THE USE OF MONOCLONAL ANTIBODIES TO DEMONSTRATE ANTIGENIC VARIATION BETWEEN INFLUENZA A (H_1N_1) ISOLATES FROM JOHANNESBURG

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ABSTRACT

Since the reappearance of the influenza A (H_1N_1) subtype in 1977, the virus has shown progressive antigenic variation in isolates from other parts of the world. The virus showed more rapid evolution in the latter part of the 1980 decade. To determine if this variation of the virus was present in local influenza outbreaks, sequential isolates from the 1989 influenza season were studied. The 1989 season saw increased activity of both the H_1N_1 and H_3N_2 subtypes. The majority of the H_1N_1 isolates were from subjects born after 1957.

A panel of monoclonal antibodies showed that co-circulation of antigenic variants occurred during 1989 with the emergence of a dominant strain later in the season.

DECLARATION

I declare that this dissertation is my own, unaided work and has not been submitted for any degree or examination at any other university.

Desmond James Martin

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1. INTRODUCTION

The potential of the influenza virus as a major and unpredictable threat to public health is highlighted by historic events. During the weeks from 19 October 1918 to the end of February 1919, an influenza A virus which was antigenically related to a swine influenza A virus killed at least 20 million persons and caused disease in humans one hundred times that number (Webster *et al*, 1982).

Antigenic variation has been a hallmark of the influenza virus which led to the paradox of an "unvarying disease caused by a varying virus". The study of this antigenic variation has led to the application of a number of molecular techniques to further our understanding of the "molecular epidemiology" of influenza viruses. Among these techniques monoclonal antibodies have been used to define important antigenic epitopes of the virus and are still used to detect variation in virus isolates. Monoclonal antibodies have been used, both to analyze naturally occurring isolates and to generate mutants in the laboratory by growing influenza virus in the presence of monoclonal antibodies.

1.1 THE VIRUS

1.1.1 Nomenclature and classification of influenza viruses

Influenza viruses are classified into 3 types - A, B and C - on the basis of their type-specific nucleoprotein and matrix protein antigens. Type A influenza viruses are further classified into subtypes based on the antigenic characteristics of their surface antigens, haemagglutinin (HA) and neuraminidase (NA). Twelve distinct HA subtypes and nine NA subtypes are now recognised in the nomenclature

systems recommended by the World Health Organization (Bulletin World Health Organization, 1980) and a thirteenth HA subtype has subsequently been identified (Hinshaw *et al*, 1982). During the present century three distinct subtypes of influenza A virus have caused pandemics in man - the H_1N_1 subtype which appeared in 1918, the H_2N_2 ('Asian') subtype which appeared in 1957 and the H_3N_2 ('Hong Kong') subtype which appeared in 1968. In addition the reappearance of the H_1N_1 subtype occurred in 1977 and has co-circulated with H_3N_2 subtype until the present time.

1.1.2 Morphology and composition of influenza A virus

Influenza A viruses are pleomorphic-enveloped viruses approximately 80-100nm in diameter. The host cell derived lipid envelope contains two morphologically distinct glycoprotein peplomers, the HA and the NA. The NA is mushroom shaped with a narrow stalk (Wrigley *et al*, 1973), while the HA is triangular in cross section and extends approximately 12 nm from the envelope. Both peplomers penetrate the lipid bilayer and are thought to interact with the matrix (M) protein. The M protein provides structural integrity to the virion and is involved in the assembly of the virus at the plasma membrane before release from the cell by a process of budding. Within the M protein shell are the nucleoprotein (NP) and polymerase proteins (PB1, PB2 and PA) which are closely associated with the RNA of the virus. The virus genome consists of eight distinct segments of single stranded RNA of different coding potentials (Inglis *et al*, 1976). The virus RNA is of noncoding sense and non-infectious, i.e. is "negative stranded".

Messenger RNA is transcribed from the virus RNA by the virion-associated transcriptase enzyme.

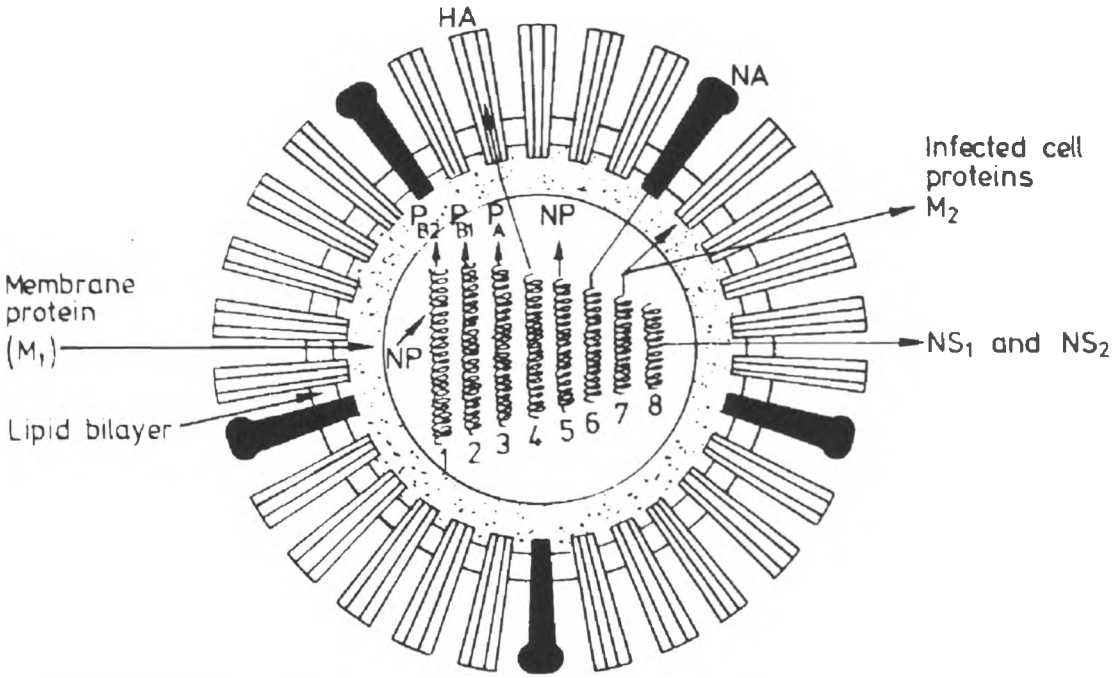

Figure 1 Model of Influenza virus showing protein and RNA composition (after Lamb RA, 1983)

1.1.3 The Haemagglutinin Molecule

The HA of influenza virus is a highly variable antigen, undergoing antigenic changes which enable the virus to escape neutralisation by antibody. It also plays an essential role in the biological properties of the virus, including attachment to cells and initiation of infection (Lazarowitz and Choppin, 1975) and virulence (Bosch *et al*, 1979). The HA contains important antigenic determinants which stimulate the production of neutralising antibody, following either infection or immunization. It is the variation in the HA glycoprotein which is mainly responsible for the continually occurring outbreaks of influenza. The HA is a

trimer of 224,640 daltons molecular weight (Wiley *et al*, 1977) and comprises 25% of the protein of the virion. Each HA monomer is coded by the fourth largest RNA segment and is synthesized as a single polypeptide chain which undergoes post-translational cleavage to give two polypeptide chains called HA_1 and HA_2, with polypeptide molecular weights of 36,000 and 27,000 respectively. Each monomer is linked by disulphide bonds (Skehel, 1972). Cleavage of the HA polypeptide into HA_1 and HA_2 is necessary for the virus particles to be infectious. The three-dimensional structure of the HA from an H_3N_2 subtype has been further studied, using X-ray crystallography (Wilson *et al*, 1981). Together with data on the amino acid sequence of HA (Porter *et al*, 1979; Gething *et al*, 1980) these investigations have enabled precise location of the cell binding site, antigenic determinants and fusion sequence within the three dimensional structure of the HA. As regards binding sites for antibody, at least four (labelled A, B, C, D, see figure 2) have been identified by analyzing

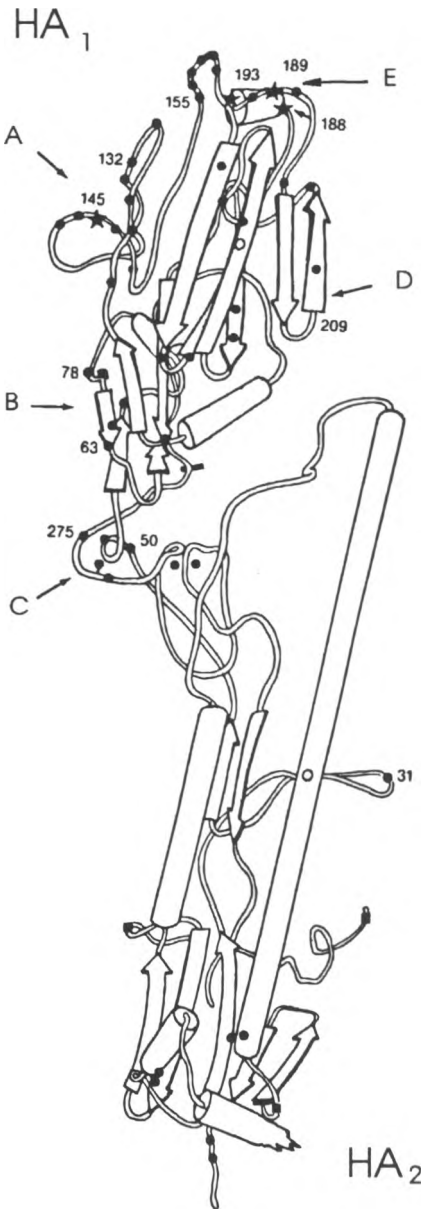

Figure 2 Structure of the haemagglutinin monomer (H_3) showing folding of the HA_1 and HA_2 polypeptide (after Wiley *et al*, 1981)

antigenic mutants selected in the laboratory under immunological pressure from monoclonal antibodies (Yewdell *et al*, 1979) and by comparison of the HA sequences of naturally occurring variants (Wiley *et al*, 1981). Site A has an unusual protruding loop which projects from the molecular surface and forms the centre of the most obvious antibody binding site. Antigenic site B occurs along the upper edge of a pocket tentatively implicated in virus receptor binding. Site C is formed by the outer cleft between adjacent HA monomers and lies on the side and approximately halfway down the globular head. In these antigenic sites, the amino acid substitutions that cause antigenic variation are located externally, but antigenic site D departs from this. Several amino acid substitutions in the HAs of both natural and laboratory-selected antigenic mutants occur in the interface region between subunits in the HA trimer. These amino acids may be recognized as a result of relative movement of the globular regions of HA_1 to expose the interface regions. However, it is possible that the actual antibody binding site is remote from amino acids but affected by the exact fit at the interface, and might be disturbed by amino acid substitutions. The earlier studies were carried out on the H_3 HA subtype (Wiley *et al*, 1981) but a later study (Caton *et al*, 1982) showed that the antigenic sites of the H_1 subtype occurred in identical regions of the HA. Furthermore, in this study it was considered that site A was of the greatest importance, as judged by the epidemiological significance of viruses expressing altered antigenicity at this site. In addition a predominance of monoclonal antibodies generated from mice after immunization with virus were directed against site A (Webster and Laver, 1980). A further antigenic site, E was first suggested by Caton *et al* (1982). This additional immunodominant site

is present on both the H_3 and H_1 subtype. Using monoclonal antibodies in competitive binding assays, it was shown that sites C and E are topographically distinct from B and D, but these two separate regions (C and E) are linked together by site A (Brown *et al*, 1990). It has been suggested that 4 distinct topographical antigenic regions A, B, C and D exist and that site E is formed when two regions that are widely separated in the HA monomer associate in the assembled trimer. Sequences of the HA gene of several field isolates of influenza B have been determined (Krystal *et al* 1982, 1984). It appears that the variable areas in the influenza B HA structure correspond to antigenic regions, A, B and D, of influenza A viruses. No changes were found in the B HAs in areas corresponding to antigenic region C of influenza viruses. The three-dimensional structure of the influenza B HA is not known but comparison of sequence data and computer-assisted model building suggests a structural similarity between influenza A and B HAs (Krystal *et al*, 1982). However the sequence data of laboratory-generated variants of influenza B suggest that there is a considerable overlap between the antigenic regions of the influenza B HA molecule. Distinct antigenic sites as are found in the HA of influenza A may not occur in the HA of influenza B viruses. The HA_1 in B viruses may be folded into a more compact structure, such that a relatively larger proportion of the sequence is recognized by a single monoclonal antibody.

1.2 ANTIGENIC VARIATION IN INFLUENZA

Two distinct kinds of antigenic variation have been demonstrated in influenza viruses, antigenic drift and major antigenic shifts. The first consists of relatively

minor changes in the surface glycoproteins that occur gradually within a family of strains, all of which are clearly related to each other with respect to both internal and surface antigens. Antigenic drift is now considered to occur by an accumulation of a series of point mutations in the gene coding for HA (or NA), resulting in an accumulation of amino acid sequence changes that alter the antigenic sites of the molecule. Progressive antigenic drift has been observed consistently for all subtypes of influenza A virus. The majority of the amino acid changes leading to antigenic drift occur in the HA molecule and fewer differences are noted in other antigenic domains (Laver, 1964). There is evidence that changes in a single epitope may be enough to allow the virus to escape neutralisation by antibody in human sera (Natali *et al*, 1981). Among influenza A strains infecting man, each successive variant replaces the existing ones. This may be due to a selective advantage possessed by antigenic variants in overcoming immunological host resistance.

It is apparent that field isolates with minor antigenic changes do co-circulate and cause disease and have therefore epidemiological importance (Wiley *et al*, 1981). Many different co-circulating variants can be detected during an epidemic year with monoclonal antibody to the HA (Webster *et al*, 1979). The second kind of antigen variation which has only been described for influenza A is a major antigenic shift. When this occurs a "new" virus suddenly appears in the human population with HA and sometimes NA molecules of a different subtype from those of the virus circulating before the new virus appeared. It is these "new" viruses which caused the major classical pandemics of influenza. All major

antigenic shifts in influenza A virus have had their origin in China and it is thought that "new" antigens arose by a reassortment of genes between viruses of animal and avian origins. The Hong Kong strain of human influenza has an HA that is closely related to that of duck/ukraine and equi-2 virus (Laver and Webster, 1973).

1.2.1 Antigenic Drift

Because of antigenic drift it has proved difficult to control influenza by vaccination and variations in the virus have led to altered virulence, pathogenicity, host range and transmissibility of the virus. These factors have combined to lead to the spread and severity of influenza epidemics. It is commonly thought that antigenic variation confers a selective advantage to the virus, allowing it to escape the population immunity which has been derived from previous infection or influenza immunization (Wiley *et al*, 1981; Kendal, 1987).

Amino acid changes in the HA molecule can be classified as buried or superficial (Wilson *et al*, 1981). Furthermore, the amino acid changes can be characterized as "main stream", which become "fixed" in subsequent strains, and "side stream" mutations, which are usually specific to that strain (Both *et al*, 1983). Main stream changes become fixed through a process of natural selection and can lead to the emergence of a dominant strain which has a tendency to replace co-circulating strains in a community. It is thought that superficial residues which are more exposed to antibodies in the host are therefore more hypervariable and likely to undergo antigenic drift, often in a sequential fashion. Buried residues

which are less likely to be subject to immunological pressure tend to undergo less antigenic drift. However, a study by Raymond *et al* (1986) on the HA derived from 19 field strains of the H_1 subtype showed that 11 of the 54 amino acid changes (using USSR/77 as the standard sequence) were in buried residues of the HA trimer and in positions unavailable to direct antibody selection pressure, suggesting that not all mutations are related to antigenicity. Mutations in these hidden residues may be related to the inherent mutability of certain sequences of the HA molecule. However, Kilbourne *et al*, 1990 showed that HA and NA proteins have demonstrated varying rates of antigenic evolutions. With H_1N_1 viruses, an arrest of significant evolution of the NA discordant with the continuing antigenic drift of the HA was found in isolates from the 1980 - 1983 period. They felt that it is probable that the different and independent rates of evolution of HA and NA reflect the greater selective pressure of HA antibodies, which forces the more rapid emergence of HA escape mutants.

The concept of immunological pressure in the host or community being the major pathway responsible for antigenic drift in influenza virus has been challenged by Hope-Simpson and Golubev (1987). They argued that if the infected person was transmitting the virus to susceptibles, these susceptibles would not possess immunity to the infecting strain, as second infections are rare. Thus if these susceptibles became infected, they would not be in a position to exert immunological pressure. They further suggested that latent residues from a previous infection persist in the host and are later seasonally reactivated and so must inevitably encounter the immunity that the virus has itself engendered in the

person to whom it had caused influenza. During this persistent infection, mutants are generated. Further work by Patterson and Oxford (1986) has supported this concept by showing that host cells themselves do exert considerable selection pressure on the virus. In this process, variants are favoured in comparison to the parent strain.

Studies by Natali *et al* (1981) and Wang *et al* (1986) show that most humans, especially children, mount a limited antibody response to influenza virus, which, in the case of the HA, is restricted to one or two antigenic sites. Moreover, the spectrum of antibodies produced differs between individuals. These authors, together with Wiley *et al* (1981), feel these findings provide an explanation for how new epidemiologically-important field strains, which are thought to require at least one mutation in each of the major antigenic sites, can arise. In view of the fact that the calculated probability of a mutant arising with a substitution in every antigenic site is too low to allow the introduction of a viable new strain within a single immune individual (Gerhard *et al*, 1981; Webster and Laver, 1980), it is likely these viruses evolve in a step-wise manner by sequential infection of individuals possessing different and limited antibody repertoires.

A further mechanism that can contribute to antigenic variation in H_1N_1 virus is the reassortment with co-circulating H_3N_2 strains in human populations. H_1N_1 strains circulating prior to 1957 were not subject to reassortment events whereas some isolates in the 1978-1979 season arose by reassortment with co-circulating H_3N_2

strains (Young, Desselberger and Palese, 1979; Young and Palese, 1979). The genes for HA, NA, M and NS came from the H_1N_1 parent, so maintaining the H_1N_1 serotype.

Two distinct laboratory approaches have been utilised in the analysis of antigenic drift occurring in influenza viruses. Antigenic variants of the HA (and NA) have been selected by growing virus in the presence of monoclonal antibody to the HA (or NA). The HA variants were present at a frequency of 10^{-5} in stock virus (Yewdell *et al*, 1979; Webster *et al*, 1980) and did not bind at all to the antibody used for their selection. The change in antigenicity was found in most cases to be associated with single changes in the amino acid sequence of HA_1. Such variants have been used to construct an operational antigenic map of the HA molecule by comparative antigenic analysis of the mutant virus with the monoclonal antibody.

The second approach has been that of studying naturally occurring influenza isolates. Most of the sequence changes, leading to altered antigenicity, in the Hong Kong HA between 1968 and 1979, were in the HA_1 portion of the HA molecule, only three changes being found in HA_2 (Both and Sleigh, 1981). Although the changes are distributed over the HA_1 they are clustered in the four regions mentioned previously (Wiley *et al*, 1981).

Data derived from studies on influenza B viruses have shown that amino acid substitutions tend to come and go over time, in contrast to the steady

accumulation of amino acid substitutions that occur in HA_1 of influenza A virus of the H_3 subtype (Both *et al*, 1983; Skehel *et al*, 1983). Furthermore antigenic analysis (Lee *et al*, 1983) indicates that antigenic drift does not occur sequentially over time as in influenza A viruses. A noticeable feature of the sequences of the HA genes of monoclonal variants of B/Hong Kong/8/73 is the relatively high incidence of multiple nucleotide changes; four of the eight monoclonal variants sequenced had more than one nucleotide change.

1.2.2 Host Cell Selection of Influenza Virus Antigenic Variants

Human influenza viruses are routinely adapted to growth in fertile hens' eggs. Early studies by Burnet and Clarke (1942) established that even limited passage of influenza virus in the allantoic cavity of eggs, rather than in the amniotic cavity of the same egg, could lead to selection of biologically diverse viruses termed 'O' original (original phase in the amniotic cavity) or D (derived phase in the allantoic cavity).

Using monoclonal antibodies Schild *et al* (1983) demonstrated that influenza B virus adapted to growth in eggs is antigenically distinguishable from virus grown in mammalian cells. It was clear that the host cells were selecting antigenically variant HAs, a phenomenon referred to as 'laboratory drift'. Robertson *et al* (1985) reported that in four of five influenza B viruses adapted to egg growth a potential glycosylation site present in the Madin-Darby canine kidney (MDCK)-

cell-grown virus was lost in egg-grown virus. The associated amino acid changes were noted particularly in the tip region of the HA, in and around the receptor binding site and the important epitope B.

Oxford (1991), in antigenic studies of influenza virus isolated in defined community epidemics, showed that the HA of the cell-grown virus was more antigenically related to the natural epidemic virus since post-infection human antisera reacted preferentially with the HA of this virus. This was suggested previously by Pyhälä (1987) who in an epidemiological study showed that antibody to MDCK-grown virus was a more accurate indicator of immune status of a community than antibody to egg grown virus variants.

1.2.3 Receptor-Binding Variants

Human influenza A isolates of the H_3 subtype preferentially bind sialic acid (SA) α 2,6 Gal linkages; animal isolates preferentially bind SA α 2,3 Gal linkages on cell surface sialoligosaccharides. Daniels *et al* (1984) used monoclonal antibodies to compare the antigenicities of the HAs of two receptor binding mutants of X-31 influenza virus (containing the H_3 HA of A/Aichi/2/68). Differences in antibody-binding properties was related to the proximity of the antibody-binding sites and the sialic acid binding site in the three-dimensional structure of the H_3 HA (Wilson *et al*, 1981; Wiley *et al*, 1981).

1.2.4 Variations in the Antigenic Sites of the HA at low pH

In addition to its function in binding to cell receptors, the HA mediates fusion between viral membrane and cell membrane. *In vitro*, membrane fusion and haemolysis are maximal at a pH of about 5.0, which is close to that of endosomes, and fusion does not normally occur at the cell surface. The fusion involves those amino acids which become the N terminus of HA_2. Cleavage of the HA into HA_1 and HA_2 is an absolute requirement for fusion. The HA undergoes a low pH-induced conformational change which exposes the N-terminal region of HA_2, with an opening of the trimer interface. This results in protein domains' moving relative to each other. Not surprisingly this dramatic change in conformation is accompanied by antigenic changes (Webster *et al*, 1983). Antigenic sites B and D (figure 2) are altered by exposure to pH to the extent that antibodies which recognize these sites have much reduced or no activity in a haemagglutination inhibition test.

1.2.5 Effect of Carbohydrate on Antigenic Sites

It has been suggested (Wiley *et al*, 1981) that carbohydrate addition might mask antigenic sites. Direct evidence was obtained by Skehel *et al*, (1984). A single amino acid substitution was found in a variant of X-31 (Aichi/68/H_3) HA selected with a monoclonal antibody. This mutation generated an oligosaccharide binding site which subsequently prevented attachment of the monoclonal antibody. Further studies on the 1969 epidemic virus A/England/878/69 provided similar evidence and demonstrated that epidemiological significance is attached to carbohydrate-mediated modulation of HA antigenicity.

1.2.6 Antigenic Variation in Neuraminidase

The neuraminidase (NA) accounts for about 5-10% of the virus protein and exists as a mushroom-shaped spike on the surface of the virion. The NA molecule is composed of a single polypeptide chain coded by RNA segment 6 and is oriented in the virus membrane in the opposite way to HA. Chemically, the enzyme catalyses cleavage of the α- ketosidic linkage between terminal sialic acid and an adjacent sugar residue. This enzymatic reaction probably permits transport of virus through mucin on cells, during both viral infection and virus release and destroys the HA receptor on the host cell, thus facilitating release of virus particles from infected cells. The removal of sialic acid from the carbohydrate moiety of newly synthesized HA and NA may prevent self-aggregation of the virus. The removal of the sialic acid may expose the HA to proteolytic cleavage and hence alter viral virulence (Schulman and Palese, 1977). Antibodies to NA do not neutralize or prevent virus infection but act in preventing virus release and hence spread from infected cells (Rott *et al*, 1974), thus modifying the pathology in the host.

In general, the role of NA may be to facilitate mobility of the virus both to and from the site of infection. A number of monoclonal antibodies to NA of the N_2 subtype have been obtained and competitive radio-immunoassays with these antibodies showed that the NA possessed at least four overlapping antigenic regions. Antigenic variants of the NA have been selected using monoclonal antibodies at a frequency of 1 in 10^5, similar to the frequency of variants in the HA molecule (Yewdell *et al*, 1979).

Figure 3 shows the location in the three-dimensional structure (Varghese *et al*, 1983; Colman *et al*, 1983) of the changes in NA of variants of N_2 and N_9 subtypes selected with monoclonal antibodies. Nearly all the changes are clustered on the upper surface of the molecule, surrounding the active site pocket. Amino acid changes at these sites have been noted in 'drifted' field strains and in laboratory-derived mutants and thus contribute to antigenic variation of the virus (Colman *et al*, 1983).

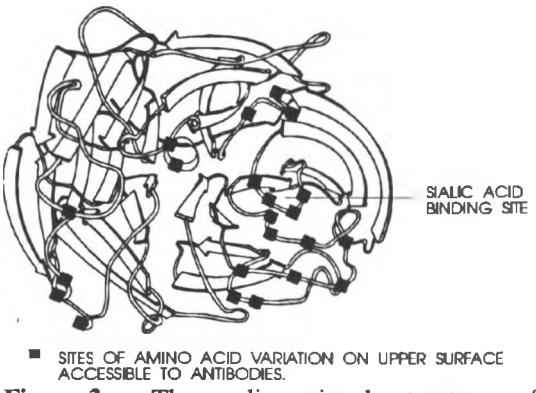

Figure 3 Three dimensional structure of neuraminidase (after Varghese *et al*, 1983)

1.2.7 Antigenic Variation in other Influenza Proteins

The internal and non-structural proteins of influenza virus are less accessible to antibodies and hence would be expected to show less variation than the HA and NA antigens.

a) Nucleoprotein

The nucleoprotein (NP) is the classical type specific antigen, providing the basis for typing of virus into influenza A, B and C. Genetic analysis of a large number of influenza strains has revealed that the NP genes can be placed in five different groupings (Bean, 1984). All the avian strains fall into two groups, the equine virus strains form two other groups, and one group contains all the human and bovine influenza viruses. This

restriction of groups of genetically-related NP genes to viruses infecting certain species suggests that this protein may play a role in determining species-specificity. The genes coding for the NP of influenza viruses of two subtypes, namely A/PR/8/34 (H_1N_1) and A/NT/60/68 (H_3N_2) have been completely sequenced. The data would suggest a high degree of conservation of the gene over the 34-year period which separated these viruses despite the antigenic 'shifts' which occurred in 1957 and 1968. Serological analyses of NP of influenza viruses using polyclonal (Schild *et al*, 1979) and monoclonal antibodies show some changes in antigenic determinants of the molecule. Studies with monoclonal antibodies to the NP of A/WSN/33 (H_1N_1) virus have shown that antigenic variation occurs in this molecule. The NP possesses at least three non-overlapping antigenic areas, one area being the same on all strains tested (van Wyke *et al*, 1980).

b) Matrix Protein

The matrix (M) protein is a type-specific antigen of influenza virus and is coded for by gene segment 7. Antigenic analysis of M_1 protein, using various techniques, has established that M_1 protein is type-specific and antigenically closely similar for all influenza A viruses tested (Schild, 1972). The accumulation of point mutations appears to occur at an approximately constant rate of 1-2 x 10^{-3} per year per nucleotide. This value is two to seven times smaller than the rate observed in the same period for gene segment 4 which codes for HA. Indeed, the sequence of

gene segment 7 appears to be more highly conserved than other segments of influenza A viruses. This may reflect a strong factor in the conservation of the gene product M_1, relating to its probable involvement in virus morphogenesis (Ortin *et al*, 1983). However studies with a panel of monoclonal antibodies to M protein of A/WSN/33 (H_1N_1) indicate that there are three antigenic sites on this molecule and that antigenic differences can be detected both within and between subtypes (van Wyke *et al*, 1984).

c) Non-Structural Proteins

RNA segment 8 codes for at least two non-structural polypeptides, NS_1 and NS_2. The function of NS_1 or NS_2 has not been established. NS_1 is made in large amounts early in infection and accumulates in the nucleus (Lazarowitz *et al*, 1971). NS_2 is made late in infection and is found predominantly in the cytoplasm (Lamb and Lai, 1980). Studies with a panel of monoclonal antibodies of NS_1 of A/WSN/33 (H_1N_1) indicate that there are three overlapping antigenic sites on the molecule (Brown *et al*, 1983). Because NS_1 and NS_2 are internal proteins of infected cells and hence less available to antibodies, they would be expected to show less sequence variation than the surface glycoproteins. This has been shown in sequencing studies (Lamb and Lai, 1980).

d) Polymerase Proteins

The three largest proteins of the virion - PB_1, PB_2 and PA - are found in association with the NP and virion RNA. To date no information is available about antigenic variation in these proteins.

1.3 THE PLACE OF MONOCLONAL ANTIBODIES IN THE STUDY OF INFLUENZA VIRUSES

1.3.1 General considerations

The basic property of a monoclonal antibody is that of combining specifically with a single antigenic determinant (epitope). Monoclonal antibodies are capable of detecting differences of one amino acid, formerly only demonstrated by recourse to peptide fingerprinting or oligonucleotide mapping of the viral genome (Carter and Ter Meulen, 1984). Production of such antibodies is now possible using the hybridoma technology introduced by Köhler and Milstein (1975), whereby immunocompetent lymphocytes are rendered "immortal" by fusion with myeloma cells. The latter are mutant plasmacytoma cell lines which have been selected to lack the enzyme hypoxanthine guanosine phosphoribosyl transferase. Such mutants cannot grow in medium containing aminopterin and supplemented with hypoxanthine and thymidine (HAT medium) because they are unable to utilize the salvage pathway (Littlefield, 1964). Hybrids between the myeloma cells and spleen cells can be selected from the parental components as they are the only cells that actively multiply in HAT selective medium. The antibodies produced by the resulting cell hybrid (hybridoma) clones are by definition monoclonal

antibodies and are, in effect, homogenous immunological reagents of defined specificity, avidity, high specific activity and selected isotype (Nowinski *et al*, 1983).

1.3.2 Current Status of Monoclonal Antibodies in Influenza Research

Many techniques are currently in use to detect antigenic variation in influenza viruses; specifically, oligonucleotide mapping of the RNAs (Desselberger *et al*, 1978), sequencing of RNAs and cloned genes (Air, 1981), peptide mapping, protein and RNA gels (Laver and Webster, 1973; Hinshaw *et al*, 1980).

Although direct nucleotide sequencing remains the most sensitive research tool, its use is not without certain drawbacks. Sequencing is a tedious and material-consuming procedure. A further drawback is that sequence analysis of amino acids (sequential determinants) do not always correlate with the three-dimensional arrangements of amino acids (configurational determinants). Such antigenic sites are specified by the secondary and tertiary folding of the molecule. Hence changes in nucleotide sequence do not always translate to change in antigenicity of the molecule. Consequently nucleotide sequence-changes may represent neutral or irrelevant mutations in the molecule (Daniels *et al*, 1985). In addition, the attachment of carbohydrate at a mutant antigenic site may not lead to an altered antigenicity but sequence analysis will be altered (Caton *et al*, 1982).

Monoclonal antibodies are more suited to study the configurational determinants, particularly with reference to the HA molecule of the influenza virus.

Monoclonal antibodies are often used in combination with sequencing studies or other techniques (Caton *et al*, 1982; Nakajima *et al*, 1983).

1.3.3 Newer Initiatives utilising Monoclonal Antibodies

a) Use of IgA Monoclonal Antibodies

Most studies, using monoclonal antibodies in the study of influenza, have utilized antibodies of the IgG class. Secretory IgA is thought to be of particular importance in immunity to influenza. IgA - secreting hybridomas were produced following intranasal infection of mice with influenza A virus X-31 (Maoliang, 1986). In this study it was demonstrated that the specificities of IgA monoclonal antibodies are similar to those of IgG monoclonal antibodies.

b) Immunogold Labelled Monoclonal Antibodies

An electron microscopic immunogold labelling technique employing monoclonal antibodies has been applied to the antigenic analysis of influenza A and B viruses (Patterson and Oxford, 1986). Reassortant influenza A (H_3N_2) viruses containing HA molecules from viruses isolated between 1968 and 1982 were analyzed with a panel of monoclonal antibodies raised against viruses which appeared over the same period. The immunogold labelling technique clearly demonstrated the antigenic drift in the HA molecule that occurred between 1968 and 1982. When the technique was applied to the examination of viruses from a more geographically restricted influenza epidemic in a semi-closed community,

antigenic variants were found. Furthermore, the technique enabled the identification of distinct antigenic-variant subpopulations within a single clinical isolate. Analysis of the HA of MDCK cell or egg-grown virus by this procedure provided data to support the hypothesis that the host cell exerts selective pressure on subpopulations of virus resulting in the emergence of antigenic variants.

c) Crystals of Influenza Virus Neuraminidase Complexed with Monoclonal Antibodies

Influenza virus NA provides the ideal system to investigate the structure of epitopes on proteins. The three-dimensional structure of the NA is known (Varghese *et al*, 1983) and the ready availability of antigenic variants with known sequence changes, as well as a large number of monoclonal antibodies to different regions of the NA, will enable identification of the nature and extent of antigen-antibody binding surfaces. Experiments in progress aim to show how the three-dimensional structure of the epitopes changes during antigenic drift: in particular, how a single amino acid sequence change in the NA polypeptide renders it unrecognizable to antibodies which previously bound very well. This is being done by X-ray diffraction analysis of crystalline NA molecules of influenza virus variants selected with those MAbs to the NA which were used in the crystalline complex experiments (Tulloch *et al*, 1986).

d) Monoclonal Antibodies used in Indirect Immunofluorescence Assays

Monoclonal antibodies have been used to detect influenza A and B in clinical specimens by indirect immunofluorescence assays using Madin-Darby canine kidney monolayers in shell vials (Espy *et al*, 1986). This technique is a refinement of direct immunofluorescent staining of nasopharyngeal smears. The latter specimens, however, often contain debris which leads to non-specific staining. Shell vial centrifugation techniques have shown a remarkable sensitivity (83%) compared to conventional tube culture, if staining is carried out at 48 hours (Guenthner and Linnermann, 1988). A modification of the centrifugation technique utilizes a 24-well tissue culture plate with a monolayer of MDCK cells. An indirect immunofluorescence test using a pool of monoclonal antibodies is carried out after a 40 hour-incubation. The sensitivity of this assay compared to conventional tube culture is 82% (Mills *et al*, 1989).

1.4 INFLUENZA A (H_1N_1) VIRUSES

An influenza A virus caused outbreaks throughout the world in 1977 and 1978. The first outbreaks occurred in Tientsen, China (Kung *et al*, 1978). A unique feature of this event was that the H_1N_1 infection was virtually absent among adults and affected children and young persons. Although sporadic infections were reported among adults born before 1957, immunity resulting from previous H_1N_1 infections prevented infection and/or illness among adults. Unexpectedly, these viruses were shown to possess HA and NA antigens very closely related to those which circulated some 27 years previously in the 1950's (Kendal *et al*, 1978;

Zhdanov *et al*, 1978). The successful spread of these H_1N_1 viruses also challenged previous assumptions that only a single subtype of influenza virus could circulate at a particular time. Indeed, the years from 1977 until the present time have witnessed the concurrent spread of influenza A H_1N_1 and H_3N_2 viruses and reassortants possessing gene segments derived from both these viruses have been identified.

Analysis of the RNAs of several of the newly emerged H_1N_1 viruses by oligonucleotide mapping revealed that the RNAs of most of the eight gene segments of the 1977 strains showed close identity to those of earlier human strains of the 1950's (Nakajima *et al*, 1978; Kozlov *et al*, 1981). Monoclonal antibodies were used to demonstrate that the antigenic determinants of the 1977 strains were homologous to those of 1950 strains (Webster *et al*, 1979). Thus it was not surprising that previous exposure to the antigen provided protection against the 1977 H_1N_1 strains. These results implied that the viruses of previous epidemic periods could reappear after an absence of several years and that their re-emergence did not involve gene reassortment. At present, the precise origin of the re-emerged A/USSR/90/77 (H_1N_1) strains remains a mystery. Several hypotheses are possible, including latency or persistence of the original 1950 virus in animals and humans, or an accidental laboratory escape.

The severity of clinical infection with the H_1N_1 virus was noted to vary considerably during the 1978 epidemic in England and Wales (Hoskins, 1979). Some children were severely ill with chest pains, while others displayed only

minor symptoms. In addition, some schoolchildren had an asymptomatic seroconversion. There was a low mortality attributable to the appearance of the new subtype in Britain in 1978. This was probably due to the age limitation of the illness, with pre-existing immunity present in older subjects who were exposed to the virus prior to 1957 (Chakraverty *et al*, 1982). It is also thought that older adults readily acquire immunity to new antigenically-altered variants, probably as a result of immunologic priming that occurred in childhood (Glezen *et al*, 1991). An additional factor contributing to the low mortality is a possible variation in the intrinsic virulence of the virus (Oxford *et al*, 1980). Inability to replicate at temperatures of 38°C or more is a feature of virus strains which have been artificially attenuated in virulence. A higher proportion of these temperature-sensitive (ts) mutants was found among influenza A (H_1N_1) isolates recirculating from 1977 onwards in comparison with isolates of the H_3N_2 subtype. Furthermore, a comparison of isolates occurring after 1977, with pre-1957 isolates, found a higher proportion of ts viruses in the later strains. However, reduction in virulence in the re-emergent 1977 H_1N_1 viruses was not universal as fatal illnesses due to H_1N_1 were reported in New Zealand (Eason and Sage, 1980).

2. AIM OF THIS STUDY

Active surveillance of respiratory viruses and influenza in particular, provides valuable diagnostic information to the health care system. The National Institute for Virology (NIV) maintains an influenza surveillance system carried out by a network of "viral watch" medical practitioners. There are 16 "viral watch" monitoring centres and these are located in both adult and paediatric hospital outpatient departments, clinics and general practices in and around Johannesburg. The monitoring centres are representative of a cross-section of people of varying age, race and socio-economic background. The value of an active surveillance system was illustrated during the 1984 influenza epidemic on the Witwatersrand when important information was provided by the "viral watch" monitoring centres (Schoub *et al*, 1986).

In 1989 there was increased influenza activity in the community and this was reflected by an increase in influenza isolations in the diagnostic laboratory at the NIV. Typing of the isolates with reference antisera revealed that both H_1N_1 and H_3N_2 subtypes were circulating in the community. The majority of these isolates were of the H_1N_1 subtype (see figure 4). The H_1N_1 isolates were typed specifically as influenza A/Victoria/36/88 (H_1N_1). During the 1988-89 worldwide influenza season more than 600 isolates were characterized by the WHO collaborating centres for influenza: 46% were influenza B viruses, 30% were influenza A (H_3N_2) and 24% were influenza A (H_1N_1). The majority of these $H1N_1$ isolates occurred in the southern hemisphere and were reported from

Oceania and South America. Some H_1N_1 activity occurred in Asia. No H_1N_1 isolates were reported from Europe, Canada or the United States. The predominant A (H_1N_1) strains were A/Taiwan/1/86-like viruses. (MMWR December 1, 1989).

In view of the increased activity of the influenza A (H_1N_1) subtype during the 1989 season, it was decided to develop a panel of monoclonal antibodies against the reference strain A/Taiwan/1/86. Sequential isolates typed as H_1N_1 were selected from early season (April/May), mid season (June) and late season (August). The patterns of reactivity of these isolates with the monoclonal panel would be analyzed to determine if, and to what extent, antigenic variation within the strains was present.

3. MATERIALS AND METHODS

3.1 VIRUS STRAINS

3.1.1 For MAb

A/Taiwan/1/86 (HA titre 1:2560) was used as the immunizing antigen for the generation of monoclonal antibodies.

3.1.2 Sequential Isolates made at NIV during 1989 typed as Influenza A (H_1N_1)

	Lab Number	HA Titre*
Influenza A (H_1N_1)	461/89	1:320
	553/89	1:640
	568/89	1:640
	685/89	1:320
	688/89	1:640
	826/89	1:640
	828/89	1:160
	832/89	1:640
	857/89	1:1280
	867/89	1:640
	1018/89	1:640
	1044/89	1:320
	1053/89	1:640
	1028/89	1:640
	1072/89	1:640

* HA = haemagglutination titre of each isolate

Isolates used to Test for Cross Reactivity

Isolates made at NIV		HA Titre
Influenza A (H_3N_2)	1131/90	1:640
	1188/90	1:320
	1205/90	1:640

Titration of WHO Reference Strains *		
Influenza B	Victoria/2/89	1:640
	Panama/45/90	1:640
	Yamagata/16/88	1:640

* WHO reference strains kindly submitted by Dr J J Skehel, WHO Influenza Reference Centre, London

3.2 PURIFICATION OF VIRUS

The method used was an adaptation of the method of Johanssen *et al* (1989). Allantoic harvest of virus was clarified by centrifugation, using a Sorvall centrifuge with a SS34 rotor, at 12,000g for 15 minutes. Virus was pelleted from the supernatant by ultracentrifugation (Beckman ultracentrifuge) at 200,000g for 2½ hours at 4°C. The pellet was resuspended in 0,5 ml cold 50mM TN buffer pH 7,5 (50 mM Tris HCl + 0,1M NaCl) and left for 12 hours at 4°C. The virus was then purified twice by centrifugation through a continuous sucrose density gradient (20-60%) for 2½ hours at 150,000 at 4°C. Visible virus peak was removed with a 21-gauge needle and syringe and diluted with TN buffer and pelleted by further centrifugation at 150,000g for 2½ hours. Virus was quantified by reading absorbence at OD 280 using a U.V. Spectrometer (Bausch and Lomb, Spectronic 1001). Concentration of virus was calculated according to the Beer-Lambert law governing the relationship between light absorption and concentration of a given solution. This states that

$A = \Sigma c \ell$ where Σ = extinction coefficient which is constant for a given substance

ℓ = cuvette pathlength (constant; 1 cm)

c = unknown concentration

A = absorbance

For influenza Σ is known and at 280nm is $\Sigma^{0.1\%} = 3.0$ (Cautrecases, 1971) therefore concentration can be calculated by this formula.

3.2.1 Preparation of Immunising Antigens

2 ml of purified virus A/Taiwan/1/86 (857 μg/ml, HA titre 1:160,000) was emulsified in 2 ml of Freund's complete adjuvant. This was used for the initial immunization of Balb/c mice. Purified virus was aliquotted and stored in 50 mM TN buffer pH 7,5 at - 70°C and was subsequently used for booster immunizations.

3.3 PRODUCTION OF MONOCLONAL ANTIBODIES

3.3.1 Cells

a) Myeloma cells

The FO-clone of SP2/0 (Fazekas de St. Groth and Scheidegger, 1980) was obtained from American Tissue Culture Collection (ATCC). The cells were grown in standard medium consisting of RPM1 - 1640 with 15% foetal calf serum, .01% sodium pyruvate, 0,0375% sodium bicarbonate, 0,05% of 0,1M 2-mercaptoethanol, 0,01% amphotericin B and antibiotics. The cells were inspected daily and subculturing was performed as required. This was done to maintain the myeloma cultures in an exponential growth phase which would in turn optimize the amount of fusion competent cells. In the case of the FO myeloma line, which has a doubling rate of 8,7h (Fazekas de St. Groth and Scheidegger, 1980), this entailed subculturing daily.

b) Immune Spleen cells

Balb/c mice were immunized with 0,25 ml purified A/Taiwan/1/86 (H_1N_1) virus (857 μg/ml, HA titre 160,000) emulsified in Freund's complete adjuvant by means of intraperitoneal inoculation (i.p.) Further i.p. booster inoculations of 0,25 ml of purified virus suspension took place 5, 8 and 12 days after the initial inoculation. After a further 14 days, the mice were given 0,05 ml of purified virus suspension intravenously together with 0,2 ml i.p. for 2 successive days. Spleens were removed 3 days later for fusion.

c) Peritoneal Macrophages

Adult Balb/c mice were killed and the abdominal skin was removed using sterile technique. Five ml of 11,6% sterile sucrose solution was injected peritoneally, the abdomen gently massaged and the fluid withdrawn using sterile plastic pipettes. The fluid was collected in polythene centrifuge tubes (Falcon, Becton and Dickinson and Company, Oxnard, USA). The fluid was centrifuged at 1000 rpm and resuspended in HAT medium. The count was adjusted to 2 x 10^5/ml macrophages.

3.3.2 Fusion

The method for production and maintenance of monoclonal antibodies was based on that of Fazekas de St. Groth and Scheidegger (1980).

A. On the day before fusion

i) Preparation of fresh HAT selective medium

To standard medium consisting of RPM1 - 1640 with 15% foetal calf serum, 1 mM pyruvate, 0,0375 sodium bicarbonate, 0,05% 0,1M 2-mercaptoethanol, 0,01% amphotericin B and antibiotics was added 50 X hypoxanthine aminopterin thymidine (HAT) concentrate (Flow Laboratories) to give a final concentration of 1%.

ii) Preparation of micro cultures

0,15 ml freshly prepared HAT-medium was added to the wells of 8 microtitre tissue culture plates (Sterilin Ltd, Middlesex, UK).

iii) Peritoneal macrophages

These were prepared from 4 Balb/c mice by the method described previously and the count adjusted to 2 x 10^5 cells/ml. 0,05 ml drops were distributed in the microtitre wells and the trays placed in a humidified container at 37°C.

iv) Myeloma cells

Myeloma cultures were trypsinised to ensure that the cells were in a logarithmic growth phase for fusion the following day.

A. On the day of the fusion

i) Preparation of parental cells for fusion

a) Myeloma cells

FO myeloma cells were harvested and the suspensions were transferred to 50 ml centrifuge tubes. The cells were centrifuged at 1000 rpm for 7 minutes. The pellets were pooled and reconstituted to 50 ml with Dulbecco buffer (with 0,28% glucose pH 7,2) and an aliquot mixed with trypan blue and counted to determine that the viability was greater than 90%. The remaining cells were centrifuged again and resuspended in Dulbecco buffer at a concentration of 10^7 cells/ml.

b) Spleen cells

Using aseptic technique, spleens from two hyperimmune Balb/c mice were removed and washed in Dulbecco buffer. Tissue was trimmed and by blunt dissection the spleen cells were gently teased into 10 ml of Dulbecco buffer and transferred to a 15 ml centrifuge tube. The cells were dispersed by pipetting using a plastic pipette and left to stand for 10 minutes. A cell count of the supernatant in Türks solution was performed. Saline was added to the pellet and the concentration adjusted to 10^8 cells/ml.

ii) Fusion

5 ml FO cell suspension (concentration 10^7 cells/ml) was combined with 3 ml spleen cell suspension (concentration 10^8 cells/ml) in a 50 ml centrifuge tube and saline was added to a final volume of 50ml. The suspension was centrifuged at 1000 rpm for 7 minutes. The supernatant was removed and the pellet was loosened by flicking the tip of the tube. 1 ml polyethylene glycol fusing solution (PEG-4000; Merck Chemicals, Hohenbrunn, Germany) was added over 1 minute under constant agitation of the tube. The tube was then immersed for 60 seconds in a 37°C water bath, keeping the contents swirling all the time. The fusion was stopped by slowly adding 20 ml saline, 1 ml over the first 30 seconds, 3 ml over the next 30 seconds and the rest over the second minute. The mixture was topped up with saline, allowed to stand for 5 minutes and then centrifuged at 1000 rpm for 7 minutes. The saline supernatant was discarded and replaced with standard medium and centrifuged again. The cells were taken up in 30 ml HAT medium and resuspended by gentle pipetting. 0,05 ml drops were distributed in the wells of the microtitre plates prepared the previous day and the plates returned to the humidified container at 37°C.

3.3.3 Maintenance of Hybridomas

On the sixth day after fusion, approximately 50% of the supernatant fluid from each well was removed and replaced with fresh HAT medium. Feeding of the cultures was subsequently carried out every second day.

3.3.4 Screening of Colonies

The cultures were inspected daily from the sixth day and growing colonies scored as follows:

>50 cells = 1+ ; 50-500 cells = 2+ ; 500-1000 = 3+ ;

>1000 cells = 4+. An aliquot of the supernatant fluid of wells containing 3+ and 4+ sized colonies was removed for screening for antibody secretion using the indirect immunofluorescent antibody assay.

3.3.5 Cloning and Expansion

Positive hybridoma colonies were cloned by limiting dilution by preparing 10^{-1} and 10^{-2} dilutions of the cells in HAT-selective medium. 0,05 ml of the resulting cell suspensions were then distributed in a number of wells in a 24-well tissue culture plate (Costar, Cambridge, Mass., USA) each containing 1 ml medium and 2 x 10^4 macrophages. The medium was replaced every third day until the secondary colonies were large enough to be retested by indirect immunofluorescence. Those colonies producing influenza antibodies were transferred to 25 cm^2 tissue culture flasks (Costar). The medium was changed to HT- selective medium at this stage as the aminopterin was no longer necessary since the only surviving cells were the myeloma-lymphocyte hybridomas. The

cells were expanded by passaging them at first into 75 cm^2 and later into 150 cm^2 flasks. The medium was supplemented by the addition of a 5% macrophage extract to facilitate the expansion of the cells. The cells were used to prepare both frozen stocks of hybridoma clones and monoclonal antibody - rich ascitic fluid.

3.3.6 Preparation of Macrophage Extract

Peritoneal macrophages were obtained as described previously and the resultant cells were cultured in standard medium with 5% foetal calf serum in a 150 cm^2 flask. The supernatant fluid was harvested after 7 days. This was passed through a sterile 22 μcm Millex GS filter unit (Millipore Corp., Bedford, USA) and stored at -70°C as a stock of growth-enhancing medium.

3.3.7 Preservation of Clones

The hybridoma cells were harvested, centrifuged and adjusted to a concentration of 10^7/ml in standard media containing 10% dimethyl sulphoxide (DMSO). The cell suspensions were dispensed in 1 ml volumes and frozen down slowly by cooling first at 4°C for 1 hour, storing in a closed polystyrene container at -70°C overnight before transferring them to liquid nitrogen. Recovery of viable cells is better than 50% if the tubes are quickly thawed, the cells washed in about 10 ml of complete medium and cultured directly.

3.4 PRODUCTION OF ASCITIC FLUID

Balb/c mice were injected i.p. with 0,5 ml Pristane (2,6,10,14 - tetramethylpentadecane; Sigma chemicals, St. Louis, Mo, USA) as this is essential for ascites formation (Brodeur *et al*, 1984). Two weeks after priming, the mice were injected i.p. with 10^5 FO - hybridoma cells which correspond to the optimum number of hybrid cells for ascitic fluid production as determined by previous titration. Ten to 14 days later the ascitic fluid was collected by aseptic technique and centrifuged at 3000 rpm for 15 minutes. The fluid was stored at 4°C overnight and then aliquotted and frozen at -70°C.

3.5 PREPARATION OF IMMUNOFLUORESCENT SPOT SLIDES

Flasks of Madin Darby canine kidney (MDCK) cells were individually infected with the strains of influenza A (H_1N_1), influenza A (H_3N_2) and influenza B that were listed previously. Infection of these flasks was carried out at an input multiplicity of 0,1 - 1 and harvested when early signs of cytopathic effect were noted. The cells were adjusted to 1,5 x 10^6/ml and mixed in a 1:3 ratio with uninfected cells. The cell suspensions were spotted onto 8-well multitest slides (Flow Laboratories). The slides were air-dried and then fixed with cold acetone. The quality of fluorescence was checked using a commercial influenza A and B monoclonal antibody kit (Monofluo Kit Influenza; Diagnostics Pasteur). The slides were stored at -70°C until use.

3.6 INDIRECT IMMUNOFLUORESCENT ANTIBODY ASSAY (IIF)

Immunofluorescent tests were carried out by adding an appropriate dilution of monoclonal antibodies (MAbs) to the fixed infected cells. For the purpose of screening colonies that were excreting antibodies, hybridoma colony supernatants were tested without dilution. For the testing of MAb-rich ascitic fluid, a dilution of 1:100 was used.

For conventional IIF tests, once the slides had been incubated with the MAbs for 30 minutes at 37°C, they were washed in phosphate-buffered saline (PBS) and treated with affinity-purified anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate (Cappel Laboratories). After a further 30 minutes' incubation at 37°C, the slides were washed, mounted with glycerol and viewed with a Nikon HFX-II Optiphot ultra-violet microscope.

3.7 CHARACTERIZATION OF MABS

3.7.1 Isotyping of Monoclonal Antibodies

The mouse monoclonal antibody isotyping kit (Amersham, UK) was used to determine the isotype of the MAbs. Stored hybridoma supernatants were tested. For each hybridoma supernatant one typing stick was placed into a labelled container. 2 ml of hybridoma supernatant was diluted in 6 ml TBS-T (0.1% Tween 20 in Tris Buffered Saline pH 7.6). 3 ml of this diluted hybridoma sample was added to the typing stick in the container and incubated at room temperature for 15 minutes with constant agitation. The hybridoma solution was poured off and the typing stick was washed by adding 5 ml of TBS-T to the container.

Incubation with agitation was carried out for 5 minutes. The washing procedure was repeated once. 3 ml of 1:500 peroxidase-labelled anti-mouse antibody was added to the container and incubated for 15 minutes with agitation. The stick was washed as described previously. A substrate solution was prepared by dissolving one 4-chloro-1-naphthol tablet in 10 ml of cold (2-8°C) methanol. To this was added 1 drop of hydrogen peroxide solution (30% v/v) to 50 ml of TBS. The two solutions were thoroughly mixed before use. 3 ml of substrate solution was added to each container and incubated with agitation for 15 minutes. After discarding the substrate solution, 3 washes using distilled water were carried out. After drying the results were interpreted.

3.7.2 Radio-Immunoprecipitation Assay (RIPA)

a) Labelling of virus

Flasks of MDCK cells were infected with influenza A/Taiwan/1/86 at an input multiplicity of 10^{-1}. The flasks were centrifuged for 1 hour at 1000 rpm. Thereafter the cells were incubated for 2 hours with serum-free Eagles minimum essential medium with Earles Salts (EMEM). After washing thoroughly with PBS, methionine free EMEM was added to each flask, L-(^{35}S) methionine (10μCi/ml, SP. act. 943 μCi/mml) was then added and incubated overnight at 37°C. The cells were then harvested, washed in cold PBS and lysed with NET-BSA buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7,4, 1 mg/ml BSA).

The lysates were disrupted with a probe sonicator and topped up with NET-BSA buffer. MAbs shown by isotyping to be of the IgG_1 subclass

were allowed to react with 50μl of anti-mouse immunoglobulin (Cappel Laboratories) for 1 hour.

b) Immunoprecipitation

For the immunoprecipitation 50μl of monoclonal immune ascitic fluid was added to the labelled infected cell lysates. The mixture was allowed to stand at room temperature for 6 hours. Immune precipitates were recovered using Protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the method of Struthers and Swanepoel (1982), resuspended in solubilising buffer (Laemmli, 1970) and analyzed in linear 12% gels. A polyclonal guinea pig influenza antiserum was used as a positive control. Uninfected cell lysates were used as a negative control. In addition radio-labelled protein standards (Amersham) were subjected to electrophoresis in parallel for estimation of molecular weights of the viral proteins. The gels were treated with Amplify (Amersham), dried and exposed at room temperature, using Hyperfilm B-Max (Amersham).

4. RESULTS

4.1 PRODUCTION OF MONOCLONAL ANTIBODIES (MABS)

4.1.1 Isolation of MAbs

Eight positive excreters were identified by indirect immunofluorescence from among 69 colonies screened (11,6%).

4.2 CHARACTERIZATION AND APPLICATION OF MABS

4.2.1 Determination of Immunoglobulin Subclass

The immunoglobulin subclasses of the 8 MAbs are shown in Table 1. Antibodies representing all the IgG subclasses were obtained.

4.2.2 Determination of MAb Specificity

The optimum period for radiolabelling the infected cells for the detection of the virus-specified proteins by polyacrylamide gel electrophoresis was found to be 24 hours post-infection. Examples of the banding patterns of the HA and NP MAbs are shown in Figure 5.

It is interesting to note that a double but discrete banding pattern is associated with the MAb directed against the NP. This is consistent with the findings of Zhirnov and Bubrinskaya (1981), who described two forms of NP of different molecular weight produced in virus infected cells by post translational modification. The higher molecular weight form (NP_{56}) is preferentially incorporated into the ribonucleoprotein of new budding virions. The second form (NP_{53}) is a secondary product of NP_{56} and accumulates in cells late in infection.

The specificity of the MAbs is shown in Table 2. Six MAbs precipitated the HA protein. The remaining 2 MAbs precipitated the NP protein. Differing patterns of fluorescence were noted between the MAbs directed against the NP proteins compared with the fluorescence of the MAbs against the glycoproteins. The fluorescence associated with the NP MAbs was more compact and concentrated in the perinuclear region of the cell whereas the glycoprotein MAbs produced more generalised, scattered fluorescence oriented towards the cytoplasmic membrane of the cell.

4.3 AGE PREVALENCE OF PATIENTS WITH INFLUENZA A (H_1N_1) ISOLATES DURING THE 1989 SEASON

A total of 45 influenza A (H_1N_1) influenza isolations were made during the 1989 influenza season. Of these, details regarding age were available from 38 patients. The majority, 32 of the 38 (84%), were born after 1957 and were younger than 32 years of age (figure 6).

4.4 ANALYSIS OF INFLUENZA A (H_1N_1) ISOLATES BY INDIRECT IMMUNOFLUORESCENCE ASSAY USING THE MAB PANEL

An analysis of 15 sequential influenza A (H_1N_1) isolates from the 1989 influenza season was carried out. Representative samples from early in the season (April and May) from mid season (June) and the latter part of the season (August) were selected. To establish if cross reactivity patterns with influenza A (H_3N_2) and influenza B viruses occurred, isolates of these viruses were tested. Influenza A (H_3N_2) isolates 1131/90, 1188/90 and 1205/90 were specifically chosen because

these isolates were originally typed using reference antisera as influenza A (H_1N_1)-like viruses. The titre in the haemagglutination inhibition test was noted to be low. These isolates were subsequently typed by Dr J. Skehel (National Institute for Medical Research, London) as influenza A/Beijing/352/89 (H_3N_2). Specific antisera for influenza A/Beijing/352/89 (H_3N_2) were not available in our laboratory at that time. These were among the first isolates of this particular virus which were also isolated at the same time in Australia. (Personal communication Dr J.J. Skehel). The cross reactivity with H_1N_1 antisera highlights the problems that can arise with antisera in the haemagglutination inhibition test (Dowdle and Schild, 1975), mainly due to the presence in many sera of non specific inhibitory substances.

The results of the antigenic analysis of the influenza isolates using monoclonal antibodies in an indirect immunofluorescent assay are shown in Tables 3 and Table 4 and in figure 7.

6. DISCUSSION

The global re-emergence of influenza A (H_1N_1) occurred in 1977 but illness was detected only in young subjects. A similar pattern was noted in the 1978-1979 season in the United States of America. Since that time the incidence of illness has been low among older adults and such illness has generally been mild (Monto *et al*, 1985). In the years following the 1983-1984 influenza season influenza A (H_1N_1) strains were isolated sporadically in most parts of the world other than in Asia, Oceania and South Africa (MMWR, 1987; NIV record's figure 4). Low levels of A (H_1N_1) activity continued to be reported throughout the world in the 1988-89 season, however, and reports of H_1N_1 activity continued to be reported from the countries mentioned previously (MMWR Dec 1, 1989). This trend continued throughout the 1989-90 season (MMWR May 4, 1990).

In South Africa H_1N_1 influenza activity was noted in 1986 and 1987 (NIV records, figure 4). 1988 saw the circulation of both the influenza A/H_3N_2 subtype and influenza B. The influenza season under study, 1989, saw fairly intense influenza activity with the co-circulation of both H_1N_1 and H_3N_2 subtypes. The majority of the H_1N_1 isolations were from patients who were 32 years and younger, having been born after 1957 (figure 6). These patients are unlikely to have had prior exposure to the influenza A (H_1N_1) subtype. Regrettably, no clinical details are available on these patients concerning the severity of the illnesses nor the frequency and nature of any complications. As all the specimens referred for isolation were from patients who attended hospital outpatient clinics

or were from general practices, rather than from hospitalised inpatients, it can be inferred that the patients were treated on a ambulatory basis. By implication, therefore, the illnesses were likely to be less severe. It must be emphasized that the distribution of the patients from whom the samples were obtained is not necessarily representative of the community at large. They do, however, provide valuable information as to the nature of the influenza activity during any given influenza season.

The monoclonal panel whose specificities are shown in table 2 were reacted against 15 H_1N_1 isolates chosen to be representative of the early, mid and late influenza season. Four distinct patterns emerged (Tables 3 and 4). These patterns did not reflect geographical clustering of isolates. Pattern A was the most prevalent encountered and seemed to emerge as the "dominant" strain as the epidemic progressed. Pattern B was most prevalent during the mid-season. As expected, the monoclonal antibodies specific for the NP ($3G_8$ and $3H_2$) reacted with all the isolates. The variable patterns were associated with the MAbs reactive with the HA molecule. It is interesting to note that the NP MAbs reacted in the IIF test with the isolates of the H_3N_2 subtype. The commercial monoclonal antibody kit (Monofluo Kit Influenza, Diagnostics Pasteur), which was being used as a positive control in the IIF test, failed to react with the H_3N_2 isolates (1131/90, 1188/90 and 1205/90). An awareness should be present of the lack of sensitivity of this commercial type A-specific MAb, which is directed against the NP protein, when used in tests for rapid identification of local influenza isolates. Previously this kit had been used in the routine diagnostic laboratory at the NIV

for rapid identification of influenza isolates using the shell-vial centrifugation technique. This has been replaced by the monoclonal antibody $3G_8$ in this routine assay.

Growth of influenza virus in embryonated hens' eggs can introduce artefactual mutations, probably in surface positions of the HA, which affect binding to antibody and to cell receptors (Schild *et al*, 1983). Such mutations could affect the antigenicity in laboratory tests and could artificially link strains which, in fact, are not closely related. This phenomenon is referred to as "laboratory drift". This variable was considered during this present study, because virus was passaged in hens' eggs prior to inoculation onto MDCK monolayers for the production of spot slides suitable for use in the IIF test. However, it was decided that the limited passage of the panel of viruses in hens' eggs, together with a single passage on MDCK cells would not exert selective pressure to promote laboratory-induced antigenic variation. In addition, the discrete and limited reactivity patterns on IIF testing would tend to support this.

In recent years, the epidemiology of the influenza A (H_1N_1) subtype has revealed a steady decline in activity of this virus in the form of epidemics or outbreaks. This has been particularly noticeable in the northern hemisphere. Sporadic outbreaks continue to occur in Asia and countries in the Southern hemisphere including South Africa. This is in contra-distinction to influenza A (H_3N_2) and influenza B activity which has continued to be reported on an annual basis worldwide. Immunity to the H_1N_1 subtype appears to be solid and long-lasting;

(Chakraverty *et al*, 1982) thus the disease tends to manifest in young subjects. Glezen (1991) showed that wide differences in the distribution of H_1N_1 infections were apparent when comparing age-specific infection rates in Houston for a 12-year period ending June 1989. More than 50% of H_1N_1 infections were detected among persons aged 10 - 34 years, compared with 28% of influenza A/H_3N_2 and 35% for influenza B. Over the age of 35 years, the contribution of H_1N_1 viruses dropped to only 4%, compared with 20% and 16% for H_3N_2 and influenza B, respectively. Thus most of the H_1N_1-positive persons were born after 1956. The present study confirms these findings. An ever-decreasing pool of susceptible subjects is thus created with consequent reduction in the circulation of the H_1N_1 subtype. A similar situation may have existed from 1940 to 1957 where in Great Britain a gradual decrease in influenza A activity was noted during this time, apart from an extensive epidemic in 1951 (Semple, 1951). This virus was found to deviate antigenically from the 1950 viruses (Isaacs and Andrews, 1951). Long-standing immunity is not associated with viruses of the H_3N_2 subtype so that a similar situation is not evident with that virus subtype.

The reduction in the pool of susceptible subjects can exert immunological pressure on the virus, leading to antigenic variation. Evidence for this is to be found in a study by Raymond *et al* (1986). The sequence determination and analysis of the HA_1 regions for 19 strains of the H_1N_1 subtype were studied. Nine strains for the period 1950-1957 and thirteen for the period 1977-1983 were analyzed. The results obtained confirmed that the sequence of the HA of a 1977 reference strain was virtually identical to the 1950 reference strain. Thus, the evolution of one

gene on two separate occasions was followed. The analyses of the earlier 1950-1957 strains confirmed the close relationship between A/USSR/90/77 and A/Fort Warren/1/50. This confirmed work done earlier by Young *et al* (1979), which showed differences between these viruses at only 4 amino acid positions. The five 1950-1957 strains were very obviously different from one another in many positions. Indeed, there were 33 amino acid changes between A/Fort Warren/1/50 and the last member of the series - A/Denver/1/57. Antigenic variation was confirmed by analysis using ferret antisera. The rapid rate of evolution in the eight-year period, 1950-1957 (1,2% amino acid change/HA_1 domain/year), contrasts with the slower rate in the 7 year-period, 1977-1983. Here, only 18 amino acid changes separate the two strains, A/USSR/90/77 and A/Dunedin/27/83 which are farthest apart (0,8% amino acid change/HA_1 domain/years). For comparison, Both *et al* (1983) reported 0,7% amino acid change/HA_1 domain/year in H_3 strains between 1968 and 1979. In these studies sequential changes occurred and a "fixation" of these main stream changes occurred, leading to a strain or strains that enjoyed a selective advantage. These strains tended to replace other co-circulating strains and led to the emergence of a "dominant" strain or strains. Raymond (1986) suggests that the more rapid evolution of the strains between 1950-1957 correlates with the circulation of the virus in the presence of a higher overall level of population immunity at the time, compared to the situation in the subsequent post-1977 era. High antibody pressure can result in the emergence of highly specialised strains with many unique features. These features could have implications with regard to the virulence or transmissibility of the virus. This may have been the situation in

Liverpool, Great Britain, in 1951 (Isaacs and Andrews, 1951). This outbreak was associated with a virus that displayed an unusually increased virulence which occasioned a huge mortality in Liverpool. The unusually high attack rates and high mortality rates among the elderly are reflected in the records of the Medical Officer of Health of the City of Liverpool (Semple, 1951). The present study confirms the presence of 4 antigenically dissimilar strains co-circulating in the 1989 influenza season. A dominant strain appears to have emerged during the latter part of the season.

Genetic reassortment between co-circulating viruses of the H_1N_1 and H_3N_2 subtypes can occur (Young and Palese, 1979 : Bean *et al*, 1980). Four genes of the H_1N_1 subtype which maintain the H_1N_1 phenotype, namely the genes coding for the HA, NA, M and NS proteins are retained in the new reassortant. Four genes are donated by the H_3N_2 subtype. These viruses do appear to have epidemiological significance (Nakajima *et al*, 1980). This may have been the mechanism responsible for the 1951 epidemic in Great Britain, although this has never been elucidated. It is of interest that in the present study the 1989 influenza season saw the co-circulation of viruses of both the H_1N_1 and H_3N_2 subtypes (figure 4).

7. CONCLUSION

(H_1N_1) antigenic variation of influenza A virus isolates from 1989 was studied by using a panel of monoclonal antibodies. This study showed that co-circulation of antigenic variants of the virus occurred in that year. It is postulated that this has occurred in the face of an ever-decreasing pool of susceptible subjects, thus exerting considerable immunological pressure on the virus. This situation is analogous to that which existed in the period between 1950 and 1957. It is further postulated that this immunological pressure on the virus could lead to the emergence of an unique strain which could display increased virulence and/or transmissibility. Once again a precedent for this may have occurred in Great Britain in 1951. The future of the H_1N_1 subtype is uncertain, as the cumulative evidence mirrors that of the 1950-1957 period where a reassortment led to the emergence of a new subtype. The above has clear implications regarding influenza virus surveillance and vaccine recommendations. To this end it is recommended that this study be extended into an ongoing surveillance of the H_1N_1 subtype. Finally, an extension of this study would be the use of nucleotide sequencing as an additional molecular tool to detect intrastrain variations of the virus. This study would have importance in following the evolution and future development of the influenza A (H_1N_1) subtype.

TABLE 1. : IMMUNOGLOBULIN SUBCLASS OF MABS

MONOCLONAL ANTIBODY	SUBCLASS
5F12, 6C7	IgG1
4F9	IgG2a
3G8, $3H_2$	IgG2b
2E2, 8C6, 8E9	IgG3

TABLE 2: SPECIFICITY OF THE INFLUENZA A (H_1N_1) MABS AS DETERMINED BY THE RADIO-IMMUNE PRECIPITATION ASSAY

MONOCLONAL ANTIBODY	SPECIFICITY
2E2	Haemagglutinin
4F9	Haemagglutinin
5F12	Haemagglutinin
6C7	Haemagglutinin
8C6	Haemagglutinin
8E9	Haemagglutinin
3H2	Nucleocapsid
3G8	Nucleocapsid

TABLE 3. ANTIGENIC ANALYSIS OF INFLUENZA ISOLATES USING MONOCLONAL ANTIBODY PANEL

Monoclonal Antibody

	Laboratory isolate	8C6	2E2	8E9	4F9	3G8	3H2	5F12	6C7
Influenza A (H1N1)	461/89	▨			▨	▨	▨		▨
	553/89	▨	▨	▨	▨	▨	▨	▨	▨
	568/89	▨	▨	▨	▨	▨	▨	▨	▨
	685/89			▨	▨	▨	▨		
	688/89	▨			▨	▨	▨		▨
	826/89	▨			▨	▨	▨		▨
	828/89			▨	▨	▨	▨		
	832/89	▨	▨	▨	▨	▨	▨	▨	▨
	857/89	▨			▨	▨	▨		▨
	867/89	▨		▨	▨	▨	▨		▨
	1018/89	▨	▨	▨	▨	▨	▨	▨	▨
	1044/89	▨	▨	▨	▨	▨	▨	▨	▨
	1053/89	▨	▨	▨	▨	▨	▨	▨	▨
	1058/89	▨	▨	▨	▨	▨	▨	▨	▨
	1072/89	▨			▨	▨	▨		▨
Influenza A (H3N2)	1131/90					▨	▨		
	1188/90					▨	▨		
	1205/90					▨	▨		
Influenza B	B/VICT/2/87								
	B/PAN/45/90								
	B/YAM/16/88								

▨ Reactive to Monoclone

TABLE 4. PATTERNS OF REACTIVITY OF INFLUENZA A (H_1N_1) ISOLATES USING MONOCLONAL ANTIBODY PANEL

	Monoclonal Antibody								
	8C6	2E2	8E9	4F9	3G8	3H2	5F12	6C7	
Pattern A	▨	▨	▨	▨	▨	▨	▨	▨	n=7
Pattern B	▨			▨	▨	▨		▨	n=5
Pattern C			▨	▨	▨	▨			n=2
Pattern D	▨		▨	▨	▨	▨		▨	n=1

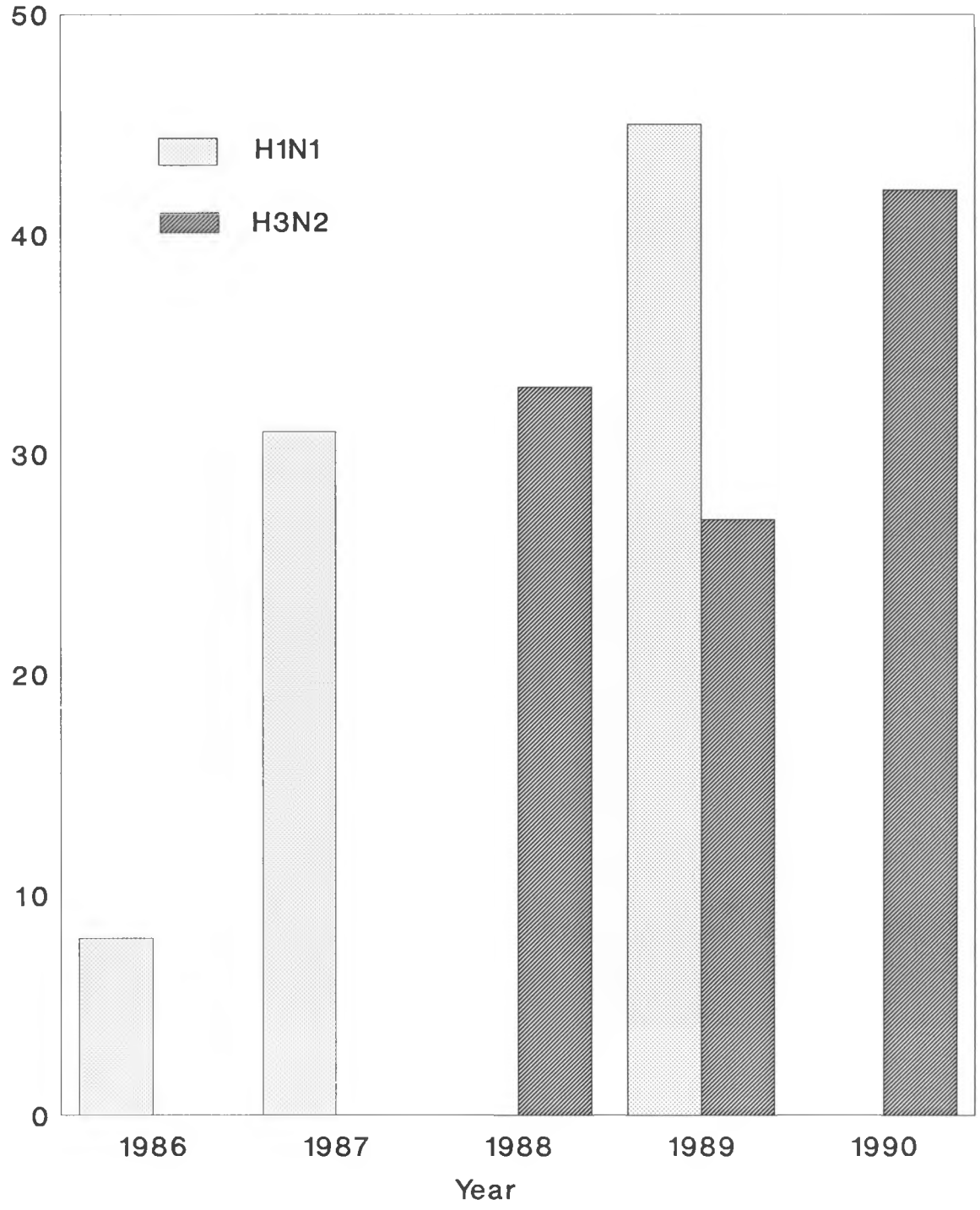

Figure 4. Influenza A isolations from 1986 to 1990

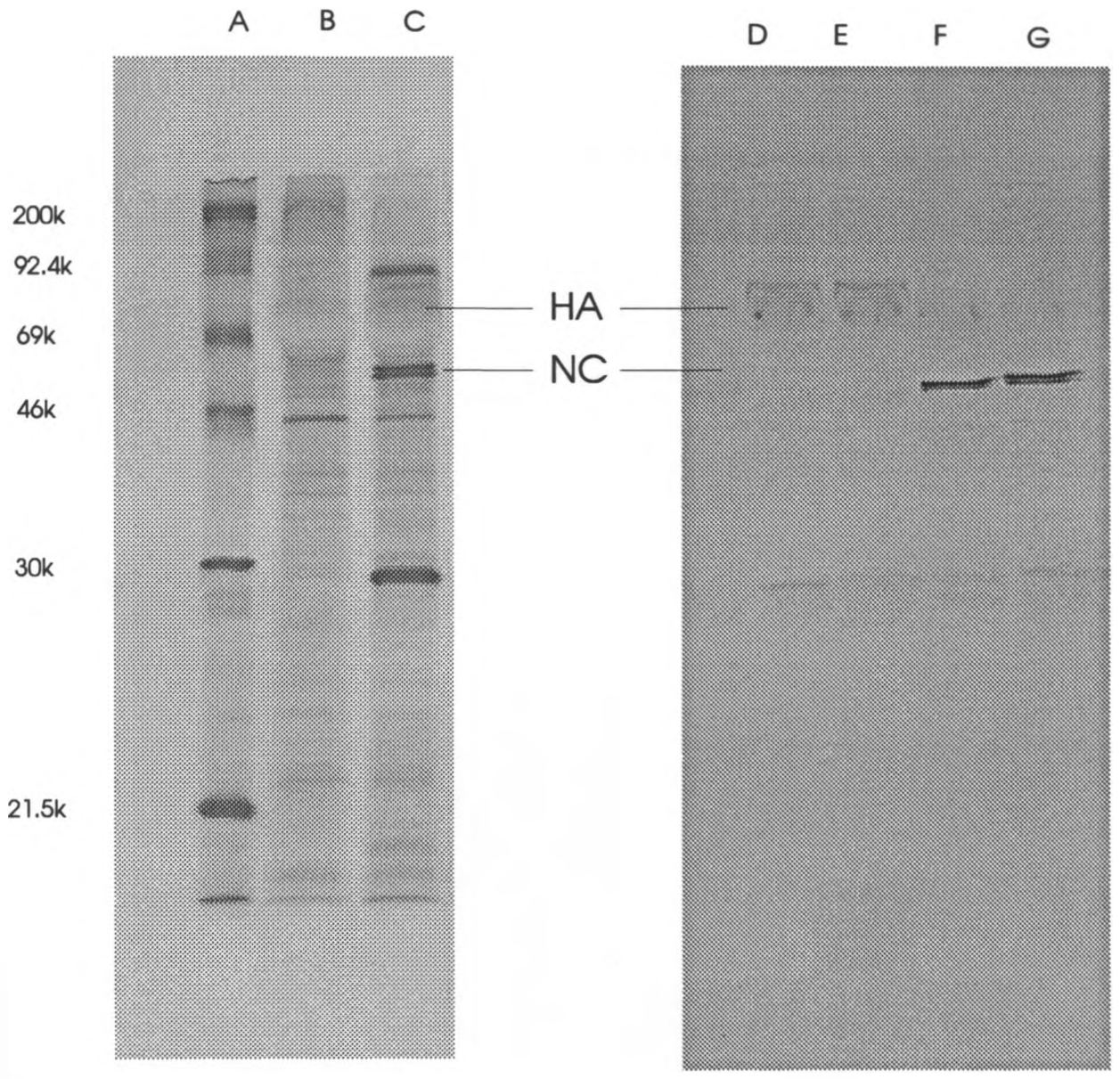

Figure 5. Examples of an immunoprecipitation of [^{35}S] methionine-labelled influenza protein by monoclonal antibodies.

Lane A Molecular weight marker
Lane B Uninfected cell lysate
Lane C Proteins precipitated by polyclonal guinea pig antiserum
Lane D Haemagglutinin protein precipitated by MAb 8C6
Lane E Haemagglutinin protein precipitated by MAb 5F12
Lane F Nucleocapsid protein precipitated by MAb 3H2
Lane G Nucleocapsid protein precipitated by MAb 3G8

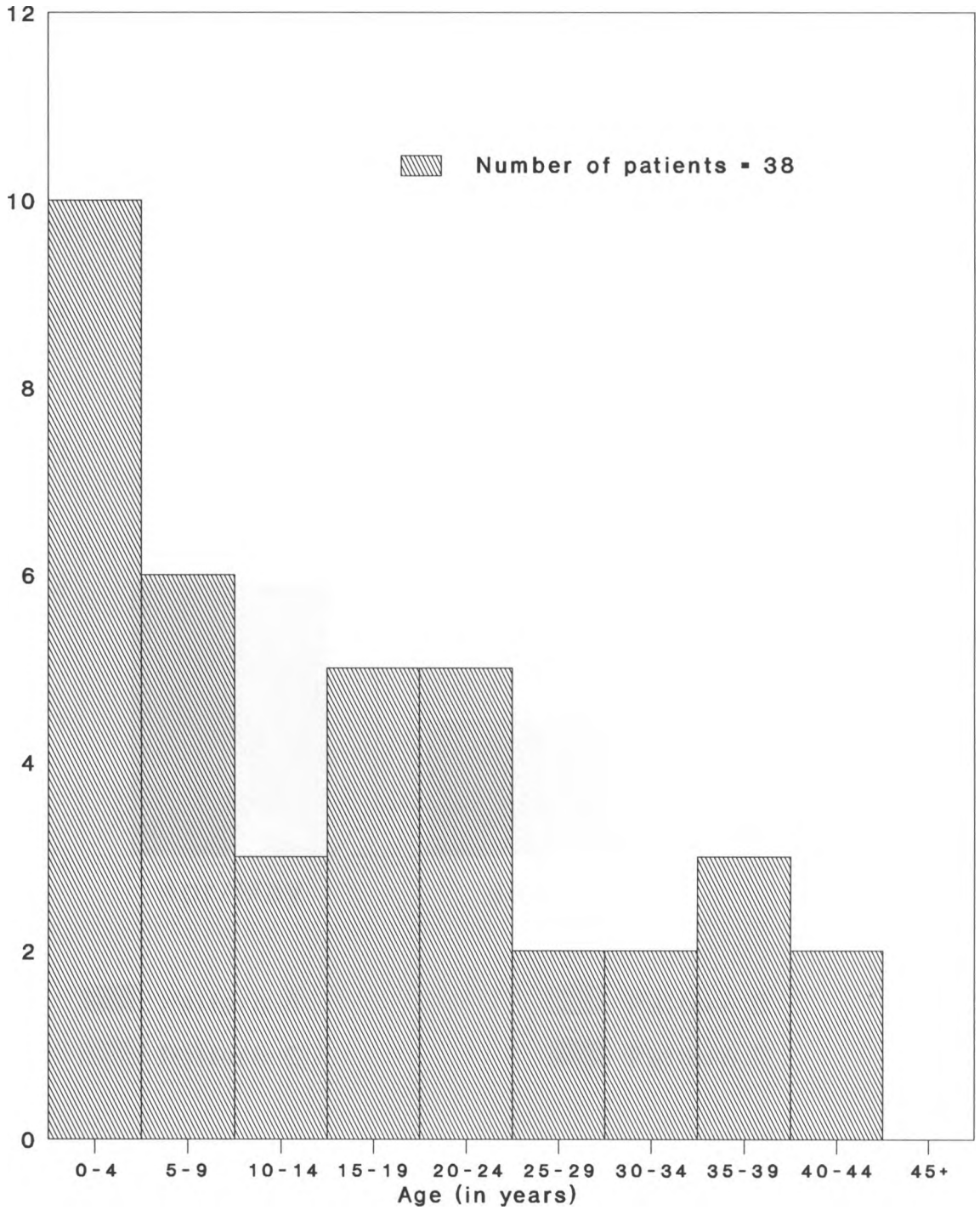

Figure 6. Age prevalence of patients with influenza a (H_1N_1) isolates during the 1989 season

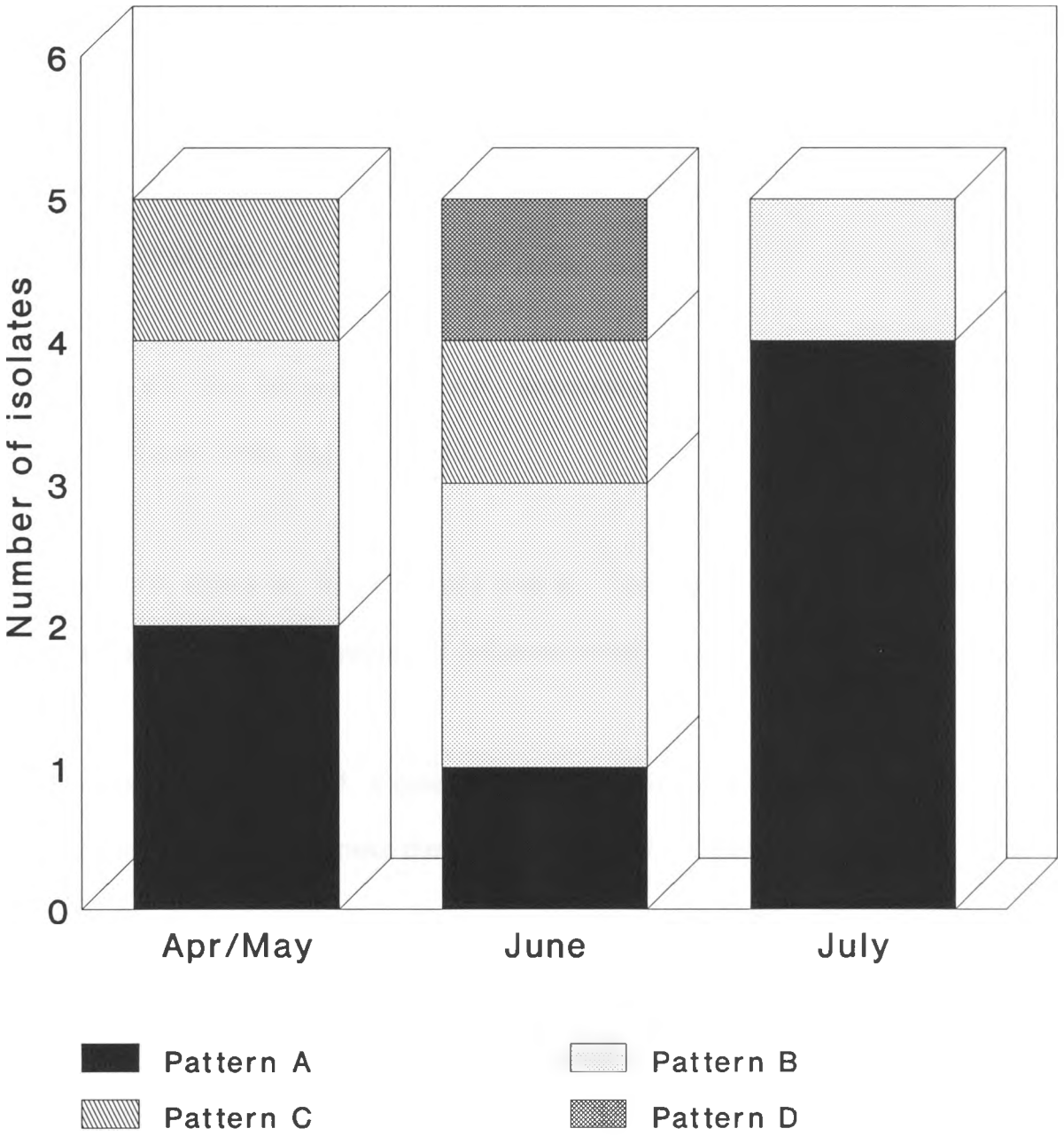

Figure 7. Patterns of Reactivity of Influenza A (H_1N_1) Isolates using Monoclonal Antibody Panel showing different Patterns during 1989 Influenza Season

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