne R.W., Waterson A.P., Wildy P., and Farnham A.E. (1960). The structure and composition of the myxoviruses. I. Electron microscopic studies of the structure of myxovirus particles by negative staining techniques. Virology 11: 79-98.

Hoyle L., Horne R.W., and Waterson A.P. (1961). The strucure and composition of the myxoviruses. II. Components released from the influenza virus particle by ether. Virology 13: 448-459.

Hsia C-N.L., Foy H.M., and Cooney M.K. (1980). Relationship between surface antigens of two variants of influenza A(H3N2) virus revealed by hemagglutination-inhibition, kinetics neutralization and neuraminidase-inhibition. Infect. Immun. 30: 467-472.

Huang R.T.C., Rott R., Wahn K., Klenk H-D., and Kohama T. (1980a). The function of the neuraminidase in membrane fusion induced by myxoviruses. Virology 107: 313-319.

Huang R.T.C., Rott R., and Klenk H-D. (1981). Influenza viruses cause hemolysis and fusion of cells. Virology 110: 243-247.

Huang R.T.C., Wahn K., Klenk H-D, and Rott R. (1979). Association of the envelope glycoproteins of influenza virus with liposomes. A model in viral envelope assembly. Virology 97: 212-217.

Huang R.T.C., Wahn K., Klenk H-D., and Rott R. (1980b). Fusion between cell membrane and liposomes containing the glycoproteins of influenza virus. Virology 104: 294-302.

Hudson J.B., Flawith J., and Dimmock N.J. (1978). Early events in influenza virus infection. III. The formation of a nucleoplasmic ribonucleoprotein complex from the input virion. Virology 86: 167-176.

Inglis S.C., Barrett T., Brown C.M., and Almond J.W. (1979). The smallest genome RNA segment of influenza virus contains two genes that may overlap. Proc. Natl. Acad. Sci. USA. 76: 3790-3794.

Inglis S.C., Carrol A.R., Lamb R.A., and Mahy B.W.J. (1976). Polypeptides specified by the influenza virus genome. I. Evidence for eight distinct gene products specified by fowl plague virus. Virology 74: 489-503.

Jackson D.C.T., Dopheide T.A., Russel R.J., White D.O., and Ward C.W. (1979). Antigenic determinants of influenza virus hemagglutinin. II. Antigenic reactivity of isolated N-terminal cyanogen bromide peptide of A/Memphis/72 hemagglutinin heavy chain. Virology 93: 458 - 465.

Jahiel R.I., and Kilbourne E.D. (1966). Reduction in plaque size and reduction in plaque number as differing indices of influenza virus - antibody reactions. J. Bacteriol. 92: 1521-1534.

Jennings L.C., and Miles J.A.R. (1978). A study of acute respiratory disease in the community of Port Chalmers. II. Influenza A/Port Chalmers/1/73: intrafamilial spread and the effect of antibodies to the surface antigens. J. Hyg. Camb. 81: 67-75.

Kendal A.P., Biddle F., and Belyavin G. (1968). Influenza virus neuraminidase and the viral surface. Biochim. Biophys. Acta. 165: 419-413.

Kendal A.P., Bozeman F.M., and Ennis F.A. (1980). Further studies of the neuraminidase content of inactivated influenza vaccines and the neuraminidase antibody responses after vaccination of immunologically primed and unprimed populations. Infect. Immun. 29: 966-971.

Kendal A.P., and Eckert E.A. (1972). The preparation and properties of 14C-carboxamindomethylated subunits from A2/1957 influenza neuraminidase. Biochim. Biophys. Acta 258: 484-507.

Kendal A.P., Madely C.R. and Allan W.H. (1971). Antigenic relationships in Avian Influenza viruses: identification of two viruses isolated from turkeys in Great Britain during 1969-1970. J. Gen. Virol. 13: 95-100.

Kendal A.P., Minuse E., Maassab H.F., Hennessy A.V. and Davenport F.M. (1973). Influenza Neuraminidase Antibody Patterns of Man. Amer. J. Epidemiol. 98: 96-103

Kendal A.P., Noble G.R. and Dowdle W.R. (1977). Neuraminidase content of influenza vaccines and neuraminidase. Antibody response after vaccination of immunologically primed and unprimed population. J. Infect. Dis. 136 (Suppl.) S415 -S425.

Kharitonenkov I.G., Zakomirdin J.A., Gitelman A.K., and Bukrinskaya A.G. (1980). Influenza virus proteins and interaction with planar lipid bilayers. Voprosi Virusologii (USSR), No. 6, 708 - 712. English translation.

Kilbourne E.D. (1968). Recombination of influenza A viruses of human and animal origin. Science 160: 74.

Kilbourne E.D. (1974). The molecular epidemiology of influenza. J. Infect. Dis. 127: 478 - 486.

Kilbourne E.D. (1975). Influenza Viruses and Influenza. (E.D. Kilbourne, ed.). Academic Press, New York.

Kilbourne E.D. (1976). Comparative efficacy of neuraminidase-specific and conventional influenza virus vaccines in induction of antibody to neuraminidase in humans. J. Infect. Dis. 134: 384-394.

Kilbourne E.D. (1978). Opinion. Influenza as a problem in immunology. J. Immunol. 120:1447-1452

Kilbourne E.D., Laver W.G., Schulman J.L. and Webster R.G. (1968) Antiviral activity of Antiserum specific for an influenza neuraminidase. J. Virology 2: 281-288

Kilbourne E.D., Schulman J.L., and Couch R.B., and Kasal J.A. (1972). Mono-antigenic immunization and the induction of partial immunity. A new approach to influenza immunization. In J.L. Melnick (ed.) International virology 2. Proceedings of the 2nd international congress for virology. S. Karger, Basel. p. 118-119.

Klenk H-D, and Rott R. (1973). Formation of influenza virus proteins. J. Virol. 11: 823-831.

Klenk H-D., Rott R., Orlich M., and Blodorn J. (1975). Activation of influenza viruses by trypsin treatment. Virology 68: 426-439.

Krug R.M., and Soeiro R. (1975). Studies on the intracellular location of influenza virus-specific proteins. Virology 64: 378-387.

Lamb R.A., and Choppin P.W. (1979). Segment 8 of the influenza virus genome is unique in coding for two polypeptides. Proc. Natl. Acad. Sci. USA. 76: 4908-4912.

Lamb R.A., Choppin P.W., Chanock R.M., and Lai C-J. (1980). Mapping of the two overlapping genes for polypeptides NS1 and NS2 on RNA segment 8 of influenza virus genome. Proc. Natl. Acad. Sci. USA. 77: 1857-1861.

Lamb R.A., and Choppin P.W. (1981). Identification of a second protein (M2) encoded by RNA segment 7 of influenza virus. Virology 112: 729-737.

Lamb R.A., Etkind P.R., and Choppin P.W. (1978). Evidence for a ninth influenza viral polypeptide. Virology 91: 60-78.

Larson H.E., Tyrell D.A.J., Bowker C.H., Potter C.W., and Schild G.C. (1978). Immunity to challenge in volunteers vaccination with an inactivated current or earlier strain of influenza A (H3N2). J. Hyg. (Camb.) 80:243-248.

Laver W.G. (1963). The structure of influenza viruses.3. Disruption of the virus particle and separation of neuraminidase activity. Virology 20: 251-262.

Laver W.G. (1971). Separation of two polypeptide chains from the hemagglutinin subunit of influenza virus. Virology 45: 275-288.

Laver W.G. and Air G.M. (1980). "The Structure and Variations in Influenza Virus". W.G. Laver and G.M. Air (eds.). Elsevier North Holland.

Laver W.G., Air G.M., Dopheide T.A., and Ward C.W. (1980). Amino acid sequence changes in the hemagglutinin of A/Hong Kong (H3N2) influenza virus during the period 1968-1977. Nature 283: 454-461.

Laver W.G., and Baker N.J. (1972). Amino acid composition of polypeptides from influenza virus particles. J. Gen. Virol. 17:61-67.

Laver W.G., Downie J.C., and Webster R.G. (1976). Diversity of antibody response to the antigenic determinants of the hemagglutinin subunits of influenza viruses. J. Immunol. 116: 336-341.

Laver W.G., and Kilbourne E.D. (1966). Identification in a recombinant influenza virus of structural proteins derived form both parents. Virology 30: 493-501.

Laver W.G., and Valentine R.C. (1969). Morphology of the isolated hemagglutinin and neuraminidase subunits of influenza virus. Virology 38: 105-119.

Laver W.G., and Webster R.G. (1979). Ecology of influenza viruses in lower mammals and birds. Br. Med. Bull. 35: 29-33.

Lazar A. and P.F. Wright (1980). Cell-mediated immune response of human lymphocytes to influenza A/USSR (H1N1) virus infection. Infect. Immun. 27: 867-871.

Layde P.M., Engelberg A.L., Dobbs H.I., Curtis A.C., Craven R.B., Graitcer P.L., Sedmak G.V., Erickson J.D., and Noble G.R. (1980). Outbreak of influenza A/USSR/77 at Marquette University. J. Infect. Dis. 142: 347-352.

Lazarowitz S.G., and Choppin P.W. (1975). Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology 68: 440-454.

Lief F.S., and Henle W. (1959). Methods and procedures for use of complement-fixation technique in type- and strain-specific diagnosis of influenza. Bull. W.H.O. 20:411-420.

Lubeck M.D. and Gerhard W. (1981). Topological mapping of antigenic sites on the influenza A/PR/8/34 virus hemagglutinin using monoclonal antibodies. Virology 113: 64-72.

Luther P., Adamczyk B., and Bergman, K.C. (1980). Simple test for detection of virus neuraminidase and anti-neuraminidase using lectins (lectin-neuraminidase-testsystem). Zbl. Bakt. Hyg. I. Abt. Orig. (Abstract). A 248: 281-285.

Meada T., Kawazaki K. and Ohnishi S-I (1981) Interaction of influenza virus hemagglutinin with target membrane lipids is a key step in virus-induced hemolysis and fusion at pH 5.2, Proc. Natl. Acad. Sci. U.S.A. 78: 4133-4137.

McGeoch D., Fellner P., and Newton C. (1976). Influenza virus genome consists of eight distinct RNA species. Proc. Natl. Acad. Sci. USA. 73: 3045-3049.

Meier-Ewert H., Petri T., and Bishop D.H.L. (1981). Oligonucleotide fingerprint analysis of influenza C virion RNA recovered from five different isolates. Arch. Virol. 67: 141-147.

Min-Jou W., Verhoeyen M., Devos R., Saman E., Ronxiang F., Huylebroeck D., Van Rompuy L., Fang R.X., and Fiers W. (1980). Cloning and DNA nucleotide sequence analysis of the hemagglutinin and neuraminidase genes of influenza A strains. In: Structure and Variation in Influenza Virus. Laver W.G. & Air G. (eds.) p. 63-68. Elsevier, New York.

Monto A.S., and Kendal A.P. (1973). Effect of neuraminidase antibody on Hong Kong influenza. Lancet 1:623-625.

MMWR (Morbidity Mortality Weekly Report) (1981). Influenza. 30 (No. 23): 279-287.

MMWR (Morbidity Mortality Weekly Report) (1982). Influenza. 31 (No. 9): 110-111.

Moss R.A., Underwood P.A., Bender V.J., and Whittaker R.G. (1980). Antigenic drift in the hemagglutinin from various strains of influenza A/Hong Kong/68 (H3N2). In: Structure and Variation in Influenza Virus. (W.G. Laver and G. Air, eds.). pp. 329-338. Elsevier/North-Holland, New York.

Mostow S.R., Schild G.C., Dowdle W.R., and Wood R.J. (1975). Application of the single radial diffusion test for assay of antibody to influenza type A viruses. J. Clin. Microbiol. 2: 531-540.

Murphy B.R., Kasal J.A., and Chanock R.M. (1972). Association of serum anti-neuraminidase antibody with resistance to influenza in man. New Engl. J. Med. 286: 1329-1332.

Murphy B.R., Markoff L.J., Hosier N.T., Rusten H.M., Chanock R.M., Kendal A.P., Douglas R.G., Betts R.F., Cate T.R., Couch Jr. R.B., Levine M.M., Watternman D.H., and Holley Jr. H.P. (1978). Temperature- sensitive mutants of influenza A virus: evaluation of A/Victoria/3/75-ts-1[E] recombinant viruses in volunteers. Infect. Immun. 20: 671-677.

Murphy B.R., Tierney E.L., Barber B.A., Yolken R.H., Alling D.W., Holley H.P.Jr., Mayner R.E., and Chanock R.M. (1980). Use of the enzyme-linked immunosorbent assay to detect serum antibody responses of volunteers who received attenuated influenza A virus vaccines. Infect. Immun. 29: 342-347.

Nakajima K., Desselberger U., and Palese P. (1978). Recent human influenza A (H1N1 viruses) are closely related genetically to strains isolated in 1950. Nature (London). 274: 334-339.

Nakamura K., and Compans R.W. (1978). Glycopetide components of influenza viral glycoproteins. Virology 86: 432-442.

Noll H., Aoyagi T., and Orlando J. (1962). The structure relationshop of sialidase to the influenza virus surface. Virology 18: 152-157.

Ogra P.L., Chow T., Beutner K.R., Rubi E., Strussenberg J., DeMello S., and Rizzone C. (1977). Clinical and immunoligical evaluation of neuraminidase specific influenza A virus vaccine in humans. J. Infect. Dis. 135: 499-506.

Oxford J.S., McGeoch D.J., Schild G.C., and Beare A.S. (1978). Analysis of virion RNA segments and polypeptides of influenza A virus recombinants of defined virulence. Nature (London). 273: 778-

Oxford J.S. and Schild G.C. (1979). The specificity of antihemagglutinin antibody response induced in man by inactivated influenza vaccines and by natural infection. J. Hyg. Camb. 82: 51-61.

Oxford J.S., Schild G.C., Potter C.W., and Jennings R. (1979). The specificity of the anti-hemagglutinin antibody response induced in man by inactivated influenza vaccines and by natural infection. J. Hyg. Cambr. 82: 51-61.

Palese P., and Schulman J.L. (1976). Differences in RNA patterns of influenza A viruses. J. Virol. 17: 876-884.

Palese P., Tobita K., Veda M., and Compans R.W. (1974). Characterization of temperature-sensitive influenza virus mutants defective in neuraminidase. Virology 61: 397-410.

Paniker C.K.J. (1968). Serological relationships between the neuraminidases of influenza virus. J. Gen. Virol. 2: 385-394.

Pereira R.C. (1979). Global surveillance of influenza. Br. Med. Bull 35:9-14.

Pons M.W. (1976). A reexamination of influenza single-and double-stranded RNAs by gel electrophoresis. Virology 69: 789-792.

Pons M.W. and Hirst G.K. (1968). Polyacrylamide gel electrophoresis of influenza virus RNA. Virology 34: 385-388.

Porter A.G., Smith J.C., and Emtage J.S. (1980). Nucleotide sequence of influenza virus RNA segment 8 indicates that coding regions for NS1 and NS2 proteins overlap. Proc. Natl. Acad. Sci. USA 77: 5074-5078.

Potter C.W., Jennings R., Phair J.P., Clark A., and Stuart-Harris C. (1977). Dose-response relationship after immunization of volunteers with a new surface-antigen-adsorbed influenza virus vaccine. J. Infect. Dis. 135: 423-431.

Potter C.W. and Oxford J.S. (1979). Determinants of immunity of influenza in man. Br. Med. Bull. 35: 69-75.

Rafelson M.E., Gold S., and Priede I. (1966). Neuraminidase (Sialidase) from influenza virus. "Methods in Enzymology" 8: 677-680.

Rees P.J., and Dimmock N.J. (1981). Elecrophoretic separation of influenza virus ribonucleoproteins. J. Gen. Virol. 53: 125-132.

Ritchey M.B., and Palese P. (1976). In vitro translation of influenza virus messenger RNAs. Virology 72: 410-420.

Rohde W., Harms E., and Scholtissek C. (1977). Biochemical studies on influenza viruses. I. Comparative analysis of equi 2 virus and virus N genes and gene products. Virology 79: 393-404.

Rott R. (1980). Studies on structure - Function relationships of influenza virus glycoproteins. In "Stucture and Variation in Influenza Virus". W.G. Laver and G.M. Air (eds.). pp. 201-209. Elsevier North Holland.

Rott R., Huang R.T.C., Wahn K., and Klenk H-D. (1981). On the initiation of myxovirus infection. In: Replication of negative strand viruses. D.H.L. Bishop and R.W. Compans (eds.) pp. 269-276. Elsevier/North-Holland, New York.

Rott R., Reina Cher M., Orlich M., and Klenk H-D. (1980). Cleavability of hemagglutinin determines spread of avian influenza viruses in the chorioallantoic membrane of chicken embryo. Arch. Virol. 65: 123-233.

Saratenau D.E., Ehrengut W., Pressier K., Peukert M., and Schenk K.D. (1980). Serologic response to whole, split and subunit influenza vaccines of persons with and without immunological experience towards influenza A/USSR/90/77 virus. Comp. Immunol. Microbiol. Infect. Dis. 3: 225-236.

Schild G.C. (1970). Studies with antibody to the purified hemagglutinin of an influenza Ao virus. J. Gen. Virol. 9: 191-197.

Schild G.C. (1972). Evidence for a new type of specific structural antigen of the influenza virus particle. J. Gen. Virol. 15: 99 -104.

Schild G.C. (1979). The influenza virus: Antigenic composition and immune response. Postgrad. Med. J. 55: 87-97.

Schild G.C., Newman R.W., Webster R.G., Major D., and Hinshaw S. (1980). Antigenic analysis of influenza A virus surface antigens : considerations for the nomenclature of influenza virus. Comp. Immunol. Microbiol. Infect. Dis. 3: 5-18.

Schild G.C., Oxford J.S., Dowdle W.R., Coleman M., Pereira M.S., and Chakraverty P. (1974). Antigenic variation in current influenza A viruses: evidence for a high frequency of antigenic "drift" for the Hong Kong virus. Bull. W.H.O. 51: 1-11.

Schild G.C., Oxford J.S., and Newman R.W. (1979). Evidence for antigenic variation in influenza A nucleoprotein. Virology 93: 569-573.

Schild G.C., and Pereira H.G. (1969). Characterization of the ribonucleoprotein and neuraminidase of influenza A viruses by immunodiffusion. J. Gen. Virol. 4: 355-363.

Schild G.C., Smith J.W.C., Cretescu L., Newman R.W., and Wood J.M. (1977). Strain-specificity of anitbody to hemagglutinin following inactivated A/Port Chalmers/1/73 vaccine in man: evidence for a paradoxical strain specific antibody response. Dev. Biol. Stand. 39: 273-281.

Schild G.C., Winter W.D., and Brand C.M. (1971). Serological diagnosis of human influenza infections by immunopreiciptin techniques. Bull. W.H.O. 45: 465 - 471.

Scholtissek C. (1978). The genome of the influenza virus. Curr. Top. Microbiol. Immunol. 80: 139-169.

Scholtissek C. (1979). Influenza virus genetics. Advanced Genetics 20:1-36.

Scholtissek C., Harms E., Rohde W., Orlich M., and Rott R. (1976). Correlation between RNA fragments of fowl plaque virus and their corresponding gene functions. Virology 74: 332-344.

Schulman J.L., Khakpour M., and Kilbourne E.D. (1968). Protective effects of specific immunity to viral neuraminidase on influenza virus infection of mice. J. Virol. 2: 778-786.

Schulman J.L., and Kilbourne E.D. (1969). Independent variation in nature of hemagglutinin and neuraminidase antigens of influenza viruses: Distinctiveness of hemagglutinin antigen of Hong Kong/68 viruses. Proc. Natl. Acad. Sci. USA 63: 326-333.

Schulze I.T. (1970). The structure of influenza virus. I. The polypeptides of the virion. Virology 42: 890-904.

Schulze I.T. (1972). The structure of influenza virus. II. A model based on the morphology and composition of subviral particles. Virology 47: 181-196.

Schwarz R.T., and Klenk H-D. (1981). Carbohydrates of influenza virus. Virology 113: 584-593.

Schwarz R.T., Schmidt M.F.G., Anwer U., and Klenk H-D. (1977). Carbohydrates of influenza virus. I. Glycopeptides derived from viral glycoproteins after labelling with radioactive sugars. J. Virol. 23: 217-226.

Shaw M.W., and Compans R.W. (1978). Isolation and characterization of cytoplasmic inclusions from influenza A virus infected-cells. J. Virol. 25: 608 - 615.

Siekmann U., Schoop H.J., and Kuwert E. (1975). Contribution to the standardization of influenza neuraminidase inhibition tests, based on enzyme-antienzyme kinetic studies. Dev. Biol. Stand. 28: 324-335.

Skehel J.J. (1972). Polypeptide synthesis in influenza virus - infected cells. Virology 49: 23-36.

Skehel J.J. (1973). Early polypeptide synthesis in influenza virus - infected cells. Virology 56: 394-399.

Sleigh M.J., Both G.W., BrownLee G.G., Bender V.J., and Moss B.A. (1980). The hemagglutinin gene of influenza A virus : Nucleotide sequence analysis of cloned DNA copies. In: Structure and Variation in Influenza Virus. Laver W.G. and Air G.M. (eds.) pp. 69-80. Elsevier, New York.

Sleigh M.J., Both G.W., Underwood P.A., and Bender N.J. (1981). Antigenic drift in the hemagglutinin of the Hong Kong influenza subtype: Correlation of amino acid changes with alterations in viral antigenicity. J. Virol. 37: 845-853.

Slepushkin A.N., Schild G.C., Beare A.S., Chinn S., and Tyrrell D.A.J. (1971). Neuraminidase and resistance to vaccination with live influenza A2 Hong Kong vaccines. J. Hyg. Camb. 69: 571-578.

Smith W., Andrews C.H., and Laidlaw P.P. (1933). A virus obtained from influenza patients. Lancet ii: 66-68.

Stuart-Harris C., and Schild G.C. (1976). Influenza: The viruses and the disease. Edward Arnold. London PP. 50-77.

Taylor R.N., Hueng A.Y., Fulford K.M., Przybyszewski V.A., and Hearn T.L. (1979). Quality control for immunologic tests. U.S. Department of Health, Education, and Welfare. Atlanta, Georgia.

Thraenhart O. and Kuwert E.K. (1976). Standardization of a rapid modified micro neuraminidase inhibition test (Essen-NIT) for influenza virus-neuraminidase antibody assay and comparison with the W.H.O. method. J. Biol. Stand. 4: 225-241.

Tyrrell D.A.J. and Smith J.W.G. (1979). Vaccination against influenza A. Br. Med. Bull. 35: 77-85.

Van Wyke K.L., Hinshaw V.S., Bean W.J., and Webster R.G. (1980). Antigenic variation of influenza A virus nucleoprotein detected with monoclonal antibodies. J. Virol. 35: 24-30.

Virelizier J.L., Allison A.C., and Schild G.C. (1979). Immune response to influenza virus in the mouse and their role in the control of infection. Br. Med. Bull. 35: 65-68.

Virelizier J.L., Oxford J.S., and Schild G.C. (1976). The role of humoral immunity in host defence against influenza A infection in mice. Postgraduate Med. J. 52: 332 - 337.

Ward C.W., and Dopheide T.A. (1980). Completion of the amino acid sequence of a Hong Kong influenza hemagglutinin heavy chain: sequence of cyanogen bromide fragment CN-1. Virology 103: 37-53.

Warren L. (1959). The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234: 1971-1975.

Waterfield M.D., Espelie K., Elder K., and Skehel J.J. (1979). Structure of the hemagglutinin of influenza virus. Br. Med. Bull. 35: 57-64.

Webster R.G., and Darlington R.W. (1969). Disrupion of myxoviruses with Tween 20 and isolation of biologically active hemagglutinin and neuraminidase subunits. J. Virol. 4: 182-187.

Webster R.G., and Laver W.G. (1975). Antigenic variation of influenza viruses. In: Influenza Viruses and Influenza (Ed. by Kilbourne E.D.). pp. 270-314.

Webster R.G., and Laver W.G. (1980). Antigenic drift in Hong Kong (H3N2) influenza viruses: Selection of variants with potential epidemiological significance using monoclonal antibodies. In: Structure and Variation in Influenza virus. W.G. Laver and G.M. Air (eds.) pp. 283-292. Elseviers/North-Holland, New York.

White D.O. (1974). Influenza viral proteins : identification and synthesis. Curr. Top. Microbiol. Immunol. 63: 1-48.

White J., Kartenbeck J., and Helenius A. (1982). Membrane fusion activity of influenza virus. EMBO Journal 1: 217-222.

White J., Matlin K. and Helenius A. (1981). Cell fusion by semliki forest, influenza and vesicular stomatitis viruses. J. Cell Biol. 89: 674-679.

Wiley D.C., Skehel J.J., and Waterfield M.D. (1977). Evidence from studies with cross-linking reagent that the hemagglutinin of influenza virus is a trimer. Virology 79: 446-448.

Wilson I.A., Skehel J.J., and Wiley D.C. (1981). The hemagglutinin membrane glycoprotein of influenza virus: Structure at 3A resolution. Nature 289: 366-373.

Winter G., and Fields S. (1980). Cloning of influenza DNA into M13: The sequence of the RNA segment encoding the A/PR/8/34 matrix protein. Nucleic Acid Res. 8: 1965-1974.

Winter G., and Fields S. (1981). The structure of the gene encoding the nucleoprotein of human influenza virus A/PR/8/34. Virology. 114: 423-428.

Wrigley N.C., Skehel J.J., Charlwood P.A., and Brand C.M. (1973). The size and shape of influenza virus neuraminidase. Virology 51: 525-529.

Yewdell J.W., Frank E., and Gerhard W. (1981). Expression of influenza A virus internal antigens on the surface of infected P815 cells. J. Immunol. 126: 1814-1819.

Young J.F., Desselberger U., and Palese P. (1979). Evolution of human influenza A viruses in nature: sequential mutations in the genomes of new H1N1 isolates. Cell 18:73-83

Young J.F. and Palese P. (1979). Evolution of human influenza A viruses in nature: Recombination contributes to genetic variation of H1N1. Proc. Natl. Acad. Sci. U.S.A. 76: 6547-6551.

APPENDIX A

0.01M Phosphate - buffered Saline (PBS) pH7.2 - (CDC Manual, 1975)

8.5 g NaCl
Na_2HPO_4 (anhydrous) (Fisher Scientific Co., S-374)
$NaH_2PO_4.H_2O$ (Fisher Scientific Co., S-369)
double distilled water

stock PB solution: 5.48 g Na_2HPO_4 + 1.575 g $NaH_2PO_4.H_2O$: dissolve in 170 ml double distilled water and make up to 200 ml.

Working solution: 40 ml stock + 8.5 g NaCl: dissolve in 800 ml double distilled water and adjust to 1 litre. This is 0.01 M PBS.

Adjust pH to 7.2 with 1N NaOH or 1N HCl

Penicillin - Streptomycin solution.

6.150 g Penicillin G(M.W. 356.4 at 1,625 units/mg)

+

12.5 g Dihydrostreptomycin sulfate (M.W. 146.24 at 800 ug/mg)

Add double distilled water to 100 ml.

Therefore the solution contains 100,000 units of Penicillin per ml & 100,000 ug Streptomycin per ml of double distilled water.

Sterilize by filtration using 0.22 um porosity filter. Store at -20°C.

0.05M STE (Sodium, Tris, EDTA)- (Pons and Hirst, 1968)

0.1 M NaCl, 0.001 M EDTA, 0.05 M Tris-HCl, pH 7.2

NaCl	5.84 g
Trizma-HCl (Sigma)	13.22 g
Trizma Base (Sigma)	1.94 g
EDTA	0.3362 g

Add double distilled water (DDW) to 1 litre

Phosphate-Buffer (PB) (CDC Manual, 1975)

Stock solution: A. 0.4 M Na_2HPO_4 ..... 56.78 g/L H_2O
B. 0.4 M $NaH_2PO_4.H_2O$ ..... 55.20 g/L H_2O

To make 0.4 M PB, pH 6.0

A. 180 ml 0.4 M Na_2HPO_4
B. 820 ml 0.4 M $NaH_2PO_4.H_2O$

Add double distilled water to 1 litre.
Adjust with either A or B to obtain pH 6.0 exactly

0.85% - Saline

8.5 g NaCl (Fisher Scientific Co., S-671)

Add double distilled water to 1 litre

Thiobarbituric Acid Reagent (CDC, 1975)

Thiobarbituric acid (Baker analyzer reagent, 5-V774)
Sodium sulfate (anhydrous) (Fisher Scientific Co., S-421) DDW.

Dissolve 3.0 g thiobarbituric acid in 500 ml DDW.
Add 35.5 g anhydrous sodium sulfate - dissolve by heating until completely dissolved.
Cool and store at room temperature (RT) in a bottle.
Reagent was used within 24-48 hours.

Butanol Reagent (CDC, 1975)

1- Butanol (n-butyl alcohol) (Baker Analyzer Reagent - No.3-9054)
Concentrated hydrochloric acid (HCl) (Baker Analyzer Reagent - No.2 - 9535)

Add 50 ml concentrated HCl to one litre butanol. Mix.
Reagent can be stored at RT in a brown bottle.
Reagent was used within 48 hours.

2.5% Sodium Citrate (CDC, 1975)

Add 2.66 g sodium citrate ($Na_3C_6H_5O_7.2H_2O$) to 100 ml DDW.
Sterilize by filtration through 0.22 u Millipore membrane.
Store at 4° C.

Liquid Paraffin (oil) (Fisher Scientific Company).
Saybolt viscosity 125/135 (No. 0-119).

Reconstituted Fetuin

Reconstitute lyophylized fetuin (Gibco. No. R68751 - Grand Island Biologicals) in sterile double distilled water (DDW) to give a final concentration of 48-50 mg protein per ml. Store at 4°C and use within 48 hours.

Periodate Reagent

Sodium metaperiodate ($NaIO_4$) (Sigma Chemical Co. -S-1878)
Syrupy orthophosphoric acid (Fisher Scientific Co., A-242).
Double distilled water (DDW).

Dissolve 4.28 gm $NaIO_4$ in 38 ml DDW.
Add 62 ml Syrupy orthophosphoric acid and mix thoroughly.
Reagent can be stored at room temperature (20-25°C) (RT) in a brown bottle, indefinitely.

Arsenite Reagent

Sodium arsenite ($NaAsO_2$) (Fisher Scientific Co., S-225).
Sodium sulfate (anhydrous) (Fisher Scientific Co., S-421)
Concentrated sulfuric acid (Fisher Scientific Co.)

Dissolve 10 g $NaAsO_2$ in 100 ml DDW
Add 7.1 g anhydrous sodium sulfate to dissolve.
Add 0.3 ml concentrated sulfuric acid
Reagent can be stored indefinitely at RT in a brown bottle.

Appendix B

Back Titration of Hemagglutinin Antigen for HI Test (CDC, 1975)

Add 0.05 ml of PBS pH 7.2, to five wells in the same row in Linbro disposable tray. Add 0.05 ml of test antigen to the first well, and make serial two-fold dilutions through wells 2 to 5, resulting in 4, 2, 1, 0.05, 0 HAU/0.025 ml in wells 1, 2, 3, 4 and 5 respectively. Add 0.05 ml of 0.5% fowl RBC to each well. The RBC controls included contain 0.05 ml PBS and 0.05 ml of 0.5% fowl RBC. Allowed plate to stand at room temperature for 30 minute until cells in the RBC control settle at the bottom of the well. Complete hemagglutination appears in the first 3 wells, partial or no hemagglutination in well 4, and no agglutination in well 5. This indicates that the test antigen contains 4 HAU/0.025 ml of virus dilution.

Treatment (of sera for the removal of non-specific inhibitiors) with Receptor-Destroying Enzyme (RDE) Vibrio cholerae (CDC, 1975):

The titre of RDE (Microbiological Associates Company) was predetermined by R.E. Fyson (1973) and the RDE working dilution received contained 100 units per ml. The method outlined by CDC manual (1975) was followed. To each 0.1 ml of serum, 0.4 ml of the RDE working dilution was added, mixed thoroughly, and incubated overnight at 37°C in a waterbath. A volume of 0.3 ml of 2.5% sodium citrate solution was added, mixed, and incubated in 56°C waterbath for 30 minutes, then 0.2 ml PBS (pH 7.2) was added. This treatment would result in a 1:10 dilution of the sera.

Detection of Virus in Allantoic Fluid

Spot hemagglutination test

A spot hemagglutination test was used to determine the presence of the virus in allantoic fluid of chick embryo (CE-ALF). In plastic trays, one volume of V-ALF was mixed with 4 volumes of PBS and 5 volumes of 0.5% rooster RBC (FRBC) in PBS (pH 7.2). Controls included normal ALF, instead of V-ALF, mixed with PBS and FRBC as specified above. Trays were mixed on ice and HA was read after 30 minutes at 50% end point.

Cross-reaction neuraminidase-inhibition (NI) antibody titres of reference ferret specific antisera to H7N1 and H7N2.

	NI titres of ferret antiserum to	
Virus	H7N1	H7N2
H7N1	40	< 10
H7N2	< 10	160