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Antigenic competition of influenza virus glycoproteins. Immunological and cellular mechanisms responsible for the influence of hemagglutinin priming on the immunogenicity of the viral neuraminidase

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ANTIGENIC COMPETITION OF INFLUENZA VIRUS GLYCOPROTEINS. IMMUNOLOGICAL AND CELLULAR MECHANISMS RESPONSIBLE FOR THE INFLUENCE OF HEMAGGLUTININ PRIMING ON THE IMMUNOGENICITY OF THE VIRAL NEURAMINIDASE

by

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I. Abstract

ANTIGENIC COMPETITION OF INFLUENZA VIRUS GLYCOPROTEINS: IMMUNOLOGICAL AND CELLULAR MECHANISMS RESPONSIBLE FOR THE INFLUENCE OF HEMAGGLUTININ PRIMING ON THE IMMUNOGENICITY OF THE VIRAL NEURAMINIDASE

by Bert E. Johansson

Advisor: Distinguished Service Professor Edwin D. Kilbourne

In man, vaccination with neuraminidase (NA) in H7N2 influenza virus antigenic hybrids elicits greater anti-NA response than does N2 NA in H3N2 conventional vaccine, presumably because humans are H3 hemagglutinin (HA) primed and anti-H3 anamnestic response depresses concomitant N2 responses by antigenic competition. BALB/c mice were primed by different schedules of infection with H3N1, H3N2 and H3N7 viruses and given H3N2 and H7N2 vaccines equivalent in NA immunogenicity. In schedules using sequential infections, but not after a single infection with any virus, anti-N2 booster response was four-fold greater with H7N2 vaccine. Thus, HA-influenced suppression of immunologic response to viral NA requires adequate HA priming. Titration of primed helper T (T_h) cell activity by adoptive transfer of purified T cells to athymic mice and assay of the proliferative response of T cells in B and T cell mixtures stimulated by reassortant influenza viruses *in vitro* has shown: 1) HA is dominant over NA in both B and T cell priming, 2) an increase in H3 specific B cells occurs in mice boosted with H3N2 vaccine and an increase in

N2 specific B cells in those boosted with H7N2 vaccine and, 3) memory B cells function as antigen presenting cells and interact with memory T_h cells in the mediation of intravirionic HA-NA antigenic competition in favor of HA. Analysis of equivalent macrophage-T cell mixtures demonstrates macrophages from variously immunized and from unimmunized BALB/c mice are equivalent in their capacity as antigen presenting cells when stimulated in vitro with influenza virus.

Immunization of mice with graded doses of purified influenza virus HA and NA antigens demonstrated equivalent responses when HA-specific and NA-specific serum antibodies were measured by enzyme-linked immunosorbent assays (ELISA). Apparently, the antigenic competition between HA and NA glycoproteins results from the difference in their quantitative relationships on the virion surface and not from differences in intrinsic immunogenicity.

Injection of mice with purified H3 HA or N2 NA antigen resulted in immunity manifested by reduction in pulmonary virus following challenge with virus containing homologous antigens. While H3 immunization with all but the lowest doses of antigen prevented infection, immunization with N2 was infection-permissive at all antigen doses. The demonstrable immunogenicity of highly purified neuraminidase as a single glycoprotein without adjuvant and influenced by HA antigenic competition offers a novel approach for human immunization against influenza.

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V. INTRODUCTION

Influenza is an acute respiratory tract infection that can result in a mild inapparent infection or a severe incapacitating illness. Influenza viruses, the causative agents of influenza, were termed myxoviruses (Andrewes et al, 1955) to denote the affinity of the virion for mucin in the form of mucopolysaccarides and glycoprotein. Later the designation was changed to orthomyxovirus to distinguish them from paramyxoviruses. Influenza viruses are divided into types A, B, and C, a classification based on genetic differences and antigenic differences between surface proteins and the major internal proteins, M and NP. Influenza A viruses are described by a nomenclature which includes the host of origin, geographic origin, strain number and year of isolation. The antigenic classification of the HA and NA are given in parenthesis, e.g. A/PR/8/34 (H1N1). There are 13 antigenic subtypes of HA (H1-H13) and 9 subtypes of NA (N1-N9). All subtypes are found in birds, but only a few in humans (H1,H2,H3; N1-N2), swine and horses. Influenza A virus has a genome composed of eight negative sense RNA segments that encode ten viral proteins (Mahy, 1983). Two of these genes encode for the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) which appear as "spikes" on the virion surface when examined by electron microscopy (Laver and Valentine, 1969). The HA mediates the initial attachment of the virion to cells via sialic acid residues (Choppin and Tamm, 1960) and

possesses a fusion capability that enables the virus envelope to integrate with a lysoendosomal membrane allowing the interal viral components access to the cell cytoplasm (Yoshimura and Ohnishi, 1984). Antibodies to HA neutralize viral infectivity; antigenic variation in this molecule is mainly responsible for frequent outbreaks of influenza and for the poor control of infection by immunization. The HA molecule is present in the virion as a trimer. Each monomer exists as two chains, HA1 and HA2 linked by a disulfide bond (Wilson et al, 1981). The precursor single polypeptide must be cleaved to produce HA1 and HA2 for the virus particle to be infectious (Lazarowitz and Choppin, 1975). HA2 is anchored in the membrane by a sequence of hydrophobic amino acids at the C terminus. As shown by X-ray crystallography, each HA molecule contains two main regions: a triple-strand coil of alpha helices and a globular region of anti-parallel β-sheets (Wiley et al, 1981; Wilson et al, 1981). The cell receptor-binding site and the variable antigenic determinants are located on the globular domain (Caton et al, 1982).

The NA is an enzyme which cleaves sialic acid residues from any oligosaccharide chain possessing that terminal sugar, including terminal keto groups on the HA and NA molecules. NA's exact role is not certain but is probably involved in the release of new virus particles. NA exists as a spike on the virion with a "head" containing four coplanar and approximately spherical subunits, and a

centrally attached "stalk" with a transmembrane hydrophobic region (Laver and Valentine, 1969). NA is oriented in the opposite way to HA--the transmembrane segment is at the N terminus. Studies with X-ray crystallography have shown that the polypeptide chain is arranged in six topologically identical, four-stranded, anti-parallel β-sheets, giving an overall appearance of propeller blades (Varghese et al, 1983; Colman et al, 1983). The catalytic site appears to be a large pocket on the distal surface formed by 18 residues conserved among subtypes (Varghese et al, 1983; Air et al, 1985). Most of the antigenic variation occurs in sites that encircle the enzymatic active site (Air et al, 1985).

Intact virions also contain a third envelope associated protein called matrix (M) protein, which is the major virus protein and is associated with the inside of the membrane (Compans et al, 1970). Inside the viral envelope are the eight RNA segments, the polymerase proteins (PA, PB1, PB2) which supply the enzymatic machinery for viral RNA synthesis and nucleoprotein (NP) which associates with RNA segments to form ribonucleoprotein (RNP) (Krug 1971, 1972). RNPs are found in the nucleus of infected cells (Lin and Lai, 1983).

Viral Replication and Assembly

As with other enveloped viruses, influenza undergoes receptor mediated endocytosis. The cell receptor contains sialic acid (Choppin and Tamm, 1960) and the viral receptor

is the distal end of the HA molecule (Rogers et al, 1983). Uncoating of the virus occurs as a result of the endosome containing the virus particle fusing with a lysosome (Yoshimura and Ohnishi, 1984). The low pH of the lysosome catalyzes a conformational change in HA (Skehel et al, 1982) that results in the viral envelope fusing with the endosomal membrane and the viral genome being released into the cytoplasm (Simons and Garoff, 1980). The viral negative strand RNA serves two functions, one transcription and the other replication. The viral negative strand RNA within the cell directs synthesis of positive strand RNA and messenger (mRNA). Cellular mRNA contributes a capped methylated primer and 10-15 nucleotides from the 5' terminal end for incorporation into the viral mRNA (Mahy et al, 1972). This cleavage activity, priming of the nascent strand and elongation has been shown to occur with isolated RNA polymerase-RNA complexes composed of PB1, PB2, PA and vRNA in the absence of NP (Kato, Mizumoto and Ishihama, 1985). Viral transcriptase transcribes genomic RNAs to make monocistronic mRNAs (+ strand), each of which specifies a single protein. Viral polymerases direct the replication process. A full-length (+) strand is made and in turn serves as a template for synthesis of progeny (- strand) RNA (Beaton and Krug, 1984, Hay et al, 1977).

Two of the eight virus RNA segments, numbers 7 and 8, each encode at least two proteins. Segment 7 contains three open reading frames from two of which gene products

have been found; one encodes the non-glycosylated envelope protein M1 (matrix protein) in a continuous reading frame, overlapping this is a second reading frame for a smaller protein, M2 (Lamb and Choppin, 1983). M2 is not found in the virion but is expressed at the surface of infected cells (Zebedee et al, 1985). The two proteins in Segment 8 show a similar relationship. NS1 (non-structural) is derived from a continuous reading sequence, whereas NS2 results from a splicing event. Neither NS1 or NS2 is found in the virion and their functions are not clear (Greenspan et al, 1985).

The assembly of the virion occurs in two stages: assembly of the nucleocapsid and inclusion of this in the viral envelope. Nucleocapsid assembly appears to take place in the nucleus, whereas the viral envelope takes shape at the cell plasma membrane. The latter process begins in the membrane of the endoplasmic reticulum in which viral glycoproteins are first inserted and then glycosylated as they pass through the Golgi apparatus. HA and NA are both transported to the cell's apical surface (Rodriquez-Boulan and Sabatini, 1978); each contains structural features capable of directing the transport of the protein to apical cell surfaces (Jones et al, 1985). Formation of ribonucleoproteins (nucleocapsids) occurs free in the cytoplasm (Compans and Caliguiri, 1973). M1 protein acts as a bridge between the nucleocapsid and the viral envelope but how this occurs and whether there is contact

between the glycoproteins, intracytoplasmic segments, and nucleocapsids is not clear (Lenard and Compans, 1974). After assembly at the cell plasma membrane, the completed influenza virus particles push out from the plasma membrane in a budding or evagination process not yet understood (Choppin, 1963; Simons and Garoff, 1980).

The assembled virion exposes only two glycoproteins to the environment, HA and NA. HA has its most important roles before replication (i.e., attachment and fusion), whereas NA may function both before and after replication. Before replication, NA may act on non-specific inhibitors in the extracellular fluids and possibly to release virus from mucus (Kilbourne et al, 1975). After replication NA acts on substrates in or on the infected cell, and on the virion itself in accomplishing release and detachment from infected cells (Palese et al, 1974; Palese and Compans, 1976).

A subject of increasing interest is the expression of viral proteins other than HA and NA at the surface of infected cells. It has been shown that NP is expressed on the surface of infected cells (Virelizier et al, 1977; Yewdell et al, 1981), and that this may also occur in cells transfected with DNA plasmids containing coding sequences for NP (Townsend et al, 1984). Less clear results have been found for matrix (M) proteins. Workers using polyclonal antisera suggested that M was expressed at the cell surface (Ada and Yap, 1977; Reiss and Schulman, 1980);

in contrast, workers using monoclonal antibodies have found very small amounts of M protein expressed at the cell surface (Hackett et al, 1980; Yewdell et al, 1981). These results may be explained as either contamination of the polyclonal antisera with anti-HA antibodies (Reiss and Schulman, 1980) or possibly by the anti-M sera recognizing epitopes shared by M1 and M2, since M2 has been found expressed in considerable amounts at the infected cell surface (Lamb and Choppin, 1983; Zebedee et al, 1985). The extent to which other internal proteins are expressed on the infected cell surface has not been fully explored. However, recent work has shown that anti-influenza virus cytotoxic T cells recognize the three viral polymerases (PA, PB1, PB2) and a protein not incorporated into virions, NS1 protein (Bennick et al, 1987).

Antigenic Properties of Viral Proteins

B Cell Recognition:

Locations of major antigenic sites on the HA molecule have been described using two complementary techniques: 1) generation of a panel of monoclonal anti-HA antibodies for selecting viral mutants expressing antigenically changed HA molecules. Analysis of the binding resulted in construction of an antigenic map of the HA molecule, 2) an analysis of the three dimensional structure of HA molecules and a comparison of the amino acid sequences of the HA from related epidemic and mutant strains (Webster and Laver, 1980; Wiley et al, 1981; Gerhard et al,

1981). There is consensus that these approaches show four principal antigenic determinants which have been designated for the H3 molecule as: site A,B,C, or D (Wiley et al, 1981) and for the H1 molecules as: Sa, Sb, Ca or Cb (Gerhard et al, 1981, Caton et al, 1982). The antigenic sites of PR8 H1 HA correlate with the structure of the Hong Kong H3 HA, indicating that the HA molecules of these two subtypes must share many related structural features, although there is less than 25% sequence similarity between H1 and H3. Moreover, Caton et al (1982) suggest that the presence of carbohydrate moieties at certain sites could result in some of the differences in antigenicity observed between H3 and H1.

Recently, Nestorowicz et al (1985a) used a panel of anti-HA monoclonal antibodies to test for binding to different physical forms of the H3 HA molecule. The HA used for binding was in three forms--as the trimer, as the monomer and as a reduced and alkylated form. Their results indicated that antibodies, including neutralizing antibodies to important antigenic sites, react with conformational determinants, some of which result from the interface of regions between monomer subunits in formation of the HA trimer (i.e., site D).

A number of groups have synthesized short peptides corresponding to sequences in the HA molecule, induced antibody formation to these peptides and tested the ability of these antibodies to react with the HA molecule (Jackson

et al, 1982; Muller et al, 1982; Green et al, 1982; Nestorowicz et al, 1985b). The peptides aimed to mimic regions of HA that form important antigenic sites of the molecule. The results varied. Nestorowicz et al (1985b) used eight peptides varying in size from 9 to 25 amino acids, but found only a few to be immunogenic, and only one induced antibody that bound to intact virus. In contrast, Green et al (1982), reported that 18 of 20 peptides representing 75% of the HA1 molecule, could elicit antibodies that bound to HA. However, antibody raised to the intact HA did not bind to any of the peptides.

HA as the major antigen of influenza viruses possesses both specific and cross-reacting determinants (Kilbourne, 1976; Noble et al, 1977). With conventional serologic methods (e.g., HI) significant cross-reactivity is limited to strains within a subtype although minor cross-reactions can be found (Kilbourne, 1976). Russ et al (1978) found that rabbits immunized with either intact or purified HA produced antibodies reactive with either HA1 or HA2, and that some antibodies to the HA2 showed a limited amount of cross-reactivity. In particular, serum against two heterovariant H1 (A/NWS/33 and A/FM/50) viruses reacted with an HA2 purified from a virus containing H3 HA, but serum raised against another H1 heterovariant (A/Bel/42) or an H2 virus (A/Singapore/157) did not react. Using purified HA1 or HA2 in radioimmunoassay, it was observed that serum raised against H1N1 virus contained two popu-

lations of antibody binding to HA1 or HA2 (Graves et al, 1983). The HA2-specific antibodies to H1 HA had some homotypic cross-reactivity (i.e. to H3 HA). This observed cross-reactivity can be explained by the sequence similarity of HA genes; HA2 subunits of influenza A viruses are highly conserved (Krystal et al, 1982; Skehel et al, 1980).

Similar approaches to those described above are being used to study the antigenic properties of NA (Air et al, 1985; Colman et al, 1983). Variation in amino acid sequence has been observed to occur in regions which form a nearly continuous surface at the top of the subunit (Air et al, 1985; Colman et al, 1983). The catalytic site which is a large pocket of the distal surface appears to be surrounded by variable regions (Colman et al, 1983). Studies with monoclonal antibodies to laboratory derived mutants showed that these variable areas are also antigenic (Biddison et al, 1977).

Internal proteins of influenza virus have not been examined to the same extent as HA and NA. Van Wyke et al (1980) tested five monoclonal antibodies reactive to different NP determinants on a large panel of viruses of different serological subtypes (infecting humans and animals) isolated between 1930 and 1978. One antibody detected an apparently invariable region while the other four detected variable regions. These authors concluded that point mutations and genetic reassortment contributed to the antigenic variability of NP but that these changes

occurred independently of those in the surface glycoprotein. Similar findings have been made for M protein (Van Wyke et al, 1984). Monoclonal antibodies specific for M were reacted with five influenza A strains. Two antigenic sites showed antigenic variation, whereas the third appeared to be invariant. At least two of the three antigenic sites were in nonoverlapping domains. Bucher et al (unpublished manuscript) using PR8 M-protein purified from a reassortant virus generated a panel of 18 monoclonal antibodies reactive to three different antigenic sites.

T cell recognition: T helper cells

The T cell receptor, in contrast to the immunoglobulin (Ig) receptor on B cells recognizes antigen only in association with self-MHC antigen. (Benacerraf and McDevitt, 1972; Zinkernagel and Doherty, 1979). One of the important findings emerging from studies using protein antigens and oligopeptides to induce T and B cell responsiveness is that different epitopes activate T and B cells (Maizels et al, 1980; Berzofsky, 1983). Thus, B cells reactive to lysozyme (Streicher et al, 1984; Allen et al, 1985; Maizels et al, 1980), influenza A virus HA (Katz et al, 1985), myoglobin (Streicher et al, 1984; Berzofsky, 1983) and for the pre-S region of Hepatitis B surface antigen (Milich et al, 1986) utilize determinants different from those of T cells stimulated with the same antigen. Although T and B cells have different reactivities, the determinants recognized by either can be overlapping or

adjacent on the protein molecule (Milich et al, 1986).

The requirement of T helper (T_h) cells for the induction of specific immunity to influenza virus proteins has been well documented (Virelizier et al, 1974; Burns et al, 1975; Anders et al, 1979). A T_h cell function is needed for the production of neutralizing antibodies during both primary (Virelizier et al, 1974, Burns et al, 1975) and secondary (Anders et al, 1979) immune response. Immunization with whole influenza virus gives rise to populations of MHC-restricted T_h cells in both humans and mice directed toward the major surface glycoproteins, HA and NA and the internal proteins M and NP (Hurwitz et al, 1984; Hackett et al, 1985). Two major groups of T_h cells have been described, one that recognizes antigenic determinants common to all influenza A viruses and the other that is reactive only to determinants within a given subtype (Lamb et al, 1982). Analysis of a panel of human T helper clones induced with influenza A virus revealed that approximately 50% were subtype cross-reactive with the majority of these clones reactive with M or NP (Lamb et al, 1982). Comparable results have been obtained in mice, where, at the clonal level 58% of the T_h hybridomas were reactive to M or NP and were subtype cross-reactive, whereas the HA and NA specific hybridomas were subtype specific (Hurwitz et al, 1985). The specificity of the cross-reactive T_h population is not limited to internal components of the virion. It has been shown that both

subtype specific and cross-reactive T cells can be stimulated by both HA1 and HA2 polypeptide chains of the HA molecule, but that recognition of antigenic determinants on HA2 are primarily involved in subtype cross-reactivity (Katz et al, 1985). Although, an invariant epitope in HA1 capable of eliciting cross-reactive T_h has been identified using synthetic oligopeptides (residues 306-329 on H1 of PR8) (Lamb et al, 1982; Green et al, 1982). This region is distant from the four proposed antibody binding sites involved in viral neutralization. Similarly, Hurwitz et al (1984) raised several HA-specific T-cell hybridomas and tested their ability to respond to a panel of PR8 virus mutants with known amino acid changes. Their results indicated that 1) immunization with influenza virus elicits HA-specific T cells that recognize different determinants than those seen by HA-specific B cells, 2) there are three major T cell determinants on the HA1 polypeptide, one corresponding to the same region described by Lamb et al (1982).

Interestingly, both human and murine T_h cells specific for internal components of the virion are able to collaborate with B cells in the production of HA specific antibody (Russell and Liew, 1979, 1980; Lamb et al, 1982; Scherle and Gerhard, 1986). This phenomenon has been termed "intermolecular/intrastructural" help (Lake and Mitchison, 1976). Studies by Scherle and Gerhard (1986) have shown that T_h cells specific for the internal

proteins M and NP are as effective in vivo as an HA specific T_h clone in providing help for HA specific B cells during a primary response. This study also showed evidence that the observed help followed the rules of a "cognate interaction" (i.e., direct T-B interaction) and therefore, supports the concept of intermolecular/intra-structural T-B interaction in the anti-influenza response. A possible mechanism for this phenomenon is that HA specific B cells capture influenza virus particles via their surface Ig, but after internalization and processing present the various processed protein components (e.g. M NP) of the virus (Johansson et al, 1987c).

T cell recognition: Cytotoxic T cells;

Cytotoxic T cells, like T_h cells, differ from B cells in the requirement for antigen presentation in conjunction with self-MHC gene products. Shortly after demonstration that cytotoxic T cells (CTL) could be generated against influenza virus (Yap and Ada, 1977) several groups showed that CTL raised to one A strain of virus could lyse target cells infected with any A strain, but not with a B strain (Doherty et al, 1977; Zweerink, Askonas, Millican et al, 1977; Braciale, 1977). Early studies indicated that CTLs generated during the course of viral infection of mice show two types of specificity, one, specific to the stimulating virus and another, broadly reactive with other influenza subtypes (Braciale, 1977). Subsequently, limiting dilution analysis showed that the

majority of CTLs were "cross-reactive" (Owen et al, 1982). Work with influenza virus infected mice showed that CTL's can be divided into three groups; 1) those specific for HA of the stimulating virus, 2) those which recognize HA of all viruses within a subtype, and 3) those which recognize targets infected with any A strain virus (Braciale et al, 1981; Koszinowski et al, 1980).

It had been thought that CTL's would predominantly recognize surface glycoproteins and particularly HA in view of the importance of this antigen in viral attachment to cells. There can be little doubt that in both murine and human studies HA is recognized by CTL, but the evidence suggest 1) about 10%-15% CTL precursor cells recognize HA (Owen et al, 1982; Andrew et al, 1986) and 2) there is some cross-recognition between H1 and H3 (Kozinowski et al 1980; Braciale et al, 1981). The precise locations of the subtype specific and cross-reactive epitopes are not known. Wabuke-Bunoti and Fan (1983) reported that HA2 could induce a weak subtype specific secondary CTL response. Later work by Kuwano et al (1988) showed that HA2 polypeptide of H1 HA elicited CTL's capable of recognizing a cross reactive epitope on H1 and H2 subypes, but not on H3.

It is not clear whether or not viral NA is a CTL target molecule. There is a report suggesting that NA is a target but it does not unquivocally demonstrate that NA is recognized by CTL (Sterkers et al, 1985). Recent work has shown that enzymatically active NA can interfere with

sensitization of CTL to influenza virus protein (Yewdell et al, 1988; Hosaka et al, 1988). More work is necessary to elucidate the role NA plays in CTL recognition.

Several groups have carried out experiments investigating the CTL recognition of influenza virus proteins other than the surface glycoproteins. Kees and Krammer (1984) using limiting dilution analysis of early responses to reassortant viruses found that up to 90% of CTL in C57Bl/6 mice recognized internal proteins. Published literature indicates that of the influenza viral proteins HA (Ennis et al, 1981; Braciale et al, 1986), possibly NA (Sterkers et al, 1985), NP (Yewdell et al, 1985; Townsend and Skehel, 1984), M (Gotch et al, 1987) PB1, PB2, PA and NS1 (Bennink et al, 1987) are recognized by CTLs. Yet recent work has shown that the reactivity of CTLs to a given protein varies among individuals (Pala et al, 1986) and between mouse strains possessing different haplotypes (Bennink et al, 1987; Doherty et al, 1978; Pala and Askonas, 1985). Yewdell et al (1985) using murine target cells infected with a recombinant vaccinia virus containing the NP gene from PR8, reported that a major proportion of cross-reactive CTL in BALB/c mice recognize NP, but that NP was not the sole target antigen recognized by cross-reactive CTLs. Determination of CTL precursor frequency by limiting dilution analysis revealed that about 30% recognize NP (Andrew et al, 1986). There are two classes of NP-specific CTL, one which is cross-reactive

among all A strains and another which can distinguish NP between two groups of influenza A viruses, isolated between 1934-1943 and 1943-1979 (Townsend and Skehel, 1984). Townsend et al (1984), using mouse L cells transfected with a series of deletion mutants of the influenza virus NP gene, showed that human NP-specific CTL recognized three determinants on NP that are short overlapping fragments. These determinants were further defined by reactivity to short synthetic peptides (Townsend et al, 1986).

Antigenic Variation

Two distinct type of antigenic variation occur in influenza viruses, antigenic drift and major antigenic shifts. Antigenic drift consists of relatively minor alterations at the genetic level that result in gradual changes in the surface glycoproteins. Antigenic shift involves major changes in HA and/or NA.

Antigenic drift in both HA and NA occurs by an accumulation of point mutations, that result in amino acid sequence changes. In the case of H3 HA, heterovariants isolated between 1968 and 1977, most changes occurred in the HA1 molecule, with only three changes in HA2 (Both et al, 1983). A compilation of various viral protein sequences showed that HA and NA are most susceptible to antigenic drift, having amino acid substitutions at a rate of 0.97% amino acids/year and 0.91% amino acids/year, respectively, whereas NP and M drifted more slowly at 0.18%

amino acids/year and 0.07% amino acids/year, respectively (Huddleston and Brownlee, 1982). In nature, HA and NA apparently undergo relatively independent antigenic variation (Schulman and Kilbourne, 1969). Drift can be mimicked in the laboratory by growing virus in the presence of monoclonal antibody. Variants of both HA and NA that do not bind the selecting antibody appear at a frequency of about 10^{-5} (Portner et al, 1980). The change in antigenicity was found, in most cases, to be associated with a single amino acid change, and, as with naturally occurring HA mutants, these changes were clustered in the four known antigenic sites in HA1 (Gerhard et al, 1981; Caton et al, 1982). Similar studies to select NA variants have also shown single amino acid changes that are clustered in or near the major antigenic sites (Air et al, 1985; Laver et al, 1982). When tested with polyclonal antisera, antigenic variants with only one amino acid change seems to have little effect on the total antigenic properties of HA or NA (Air, Laver, Webster, 1987) suggesting that a significant change from an epidemiological viewpoint requires changes in two or more sites (Cruse and Lewis, 1987). Possibly, mutations occur sequentially during the spread of a virus, so that a change in only one epitope can give some selective advantage.

Two mechanisms have been proposed to explain antigen shift. First, it has been shown that genetic reassortment between different human viral strains (Palese and Young,

1982) or between human virus and animal or avian influenza viruses (Webster et al, 1971) can occur in vivo. The sudden replacement of H2N2 by H3N2 in 1968, can be explained by this mechanism. Examination of the A/Hong Kong/1/68 virus revealed that the shift contained 7 genes (including NA) of a human Asian (H2N2) strain (Scholtissek et al, 1978a) and an HA gene with strong sequence similarity (96%) and antigenically related to A/Duck/Ukraine/63 (Fang et al, 1981).

A second explanation for the origin of pandemic strains is that the "new" virus is actually the "recycling" of an earlier strain that remained hidden and unchanged in some unknown place. Evidence for this kind of event was obtained from the appearance of an H1N1 virus in northern China in 1977 that spread worldwide. This "Russian flu" seems to be identical in all genes to a virus that circulated in 1950 (Scholtissek et al, 1978b). Was this virus preserved in a frozen state or conserved in an animal reservoir?

Antibody Response to Influenza Virus

Antibody response to influenza infection has been thoroughly studied in humans and animals, although some observations from animal studies have yet to be shown in man. Nonetheless, much of the data pertaining to the role of antibody in protection and recovery have been derived from animal studies.

Infection or artificial immunization with influenza

virus elicits an antibody response to HA, NA, M, and to a lesser extent NP. The anti-HA response has been best investigated by study of hemagglutination inhibiting (HI) antibodies and neutralizing antibodies. The isotype-specific antibody response to HA using ELISA, has been studied in people immunized with live cold-adapted (ca) influenza virus vaccines (Murphy et al, 1982). A characeristic primary serological response was seen in children experiencing their first influenza infection. Serum IgM and IgG responses occurred in all cases, whereas IgA responses occurred much less frequently and at a lower magnitude. IgA was the major Ig response in nasal secretions and occurred in a majority of cases. IgG responses in nasal secretions were infrequent and of lower magnitude than IgM and IgA responses (Murphy et al, 1982). A secondary antibody response was observed in adults primed by natural infection and subsequently infected with a live attenuated heterovariant virus (Burlington et al, 1983). Serum IgG and IgA responses were seen in most cases; there was a high correlation between serum and secretory IgA responses. IgM responses were infrequently detected in serum and not at all in nasal secretions (Burlington et al, 1983).

The kinetics of serum and secretory antibody response to the HA of intranasally inoculated attenuated influenza A virus was described by Murphy et al (1982) using an ELISA sensitized with purified HA. Serum IgM

response peaked approximately 2 weeks after inoculation and declined by 4 to 7 weeks. The serum IgG response peaked at around 4 to 6 weeks. IgA in both serum and nasal washings peaked 2 weeks after immunization. IgM is found after either experimental immunization or natural infection with influenza virus but IgM responses during reinfection occur variably, presumably related to the degree of antigenic variation between succeeding viruses (Gonchoroff et al, 1982). Serum HI titers gradually decrease over the first 6 months subsequent to infection, then may persist for several years. Succeeding infections by closely related virus strains boost immunity to shared epitopes (Couch and Kasel, 1983). Francis et al (1953) recognized that immunologic response in influenza virus infection was directed towards the initial priming antigens experienced during first infection ("original antigenic sin"). Therefore, in subsequent infections, antigenic determinants shared by the original and reinfecting virus will stimulate a predominant, cross-reactive secondary antibody response, whereas new determinants in the reinfecting variant induce a primary response (Virelizer et al, 1974).

Antibody response to viral NA has not been as thoroughly investigated as antibody response to HA. Antibody to NA can be detected in serum and nasal secretion after immunizations or infection with influenza virus (Murphy et al, 1982; Slepushkin et al, 1971; Schild, 1969; Kilbourne et al, 1968). Using neuraminidase inhibition

(NI) assay and ELISA, antibody to NA can be detected in serum during both primary and secondary infections and may persist for many years (Murphy et al, 1982). The dynamics of anti-NA antibody formation and duration in serum and in nasal secretions are similar to those for the anti-HA response (Hruskova et al, 1976). Antibody responses to M protein and NP have also been found after immunization with influenza virus (Webster and Hinshaw, 1977; Schild, 1972; Johansson et al, 1987a). There are no reports of antibody response to minor viral proteins subsequent to infection although polyclonal antisera and monoclonal antibodies to the polymerase (Shaw et al, 1982; Young et al, 1983), NS1 and NS2 (Greenspan et al, 1985) proteins have been prepared in animals.

Antiviral Activity of Antibody Response

Antibody directed against influenza virus HA can be neutralizing. Presumably, anti-HA antibody exerts its effects by preventing attachment of the virus to host cells (Davenport et al, 1964) or by possibly interfering with the fusion event subsequent to endocytosis (Yoden et al, 1986; Kida et al, 1983). Antibody to NA exerts its effects at a later stage of multicycle infection and is therefore infection-permissive over a broad range of antibody concentrations (Schulman, 1969; Kilbourne et al, 1975). Anti-NA antibody may cause: 1) steric inhibition of enzyme function, thereby delaying viral release from the host cell: 2) cross-linking virions resulting in aggregation of

viral particles, thus reducing the number of effective infecting units: 3) cementing of free viral particles to host-cell associated NA antigen: or 4) inhibition of final detachment of the budding virion (Kilbourne et al, 1975).

Cell Mediated Immunity to Influenza Virus

Specific cell-mediated immunity to influenza antiens has been demonstrated in man (Chow et al, 1979) and in mice (Reiss and Schulman, 1980). It has been shown that functional T cells are required for production of antibody to influenza virus proteins (Virelizer et al, 1974; Anders et al, 1979; Burnes et al, 1975) and that infection is more protracted in their absence (Wells et al, 1983). Depletion of $L3T4^+$ T_h cells in mice resulted in suppression of the formation of antibodies to influenza virus and a reduction of influenza virus specific CTL activity (Lightman et al, 1987). This latter finding confirms studies that show T_h cells play a role in the generation of influenza virus specific CTL response (Reiss and Burakoff, 1981). In further studies, Lightman, et al (1987) transferred $Lyt2^+$ or $L3T4^+$ T cells sorted from spleens of influenza virus infected mice to athymic recipient mice. The $L3T4^+$ T cells were not as effective as Lyt2 cells in clearing virus from the lungs of recipient mice infected with influenza virus. $Lyt2^+$ CTL have been shown to protect against lethal infection of mice and function to clear virus from lungs of infected animals (Lin and Askonas, 1981; Taylor and Askonas, 1983).

A significant proportion of CTLs and T_h cells recognize internal proteins (Johansson et al, 1987c; Gotch et al, 1987; Hurwitz et al, 1985; Kees and Kramer, 1984) and do not discriminate between different influenza virus strains (Yewdell et al, 1985; Zweernink et al, 1977; Koszinowski et al, 1980).

Vaccination Against Influenza

Disease caused by influenza virus infection is not a trivial matter. In the United States influenza can kill 10,000 people in a non-pandemic year and 30,000 people after an acute epidemic (Frank et al, 1985). Additionally, in terms of physician/hospital expenses and the time away from work, the dollar cost to the American economy is immense. The Surgeon General of the United States Public Health Service estimated that at least 43 million Americans are at risk of death from influenza by virtue of their underlying medical illness and should be immunized (Mostow, 1986). Persons over 60 years of age have an increased case-fatality rate for influenza, probably due to waning immunity with age and complicating cardiac or pulmonary disease. As the proportion of elderly persons increases so will the need for control of influenza. Additionally, pregnant women, asthmatics, diabetics, and patients receiving immunosuppressive drugs for organ transplants or neoplastic diseases are at high risk for severe influenza.

Influenza A virus infection remains a major public health problem in spite of the availability of specific

whole virus influenza vaccines. Traditional influenza A vaccines comprise inactivated or attentuated viral strains which possess the prevalent HA and NA antigens. Antibody to both major surface antigens can be found in the serum of individuals immunized with these vaccines (Murphy et al, 1982; Couch et al, 1971). When these vaccines are effective illness is prevented usually by preventing infection (Couch et al, 1971; Kilbourne, 1978). These conventional vaccines are effective only if the HA of the vaccine strain is closely matched in antigenic structure to the expected wild type strain HA. Furthermore, immunity produced in this way is of short duration (Couch et al, 1974); consequently, annual vaccination may be necessary to perpetuate immunity.

Inactivated Virus Vaccines

Inactivated virus vaccines are prepared from the allantoic fluid of virus-infected eggs. Virus is purified and concentrated by zonal centrifugation or chromatography. Purified virus can be inactivated by a number of procedures including treatment with formalin, β -propionolactone or U.V. irradiation. Whole-virus vaccines contain intact inactivated virus. Split-product vaccines are prepared from purified formalin-treated virus disrupted with organic solvents or detergents to solubilize the surface glycoproteins. The principle value of split-product vaccines is their low toxicity. Virtually all contemporary inactivated influenza vaccines are derived from reassortant viruses

(Kilbourne, 1987).

Questions have been raised concerning the immunogenicity and efficacy of inactivated virus vaccines. Additionally, concerns about inactivated virus vaccines include potential toxicity, possible escape from inactivation, brevity of immunity, and the presentation of influenza virus antigens by an abnormal route (i.e. parenteral injection). Sufficient data are now available to make an assessment of the immunogenicity of inactivated virus vaccines (Hirst et al, 1942; Salk, 1948; Potter, 1982; Murphy and Chanock, 1985). Parenteral administration of inactivated virus vaccine can induce antibody formation in man (Chenoweth et al, 1936) and mice (Balkovic and Six, 1986). In primed individuals, (e.g. people with serologic evidence of exposure to H3N2 influenza virus) parenteral vaccination with either an H3N2 whole virus vaccine or split-product vaccine induced "protective" levels of HI antibody, which prevented infection in 89% of recipients shortly after vaccination. In contrast, in unprimed recipients of whole-virus vaccine, only 65% developed protective levels of serum HI antibody (Murphy and Chanock, 1985). Similar findings were reported on the serologic responses to H1N1 virus (A/NJ/70). Wise et al (1977) observed that in general, levels of HI antibody were low after a single dose of vaccine but increased significantly in response to a second dose. It was noted, however, that whole-virus H1N1 vaccines prepared by Merck,

Sharpe and Dohme were effective after a single dose of vaccine, whereas two doses of whole-virus vaccine prepared by Merrell-National Laboratories were required to produce an equivalent serologic response. These different serologic responses correlated with the viral HA content of each vaccine. Additionally, split-virus vaccines were less immunogenic than whole-virus vaccines, especially in younger vaccinees (Wise et al, 1977; Meiklejohn et al, 1977). Other studies showed no difference between these two vaccine types (Meiklejohn et al, 1977). Results from several studies demonstrate that the serologic response to influenza virus vaccine varied according to the age of recipient and previous exposure to influenza virus (Wise et al, 1977; Meiklejohn et al, 1977; McLaren et al, 1977; Gonochoroff et al, 1982). Results of studies in mice are concordant with those found in humans. That is, split-virus and whole-virus vaccines were less immunogenic in unprimed subjects, however, the immunogenicity of both types of vaccine were enhanced in subjects primed by previous influenza virus infection (McLaren et al, 1977).

Vaccination with conventional whole-virus or split-virus vaccines can also induce an antibody response to the viral NA (Couch et al, 1971; Kendal et al, 1977; Kilbourne et al, 1971). In one study, a single dose of H1N1 vaccine induced NA antibody in 20% of children less than 18 years old (Kendal et al, 1977). In immunologically primed adults, the NA antibody responses to a single dose of H1N1

or H3N2 vaccine were 38% and 54%, respectively. 70% of the children given two doses of H1N1 vaccine had significant NA antibody levels, but only 48% of the children given two doses of H3N2 vaccine produced antibody to NA (Kendal et al, 1977). Studies with a reassortant influenza A vaccine (X-31: A/Aichi/2/68[R]) showed that the reassortant vaccine was as effective as a "standard" influenza vaccine. In two separate studies, 87%-100% of the X-31 vaccinated volunteers developed a significant rise in antibody to either NA or HA (Kilbourne et al, 1971; Couch et al, 1971).

The duration of serum HI antibody response after vaccination varies according to the recipient's prior antigenic experience. Primed subjects retained protective levels of antibody for approximately one year, whereas antibody levels decline rapidly in unprimed subjects (Jones and Ada, 1987).

It has been shown that T_h cells are stimulated in response to influenza virus vaccine (Chow et al, 1979). However, several groups have shown that inactivated virus is not as effective as infectious virus in induction of CTL (Braciale and Yap, 1978; Webster and Askonas, 1980). Differences in the immunogenicity of inactivated virus vaccines with respect to ability to stimulate the host for a CTL response, have been related to different methods of inactivation (Ada et al, 1981; Rouse et al, 1988). In studies with mice, virus inactivated by U.V. irradiation or formaldehyde induced a poor primary CTL response, and was

relatively ineffective in priming mice for a secondary CTL response (Ada et al, 1981; Webster and Askonas, 1980). Virus inactivated by gamma-irradiation stimulated a cross-reactive primary CTL response (Wraith and Askonas, 1985) and a secondary CTL response (Owen et al, 1988) in mice. In primed humans, inactivated virus stimulated a cross-reactive CTL response (McMichael et al, 1983). The ability of inactivated vaccine to stimulate a CTL response in unprimed humans has not been determined.

The protective efficacy of inactivated vaccine against influenza virus infection has been 70-90% in studies of vaccinated military personnel, but protection of persons in high risk categories (e.g., the elderly) has been variable (0-80%) (Barker and Mullooly, 1980; Keitel et al, 1988; WHO Memorandum, 1987). In view of antigenic drift, how effective is inactivated vaccine in induction of protection against challenge with a heterovariant virus? This question was addressed in a study by Hoskins et al (1979), who showed that the first vaccination of young school boys with a prevailing influenza virus strain provided protection against that strain, but that the next annual vaccination with a subsequent variant did not induce protection against the second strain. The cumulative influenza attack rate was the same for unvaccinated boys as for vaccinated boys, however, disease in vaccinated boys was shifted toward later epidemics (Hoskins et al, 1979).

Live attenuated virus vaccines

Live attenuated influenza vaccines are being studied in several countries, however, the World Health Organization has not formulated recommendations on their routine use (WHO Memorandum, 1987). Live attenuated vaccines have a theoretical advantage over inactivated vaccines in that they mimic natural infection and therefore, could produce a longer lasting immunity.

A live vaccine virus must be attenuated and not produce clinical illness in vaccinees or their contacts; the virus should also be infectious and induce a protective immunity; furthermore, the vaccine virus should be genetically stable. There are four main approaches for the production of live attenuated influenza virus vaccine. They are: 1) generation of virus "de-adapted" to man by passage of the virus in a non-human host (e.g., chick embryo); host range mutants can be derived by use of this strategy; 2) selection of viruses with specific genetic markers for viral attenuation. Certain markers such as temperature sensitivity (ts) and cold-adapted (ca) mutants would restrict viral growth to the relatively cooler upper respiratory tract (Jones and Ada, 1987); 3) recombine naturally occurring avian influenza viruses attenuated for replication in non-humans with human influenza viruses, with the expectation that genetically stable attenuated reassort-ants would emerge (Murphy et al, 1984; Clements et al, 1986); 4) use of genetic engineering techniques to

create attenuated mutants containing lesions or deletions in one or several genes.

Of course, with any of the approaches mentioned above, the biological markers associated with attenuation could be transferred reliably by genetic reassortment from a "master" attenuated influenza strain to a strain containing the prevailing HA and NA. The ability of influenza viruses to genetically reassort also represents a major problem with the use of live virus vaccine, in that reassortment with other viral strains could result in escape from attenuation.

Clinical studies in man suggest that live influenza A vaccines can afford protection from disease, however, the type and amount of protection afforded varies with the sort of live virus agent used (Jones and Ada, 1987; Clements et al, 1986; King et al, 1987). Recently, a study in children comparing protective efficacy of ca-mutants with inactivated influenza vaccine has shown that ca-mutants can induce an immunity equivalent to that induced by conventional inactivated vaccine (King et al, 1987). However, in another study the ca-mutant was 100-fold less efficient than its inactivated counterpart in the induction of influenza virus-specific antibody secreting cells (Jones and Ada, 1987). Clements et al (1986) evaluated an avian-human reassortant influenza in adult volunteers. The avian-human reassortant influenza virus, like its avian influenza A parent, (A/Pintail/119/79) was restricted 100-fold in replication compared with wild-type human influenza A virus human

influenza A virus (A/Washington/897/80). Despite this restriction of replication, infection of human volunteers with the reassortant virus induced resistance to an antigenically homologous wild-type human influenza A virus. However, difficulty in confirming attenuation and genetic instability may restrict this approach. The ts mutant strains have not been used directly as vaccines but have contributed genes with ts defects to mutant reassortant viruses (Murphy et al, 1972). Although influenza A reassortant viruses bearing ts genes were shown to be attenuated, their genetic instability, leading to reversion to virulence, has precluded further use.

New approaches to influenza virus vaccine development include: 1) the synthesis of the appropriate oligopeptides; 2) the synthesis of influenza virus proteins in transformed prokaryotes or eukaryotes; 3) the production of anti-idiotype antibodies; and 4) the use of infectious organisms as vectors of influenza viral genes.

Green et al (1982) demonstrated that antibodies could be formed against synthetic peptides representing various epitopes on the HA1 molecule and that such antibodies would bind to the HA molecule. As yet, immunization with synthetic oligopeptides has been only marginally effective in protecting experimental animals (Shapira et al, 1984). Additionally, Hackett et al (1985) described a synthetic decapeptide made from influenza virus HA that elicits T helper cells with the same fine recognition specificities

that occur in response to the whole HA molecule. There remains a large gap between these experimental results and what would be required to form an effective vaccine.

DNA copies of all eight influenza virus genomic RNA's have been cloned into E. coli. (reviewed by Palese and Kingsbury, 1983). Proteins made in these transformed bacteria have been produced in high levels. However, these bacterial products often elicit antibodies different from these elicited by the native protein or virus, possibly because of differences in glycosylation (Nayak et al, 1984). Better results have been obtained with mammalian (Gething and Sambrook, 1984) and insect (Kuroda et al, 1986) cells transfected with the cloned HA gene. The product from either mammalian or insect cells was immunogenic and induced a protective immunity in experimental animals. The use of these products as vaccines has not proceeded very far.

Newly proposed anti-idiotype or internal image vaccines (Dreesman and Kennedy, 1985; Bona and Moran, 1985), like those incorporating synthetic oligopeptides, would have the potential for low toxicity and antigenic specificity. Presently, there are no anti-idiotype vaccines developed as influenza virus vaccines. But recent work has shown that monoclonal antibodies recognizing an idiotype on anti-NA antibodies increased anti-NA response when injected prior to infection (Mayer et al, 1987).

A fourth approach involves the use of infectious agents, such as existing viral vaccines, as vectors of DNA

coding for influenza virus proteins. Three viruses have been considered: 1) vaccinia (Smith et al, 1983; Panicali and Paolietti, 1982), herpes (Roizman and Jenkins, 1985) and adenovirus (Davis et al, 1985). Most work has been done with vaccinia. DNA coding for all influenza virus structural proteins and the three nonstructural proteins have been inserted into vaccinia (Bennink et al, 1987; Andrew et al, 1987). Recombinant vaccinia virus vectors containing NP or HA have induced a primary antibody response and primed for a CTL response in mice which was not protective (Andrew et al, 1986). Use of these vectors as vaccines for human requires a great deal of development.

The usual object of immunization against influenza virus is complete inhibition or restriction of infection. Another strategy is infection-permissive immunization. The basic premise of this approach involves immunization with viral proteins that do not elicit neutralizing antibody but do restrict viral replication, thereby allowing infection but not disease (Kilbourne et al, 1968). In the case of influenza virus, antibodies directed against the viral NA are not neutralizing but can reduce viral replication below a pathogenic threshold (Kilbourne et al, 1968; Couch et al, 1974). Preliminary vaccine studies in humans (Beutner et al, 1979; Couch et al, 1974; Murphy et al, 1972) and mice (Schulman, Khakpour and Kilbourne, 1968; Schulman, 1969) have shown that anti-NA immunity induced either by a reassortant antigenically hybrid virus vaccine or purified

NA preparation, protected against disease from a virus containing antigenically homologous NA. Viral challenge resulted in the development of fully protective immunity capable of preventing subsequent infection. Studies are in progress to determine the feasibility of using NA-specific infection-permissive vaccines in humans.

Antigenic Competition Between Hemagglutinin and Neuraminidase

Both hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins of influenza A virus are immunogenic and are the antigens primarily involved in induction of specific immunity to influenza. HA is generally the superior immunogen, inducing antibody that directly neutralizes virus infectivity; antibody to the NA, although not neutralizing, limits viral replication in multicycle infections (reviewed by Kilbourne, 1975). "NA specific" immunization can be carried out with antigenically hybrid viruses that contain the HA of an animal strain irrelevant to human experience. Comparative studies of such "NA-specific" and conventional vaccines in populations primed to prevalent influenza virus subtypes have shown that H7N2 and H7N1 reassortant vaccines (NA-specific) evoke greater NA antibody response than H3N2 vaccines equivalent in NA immunogenicity (Kilbourne, 1976; Beutner et al, 1979; Chow et al, 1979). Diminished NA antibody response in HA primed populations has also been remarked by Kendal et al (1977).

The first observation of this phenomenon was in groups

of college students with serologic evidence of experience with H3N2 viruses, who then received either a conventional H3N2 vaccine (A/England/42/72), an antigenic hybrid H7N2 virus vaccine (A/Equine/Prague/56 x A/England/42/72) or a placebo injection (Kilbourne, 1976). All subjects had antibody to N2 before immunization and the mean initial HI and NI titers were comparable in both vaccine groups. Yet, mean antibody response to N2 was two-fold greater in those vaccinated with the H7N2 reassortant virus vaccine. In a similar study, school age children were immunized with an H3N2 (A/Port Chalmers/73) vaccine, a NA-specific H7N2 reassortant virus vaccine (A/Equine/Prague/56 x A/Port Chalmers/73) or a placebo (Beutner et al, 1979). Before vaccination, all of the children were seronegative for H7, 80% were seronegative for H3-Port Chalmers, and 90% were negative for N2-Port Chalmers. Vaccination induced seroconversion for the Port Chalmers HA and NA specific antibody in children receiving the conventional vaccine, while the NA specific vaccine induced only N2-Port Chalmers specific antibody. Two natural outbreaks of H3N2 influenza A virus infection occurred during the study period: one caused by the Port Chalmers strain in the winter of 1974-75; the other by the Victoria strain in the winter of 1975-76. Analysis of antibody titers after infection with the Victoria strain indicated that uninfected children had relatively high titers of anti-H3 Port Chalmers and anti-N2 Port Chalmers specific antibody regardless of the vaccine they had

received. It was observed that the antigenically hybrid H7N2 virus vaccine induced a greater frequency and magnitude of response to the NA antigen. Children who were asymptomatically infected with the Victoria strain had mean N2-Port Chalmers specific NI titers three- to five-fold greater than those symptomatically infected. In fact, children vaccinated with H7N2 demonstrated significantly higher N2 Port Chalmers NI antibody titers than children who received H3N2 vaccine, both before and after exposure to the Victoria strain. All groups demonstrated equivalent anti-H3 Victoria HI antibody titers. These data support the role of NA-specific immunization in protection against influenza. Furthermore, cell-mediated immunity measured by lymphocyte transformation assay showed that blood lymphocyte cultures from children immunized with H7N2 virus vaccine proliferated to two and one half-times greater number than cells from H3N2 virus vaccinees in response to <u>in vitro</u> stimulation with N2 antigen (Chow et al, 1979). The children were initially seronegative or manifested low titers of H3-Port Chalmers or N2-Port Chalmers antibody activity before immunization. The H7N2 vaccine induced no increase in H3 antibody but all subjects demonstrated an increase in N2-Port Chalmers antibody titers after immunization. Vaccination with the conventional (H3N2) vaccine resulted in seroconversion for H3-Port Chalmers and N2-Port Chalmers in all subjects, but the NI antibody titers in the H3N2 vaccine group were four- to ten-fold less than in the H7N2 vaccine

group despite, the two-fold higher NA activity of the H3N2 vaccine (Chow et al, 1979).

Recently, the immunologic response to influenza virus NA was examined in initially primed and unprimed vaccinees in whom vaccination with a conventional H1N1 vaccine or H7N1 antigenic reassortant vaccine was followed by natural infection with an H1N1 influenza virus (Kilbourne et al, 1987). Both vaccines were only marginally NA immunogenic in initially seronegative subjects, yet in vaccinees initially seropositive for H1, significant titer increases occurred with H7N1 vaccine. Furthermore, subsequent infection with an H1N1 influenza virus boosted NA antibody less effectively in the initially infection-primed vaccinees than in the initially seronegative subjects primed by vaccination. Kilbourne et al (1987) concluded that prior experience with influenza virus HA has a suppressive influence on immune response to NA. This is consistent with an earlier proposal that the superiority of the NA specific vaccine (H7N2 or H7N1) as an immunogen for antibody to NA might reflect different processing of NA when it is associated with a hemagglutinin to which the study population had not been primed, presumably because subjects were primed to the HA (H1 or H3) and the anti-HA anamnestic response depresses the concomitant NA response apparently by antigenic competition (Kilbourne, 1976).

Mechanisms of Intermolecular Antigenic Competition

Intermolecular competition with antigen mixtures

apparently is dependent on an activity of T cells. Evidence that T cells are involved in intermolecular competition indicates that antigen competition may be a side effect of T-B cell cooperation (Taussig, 1977; Johansson et al 1987c). Work with synthetic polypeptide antigens has provided evidence that the mechanism of intermolecular competition is in part a T cell effect (Taussig et al, 1972; Taussig et al, 1973). The molecules used were the multi-chain polypeptides:poly-L-(phe-glu)-poly-DL-ala-poly-L-lys [i.e., (phe-G) A-L] and poly-L-(tyr-glu)-poly-L-pro-poly-L-lys [i.e. (T-G) pro-L]. When mixtures of these synthetic polypeptides were injected into C3H/Hej mice, which are high responders to both molecules, the specificity of the ensuing antibody response depended on the relative amount of anti-gens in the mixture (Taussig et al, 1973; Taussig, 1977). When the immunizing mixture contained a molar excess of (T-G) pro-L over (phe-G) A-L the resulting immune response was mainly against (T-G) pro-L; the primary response to (T-G) pro-L was suppressed when the molar ratio was reversed. These polypeptides were used to immunize mice that are genetic low responders to (T-G) pro-L (DBA/1 mice) due to a B cell defect not linked to H-2 (Taussig et al, 1973; Shearer et al, 1972). It was shown that (T-G) pro-L was just as effective in competition against (phe-G) A-L in the low responder strain, DBA/1 as it was in the high responder strain CH3/Hej, despite the low production of pro-L antibody in DBA/1 mice (Taussig et al, 1973). These

results indicate that intermolecular competition between these synthetic polypeptides depends on the presence of T cells specific for the dominant antigen rather than on the amount of specific antibody produced. Furthermore, it was shown that the carrier molecule A-L was as effective as (phe-G) A-L as a competitor against (T-G) pro-L, even though A-L is a very poor immunogen in terms of antibody production. When a mixture containing A-L and (T-G) pro-L with a molar excess of A-L was injected into high responder mice, the response to (T-G) pro-L was inhibited despite the fact that no antibody production to A-L was detectable. There is reason to conclude that A-L is efficiently recognized by T cells since it is able to act as a carrier for haptenic groups (Taussig et al, 1972). Therefore, these results indirectly support a T-cell dependence of intermolecular competition (Taussig et al, 1973; Taussig, 1977). Additional studies with mixtures of rabbit Fc and rabbit Fab' have also been used to study intermolecular competition (Taussig, 1971, 1972). When these fragments were administered into mice in a mixture, in Freud's complete adjuvant, it was found that Fc is the dominant antigen, suppressing the anti-Fab' response provided that Fc was at least in a three to one molar excess to Fab' (Taussig, 1971). When an equimolar ratio of Fc to Fab' was injected, good primary antibody responses were obtained to both fragments. These results are consistent with the data obtained from the studies using synthetic polypeptides (Taussig et al, 1973; Brody and Siskind,

1969). Apparently, the degree and direction of intermolecular competition are dependent on the relative amounts of the immunogens in the mixture.

The exact mechanisms of intermolecular antigenic competition have not been elucidated. However, assuming the genetic precommitment of antigen-sensitive cells, antigens which are not linked together on the same molecule should be recognized separately. Intermolecular competition, on the other hand, implies a common event in the recognition or response to different antigens. Experiments in which competition was abolished in mice rendered tolerant or passively administered antiserum to the dominant antigen (Taussig and Lachman, 1972) lead to the conclusion that antigen recognition is necessary for dominance in competition. In reconciling these ideas, one must invoke a model that contains an antigen processing system through which many antigens must pass, such as the macrophage processing of antigen (Taussig et al, 1972). Some authors argue that this could be a "rate limiting step" of antibody production at which competition between antigens could occur (Brody and Siskind, 1969). However, the results from Fc/Fab competition experiments where animals were tolerized to the dominant antigen (Taussig and Lachman, 1972) are not consistent with such a hypothesis. If processing is regarded as non-specific antigen handling by macrophages after phagocytosis, such a step would have to be missing in tolerant animals, but macrophage function is not affected by

specific tolerance (Mitchison, 1969). A more specific event, and one missing in tolerance must be involved. Taussig and Lachman (1972) argue that antigen competition is the result of an early recognition event of the dominant antigen, which inhibits the early phases of immune induction to the suppressed antigen. Possibly the initial recognition of antigen by T cells on the surface of an antigen presenting cell (APC) blocks the presentation of other antigens. Werdelin found that two non-cross reacting co-polymers containing poly-L-lysine could compete with one another, apparently at the level of antigenic presentation (Werdelin, 1982). Exposure of guinea-pig responder strain APCs to the co-polymer of L-glutamic acid and L-lysine (GL) inhibited the subsequent presentation of dinitrophenylpoly-L-lysine (DNP-PLL) to DNP-PLL primed T cells. Other antigens, not under the same Ir gene control, had no such inhibitory effect. Werdelin concluded that the competitive inhibition observed between GL and DNP-PLL may reflect a competition for the Ir gene product (i.e., Ia antigens) produced by the APC. These results were confirmed by using different synthetic polymers (Rock and Benacerraf, 1983) and peptides of hen egg lysozyme (Babbitt et al, 1985). Some authors argue that sites on macrophages or dendritic cells for antigen presentation could not be so limited that competition for them could occur. If so, their function as a presentation device would be severely restricted. Guillet et al (1986) showed that stimulation of MHC Class II

restricted T cell hybridoma generated against bacteriophage cI protein could be inhibited by peptide analogues. They observed that some T cell hybridomas stimulated by a peptide comprising residues 12-26 (P12-26) differed in reactivity to truncated analogues of P12-26. These results suggest that competition depends on the T cell involved. It may well be that the peptide and the analogues compete for the same site on Class II MHC molecules but the avidity of the T cell receptor for antigen-MHC complexes ultimately determines whether competition occurs. Another possibility is that the recognition of the dominant antigen results in an expansion of a population of T suppressor cells which would act to suppress the response to other antigens (Taussig and Lachman, 1972). This explanation adequately accounts for sequential intermolecular competition (i.e., the suppression of immune response by an antigen given earlier) (Gershon and Kondo, 1971). Intermolecular competition with antigen mixtures can also be explained by the action of antigen-specific T suppressor cells. A stronger antigen, i.e., one for which more immunocompetent cells are available or an antigen given for the second time, could stimulate a sufficient degree of suppressor T cell activity to effectively inhibit the response to the weaker antigens administered at the same time (Taussig, 1977).

Statement of the Problem

It was the goal of this project to investigate the cellular mechanisms of the variable HA-conditioned immunogenicity of viral neuraminidase and the immunological parameters of the apparent antigenic competition between HA and NA.

Establishment of a murine model system, duplicating the antigenic competition observed in man, enabled us to investigate the cellular mechanisms responsible for this phenomemon. In this system, the influences of macrophages, antigenic specific B and T cells and the relative immunogenicity of HA and NA on intravirionic-antigenic competition were assessed.

VI. Immunologic Response to Influenza Virus Neuraminidase is Influenced by Prior Experience with Associated Viral Hemagglutinin: I. Sequential Infection of Mice Simulates Human Experience*

Both the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins of influenza viruses are immunogenic and are the antigens primarily involved in induction of specific immunity to influenza. As the major antigen, the HA induces antibody that directly neutralizes virus infectivity; antibody to the NA, although not neutralizing, limits viral replication in multicycle infection (Kilbourne et al, 1975). Immunization specific for the viral NA therefore is infection permissive and can reduce replication of challenge virus below a pathogenic threshold yet permit sufficient replication for definitive immunization to both viral antigens (Kilbourne et al, 1975; Couch et al, 1974). NA specific immunization can be carried out with antigenically hybrid viruses (Couch et al, 1974; Kilbourne et al, 1967; Kilbourne, 1976) that contain the NA of a human virus together with the HA of an animal virus irrelevant to human experience.

Comparative studies of such NA-specific and conventional vaccines in populations primed to prevalent influenza virus subtypes have shown that H7N2 or H7N1 reassortant (NA specific) vaccines evoke greater NA antibody response than H3N2 vaccines equivalent or greater in NA potency (Kilbourne, 1976; Kilbourne et al, 1987). Diminished

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NA antibody response in HA primed populations has also been remarked by Kendal et al (1977). Presumably the inferior response to NA seen in subjects primed to contemporary human virus HA antigens reflects some sort of antigenic competition of unknown mechanism.

This chapter describes the results of preliminary investigations of the phenomenon in a mouse model system. Having failed in earlier experiments to duplicate the phenomenon observed in man by single infections of mice, we succeeded by priming mice with two sequential infections prior to vaccination with antigenically hybrid and conventional vaccines. Establishment of a mouse model system, in which the antigenic history of the animal is known and precisely controlled has enabled us to investigate cellular mechanisms responsible for the variable immune response to NA.

MATERIALS AND METHODS

Viruses

The strains of influenza A viruses employed in these studies are identified in Table I.

Viruses were grown in the allantoic sac of 10 day old chick embryos and stored in multiple aliquots at -70°C.

Antisera

Antisera used in antigenic characterization assays were obtained by injecting 1024-2048 HA units of whole U.V. inactivated virus i.v. into rabbits. A second injection was given 40 days later and serum specimens were obtained 7 days after the second injection.

Animals

Inbred BALB/c female mice (Jackson Labs, Bar Harbor, ME) 8 weeks of age were used in the infection protocols.

Serologic Methods

Serum specimens were obtained from each mouse by retroorbital bleeding on the days specified. The sera were stored at -20°C. Non-specific inhibitors were destroyed with V. cholerae NA before use in HI or plaque inhibition tests. HI tests were performed by the microtiter method (Sever, 1962) employing the recombinant viruses $H3_{HK}N1_{PR8}$ or $H7N1_{PR8}$. Tests for neuraminidase inhibition (NI), employing the reassortant virus $H1_{PR8}N2_{HK}$, were performed as described by Kilbourne et al (1968) but with the use of an overnight incubation of virus and substrate (Kilbourne, 1976).

Enzyme-linked immunoabsorbent assays (ELISA) were performed (Khan, Gallagher, Bucher et al, 1982) on pooled sera (containing an equal volume of serum from each animal) from each infection group to determine anti-M-protein response. Detergent disrupted reassortant virus, H1N1 (containing A/PR/8/34 M protein) (Kilbourne, 1978a; Palese et al, 1976) or purified M protein (from H1N1) were used to sensitize the test plates (Khan, Bucher, Koul et al, 1982). 400 ng/ml of purified M protein or 1 µg/ml of whole virus were used at 100 µl per well.

To determine the degree of antigenic difference between $H3_{HK}$ and $H3_{PH}$ hemagglutinins, plaque inhibition

titrations were performed in Madin-Darby Canine Kidney (MDCK) cell cultures. Monolayer cultures in 60mm plastic dishes were inoculated with 50-100 pfu of virus. After 30 minutes at 37°C the agar overlay containing the appropriate antiserum was placed on the dishes. Based on 50% plaque reduction, antibody titer ratios were calculated by the method of Archetti and Horsfall (1950) (a higher R value represents a greater antigenic difference between viruses tested). Additionally, HI titrations employing heterologous and homologous H3 antisera were performed and used in calculating antibody titer ratios. The degree of antigenic difference between $N2_{HK}$ and $N2_{PH}$ was determined by NI tests and plaque size reduction (PSR) (Jahiel and Kilbourne, 1966) in MDCK cell cultures as described above.

The presence of virus in the lungs of animals exposed to virus in the aerosol chamber or by intranasal inoculation was demonstrated by methods described previously (Schulman and Kilbourne, 1963a).

Infection Procedures

Aerosol procedure: mice were randomly divided into groups of 15-20 animals and exposed to an estimated 100 MID_{50} of virus. The conditions under which the aerosol procedure was carried out were as described by Schulman and Kilbourne (1963a, 1963b) except that mice were exposed for a total of 45 minutes.

Intranasal inoculation: Mice were lightly anesthetized with ether and 50 µl of live virus or PBS was

instilled intranasally.

Vaccines

Sucrose gradient purified viruses $H3_{HK}N2_{HK}$ and H7N2 used as vaccines were inactivated by U.V. irradiation to a residual EID_{50} of $10^{-1.5}$/0.1 ml then adjusted to equal NA activity (3.0 μM of N-acetyl-neuraminic acid released/24 hrs/100 μl). Mice received 0.2 ml of virus i.p. The vaccine preparations in the Single Infection Protocol contained approximately 15 μg of total protein; vaccine preparations used in the Sequential Infections Protocol contained approximately 2 μg of total protein.

Study Design for Single Infection:

The design and results of the single infection protocol are outlined in Table II. Schedule A provides for single infection with H3N2 virus followed by i.p. injection of U.V. inactivated virus vaccine. Schedule B demonstrates the serologic response to i.p. vaccination. Animals vaccinated with sterile PBS demonstrate the serologic response to infection without vaccination. Injection of B/Lee virus vaccine demonstrates serologic changes due to non-specific effects of vaccination. The H3N2 and H7N2 vaccine groups compare the differences in N2 immunogenicity.

70 eight week old female BALB/c mice were randomly divided into two groups: 40 mice received aerosol exposure to H3N2 virus (Schedule A), 30 mice were "mock" infected with sterile PBS (Schedule B). Three days later 2 mice from Schedule A were randomly chosen and killed for determination

of their pulmonary virus titer. 30 days after exposure to virus aerosol the mice were divided into groups of 10 animals each and received i.p. injections of vaccine. The animals were bled for serum every 7 days for 4 bleedings.

Study Design for Sequential Infections

The design and results for the sequential infections protocol are outlined in Table IV. Both schedules I and II provide for sequential infection with H3 variant viruses to maximize priming to the HA antigen. Schedule I for HA specific priming entails the use of reassortant viruses (Table I) containing neuraminidase antigens N1 and N7 not contained in the vaccines to be used in final boosting. Schedule II employs sequential infection with wild-type H3N2 variants to simulate human experience and prime for both HA and NA antigens. Subgroups a and b compare the effects of H3N2 and H7N2 vaccines, while sub-group c demonstrates the serologic response to sequential infection without vaccination.

The initial infection by aerosol represents our standard procedure for insuring uniform infection of mice with an estimated 100 MID_{50} of virus (Schulman and Kilbourne, 1963a).

The intranasal route was chosen for the second infection to allow for deposition of sufficient virus in both upper and lower respiratory tracts to insure reinfection. Antigenically dissimilar heterovariant H3 viruses were chosen also to favor reinfection and to induce cross

TABLE I. VIRUSES USED IN MOUSE MODEL SYSTEM

Virus Nomenclature			
HANA Subtype	Strain or reassortant designation	Laboratory name	Specific Use
H3N1[a]	A/HongKong/1/68 - PR/8/34(R)	$H3_{HK}N1_{PR8}$	Aerosol infection: Schedule I, sequential protocol
H3N2	A/HongKong/1/68	$H3_{HK}N2_{HK}$	Aerosol infection: Schedule II, sequential protocol: Schedule A, single protocol Vaccine: in both sequential and single infection protocols
H3N7[a]	A/Philippines/2/82 - Equine/Prague/1/56(R)	$H3_{PH}N7$	Intranasal infection Schedule I
H3N2	A/Philippines/2/82	$H3_{PH}N2_{PH}$	Intranasal infection Schedule II
H7N2[a]	A/Equine/Prague/1/56-Aichi/1/68(R)	$H7N2_{HK}$	Vaccine: in sequential and single infection protocol
H3N1[a]	A/HongKong/1/68-PR/8/34(R)	$H3_{HK}N1$	Test virus in HI assay
H1N2[a]	A/PR/8/34-HongKong/1/68(R)	$H1N2_{HK}$	Test virus in NI assay
H1N1[a]	A/New Jersey/11/76 -PR/8/34(R)	H1N1	Test virus in ELISA
H7N1[a]	A/Equine/Prague/1/56-Brazil/11/78(R)	H7N1	Test virus in HI assay
--	B/Lee/40	B/Lee	Vaccine: single infection protocol

a reassortant virus

antigenic stimulation of HA antibody response.

Eight week old female BALB/c mice were randomly divided into two groups of 53 animals each. They were exposed first to aerosols of virus as shown in Table III. Three days later 6 mice from each group were randomly chosen and killed for determination of their pulmonary viral titers. Forty-two days after exposure to virus aerosols the mice were bled. The following day, the animals in Group I received reassortant virus $H3_{PH}N7$ intranasally, and, the mice in Group II $H3_{PH}N2_{PH}$ virus. Three days after infection lungs from two mice in each group were removed for demonstration of the presence of virus. 63 days post-initial infection, the mice in each group were divided into two sub-groups (a and b) of twenty animals and one subgroup of five animals (c). One group of mice received an i.p. injection of the U.V. inactivated vaccine virus H3N2 (subgroup a); the second group (subgroup b) received the reassortant virus H7N2 and the third group (c) received sterile PBS. The animals were bled for serum 7 and 14 days later.

RESULTS

Single Infection Protocol

Serologic Response to Single Infection

In this section, HI antibody to $H3_{HK}N2_{HK}$, (virus used for aerosol infection) is referred to as "heterotypic HI" to distinguish it from HI antibody to the HA of vaccine virus which is called "homotypic HI". Following aerosol

exposure to $H3_{HK}$ $N2_{HK}$ virus all animals had significant (four-fold or greater) increase in HI antibody to $H3_{HK}$ (heterotypic HI) and NI antibody to $N2_{HK}$ (Table II, Schedule A). Further, infection was confirmed by demonstration of infectious virus in lung suspensions 3 days after aerosol exposure to virus. The mean EID_{50} from these lungs was $10^{3.1}$/0.1ml ± 0.5. 30 days after aerosol infection, 82% of the animals infected had significant HI antibody to $H3_{HK}$ and 42% had NI antibody to $N2_{HK}$. Therefore, infection was accomplished and immunity to NA and HA established.

Response to Vaccine in Single Infection

Injection of $H3_{HK}N2_{HK}$ or $H7N2_{HK}$ vaccines into unprimed mice induced HI antibody to vaccine virus (homotypic HI) in all animals receiving vaccine (Table II). 82% of the animals injected with B/Lee virus vaccine made B/Lee-specific HI antibody. There were no significant differences in homotypic HI titers among vaccine groups of previously unprimed mice (Table II). In animals infected with $H3_{HK}N2_{HK}$, all vaccines induced a homotypic HI response (i.e. antibody to vaccine HA) equivalent to that seen in unprimed animals. Animals primed by infection to $H3_{HK}N2_{HK}$ then vaccinated with this same virus had significant rises (greater than 4 fold) in HI antibody typical of a secondary antibody response (Table II). There were no significant differences in H3 HI antibody (i.e., heterotypic antibody) among H3-primed animals that were

Table II: Single Infection and Vaccination of Mice With Influenza Virus: Effect of single infection on HI and NI antibody response to Vaccine

Schedule	No. Mice	Day 0 Infection[a]	Day 30 Vaccine[b]	Homotypic HI[d] Days Post-infection 30	37	42	49	Heterotypic HI[e] Days Post-infection 30	37	42	49	NI Days Post-infection 30	37	42	49
A	40	$H3_{HK}N2_{HK}$	1)B/Lee	0	1.0[f]	3.8	3.2	1.6	0.8	0.2	0.4	3.1	6.5	8.3	8.9
			2)$H3_{HK}N2_{HK}$	0.8	5.2	5.2	6.3	1.0	5.2	5.2	6.2	4.0	8.7	12.6	12.0
			3)$H7N2_{HK}$	0.3	3.0	3.2	2.3	1.2	0.3	1.2	1.8	3.8	10.5	12.3	11.9
			4)PBS	-	-	-	-	1.9	1.4	0.5	0.5	4.9	6.7	9.3	8.9
B	30	PBS[c]	1)B/Lee	0	0.6	3.0	2.4	0	0	0	0	<1	<1	<1	<1
			2)$H3_{HK}N2_{HK}$	0	2.0	2.6	2.4	0	1.8	2.8	2.2	<1	4.1	4.7	5.9
			3)$H7N2_{HK}$	0	2.4	1.7	2.3	0	0	0	0.3	<1	4.02	3.1	5.9

a: by aerosol

b: by intraperitoneal injection

c: phosphate buffered saline

d: Homotypic HI refers to the use of virus in HI that antigenically matches vaccine virus.

e: HI test antigen: $H3_{HK}N1$

f: Log 2 geometric mean titer reciprocal

vaccinated with B/Lee, $H7N2_{HK}$ or PBS.

Neuraminidase Antibody Response in Single Infection Protocol

Uninfected animals injected with a virus vaccine containing N2 NA demonstrated a gradual rise in anti-N2 antibody consistent with a primary antibody response (Table II, Schedule B). No NI antibody to N2 was detected in uninfected animals injected with B/Lee virus vaccine. Uninfected animals vaccinated with $H3_{HK}N2_{HK}$ or $H7N2_{HK}$ virus vaccine had equivalent levels of N2 antibody. Apparently, the $H3_{HK}N2_{HK}$ and $H7N2_{HK}$ were equally N2 immunogenic.

Previously infected animals had a dramatic increase in anti-N2 antibody after i.p. injection of N2-containing vaccines (Table II, Schedule A). There were no significant differences in N2 antibody levels between H3N2 vaccinated mice and mice vaccinated with H7N2. NI antibody levels in mice injected with B/Lee vaccine and PBS were indistinguishable from each other. Thus, single infection with influenza virus followed by vaccination with reassortant hybrid vaccines failed to duplicate the phenomenon observed in man of depression of NA antibody responses by HA priming.

Sequential Infection Protocol

Antigenic Characterization of Infecting Viruses

Antigenic differences between the heterovariant H3 hemagglutinins used in this study were demonstrated by HI and PI (Table III). Based on HI, an antigenic cross-reactive relationship of less than 0.8% exists between these HAs. The N2 neuraminidase antigens have an approximately

Table III

Antigenic characterization of viruses

Virus	HA Antibody Antiserum		Titer,[a] Ratio	NA Antibody Antiserum		Titer,[a] Ratio
	$H3_{HK}N2_{HK}$	$H3_{PH}N7$		$H3_{HK}N2_{HK}$	$H3_{PH}N2_{PH}$	
$H3_{HK}N1$	327,680[b]	80[b]	1:4,096[c]			
	102,400[d]	400[d]				
$H3_{PH}N2_{PH}$	16[b]	256[b]	1:16[c]			
	400[d]	102,400[d]				
$H6N2_{PH}$				20[e]	140[e]	1:20.8[f]
				800[g]	3,200[g]	
$H1N2_{HK}$				416[e]	29[e]	1:4.8[f]

[a] Heterologous titer ratio.
[b] HI.
[c] $R = 1/256 \therefore <0.8\%$ antigenically cross-reactive.
[d] PI.
[e] NI.
[f] $R = 1/10 \therefore \approx 12.5\%$ antigenically cross-reactive.
[g] psr.

12.5% antigenic cross-reactive relationship, as demonstrated by NI assay. Results from PSR and PI tests are consistent with the NI and HI tests.

Serologic Response to Sequential Infection

All animals had significant (four-fold or greater) increase in HI antibody following aerosol infection with $H3_{HK}N2_{HK}$ virus (Schedule II). Infection was further verified by demonstration of infective virus in lung suspensions 3 days after aerosol exposure to virus (Table V). Intranasal infection with viruses containing hetero-variant HA ($H3_{PH}$) 42 days later resulted in little or no increase in HI antibody to $H3_{HK}N1$ by day 70 (Groups Ic, IIc, Table IV) but small increases were evident on day 77. However, HI titers to test virus containing the $H3_{PH}$ HA homologous to the intranasal challenge virus were signif-icantly increased. Infective virus was also demonstrated in the lungs of animals sampled (Table V). Thus, reinfection and consequent restimulation of immunity were accomplished.

M-Protein Antibody Response After Sequential Infection

A significant M protein antibody response measured by ELISA occurred in all infection groups (Table VI). Schedule I animals experienced a greater anti-M protein response than Schedule II animals on day 42 (following their first infection). The evidence of greater antigenic stimulation in Group I is difficult to reconcile with the evidence of comparable levels of viral replication reflected by identical pulmonary viral titers at a single point in time

TABLE IV

Sequential infection and vaccination of mice with influenza virus antigenic reassortants: Effect of prior HA priming on NA antibody response

Schedule	No. of Mice	Day 0			Day 42, Infection No. 2[b]	Day 63, Vaccine	Day 70, Postinfection		Day 77	
		Infection No. 1[a]	HI	NI			HI	NI	HI	NI
I	19[c]	a) $H3_{HK}N1$	2.9[d] (0.94)	1.0 (0.0)	$H3_{PH}N7$	$H3_{HK}N2_{HK}$	6.5[e] (0.70)	3.0[e] (0.87)	7.5 (0.50)	4.1 (0.97)
	18	b) $H3_{HK}N1$	3.3 (0.95)	1.5 (1.53)	$H3_{PH}N7$ $H3_{PH}N7$	$H7N2_{HK}$	4.7 (0.93)	2.9 (0.97)	5.2 (0.89)	5.9 (0.83)
	5	c) $H3_{HK}N1$	3.0 (0.0)	1.2 (1.48)	$H3_{PH}N7$	None	3.0 (0.89)	1.0 (2.52)	4.2 (0.73)	1.4 (2.76)
II	18	a) $H3_{HK}N2_{HK}$	3.2 (0.46)	4.5 (1.10)	$H3_{PH}N2_{PH}$	$H3_{HK}N2_{HK}$	7.0 (0.49)	5.6 (0.73)	7.2 (0.52)	10.1 (0.32)
	19	b) $H3_{HK}N2_{HK}$	3.0 (0.96)	5.1 (1.07)	$H3_{PH}N2_{PH}$ $H3_{PH}N2_{PH}$	$H7N2_{HK}$	5.0 (0.60)	6.3 (0.62)	4.7 (0.60)	12.0 (0.61)
	5	c) $H3_{HK}N2_{HK}$	2.0 (0.0)	4.7 (1.36)	$H3_{PH}N2_{PH}$	None	3.0 (0.57)	5.0 (0.53)	3.8 (0.83)	5.6 (0.83)
III	9	None			None	$H3_{HK}N2_{HK}$	1.2 (0.88)	1.2 (2.26)	1.5 (0.82)	2.1 (1.29)
	9	None			None	$H7N2_{HK}$	0.0 (0.0)	1.0 (3.21)	0.0 (0.0)	1.9 (1.31)

[a] By aerosol.
[b] Intranasal inoculation.
[c] Number surviving until day 77.
[d] Log 2 geometric mean titer reciprocal (±SD).
[e] HI test virus: $H3_{HK}N1$. NI test virus: $H1N2_{HK}$.

Table V

Demonstration of infective virus in mouse lungs after administration of virus

Schedule	Pulmonary Virus Titers: (EID_{50})	
	Day 3, aerosol infection[a]	Day 45, intranasal infection[b]
I	3.5 ± 0.5[c]	3.2 ± 0.5
II	3.7 ± 0.5	3.4 ± 0.5

[a] Six animals tested per schedule.
[b] Two animals tested per schedule.
[c] Reciprocal mean log 10 titer ± SD.

Table VI

M protein antibody response as detected by ELISA

Test Antigen	Day					
	0[a]		42		70[b]	77[b]
Purified M protein	618[d]	I[c]	6,480	1-a	18,476	24,786
				1-b	20,501	20,871
		II	1,182	2-a	19,651	27,598
				2-b	20,136	21,310
Disrupted virus: H1N1	770	I	165,106	1-a	423,941	667,875
				1-b	467,257	524,880
		II	67,629	2-a	492,075	579,851
				2-b	561,401	553,870
Bovine serum albumin	30	I	74	1-a	89	194
				1-b	226	160
		II	82	2-a	216	244
				2-b	250	186

[a] Days after initial exposure to virus.
[b] No significant differences among subgroups.
[c] See Table II for definition of infection groups.
[d] Numbers represent reciprocal titration endpoints of pooled sera.

(Table V). However, following a second infection on day 42 and vaccination on day 63, all groups in both schedules showed an increase in anti-M protein antibody to equivalent levels. Thus, at the final observation point, there were no significant differences among subgroups (Ia, Ib, IIa, IIb) in the antibody response to M protein. M protein is a homotypically conserved protein, (i.e. relatively invariant in influenza A virus subtypes) (Lamb and Lai, 1981; Huddleston and Brownlee, 1982). The lack of significant differences in the anti-M protein response among subgroups suggests that each subgroup was antigenically stimulated by infection and vaccination to an equivalent degree, as measured by ELISA.

HA Priming Through Infection

Both infection schedules achieved the goal of H3 priming as manifested by primary serologic response after infection and secondary response following vaccination.

Response to Vaccine in Sequential Infection Protocol

Vaccines were marginal in primary immunogenicity, as shown by the geometric mean NI antibody titers in Table IV (Schedule III). However, 9 of 9 animals injected with each vaccine alone had slight increases in antibody titer by 14 days and the immunogenic effect of vaccines in animals primed by infection is clearly shown in comparisons of animals in subgroups a and b with the unvaccinated animals in the c subgroups. The $H7N2_{HK}$ vaccine stimulated $H3_{HK}$ antibody only minimally in infection primed mice (comparison

of HI responses of groups b and c) and not at all in uninfected animals. Five of 9 animals given only H3N2 vaccine had 4-fold increase in HI antibody to $H3_{HK}$ by day 77.

Neuraminidase Antibody Response

As expected, significant NA antibody response measured with H1N2 test virus occurred only in animals in schedule II infected with N2-bearing viruses. Following vaccination on day 63, all animals responded with prompt increase in NI antibody (Day 70) that continued to rise to Day 77. It is notable that vaccination with H7N2 vaccine evoked significantly greater NA antibody response with both infection schedules than did H3N2 vaccine, despite equivalence of the vaccines in NA activity. By Day 77 the anti-N2 booster response was approximately four-fold greater in groups receiving H7N2 vaccine, regardless of prior exposure to N2 antigen. A one-way analysis of variance (ANOVA) performed on the day 77 NI data ($p < 0.0001$) indicated significant differences between groups. A Tukey test (Tukey, 1953) subsequent to ANOVA ($\alpha = 0.05$) showed that subgroup IIb was significantly superior to all other groups in NI antibody production, and that subgroup Ib was significantly superior to subgroup Ia in NI antibody production. The highest level of NA antibody was found in mice primed with N2-containing viruses, then boosted with H7N2 vaccine. In both infection schedules, the magnitude of the N2 antibody response was reciprocal to the magnitude of H3 antibody response.

Unexpectedly, infection with viruses containing N1 and

N7 NA's primed the response to N2 contained in the vaccine viruses (Schedule I).

DISCUSSION

The first requirement for study of the apparent influence of HA priming on NA antibody response observed in man has been the establishment of a suitable animal model system for duplication of the effect. Simulation of human experience required initial priming by infection followed by parenteral administration of inactivated virus. Comparison of H3N2 and H7N2 vaccines in preliminary studies in mice previously infected with a single infection by H3N2 virus resulted in increased NA antibody response in H7N2 vaccinees at only one time point after vaccine administration (Table II-day 37,NI). We tried to better approximate human experience by preceding vaccination with more than one infection, on the assumption that enhanced HA priming might be required. In man, repeated heterovariant (or even homovariant) influenza virus infections are the rule (Kilbourne, 1978b).

Because earlier studies of aerosol infection had demonstrated mice to be completely refractory to homologous reinfection four weeks after initial infection (Schulman, 1967) we chose to infect sequentially with viruses of the same subtype having significant antigenic differences in their HAs. This strategem was successful, and resulted in 1) reinfection, verified by pulmonary virus isolation and homologous serologic response, and 2) stimulation of

cross-reactive HI antibody, albeit at a lower level. These results were obtained with both infection schedules, demonstrating that sequential exposure to N2 antigen (Schedule II) induced no additional immunity to reinfection over that imparted by the H3 antigen. This result is consistent with prior evidence that NA-induced immunity is infection permissive (Schulman, et al, 1968; Schulman, 1969).

With either priming schedule, approximately four-fold greater titers of NI antibody were induced by vaccine containing the heterosubtypic H7 HA antigen. Thus, both primary (Schedule I) and secondary (Schedule II) differences in NA antibody response were influenced by the HA with which the identical NA antigens were associated in the vaccine virus, rather than by prior experience with the NA antigen. The variable immunogenicity of the influenza virus surface proteins (HA and NA) can not be due to differing antigen loads between infection schedules or by antigenic competition mediated by M protein, since there were no differences among subgroups in their anti-M protein response.

Was NA antibody response enhanced by the H7 or suppressed by the H3 HA? Certain influenza virus HA antigens have been found to act as primary B cell mitogens (Anders et al, 1984); however, we have not demonstrated such activity in vitro for either the H7N2 reassortant or the H3N2 virus used in the present studies. Furthermore, primary homologous H7 antibody response has been unexceptional in humans (Kilbourne, 1976) or in mice (data not shown). Finally, our

study in mice involving only a single infection prior to H7N2 vaccination was only marginally effective in inducing greater N2 antibody response.

As suggested earlier (Kilbourne, 1976), the differing immunogenicity of the H3N2 and H7N2 vaccines is most readily explained as resulting from antigenic competition in which expanded populations of H3 specific memory cells might competitively reduce the opportunity for H3-associated N2 to bind to B or T cell antigen receptors. Competition in which a stronger antigen is given for the second time has been explained as a result of non-specific suppressor T cell activity (Taussig, 1977) inhibiting the response to a weaker antigen concomitantly administered. However, as described in detail in the following chapter, our exploration of the cellular basis of apparent antigenic competition in our system has demonstrated that an N2 specific T cell population plays a significant role (Johansson et al, 1987b). Mice boosted with H7N2 after sequential infection showed a greater N2 antibody response than mice injected with H3N2, B/Lee, or saline after infection. This enhanced ability to respond to N2 antigen can be transferred by primed T cells. These data strongly suggest that in H7N2 boosted mice there is an expanded T helper population specific for N2.

An unexpected observation was the priming effect of the H3N1 and H3N7 viruses used in Schedule I on the NA antibody response evoked by both N2-containing vaccines.

This effect probably is not related to B cell recognition of a common cross-reactive determinant shared by NAs of different subtype because antibodies reacting with N2 NA were not detected in mice sequentially infected with N1 and N7-containing viruses and were not boosted with either vaccine (Schedule Ic). Therefore, this boosting effect must be explained at the level of T cell recognition of cross-reactive determinants not serologically defined, such as cognate help from T cells recognizing cross-reactive epitopes on viral proteins other than NA. It is also possible that this effect is related to idiotype specific T cells which can increase the number of B cells specific for various antigens bearing a crossreactive idiotype. It has been shown that monoclonal antibodies specific for N1 and N2 as well as the primary and secondary antibodies elicited subsequent to infection with X-31 (H3N2) or PR8 (H1N1) viruses share cross-reactive idiotypes as defined by monoclonal antibodies recognizing the idiotype (Mayer et al, 1987).

Influenza A virus NA glycoproteins of different subtypes appear to differ markedly in amino acid sequence and significant antigenic cross-reactivity has not been defined previously with antisera. However, studies of amino acid sequence of the tetrameric head of the enzyme released from the viral membrane by protease demonstrate conservation of sequence of N2, N1 and influenza B virus NAs at crucial sites, strongly suggesting a common structure (Varghese et

al, 1983). The four catalytic sites of the enzyme have been identified on the distal surface of the tetrameric head (Colman et al, 1983). The dependence of NA inhibition by antibody in NI tests on substrate size (Fazekas de St. Groth, 1963) indicates that the catalytic site of the enzyme is not itself antigenic and that inhibition of enzyme activity by antibody is mediated by steric hindrance. Structural studies (Colman et al, 1983) demonstrate that all of seven segments of the polypeptide chain identified as antigenic sites are sufficiently close to the active site to block access of macromolecular substrates when these sites are bound to antibody. The question of shared epitopes among influenza A virus NAs of different subtypes requires investigations with additional techniques, including ELISA (Khan, Gallagher, Bucher, 1982) and plaque size reduction (Jahiel and Kilbourne, 1966).

VII. IMMUNOLOGIC RESPONSE TO INFLUENZA VIRUS NEURAMINIDASE IS INFLUENCED BY PRIOR EXPERIENCE WITH THE ASSOCIATED VIRAL HEMAGGLUTININ II. REDUCED GENERATION OF NEURAMINIDASE-SPECIFIC L3T4$^+$ HELPER T CELLS IN HEMAGGLUTININ-PRIMED MICE*

The requirement for helper T cells (T_h) for the induction of specific immunity to influenza virus surface proteins during both primary (Virelizier et al, 1974; Burns et al, 1975) and secondary (Anders et al, 1979) immune responses has been well documented. Furthermore, because T cells have been implicated as an important part of the mechanism of antigenic competition (Taussig, 1977), we began our investigation of the cellular mechanisms responsible for hemagglutinin-influenced immune response to NA by examining the effect of specifically primed T cells on neuraminidase inhibition (NI) antibody production. We have utilized a mouse model system (Johansson et al, 1987a) in which apparent HA-NA antigenic competition had been observed as the source of primed cells. Quantitation of NA-specific T_h cell activity has been effected by adoptive transfer of T cells to athymic mice in which NA antibody response was measured following subsequent exposure to NA antigen. Further, we determined that L3T4$^+$ T cells were responsible for helper activity in this system.

Materials and Methods

Viruses

The strains of influenza A viruses employed in these studies are identified in Table VII.

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Table VII

Viruses used in T cell transfer study

Virus Nomenclature		Laboratory name	Specific Use
HANA subtype	Strain or reassortant designation		
H3N2	A/HongKong/1/68	$H3_{HK}N2_{HK}$	Aerosol infection and vaccine: group 3
H3N2	A/Philippines/2/82	$H3_{PH}N2_{PH}$	Intranasal infection
H7N2[a]	A/Equine/Prague/1/56-Aichi/2/68(R)	$H7N2_{HK}$	Vaccine group 4
H3N1[a]	A/HongKong/1/68-PR/8/34(R)	$H3_{HK}N1$	Test virus in HI assay
H1N2[a]	A/PR/8/34-HongKong/1/68(R)	$H1N2_{HK}$	Test virus in NI assay
H6N2[a]	A/Turkey/Mass./75-Aichi/2/68(R)	$H6N2_{HK}$	Vaccine: athymic mice (after T cell transfer)
H6N1[a]	A/Turkey/Mass./75-India/1/80(R)	H6N1	Test virus in HI assay
B/Lee	B/Lee/40	B/Lee	Vaccine: group 5 cellular experiment

[a] Reassortant virus.

All viruses were grown in the allantoic sac of 10 day old chick embryos and stored in multiple aliquots at $-70^{o}C$. All virus strains used as vaccines were sucrose gradient purified, then inactivated by U.V. irradiation to a residual EID_{50} of less than $10^{-1.3}$/0.1 ml then adjusted to equal NA activity (3.0 μM of N-acetyl-neuraminic acid released/24 hrs/100 μl). Each animal received an i.p. injection of 0.2 ml containing approximately 2 μg of total viral protein.

Animals

Inbred BALB/c female mice (Jackson Labs, Bar Harbor, ME) 8 weeks of age were used in the infection protocols and as T cell donors. BALB/cANNCR (nu^{+}/nu^{+}) mice (NCI-Frederick Cancer Institute, Frederick, MD), age 10 weeks, were used as T cell recipients.

Serologic Methods

Hemagglutination inhibition (HI) assays, neuraminidase inhibition (NI) assays, and all other serologic procedures were performed as described in the previous chapter. The test virus for NI was $H1N2_{HK}$. The test virus for the HI test was either $H3_{HK}N1$ or H6N1. (See Table VII).

Infection Procedure

Mice used as spleen cell donors were sequentially infected with heterovariant H3N2 influenza viruses as described previously (Johansson et al, 1987a). The schedule of infection used in this study had been shown to be optimal for NI antibody production. To confirm infection after

aerosol and intranasal exposure to virus, five mice subjected to each infection procedure were randomly selected and killed for determination of pulmonary virus titers.

Preparation of B cell depleted population of spleen cells

Animals from each vaccine group (Table VIII) were killed by cervical dislocation and their spleens removed under sterile conditions. Cell suspensions were made by teasing apart the spleens in sterile phosphate-buffered saline (PBS). Erythrocytes were removed by treatment with ammonium chloride. Cell suspensions were enriched for T cells by passage over a nylon wool column (Julius et al, 1973) and effluent cells were further depleted of B cells by the panning method (Wysocki and Sato, 1978) which specifically binds Ig^+ cells. Viability counts were performed by the trypan blue exclusion method. Final concentrations of cells were suspended in PBS. Cytofluorometric analysis (Fig. 1) has shown that 97.5% of the remaining cells stain positively with fluorescein conjugated anti-Thy 1.2 monoclonal antibody (Miles Scientific, Napaville, IL).

200 µl of 1.0×10^7 purified viable T cells were injected intravenously into the tail vein of recipient athymic mice.

Preparation of $L3T4^+$ or $Lyt2^+$ depleted T cell populations

T cells were purified from splenocytes as described above. Purified T cells were treated with anti-L3T4 (to

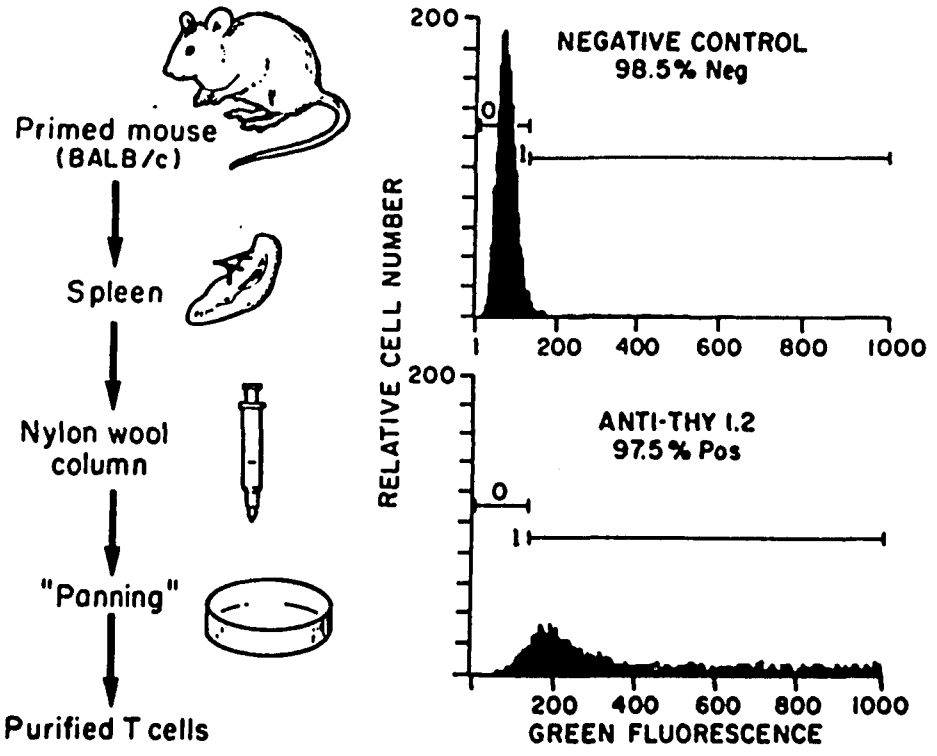


Fig. 1. T cells were purified from normal mice as shown above, left. Staining the effluent cells with fluorescein labelled anti-Thy 1.2 monoclonal antibody indicated that 97.5% were T cells

TABLE VIII

Immunization protocol and serologic response of donor mice

Group	No. of Mice	Days After Initial Infection									
		0. (Aerosol exposure)	42		42. Intranasal injection	63		63. Vaccine injection	70[a]		77	
			NI	HI		NI	HI		NI	HI	NI	HI
1	20	Mock[b]	1.0 (0)[a]	<1.0 (0)	Mock[b]	1.0 (0)	<1.0 (0)	Mock[b]	1.4 (1.01)	<1.0 (0)	1.1 (2.1)	<1.0 (0)
2	20	$H3_{HK}N2_{HK}$	4.8 (1.46)	2.5 (0.72)	$H3_{PH}N2_{PH}$	4.5 (2.17)	1.95 (0.80)	Mock[b]	5.6 (1.64)	2.85 (0.88)	7.0 (1.96)	2.6 (0.92)
3	20	$H3_{HK}N2_{HK}$	4.7 (0.96)	2.3 (0.64)	$H3_{PH}N2_{PH}$	5.02 (2.01)	1.95 (0.76)	$H3_{HK}N2_{HK}$	7.1 (0.85)	6.6 (0.96)	11.0 (0.31)	7.9 (0.42)
4	20	$H3_{HK}N2_{HK}$	4.8 (1.83)	2.3 (0.57)	$H3_{PH}N2_{PH}$	4.9 (1.96)	2.1 (0.60)	$H7N2_{HK}$	8.0 (0.92)	4.5 (0.69)	14.6 (0.79)	5.1 (0.96)
5	20	$H3_{HK}N2_{HK}$	5.2 (1.62)	2.4 (0.50)	$H3_{PH}N2_{PH}$	5.4 (2.05)	2.1 (0.64)	B/Lee	5.2 (1.87)	3.1 (0.57)	6.4 (1.81)	3.8 (0.88)
6[c]	10	None			None			$H3N2_{HK}$	1.6 (0.78)	0.9 (0.32)	3.2 (1.09)	2.0 (0.91)
									4[d]/10	9/10	10/10	10/10
7[c]	10	None			None			$H7N2_{HK}$	1.5 (0.82)	<1.0 (0.0)	2.9	<1.0 (0)
									3[d]/10	0/10	10/10	0/10

[a] Log 2 geometric mean titer (±SD).
[b] Sterile phosphate-buffered saline.
[c] Vaccine standardization groups.
[d] Number of responding animals.
HI test virus: $H3_{HK}N1$.
NI test virus: $H1N2_{HK}$.

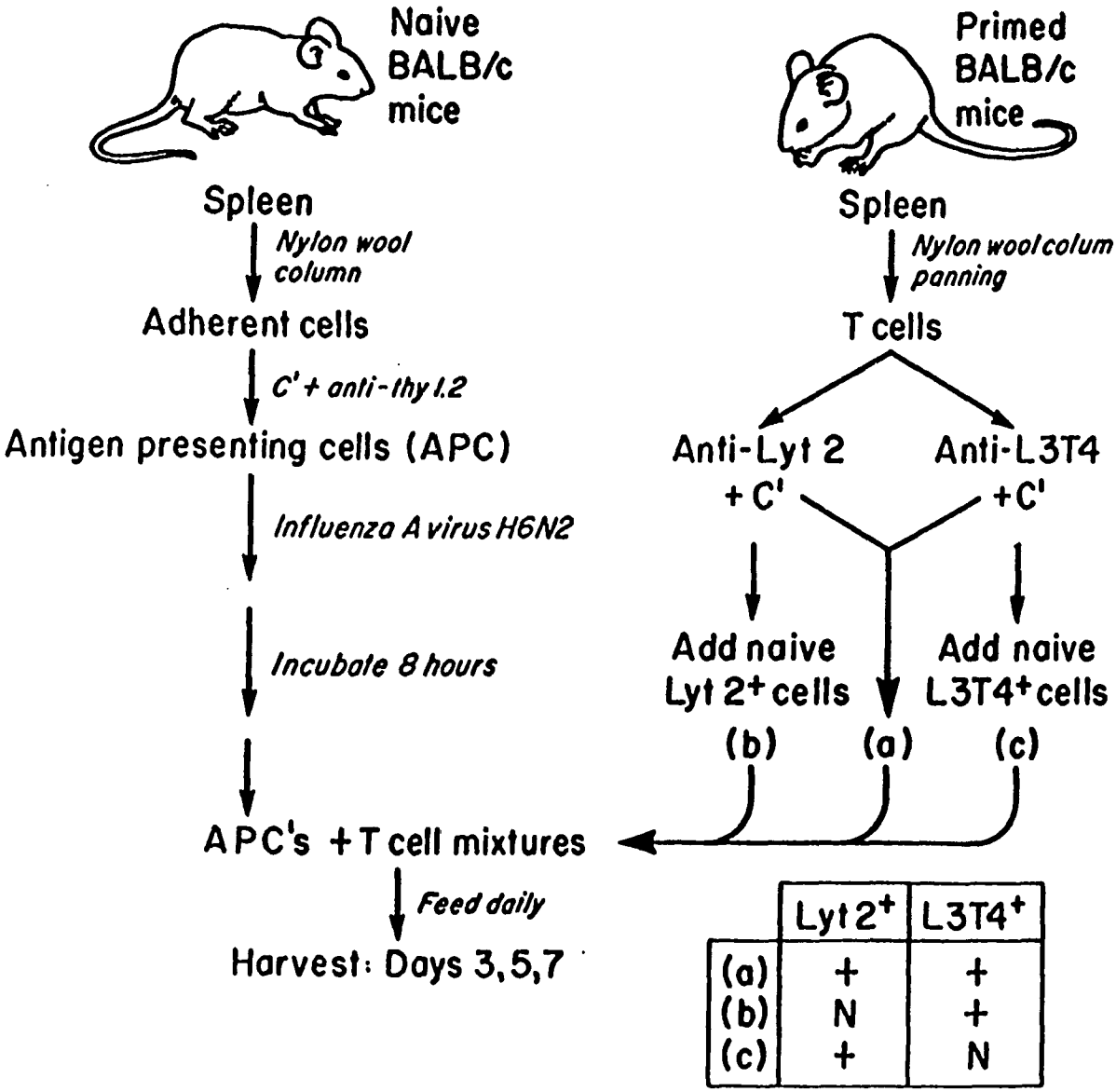
eliminate T helper cells) or anti-Lyt2 (to eliminate T suppressor cells) in the presence of complement (C'). Then each group of T cells was reconstituted in a 50-50 mixture with either naive or primed T cells of the same subpopulation previously ablated (Fig. 2). These T cell mixtures were used in in vitro spleen cultures of 2×10^7 cells/ml in a total volume of 200 µl/well (Mishell and Dutton, 1967). T cell depleted splenocytes were incubated with U.V. inactivated purified influenza virus for 8 hours prior to addition of T cells. Supernants were assayed every three days for the production of NI antibody.

Cytofluorometric analysis (Fig. 3) has shown that without treatment with specific antibody 49.7% of the T cells were $L3T4^+$ and 36.3% were $Lyt2^+$. After these unfractionated cells were treated with monoclonal anti L3T4 antibody and C', only 0.7% of the cells stained for $L3T4^+$, whereas 79.7% stained for Lyt2. Treatment of T cells with monclonal anti-Lyt2 antibody resulted in 84.1% of the T cells staining for $L3T4^+$ and 4.6% staining for Lyt2.

Vaccination Protocol

As outlined in Table VIII, 110 eight week old female BALB/c mice were either sequentially infected with heterovariant H3N2 influenza viruses (90 animals) or mock infected (20 animals). Ten of the infected animals were used to determine pulmonary virus titers after exposure to virus (see above). Twenty additional animals were held for later primary immunization with vaccines alone (Groups 6 and 7,

T CELL SUBPOPULATION EXPERIMENT



	Lyt2+	L3T4+
(a)	+	+
(b)	N	+
(c)	+	N

Fig. 2

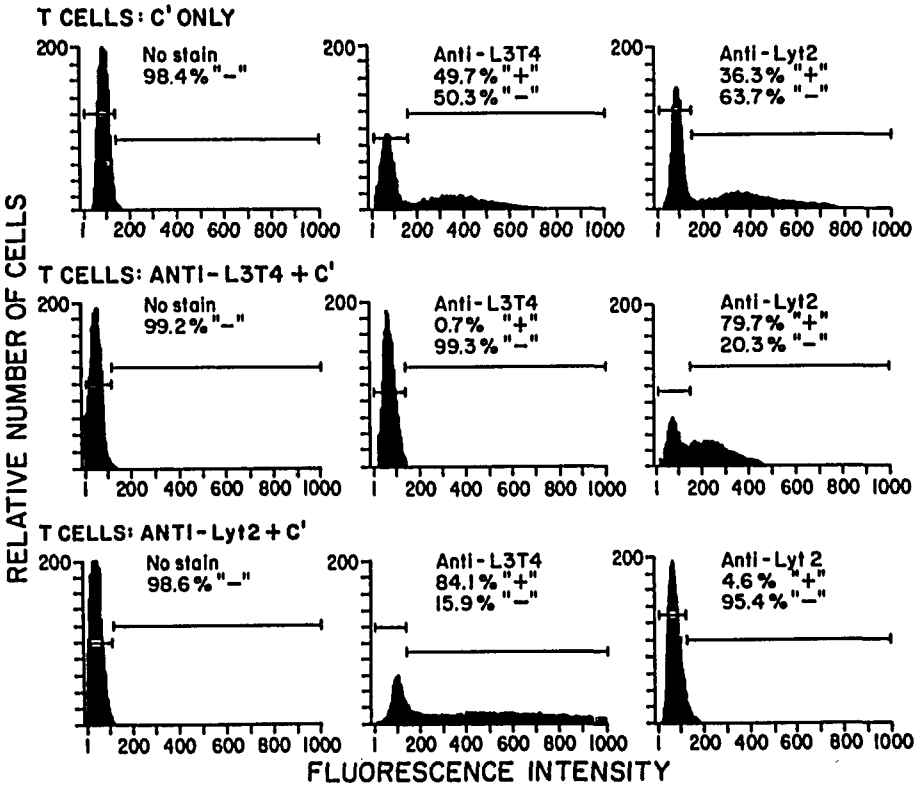


Fig. 3. Cytofluorometric analysis of T cells treated with C' (top panel), anti-L3T4$^+$ and C' (middle panel) or anti-Lyt2+C' (bottom panel)

Table VIII). 63 days after initial infection, mice were randomly divided into 4 vaccine groups of 20 animals (Table VIII). Each vaccine group received an i.p. injection of a preparation containing either PBS (Group 2), or U.V. inactivated H3N2 virus (Group 3), H7N2 virus (Group 4) or B/Lee virus (Group 5). Seven days later, 5 mice from each vaccine group were randomly selected as spleen cell donors. The remaining mice were bled and their sera tested for NI and HI activity. Purified T cells were obtained from these donor spleens and either transferred to six 10 week old female BALB/c nu^+/nu^+ athymic mice per vaccine group or used in Mishell-Dutton in vitro assays. 24 hours after T cell transfer the recipient mice were injected i.p. with U.V. inactivated H6N2 virus vaccine. The recipient mice were bled at 7 day intervals for one month.

RESULTS

Serologic Response to Sequential Infection and Vaccination of Donor Mice

Animals that were sequentially infected and vaccinated but not used as T cell donors were bled 7 and 14 days after vaccination.

The infection schedule successfully primed animals to H3 and N2 as manifested by primary serologic response after infection (Table VIII). All animals had a significant > 2 $\log_2$ (four-fold) increase in HI antibody following aerosol infection with $H3_{HK}N2_{HK}$ virus (Groups 2,3,4, and 5). Infection was also verified by demonstration of infec-

tive virus in lung suspensions 3 days after aerosol exposure to virus (Table IX). Intranasal infection with $H3_{PH}N2_{PH}$ 42 days after aerosol exposure resulted in little or no increase in HI antibody to $H3_{HK}$ or NI antibody to $N2_{HK}$ by day 63 but small antibody increases to both surface antigens unrelated to vaccination were seen by day 70 (Groups 2 and 5, Table VIII). Additionally, infective virus was detected in lung suspensions 3 days after intranasal infection, (Table IX). Therefore, reinfection and restimulation of immunity were accomplished. Priming to both HA and NA was achieved (Groups 3 and 4), as was previously described (Johansson et al, 1987a).

Only animals that received an N2-containing vaccine (Groups 3 and 4) had a prompt increase in NA antibody (day 70), that continued to rise to day 77. As seen before with this infection protocol, vaccination with H7N2 vaccine (Group 4) evoked by day 77 a significantly (fourfold) greater NA antibody response than did H3N2 vaccine (Group 3) despite equivalence of N2 immunogenicity of the vaccines in unprimed animals. Additionally, H7N2 vaccine stimulated a low level of $H3_{HK}$ cross-reactive HI antibody. Vaccination with H3N2 (Group 3) evoked a secondary response to both H3 and N2 as manifested by an increased titer to both of these antigens following vaccination. Injection with PBS (Group 2) or B/Lee virus vaccine (Group 5) did not evoke a detectable secondary response to either H3 or N2.

Table IX

Demonstration of infective virus in mouse lungs after administration of virus

Pulmonary Virus Titers: (EID_{50})	
Day 3, aerosol[a]	Day 45, intranasal[a]
4.1[b]	4.3

[a] Method of infection; five animals tested.
[b] Reciprocal mean log 10 titer.

Response to Vaccine in Unprimed Mice

In unprimed mice, both $H3_{HK}N2_{HK}$ and $H7N2_{HK}$ vaccines were marginally immunogenic as shown by geometric mean titers in Table II (Groups A and B). However, 10 of 10 mice injected with either vaccine alone had slight increases in N2 antibody 14 days after injection, and 10 of 10 animals given only $H3_{HK}N2_{HK}$ (Group A, Table II) vaccine had four-fold increases of HI antibody to $H3_{HK}$ by Day 77, The significant immunogenic effect of these vaccines in sequentially infected (primed) animals is shown in comparison of animals from Groups 3 and 4 with unvaccinated animals in Group 2. The $H7N2_{HK}$ vaccine minimally stimulated $H3_{HK}$ antibody response in infection primed animals (compare Groups 3 and 4) and in none of the uninfected animals. B/Lee virus vaccine given to animals after infection priming (Group 5) led to no increase in H3 antibody or N2 antibody in comparison to animals sequentially infected, then vaccinated with sterile PBS (Group 2).

Serologic Response of Recipient Mice

All athymic mice that received T cells responded to H6N2 vaccine virus, as shown by H6 antibody response (Fig. 4) and N2 antibody response (Table X).

H6N2 reassortant virus vaccine induced H6 specific antibody in all T cell recipient athymic mice. Only at one point (day 7 after vaccine) were significant differences among T cell recipient groups shown (Figure 4). A Tukey test ($\alpha = 0.05$) subsequent to ANOVA indicated that signi-

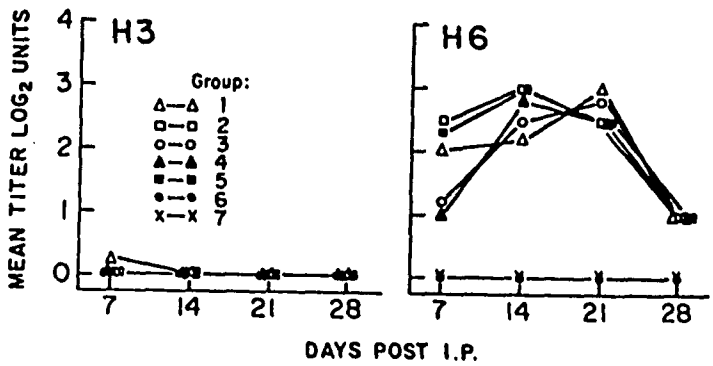
Table X.

T cell transfers: NI antibody response of recipient athymic mice

Group	Donor Experience		Recipient Experience		Days After Vaccine (NI)[b]			
	Infection	Vaccine	T cell transfer[a]	Recipient's vaccine (H6N2)	7	14	21	28
1	Mock[c]	Mock	+	+	2.23[d] (1.04)	3.70 (1.97)	3.56 (2.10)	3.78 (1.49)
2	+[e]	Mock	+	+	5.46 (1.03)	6.00 (1.45)	6.20 (3.06)	7.36 (0.80)
3	+	H3N2	+	+	5.96 (1.30)	8.00 (1.78)	9.70 (1.01)	9.46 (0.86)
4	+	H7N2	+	+	7.30 (1.39)	9.10 (0.51)	10.50 (0.42)	10.20 (0.81)
5	+	B/Lee	+	+	4.90 (2.01)	5.30 (1.11)	7.3 (0.34)	5.73[f] (0.0)
6			−	−[a]	2.50 (1.7)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)
7			−	+	1.0 (0.0)	2.8 (3.5)	1.0 (0.0)	1.0 (0.0)

[a] T cells transferred into six mice/recipient groups 7 days after donor vaccine.
[b] NI test virus: $H1N2_{HK}$.
[c] Received sterile PBS.
[d] Reciprocal log 2 geometric mean titer (±SD).
[e] See Table II.
[f] Only one survivor of six.

Fig. 4



Hemagglutinin inhibition assay on sera from T cell reconstituted athymic mice.

ficant differences did not exist between Group 3 (H3N2 vaccine) and Group 4 (H7N2 vaccine), but the anti-H6 response of Groups 3 and 4 were significantly different from Group 2 (PBS vaccine), Group 5 (B/Lee vaccine), and Group 1 (MOCK). By Day 14 through 28, there were no significant differences amongst groups, although Groups 3 and 4 remained as the weakest anti H6 responders. All groups had a precipitous drop in H6 antibody titer between day 21 and 28. Additionally, there was no measurable HI antibody to H3 antigen in any group, indicative of the specificity of the immune response in these mice toward H6 HA and N2 NA.

A two way analysis of variance (ANOVA) indicated significant differences ($p < 0.0001$) in the N2 antibody response among T cell recipient groups. A Tukey test ($\alpha = 0.05$) subsequent to ANOVA showed that animals that received T cells from donors boosted with H7N2 virus (Group 4, Table X) were significantly superior to all other groups in NI antibody production. Athymic mice that received T cells from animals vaccinated with H3N2 virus (Group 3) had the next highest N2 antibody response compared to other groups. There were no significant differences between athymic mice receiving T cells from donors injected with either PBS or B/Lee virus. Athymic mice getting unprimed T cells from immunologically naive donors (Group 1) responded to challenge virus, albeit at a lower level than primed groups.

No significant amounts of HI or NI antibody were produced in athymic mice that did not receive T cells (Grps.6,7).

In Vitro NI Antibody Production of Influenza Virus Primed B cells with L3T4$^+$ or Lyt2$^+$ cells

Studies with T cell subpopulations indicate that the helper activity seen in this system resides in an L3T4$^+$ cell subpopulation. Cultures containing primed L3T4$^+$ cells (Table XI, "b" line) were able to produce 87% to 111.9% of the NI antibody that "intact" control cultures (Table XI "a" line) produced. In cultures containing cells from mice immunized with influenza virus (Groups 2-5) Lyt2 primed cell had no significant effect on NI antibody production in comparison to unprimed controls (Table XI, Group 1c). Moreover, in any culture from any group, primed Lyt2$^+$ T cells did not enhance or suppress NI antibody production. Also, cultures containing L3T4$^+$ cells from mice vaccinated with H7N2 virus after sequential H3N2 virus infection produced the greatest amount of NI antibody, confirming findings from our studies in athymic mice.

DISCUSSION

In the present study, we have confirmed earlier observations that in mice sequentially infected with H3N2 variant viruses, vaccination with homologous H3N2 virus vaccine evokes a lesser anti-N2 antibody response than H7N2 virus vaccine (Chapter VI). Animals injected with H7N2 virus vaccine had an approximately four-fold greater anti-NA response than animals boosted with a H3N2 virus vaccine. The H3 antibody responses in these animals were inversely related to the N2 antibody response, i.e., H3N2

Table XI

MISHELL-DUTTON *IN VITRO* NI ANTIBODY PRODUCTION OF INFLUENZA VIRUS PRIMED B CELLS CULTURED WITH L3T4+ OR Lyt2+ T CELLS

	DONOR EXPERIENCE			DONOR T CELL SUBSET		*IN VITRO* NI ANTIBODY					
GROUP	INFECTION	VACCINE		L3T4+	Lyt2+	DAY 3		DAY 5		DAY 7	
1	MOCK	MOCK	a)	+	+	1.70	(100%)	2.91	(99.7%)	3.19	(109%)
			b)	+	n	1.70		2.88		3.49	
			c)	n	+	1.67		2.67		3.16	
2	+	MOCK	a)	+	+	2.47	(95.9%)	4.49	(110%)	5.06	(102%)
			b)	+	n	2.37		4.93		5.10	
			c)	n	+	1.13		2.49		3.00	
3	+	H3N2	a)	+	+	3.31	(99.8%)	5.01	(86.3%)	6.41	(101%)
			b)	+	n	2.95		4.45		6.48	
			c)	n	+	1.37		2.69		3.00	
4	+	H7N2	a)	+	+	4.09	(111.9%)	6.29	(91.0%)	8.24	(91.0%)
			b)	+	n	4.58		5.72		7.47	
			c)	n	+	1.77		3.01		3.23	
5	+	B/Lee	a)	+	+	2.52	(99.9%)	4.13	(98.0%)	5.21	(87%)
			b)	+	n	2.48		4.06		4.52	
			c)	n	+	1.67		2.98		2.97	

n = naive T cells

(%) = (NI antibody Group "b"/NI antibody Group"a") x 100

vaccine elicited a greater H3 antibody response than did H7N2 vaccine although H7N2 vaccine induced a low level of H3 cross reactive antibody.

The requirement of T helper cell activity in the anti-influenza virus immune response (Burns et al, 1975; Anders et al, 1979) and evidence that T cells may be involved in intermolecular antigenic competition (Taussig, 1977) led us to examine the role of the T cell in the phenomenon of HA-influenced response to viral NA. Transfer of influenza virus specific T helper clones to athymic mice (Scherle and Gerhard, 1986) or co-culture of unprimed B cells with influenza virus-primed T cells (Anders et al, 1981) resulted in an accelerated and greater anti-influenza virus antibody response after stimulation with influenza virus compared to the response following transfer of unprimed or non-specifically primed T cells.

In the present study, only athymic mice that received T cells were able to mount a specific antibody response to vaccine virus, confirming the dependence of immune response to influenza virus surface antigens upon helper T cells (Virelizier et al, 1974; Burns et al, 1975; Anders et al, 1979). In our study, primed T cells (Groups 2,3,4, and 5 Table X) elicited a greater anti-N2 response from naive B cells in athymic mice than did unprimed T cells. Athymic mice given purified T cells from H3N2 virus-primed donor mice injected with homologous H3N2 virus had a significantly lower N2 antibody response to H6N2 vaccine virus antigens

than athymic mice given T cells from primed donors injected with H7N2 virus vaccine, but a significantly greater N2 antibody response than animals injected with B/Lee virus or PBS. Furthermore, in in vitro antibody production assays, cell cultures containing $L3T4^+$ T cells from donor mice injected with H7N2 had the greatest NI antibody response to H6N2 test virus, an effect not seen with $Lyt2^+$ T cells from any group. These data demonstrate that there was a relatively impaired expansion of NA-specific L3T4+ T helper cells in donor mice injected with H3N2 virus after sequential infection with heterovariant (H3N2) influenza viruses of the same HA subtype, compared to donor mice injected with H7N2 virus. The lower N2 antibody response of mice injected with B/Lee virus or PBS clearly resulted from the lack of specific boosting antigens in the vaccine (i.e., vaccine did not contain H3 or N2 antigens). It is not surprising that the T helper population identified in this study is $L3T4^+$. There is a very strong correlation between expression of $L3T4^+$ and both expression of helper phenotype and class II MHC antigen reactivity (Dialynas et al, 1983; Wilde et al, 1983).

H6N2 virus vaccine induced the formation of H6 HI antibody in all athymic recipient mice provided with T cells. It is interesting that the lowest H6 HI titers were found in T cell recipient mice that had the highest NI titers . This inverse relationship between HI and NI titers among T cell recipient groups is consistent with our anti-

genic competition hypothesis. However, we must reiterate that there was a significant difference between H3N2 and H7N2 vaccination groups in N2 antibody response, although there were no significant differences between these groups in H6 antibody titers. Assuming that H6 specific T cells are in equal numbers in all groups and that HA is found in greater molar amounts than NA on influenza virions (Erickson and Kilbourne, 1980), one must conclude that the transfer of an expanded population of N2-specific T helper cells partially alleviates potential antigenic competition between HA and NA, at least in activating naive B cells present in T cell recipient athymic mice. We stress that the H3N2 and H7N2 vaccines used in the priming of donor mice induced equivalent antibody response to their homologous HA (H3 or H7) and to NA (N2) antigens in unprimed mice.

The difference in molar amounts of NA and HA on the influenza virus particle (Compans et al, 1970; Erickson and Kilbourne, 1980), may account in part for the initially superior immunogenicity of HA in conventional H3N2 and H1N1 virus vaccines (Kilbourne et al, 1987). Changes in the relative amounts of HA and NA in H3N2 and H7N2 vaccines cannot explain the decreased N2 antibody response in animals injected with H3N2 vaccine after sequential infections; therefore, alterations in the cellular components involved in the recognition of NA antigen must explain this observation. Early investigations into the cellular mechanisms of intermolecular competition have shown that

when competing antigens are sequentially presented (sequential intermolecular competition) (Taussig, 1977) the reduced response to the second antigen results from the action of a population of antigen-specific T suppressor cells (Gershon and Kondo, 1971). Intermolecular competition with antigen mixtures of synthetic polypeptides apparently is also dependent on T cell activity. Taussig (1977) argues that intermolecular antigenic competition may be a side effect of T-B cell cooperation and suggests that T helper cells compete for space on antigen presenting cells. The competition for space could be due to any of three mechanisms:

1) Lack of antigen presented in conjunction with MHC antigens. Ia^+ is not constitutively expressed on all macrophages, but increases after antigen stimulation (Unanue et al, 1984). Indeed processed antigen not associated with MHC antigens can be found on the surface of unactivated macrophages. Antigen not associated with MHC antigens cannot be recognized by T cell receptors (Schwartz, 1985),

2) Direct antigenic competition for the same Ir gene product (Werdelin, 1982; Rock and Benacerraf, 1983; Guillet et al, 1986) or,

3) A preponderance of antigen specific T effector (helper) cells that crowd out other T_h cells.

In any case, our present experiments indicate that recognition of antigen by antigen specific T cells plays a crucial role in intravirionic antigenic competition.

VIII. Antigen-presenting B cells and T_h cells cooperatively mediate intravirionic antigenic competition between influenza A virus surface glycoproteins*

Adoptive transfer experiments have shown that reduced generation of NA-specific T cells is at least partially responsible for modulation of antigenic competition in favor of HA (Johansson et al, 1987b). This work is consistent with observations that indicate intermolecular competition is dependent on T cell cooperation (Taussig et al, 1972; Taussig, 1973; Taussig et al, 1977). The exact mechanisms of intermolecular antigenic competition have not been elucidated. However, assuming genetic precommitment of antigen response cells, antigens that are not linked together should be recognized separately. Intermolecular competition, however, implies a common event in the recognition of response to different antigens. Taussig and Lachman (1972) argued that antigenic competition is the result of an event early in the recognition of the dominant antigen that inhibits the early phases of immune induction to the suppressed antigen. Furthermore, there is evidence that intermolecular antigenic competition may be a side effect of B-T cell cooperation (Taussig, 1977; Scherle and Gerhard, 1980). Scherle and Gerhard (1986) have shown that the B-T cell collaboration which results in an antiviral immune response requires a cognate (i.e., direct) T-B interaction, whether or not the determinants recognized by the T_H and B cells are located on the same viral protein or on different

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proteins within the viral particle (intermolecular/ intrastructural help). T_h cells primed to internal viral components of influenza virus can help HA-specific B cells produced antibody (Scherle and Gerhard, 1986; Russell and Liew, 1979, 1980; Lamb et al, 1982).

Recent studies suggest that antigen processing and presentation are partially a function of B-cells (Lanzavecchia, 1985). One hypothesis to explain the antigenic competition between influenza virus surface glycoproteins takes cognizance of the ability of B cells to bind, process and present antigen to T cells. This hypothesis assumes that primary immunization with influenza virus leads to preferential expansion and affinity maturation of B-cells specific for hemagglutinin (HA), the most abundant surface antigen (Webster et al, 1986). When the host is reimmunized with the same virus, HA-specific B cells would preferentially bind the virus and process and present it to T cells specific for one of the several influenza viral proteins. This B-T cell collaboration would activate the T cell and in turn induce antibody production by the B cell. Since the majority of the B cells would now be HA-specific and antigen is limiting, the anti-NA response would be competed out unless the NA is presented in association with a different HA on the virion (Johansson et al, 1987a). We have tested this hypothesis by measuring the proliferative response of purified T cells stimulated by influenza virus antigens presented by purified B cells.

These cell populations were obtained from influenza virus-primed BALB/c mice in which HA and NA specific antibody responses had been demonstrated (Johansson et al, 1987a).

MATERIALS AND METHODS

Influenza A virus strains used for infection or vaccination were as described in the previous chapter (Table VII). Antigenically hybrid, reassortant influenza A viruses H3N1 (A/Hong Kong/1/68-PR/8/34) and H6N2 (A/Turkey/Mass/76-Aichi/1/68) purified on sucrose gradients were U. V. inactivated to a residual 50% egg infective dose (EID_{50}) of less than $10^{-1.3}$/0.2 ml, then used in *in vitro* proliferation assays.

Animals. Influenza immunized BALB/c female mice (Jackson Laboratories, Bar Harbor, ME) from a previous study (Johansson et al, 1987b) were donors of B and T cells used in the present study.

Infection and Vaccination Procedure. The procedure of sequential infection with heterovariant H3N2 influenza A viruses has been described in detail in Chapter II (Table III). Vaccination procedures were as described in Chapter III (Table VIII). After two sequential H3N2 infections, animals were injected with either phosphate buffered saline (PBS), or B/Lee, A/H3N2, or A/H7N2 influenza virus vaccines. The immunization schedule and antibody responses from this study are presented in brief in Table XII.

Preparation of T cell enriched populations from spleen cells. Purified T cell populations were obtained 3 months

after final immunization from the spleens of freshly killed animals as previously described (Johansson et al, 1987a). Erythrocyte-free spleen cell suspensions were passed over a nylon wool column (Julius et al, 1975). The effluent cells were further depleted of B cells by "panning" on anti-Ig plates (Wysocki and Sato, 1978). Final concentrations of cells were suspended in fresh Dulbecco's Modified Essential Medium (DMEM) with 5% fetal calf serum (FCS). Cytofluorometric analysis using fluorescein labelled anti-thy 1.2 monoclonal antibody showed that greater than 97.5% of these cells were T-cells.

Preparation of B cell enriched populations from spleen cells. After lysis of erythrocytes with tris-ammonium chloride, splenocytes used as a B cell source were brought to a volume of 3 ml in (DMEM) with 5% FCS, then placed in 100 mm petri dishes (Fisher Scientific) coated with a 1:10 dilution of purified rabbit anti-mouse Ig antibody and normal rabbit serum (Wysocki and Sato, 1978). The cells were gently rocked for 70 minutes at $4^{o}C$. The plates were carefully washed five times with 5-10ml of PBS with 1% FCS. To recover adherent cells, the plate was filled with 20-25 ml of PBS with 1% FCS and the entire surface of the plate was flushed using a Pasteur pipette. Cells were then incubated with a 1:30 dilution of anti-thy 1.2 monoclonal antibody at $4^{o}C$ for 30 minutes, then with a 1:16 dilution of rabbit C' at $37^{o}C$ for 30 minutes. Cytofluorometric analysis of these cells showed that 95.6% stained positively

with tetramethylrhodamine labelled rabbit anti-mouse Ig+ antibody.

T cell proliferative response. Purified B cells from each experimental group were incubated with 100 hemagglutinating units (HAU) of antigenically hybrid reassortant virus, H3N1 or H6N2, for 8 hours, then gamma-irradiated (2000 rads), yielding (H3)-specific or NA (N2)-specific activated B cells. Virus-stimulated B cells (2×10^5/well) and T cells (4×10^5/well) were mixed in a microtiter plate in a total volume of 200 µl/well. These cultures were maintained at 37°C, 5% CO_2 for 36 hrs, then pulsed with 1 µCi ^{3}H-thymidine/well. After 18 hrs of incubation with label, cells were harvested and radioactivity measured. Cell cultures were maintained in DMEM containing β-mercaptoethanol (5×10^{-6}M) and 10% fetal calf serum (FCS). The proliferative response of T cells as measured by ^{3}H-thymidine uptake was studied using B cells incubated with influenza A viruses H3N1 or H6N2. In the absence of virus, the T cell proliferative response of purified B-T cell mixtures from unimmunized animals was comparable to that found with unfractionated splenocytes in other studies ($\approx$1500 cpm). However, in the presence of virus, these background levels were 10-30 x higher, possibly reflecting non-specific B cell activation by the panning procedure used in B cell isolation, unbalancing of the homeostasis established among cells in unfractionated splenocytes, or by an increased concentration of specific

antigen presenting cells in culture. Nonetheless, recent studies have shown that increased proliferation of T cells when B cells are used as antigen presenting cells is not the result of non-specific activation signals generated by the T cells or components of the medium (Gosselin et al, 1988). The use of virus reassortants (H6N2 or H3N1) containing identical internal proteins (from A/PR/8/34) and only one of the surface glycoproteins to which the experimental animals had been previously primed, enabled us to differentiate cellular responses to each surface antigen. After purification, each group of purified T cells was paired with each group of antigen stimulated B cells in a standard factorial design (Figure 5).

RESULTS

Comparative primary immunogenicity of H3N2 and H7N2 vaccines as measured by splenocyte proliferation response.

In order to determine the comparative immunogenicity and priming specificity of H7N2 and H3N2 virus vaccines, splenocytes from unprimed mice injected with either of these two vaccines were stimulated in vitro by H6N2 or H3N1 test viruses. These vaccines had induced equivalent antibody responses to their homologous HA (H3 or H7) and NA (N2) antigens in unprimed mice (Johansson et al, 1987a, 1987b). In the present study, splenocytes from these animals proliferated to equivalent levels when stimulated with H6N2 virus (Table XIII) confirming previous findings that H7N2 and H3N2 inactivated vaccines are equivalent in primary N2

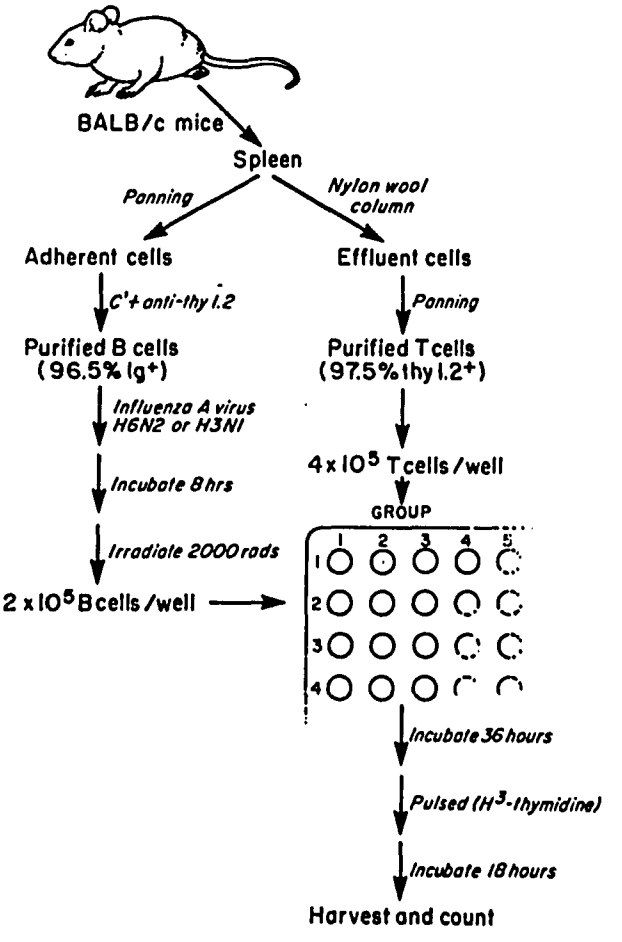

Fig. 5. Purified cell populations for in vitro proliferation assays were obtained as outlined above. See text for details.

Table XII. Immunization protocol and serologic response of mice to sequential infection and vaccination with influenza viruses.

Mouse group	Serologic response* on day PI1 (postinfection 1)								
	Titer after infection				Vaccine injection i.p. (day 63 PI1)	Titer after injection			
	1† (day 42 PI1)		2‡ (day 63 PI1)			(day 70 PI1)		(day 77 PI1)	
	NI	HI	NI	HI		NI	HI	NI	HI
1	1.0	1.0	1.0	1.0	Mock	1.4	1.0	1.1	1.0
2	4.8	2.5	4.5	1.9	Mock	5.6	2.8	7.0	2.6
3	4.7	2.3	5.0	1.9	H3N2§	7.1	6.6	11.0	7.9
4	4.7	2.3	4.9	2.1	H7N2¶	8.0	4.5	14.6	5.1
5	5.2	2.4	5.4	2.1	B/Lee	5.2	3.1	6.4	3.8
Vaccine standardization group									
A					H3N2	1.6	0.9	3.2	2.0
B					H7N2	1.5	1.0	2.9	1.0

*NA inhibition (NI) antibody titer (H3N1 test virus) is expressed as the reciprocal of the geometric mean titer; HA inhibition (HI) antibody titer (H3N1 test virus) is expressed as the reciprocal of the geometric mean titer.

†Aerosol exposure to A/Hong Kong/1/68(H3N2) virus (groups 2–5), group 1 being mock-infected (day 0).

‡Intranasal inoculation of A/Philippines/2/82(H3N2) (groups 2–5), group 1 being mock-infected (day 42).

§A/Hong Kong/1/68(H3N2).

¶A/Equine/Prague/1/56–Aichi/2/68(R)(H7N2).

immunogenicity. However, when H3N1 was used as stimulator virus, only splenocytes from animals injected with H3N2 vaccine proliferated significantly (Table XII), although splenocytes from H7N2 vaccinated mice did proliferate above background levels in response to H3N1 virus. This observation is consistent with the finding that H7 HA can induce low levels of H3 cross-reactive HI antibody (Johansson et al, 1987a, 1987b; Kilbourne, 1976). H3N1 test virus stimulated splenocytes from H3N2 vaccinated animals twice as well as an equivalent dose of H6N2 test virus, suggesting that H3 was the dominant immunogen not only after infection (Table XIV, lines 6,7 and 13, 14) but also after a single injection of vaccine.

Priming effects of sequential infection: Segregation of B and T cell responses by study of in vitro antigenic stimulation of B/T cell mixtures. Stimulation of B-T cell mixtures from unprimed (Group 1) mice with either the H3N1 or H6N2 virus resulted in equivalent T cell proliferative responses (Table XIV, lines 1 and 8).

The priming of cells to both external antigens of the infecting H3N2 viruses is shown by comparison of lines 6 and 7 (H3-specific recall) and lines 13 and 14 (N2 specific recall) with proliferative responses of unprimed cells (lines 1 and 8).

The immunodominance of H3 over N2 is seen in comparisons of proliferative response to H3N1 stimulation (lines 6, 7) with H6N2 stimulation (lines 13, 14) of cells

Table XIII

Proliferation of splenocytes from unprimed mice injected with H3N2 or H7N2 vaccine

In vitro stimulator virus*	[^{3}H]Thymidine incorporation after infection,† cpm ± SEM	
	H7N2	H3N2
H6N2	40,844 ± 1817	38,609 ± 2202
H3N1	9,095 ± 1500	74,773 ± 1129
None	1,868 ± 304	1,399 ± 119

*One hundred hemagglutinating units of virus were used as the stimulus.
†Values are mean cpm ± SD of triplicate cultures.

Table XIV

Interaction of B and T cells from sequentially infected and unimmunized mice

	Cell mixture						
	B cells		T cells				
Exp.	Mouse group	*In vivo* primed	Mouse group	*In vivo* primed	Test virus	cpm × 10^{-3}/min*	Rank¶
1	1	None†	1	None†	H3N1	49	E
2	1	None†	2	H3N2‡	H3N1	97	C
3	1	None†	5	H3N2‡ B/Lee§	H3N1	100	C
4	2	H3N2‡	1	None†	H3N1	95	C
5	5	H3N2‡ B/Lee§	1	None†	H3N1	97	C
6	2	H3N2‡	2	H3N2	H3N1	200	A
7	5	H3N2‡ B/Lee§	5	H3N2 B/Lee§	H3N1	205	A
8	1	None†	1	None†	H6N2	48	E
9	1	None†	2	H3N2‡	H6N2	94	C
10	1	None†	5	H3N2‡ B/Lee§	H6N2	85	C
11	2	H3N2‡	1	None†	H6N2	60	D
12	5	H3N2‡ B/Lee§	1	None†	H6N2	69	D
13	2	H3N2‡	2	H3N2‡	H6N2	105	B
14	5	H3N2‡ B/Lee§	5	H3N2‡ B/Lee§	H6N2	117	B

*T-cell proliferation as measured by [^{3}H]thymidine incorporation; values are mean cpm × 10^{-3}/min of triplicate cultures. Replicates were within ± 12% of the mean, so SDs are omitted.
†Animals given sterile PBS.
‡Sequential infection by H3N2 heterovariant viruses.
§Injection i.p. of virus.
¶Tukey test ($\alpha = 0.05$) subsequent to analysis of variance ($P < 0.0004$) placed significantly different groups in rank order as indicated.

from the same infected mice. This HA skewed immune response can be explained as the result of greater B cell priming. A greater proliferative response in naive T cells was induced by primed B cells stimulated by H3N1 than by primed B cells stimulated by H6N2 virus (lines 4,5 compared to lines 11,12). In contrast, T cells from infected animals exhibited no difference in proliferative response to H3 and N2-specific stimulation when mixed with naive B cells (lines 2,3 and 9,10). This result is consistent with current evidence (Zweerink, Courtneidge, Skehel et al, 1977; Ada et al, 1981) that T cell memory in influenza virus infection is primarily for internal proteins of the virus, whereas B cell memory primarily involves recognition of surface viral antigens, HA in particular. In this context, it is interesting that Group 2 and 5 B cells were only slightly better at presenting H6N2 to naive T cells than were Group 1 (unprimed) B cells (Table XIV, lines 11 and 12). This demonstrates that very little B cell memory was established for N2 even though both groups were infected twice with viruses containing N2. These results are consistent with our original hypothesis that very little expansion of NA-specific B cells occurs when NA is repetitively presented in the context of the same HA, and also suggests that internal proteins elict little or no B cell memory.

Proliferative response of T cells stimulated by H6N2: Superior proliferation in H7N2 vaccinated animals. Among T cell groups, those animals injected with H7N2 virus after

sequential infection (Table XV, Column A, Group 4) had the greatest T cell proliferative response to H6N2 and animals that received H3N2 vaccine (Group 3) had the next highest response. The T cell proliferative responses of animals that received H3N2 vaccine (Group 3) had the next highest response. The T cell proliferative response of animals injected with PBS (Group 2) or B/Lee virus (Group 5) were significantly lower than that of Groups 3 and 4 but were not significantly different from each other. Differences among proliferative responses induced by the various B cell groups reflect patterns identical to those found with T cell groups.

Proliferative response of T cells stimulated by H3N1: Animals vaccinated with H3N2 show greatest response. When H3N1 was used as stimulating test virus (Table XV, column B) a different pattern of difference among groups was found. T cells from groups of animals injected with (H3N2 or H7N2) virus after sequential infection with H3N2 variants had the greatest in vitro response to H3N1 stimulator virus. There were no significant differences among proliferation responses of T cells taken from animals sequentially infected, then injected with H7N2 (Group 4), B/Lee (Group 5) or PBS (Group 2), although these groups were significantly different from Group 3 (highest response) and Group 1 (mock infected:lowest response). The pattern of response among B cell groups from these mice was identical to the pattern found with T cell groups: greatest response from Group 3

Table XV

Rank order of mean T-cell and B-cell proliferation response to N2-specific and H3-specific antigen stimulation

Exp. A with stimulator virus H6N2			Exp. B with stimulator virus H3N1		
Mouse group	Mean cpm*	Rank†	Mouse group	Mean cpm*	Rank†
		T cells			
1	98,872	D	1	95,049	C
2	143,151	C	2	184,892	B
3	209,310	B	3	274,831	A
4	279,894	A	4	183,784	B
5	137,846	C	5	186,280	B
		B cells			
1	114,401	D	1	94,526	C
2	138,055	C	2	192,017	B
3	181,783	B	3	255,147	A
4	299,095	A	4	192,691	B
5	145,739	C	5	190,455	B

*Mean cpm of [^{3}H]thymidine incorporation in all cultures containing T cells in experiment A (or B cells in experiment B) from a given group. Each value represents 15 replicates [five B-cell groups in experiment A (or T-cell groups in experiment B) from three cultures per group = 15].

†Tukey test ($\alpha = 0.05$) subsequent to analysis of variance ($P < 0.0001$) placed significantly different groups in rank order as indicated.

(H3N2 vaccine) followed by Group 4 (H7N2), Group 5 (B/Lee), and Group 2 (PBS).

Comparative responses of primed and unprimed T cells stimulated by naive B cells: a measure of T cell memory. By culturing T cells from each group with naive B cells, we recreated in vitro a cellular situation comparable to the transfer of purified T cells to naive athymic mice (Johansson et al, 1987b) (see Table XVI). In both these experiments, segregation of effects due to T cells from each group was effected. In in vitro assays, dichotomous responses of T cells were measured by using H6N2 virus to test for N2 antigen-induced proliferation, and H3N1 virus to assay for H3 antigen-induced proliferation.

When T cells from each infection/vaccination group were exposed to naive B cells incubated with H6N2 virus, T cells from H7N2 vaccinated mice (Group 4) had a significantly higher response than cells of any other group. The T cell response of Group 3 (H3N2) was significantly greater than responses of cells of Groups 2 (PBS) and 5 (B/Lee immunized), but significantly less than that of Group 4 (H7N2) T cells. As expected, the unprimed T cells of Group 1 (MOCK infected) had the least response to stimulation by H6N2 virus.

When H3N1 virus was used in this assay instead of H6N2 virus, T cells from animals injected with H3N2 virus after sequential infection (Group 3), had the highest response. The proliferation responses to H3N1 by T cells from Group 4

Table XVI

Comparative efficacy of primed and unprimed T cells stimulated by naive B cells from mock-infected animals (group 1)

T-cell mouse group	*In vivo* priming	[^{3}H]Thymidine incorporation with test viruses*			
		H6N2		H3N1	
		cpm × 10^{-3}/min	Rank†	cpm × 10^{-3}/min	Rank†
1	None	48	D	49	C
2	H3N2‡	94	C	97	B
3	H3N2‡ H3N2§	119	B	146	A
4	H3N2‡ H7N2§	194	A	98	B
5	H3N2‡ B/Lee§	85	C	100	B

*T-cell proliferation as measured by [^{3}H]thymidine incorporation; values are mean cpm × 10^{-3}/min of triplicate cultures. Replicates were within ± 12% of the mean, so SDs are omitted.

†Tukey test ($\alpha = 0.05$) subsequent to analysis of variance ($P < 0.0001$) placed significantly different groups in rank order as indicated.

‡Sequential infection by H3N2 heterovariant viruses.

§Injection i.p. of virus.

(H7N2), Group 5 (B/Lee), and Group 2 (PBS) cannot be separated statistically. Again, cells of Group 1 (Mock infected) had the lowest in vitro response to stimulating virus.

Comparative efficacy of primed and unprimed B cells in stimulating proliferative response in naive T cells. To determine the role of B cells in the response of sequentially infected and vaccinated mice, we mixed in culture purified B cells from each group with naive T cells then exposed them to H3N1 or H6N2 test virus (Table XVII). When naive T cells were stimulated by B cells from each group pulsed with H6N2 virus the highest proliferation response was found in cultures containing Group 4 B cells. This proliferation response was significantly greater than the next highest response (Group 3). Proliferation responses from Groups 2 and 5 could not be separated statistically. However, when H3N1 virus was used to stimulate B cells from each group, the greatest proliferation of naive T cells occurred when B cells from Group 3 were used. The proliferative response of cultures containing B cells from Group 3 was significantly higher than the responses in cultures containing B cells from Group 3 was significantly higher than the responses in cultures containing B cells from Groups 4, 2 or 5, among which there were no statistically significant differences. It is noteworthy that the degree of difference between the highest responder groups was greater when the primed B cells from a group were presenting

Table XVII

Comparison of primed and unprimed B cells in stimulating proliferation response in naive T cells from mock-infected animals (group 1)

B-cell mouse group	*In vivo* priming	[^{3}H]Thymidine incorporation with test viruses* H6N2 cpm × 10^{-3}/min	H6N2 Rank†	H3N1 cpm × 10^{-3}/min	H3N1 Rank†
1	None	48	D	49	C
2	H3N2‡	60	C	95	B
3	H3N2‡ H3N2§	92	B	162	A
4	H3N2‡ H7N2§	223	A	100	B
5	H3N2‡ B/Lee§	69	C	97	B

*T-cell proliferation as measured by [^{3}H]thymidine incorporation; values are mean cpm × 10^{-3}/min of triplicate cultures. Replicates were within ± 12% of the mean, so SDs are omitted.

†Tukey test ($\alpha = 0.05$) subsequent to analysis of variance ($P < 0.0002$) placed significantly different groups in rank order as indicated.

‡Sequential infection by H3N2 heterovariant viruses.

§Injection i.p. of virus.

viral antigens than when primed T cells from that group were stimulated by naive B cells (line 4, Table XVII; line 4, Table XVI).

HA-NA antigenic competition is demonstrated by antigen-specific in vitro proliferation in B and T cell mixtures. Purified B cells and purified T cells from vaccine boosted immunization Groups 3 and 4 were mixed after B cells had been exposed to test virus. The data summarized in Table XVIII demonstrate that in mixtures of Group 3 T cells and Group 3 B cells (line 1), T cells proliferated more than twice as well as did Group 4 T cells in contact with group 4 B cells (line 2) in response to in vitro stimulation with H3N1 virus. Conversely, mixtures of B and T cells from Group 4 (H7N2 vaccine) responded more than twice as well to H6N2 virus as cells from Group 3. Although the first observation is explicable on the basis that Group 4 animals had had less H3 priming, Groups 3 and 4 had had equal exposure to the N2 antigen. These results are congruent with our previous demonstration of HA-NA antigenic competition based on antibody response (Kilbourne et al, 1987; Johansson et al, 1987a) and provide evidence that the effect is mediated by B-T cell interaction. Furthermore, the proliferative response of intergroup mixtures of B and T cells (lines 3 and 6, Table XVIII) suggests the primary importance of the B cell as APC in intravirionic antigenic competition. When T cells and B cells from Groups 3 and 4 respectively, were mixed, the higher response to H3N1 in

Table XVIII

Intra- and intergroup comparisons of B cell–T cell interaction in response to *in vitro* stimulation

	Mouse group cells		[^{3}H]Thymidine incorporation with *in vitro* viral stimulus,* cpm × 10^{-3}/min		
Exp.	B	T	*In vivo* priming	H3N1	H6N2
1	3	3	H3N2† H3N2‡	481	212
2	4	4	H3N2† H7N2‡	199	439
3	3	4	Mixed§	210	189
4	3	1	H3N2† H3N2‡	162	92
5	1	3	H3N2† H3N2‡	146	119
6	4	3	Mixed§	269	330
7	4	1	H3N2† H3N2‡	100	223
8	1	4	H3N2† H7N2‡	98	194

*T-cell proliferation as measured by [^{3}H]thymidine incorporation; values are mean cpm × 10^{-3}/min of triplicate cultures. Replicates were within ± 12% of the mean, so SDs are omitted.
†Sequential infection by H3N2 heterovariant viruses.
‡Injection i.p. of vaccine virus.
§Group 3 donors were sequentially infected by H3N2 virus and then injected i.p. with H3N2; group 4 donors were similarly infected but were injected with H7N2 virus.

vitro stimulation occurred when Group 3 B cells were used. Conversely, a greater response to H6N2 virus occurred when Group 4 B cells were used. The predominant role of B cells was most clearly seen when primed B cells from these two groups presented either H6N2 or H3N1 virus to naive T cells (lines 4 and 7). An additional point of interest is that the greatest response to in vitro stimulation with H3N1 virus was in mixtures of Group 3 B and T cells. Also, when Group 4 B cells are paired with Group 4 T cells, proliferative response to H6N2 was superior to that shown with intergroup cell mixtures.

Discussion

The relative immunogenic inferiority of N2 antigen when administered in vaccine virus containing H3, the hemagglutinin previously encountered by animals sequentially infected with H3N2 heterovariants, cannot be explained by differential immunogenicity of the H3N2 or H7N2 vaccines. Studies in unprimed mice showed that when given at equal dosage, the two vaccines induced comparable levels of NI (anti-N2) antibody and homologous HI antibody (Johansson et al, 1987a) and the present studies have shown equivalent N2-specific response of splenocytes in vitro. A likely explanation for this phenomenon posits a different immunologic response at the level of recognition and presentation of NA antigen in association with novel HA antigen. The studies presented here provide evidence for a difference in the recognition and processing of vaccine

viral particles in mice primed to H3 antigen. H3N2 viral particles are apparently preferentially captured by H3 memory B cells which reduce the opportunity for N2 to unite with B or T cell receptors that would result in N2 antibody production whereas capture of H7N2 viral particles by N2 memory B cells is unimpaired by concomitant H3 recognition. In either case processed viral antigens can then be presented to immune T cells, resulting in the activation of both the T and B cells.

In the present study we have also shown that the proliferative response of T cells from H7N2 injected animals mixed with naive B cells stimulated by H6N2 virus was superior to that of any other T cells group (Table XVI). However, when H3N1 virus was used to stimulate the same T cells mixed with naive B cells, the resulting proliferative response was significantly inferior to the proliferation of T cells from H3N2 vaccinated mice mixed with naive B cells. These results are consistent with our previous finding of reduced generation of T_h cells specific for N2 antigen in animals sequentially infected by H3N2 heterovariant viruses then injected with H3N2 vaccine. We have now demonstrated both an expansion of N2-specific B cells in mice boosted with H7N2 vaccine and an expanded H3 specific B cell population in H3N2 vaccinated mice. Furthermore, the present <u>in vitro</u> system has enabled us to segregate and assess independently the role of B and T cells in immunization by H3N2 influenza viruses and has resulted in a clear definition of

the contribution both of primed B cells and T_h memory cells in the mediation of intravirionic antigenic competition. Differences in proliferative responses among B-T cell mixtures cannot be explained as the effect of unique interfacing among cells removed from the same animal (Table XV) or among naive cells (Tables XVI and XVII). When B cells from H3N2-boosted mice were mixed with T cells from the other groups and stimulated with H3N1, it is clear that regardless of T cell group, they always yielded a better response than was observed with any other B cell group. Similarly, the proliferative response induced by H6N2 virus with B cells from H7N2 vaccinated mice was always superior to the proliferation of any other group, regardless of the T cell group employed. While this effect was seen with both H3N1-stimulated B cells as well as H6N2-stimulated B cells of H7N2 vaccinated mice, the effect was more pronounced with H6N2. This probably resulted from the fact that all B cell groups with the exception of those from naive animals were primed to H3 antigen, while B cells from H7N2 vaccinated mice were better primed to N2 antigen. This point is supported by the observation that B cells from animals that were not injected with influezna A virus vaccine antigens after sequential infection induced significantly greater T cell proliferation in response to H3N1 virus than occurred with H6N2 virus. Also, T cells from H3N2 vaccinated mice always responded better to H3N1 than did other T cells,

regardless of the B cells used in antigen presentation, and T cells from H7N2-vaccinated animals were always better responders to H6N2 virus, regardless of the B cells used. However, an interfacing effect was observed in homologous cell mixtures: the greatest proliferative response to H6N2 virus was in homologous B-T cell mixtures from H7N2 vaccinated mice and the greatest response to H3N1 virus was in B-T cell mixtures from mice given H3N2 vaccine. Taken together, all these data provide evidence that both B cells and T cells contribute to intravirionic antigenic competition between influenza virus surface glycoproteins.

The model we propose for intravirionic antigenic competition assumes that antigen presentation by macrophages probably precedes or coincides with antigen presentation by B cells. This assumption is based on recent work indicating that only activated B cells serve as efficient antigen presenters (Chesnut, Colon and Grey, 1982; Kakiuchi et al, 1983), and that B cells play this role only in secondary immune responses or late in primary response (Lanzavecchia, 1985; Hutchings et al, 1987). We postulate that in the first recognition of influenza virus antigens by the immune system, viral particles are probably engulfed by macrophages processed, and presented to T cells. Because HA is found in greater molar amounts on the virion surface than NA (Compans et al, 1970) and because the degree and direction of antigenic competition are dependent on the relative

amounts of competing immunogens, the resulting immune response is relatively HA skewed. Subsequent infections with influenza virus reinforce bias toward HA response by continuing to select HA reactive B and/or T cell clones with greater frequency than NA reactive clones. If, however, infection (or vaccination) occurs with a virus containing a previously encountered NA and an HA to which the host is immunologically naive, NA specific memory B cells, expanded by previous exposure, more efficiently capture viral particles and present antigen to memory T cells, resulting in B and T cell activation. Resting B cells recognizing the novel HA are probably competed out by the more efficient activated NA specific B cells. Clones specific for this new HA will undergo essentially a primary immune response, with an inherently slower reaction time than that which characterizes the secondary response.

Our model for intravirionic antigenic competition differs conceptually from the model proposed by Taussig (1977) for intermolecular antigenic competition that stresses T cell participation. Our model proposes not only T cell involvement but also competition at the level of B cell recognition. In intravirionic antigenic competition, the competing antigens are on the same virus particle and therefore can be taken up and presented by the same B cell, whereas studies of intermolecular competition involve mixtures of antigenic molecules which, because they are unlinked, cannot be presented by the same B cells.

Our studies have direct application in defining the complex immunologic response in influenza and reinforce earlier proposals (Couch et al, 1974) for an unconventional approach to immunization that utilizes viral NA as a primary immunogen (Couch et al, 1974; Kilbourne, 1984). To the degree that antibody to the viral NA is important in immunity to influenza (Schulman, 1975), the repeated damping of response to this antigen in favor of HA with each reinfection intrinsically and inevitably prohibits the attainment of balanced immunity to both HA and NA antigens. The situation is analogous to the well documented phenomenon of "original antigenic sin" (Francis et al, 1953) - a perversion of the anamnestic response in which primed response to conserved HA epitopes outstrips and may interfere with primary response to newly mutated antigenic sites on the HA of the notoriously changeable influenza A virus. Thus, both original antigenic sin and intravirionic antigenic competition may operate to compromise the attainment of immunity to influenza by fostering inappropriate immunologic response.

IX. Macrophages do not mediate priming-dependent intravirionic antigenic competition between influenza A virus surface glycoproteins

Macrophages play an important immunoregulatory role as phagocytes capable of ingesting and catabolizing many extracellular molecules, including viral proteins, and as secretory cells, releasing certain soluble factors continuously or in response to antigen binding and phagocytosis (Unanue and Allen, 1987). T helper (T_h) cells recognize processed antigen in association with MHC gene products on the surface of macrophages and accessory cells (Rosenthal and Shevach, 1973). It is during this interaction of T_H cells and macrophages that the initial stages of antigenic competition occur (Babbitt et al, 1986) due to direct competition for Ia molecules (Werdelin, 1982; Rock and Benacerraf, 1983; Guillet et al, 1986) or competition between T_h cells of different specificities for antigen-MHC complexes.

In the previous chapter we have shown that B cells specific for viral surface glycoproteins can present viral antigens to reactive T cells and thereby mediate antigenic competition (Johansson et al, 1987c). In the present study we confirm earlier findings that macrophages can present influenza viral antigens to immune T cells (McLaren and Pope, 1980; Rodgers and Mims, 1982; Mak and Ada, 1984) but find that after initial response to infection macrophages do not mediate HA-NA intravirionic antigenic competition.

Materials and Methods

Viruses

Influenza A virus strains used for infection or vaccination were as described earlier (Johansson et al, 1987a). Antigenically hybrid, reassortant influenza A virus H6N2 (A/Turkey/Mass/76-Aichi/2/68 [R]) (Johansson et al, 1987c) was purified on a sucrose gradient and inactivated by UV irradiation to a residual 50% egg infective dose (EID_{50}) of $10^{-1.1}$/0.2 ml, then used in *in vitro* T cell proliferation assays.

Animals

Influenza A virus primed BALB/c female mice (Jackson Laboratories, Bar Harbor, ME) from an earlier study (Johansson et al, 1987b) were donors of macrophages and T cells used in the present study.

Preparation of purified T cell populations from spleen cells

Purified T cell populations were obtained 3 months after final immunization as previously described (Johansson et al, 1987b; 1987c). Erythrocyte-free spleen cell suspensions were passed over a nylon wool column (Julius et al, 1974) and the effluent cells were further depleted of B cells by "panning" on anti-Ig^+ rabbit anti-mouse immunoglobin) plates (Wysocki and Sato, 1978). Cytofluorometric analysis with fluorescein labeled anti-thy 1.2 monoclonal antibody showed that at least 97.5% of these cells were T cells.

Infection and vaccination protocol

The procedure for sequential infections with hetero-variant H3N2 influenza A viruses has been described in detail (Johansson et al, 1987c). Vaccination procedures were as described by Johansson, et al (1987c). The immunization schedule is presented in Table XIX.

Preparation of enriched macrophage populations from splenocytes

Enriched populations of macrophages were obtained from mice of each experimental group (Table XIX) by passing splenocytes over nylon wool columns. Adherent cells were eluted off the column with cold (4°C) saline (0.85% NaCl, w/v), this fluid was forcibly pushed out of the column. These cells were then "panned" on petri dishes coated with purified rabbit anti-mouse Ig^+ (Wysocki and Sato, 1978). Cells not bound by Ig^+ were recovered from the fluid by centrifugation. Cytofluorometric analysis of these cells showed that fewer than 0.3% were stained with tetramethyl-rhodamine labelled rabbit anti-mouse Ig^+ antibody.

T cell proliferative response

Purified macrophages from each experimental group (Table XIX) were incubated with 100 hemagglutinating units (HAU) of the antigenically hybrid reassortant virus, H6N2, for 8 hours, then gamma-irradiated (2000 rads). Virus-stimulated macrophages (2×10^5/well) and T cells (4×10^5/well) from each group were co-cultured in a total volume of 200 µl/well. These cultures were maintained at

Table XIX. Immunization Experience of Mice in Macrophage Studies

Group	1st Infection	2nd Infection	Vaccine
1	$H3_{HK}N2_{HK}$	$H3_{PH}N2_{PH}$	Mock
2	$H3_{HK}N2_{HK}$	$H3_{PH}N2_{PH}$	Mock
3	$H3_{HK}N2_{HK}$	$H3_{PH}N2_{PH}$	$H3_{HK}N2_{HK}$
4	$H3_{HK}N2_{HK}$	$H3_{PH}N2_{PH}$	$H7N2_{HK}$
5	$H3_{HK}N2_{HK}$	$H3_{PH}N2_{PH}$	B/Lee

Immunization protocol for mice from which macrophages were obtained. First infection was with A/Hong Kong/1/68 (H3N2), and the second infection with A/Philippines/1/82(H3N2) (7). Mock infections and injections were with sterile phosphate-buffered saline. Vaccine preparations consisted of U.V. inactivated viruses that were injected intraperitoneally.

37°C, 5% CO_2 for 36 hours, then pulsed with 1 μ Ci ^{3}H-thymidine/well. After 18 hours of incubation with label, cells were harvested on glass wool filters and radioactivity measured. Each group of purified T cells was paired with each group of antigen pulsed macrophages in a standard factorial design (See Figure 6).

The H6N2 virus reassortant contained identical internal proteins (from A/PR/8/34) and the N2 neuraminidase to which the experimental animals have been previously primed, enabling us to compare N2 specific responses of each group.

Results

Variation in T cell proliferation response to H6N2 virus. Among T cell groups, animals injected with H7N2 virus after sequential infection (Group 4, Table XX-A and XX-B) had the greatest T cell proliferative response to H6N2 and animals injected with H3N2 vaccine (Group 3) had the next highest response. Proliferation responses among sequentially infected animals injected with phosphate-buffered saline (Group 2) were statistically indistinguishable from those of animals similarly infected but injected with B/Lee vaccine (Group 5). T cells from naive animals (Group 1) had the least response to in vitro stimulation with H6N2 virus. These results are congruent with other in vitro proliferation assays (Johansson et al, 1987c) and adoptive transfer studies (Johansson et al, 1987b) which demonstrated the role of NA-specific T memory cells in intravirionic antigenic competition.

Table XX. Comparison of macrophages and B cells as antigen presenting cells

Stimulator virus H6N2: macrophage as APC

A:

T cells of

Group	Mean cpm	Rank
1	7,347	D
2	20,750	C
3	34,714	B
4	51,225	A
5	20,866	C

C:

Macrophages of

group	Mean cpm	Rank
1	20,313	A
2	20,994	A
3	23,373	A
4	23,996	A
5	21,992	A

Stimulator virus H6N2: B cell as APC

B:

T cells of

Group	Mean cpm	Rank
1	98,872	D
2	143,151	C
3	209,310	B
4	279,894	A
5	137,846	C

D:

B cells of

Group	Mean cpm	Rank
1	114,401	D
2	138,055	C
3	181,783	B
4	299,095	A
5	145,739	C

2×10^5 APC were co-cultured with 4×10^5 T cells in triplicate wells. APC had been incubated with 100 HAU of H6N2 influenza A virus for 8 hours, then irradiated (2000 rads). Results are expressed as the mean cpm of ^{3}H-thymidine incorporation of all cultures within a given group (i.e. triplicate wells x 5 groups = 15 total wells). Note that the cells from test mice were mixed in culture in a factorial design (i.e. Group 1 T cells paired with groups 1,2,3,4 and 5 B cells, etc.) Data in columns B and D are from Chapter VII, Table XV.

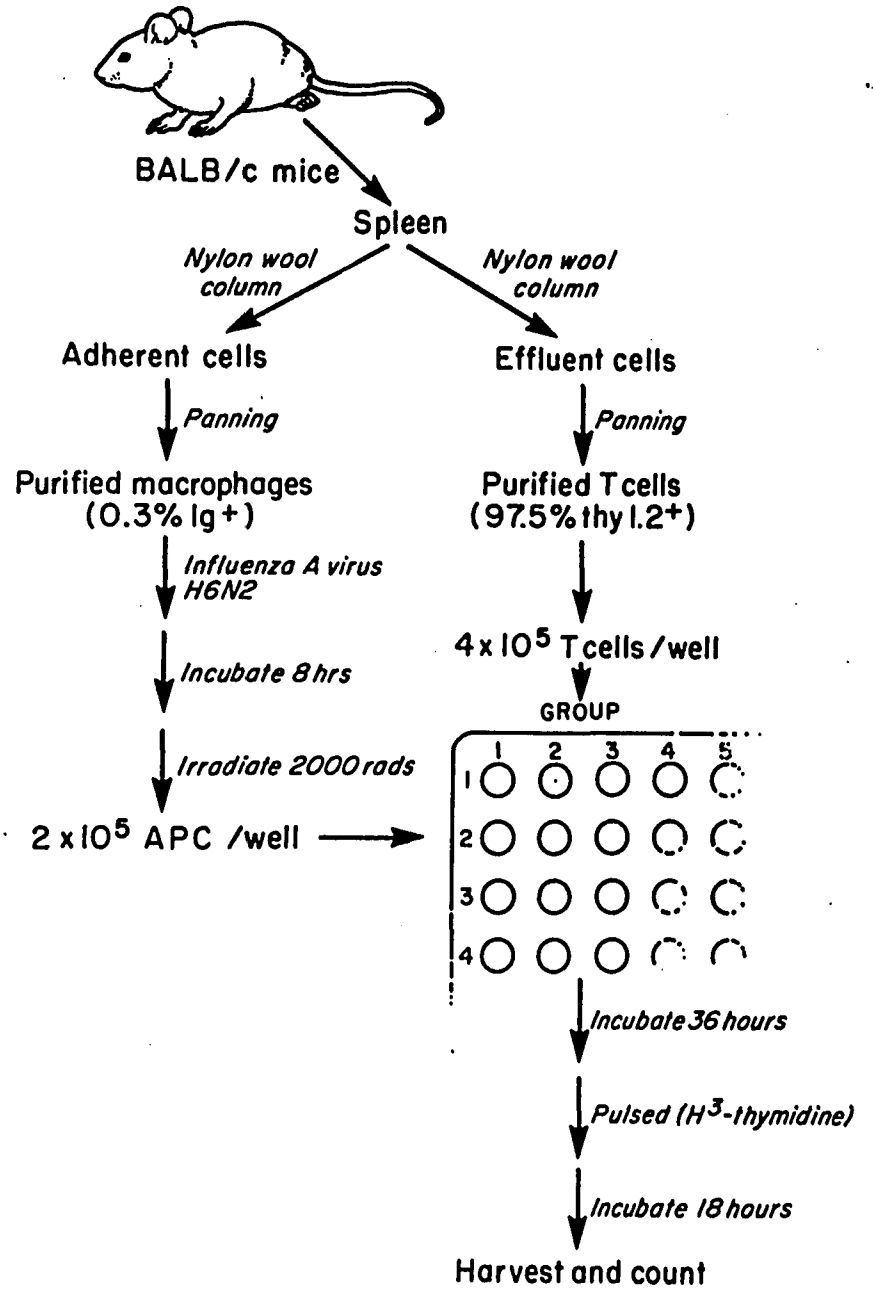

Fig. 6. Cell purification procedure and T cell proliferation assay protocol for macrophage studies.

Macrophages do not have specific antigen-presenting memory

There were no statistically significant differences in T cell proliferation among macrophage groups. Equivalent levels of T cell proliferation as measured by H^3-thymidine uptake were shown for all groups, including T cells from naive animals. Thus, macrophages do not have antigen specific memory, since there were no differences among macrophages from different experimental groups in their ability to induce T cell proliferation. This is in contrast to the variation found when B cells from each infection group were used as antigen presenting cells. B cells induced various levels of T cell proliferation dependent upon the nature of B cell immunological priming (Table XX and Johansson et al, 1987c).

DISCUSSION

Experiments described in the previous chapter indicate intravirionic antigenic competition is the result of B-T cell collaboration (Johansson et al, 1987c). In the present study we demonstrate that macrophages do not mediate HA/NA antigenic competition. Macrophages from either naive or influenza A virus (H3N2) primed mice were capable of presenting H6N2 virus to either virus primed or naive T cells. There were, however, no differences among groups in the ability of macrophages from immunized or unimmunized mice to induce T cell proliferation, although there were differences among T cell groups in their ability to proliferate in

response to H6N2 virus.

The finding that macrophages can present antigen to MHC-restricted T cells is not new, but contrasts with differences observed in T cell proliferative response when B cells are used as APC. Although initial competitive events during primary response probably occur at the level of recognition by T cells of antigen on the macrophage (Babbitt et al, 1986; Taussig and Lachman, 1972; Guillet et al, 1986; Brody and Siskind, 1969) mediation of antigenic competition subsequent to specific immunization is dominated by antigen presenting memory B cells. The superiority of B cells in antigen presentation (Chesnut et al, 1982) is confirmed directly in the present study in comparison of the amount of T cell proliferation induced by macrophages in naive (or primed) T cells to that induced by B cells in naive (or primed) T cells (Table XX).

X. Comparative immunogenicity of influenza A virus surface glycoproteins: I. Purified viral hemagglutinin and neuraminidase are equivalent immunogens

Antigenic competition exists between the HA and NA of influenza A viruses and is manifest after immunization with whole virus as a suppression of anti-NA immune response. Immunodominance of HA over NA was seen in the T cell proliferation responses of mice sequentially infected with H3N2 viruses; stimulation with H3N1 virus induced significantly greater proliferation than stimulation with non-homologous H6N2 virus (Johansson et al, 1987c). This HA skewed immune response can be explained as the result of greater B and T cell priming to HA during infection (Johansson et al, 1987c).

This difference in priming capability could be due to known differences in relative molar amounts of HA and NA in the virus or to intrinsic immunogenic differences between the two proteins. The present study compares the relative immunogenicity of influenza virus HA and NA in BALB/c mice injected with graded doses of purified H3 HA or N2 NA. Specific antibody response to each surface glycoprotein was evaluated employing reassortant viruses in hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests and purified antigens in enzyme-linked immunosorbent assays (ELISA).

Materials and Methods

Viruses: The strains of influenza A viruses used in

Table XXI. Viruses used in this study

Virus Nomenclature			
HANA subtype	Strain or reassortant designation	Laboratory Name	Specific Use
H6N2	A/Turkey/Mass/75-Aichi/2/68 (R)	$H6N2_{HK}$	Source of purified N2 neuraminidase
H3N1	A/Hong Kong/1/68-PR/8/34 (R)	$H3_{HK}N1_{PR8}$	Source of purified H3 hemagglutinin
H3N2	A/Aichi/2/68 (R)	$H3_{HK}N2_{HK}$	HI antigen
H1N2	A/PR/8/34-Hong Kong/1/68(R)	$H1_{PR8}N2_{HK}$	NI antigen

these studies are identified in Table XXI. All viruses were grown in the allantoic sac of 10 day old chick embryos and stored in multiple aliquots at -70°C.

Animals: Inbred BALB/c female mice (Jackson Labs, Bar Harbor, ME) 8 weeks of age were used in this study.

Isolation of purified HA and NA from influenza A viruses

Virus preparation: Reassortant influenza A virus strains H6N2 (A/Turkey/Mass/75-Aichi/2/68 [R]) and H3N1 (A/Hong Kong/1/68- PR/8/34 [R]) were used as sources of N2 neuraminidase and H3 hemagglutinin, respectively. Viruses were grown in the allantoic sac of 10 day old chick embryos at 37°C for 40 hours. Eggs were chilled at 4°C for at least 18 hours prior to harvesting. Cellular debris was removed by centrifugation at 11,000 rpm (Sorvall GSA rotor) for 10 minutes. Virus was pelleted from the clarified supernatant by a second centrifugation at 35,000 rpm (Beckman Ti45 rotor) for 45 min. The virus pellet was resuspended in phosphate-buffered saline (PBS; 0.01 M sodium phosphate, 0.14 M NaCl [pH7]), layered over a 30%-60% sucrose gradient and centrifuged at 25,000 rpm (Beckman SW27 rotor) for 90 min. The virus band was collected, diluted with PBS, and recentrifuged at 35,000 rpm (Beckman Ti45 rotor) for 45 min.

Extraction and purification of surface antigens: H3 HA and N2 NA were extracted and purified from influenza virus particles as described by Gallagher et al (1984) with several modifications (Fig. 7). Virus pelleted from 200 eggs

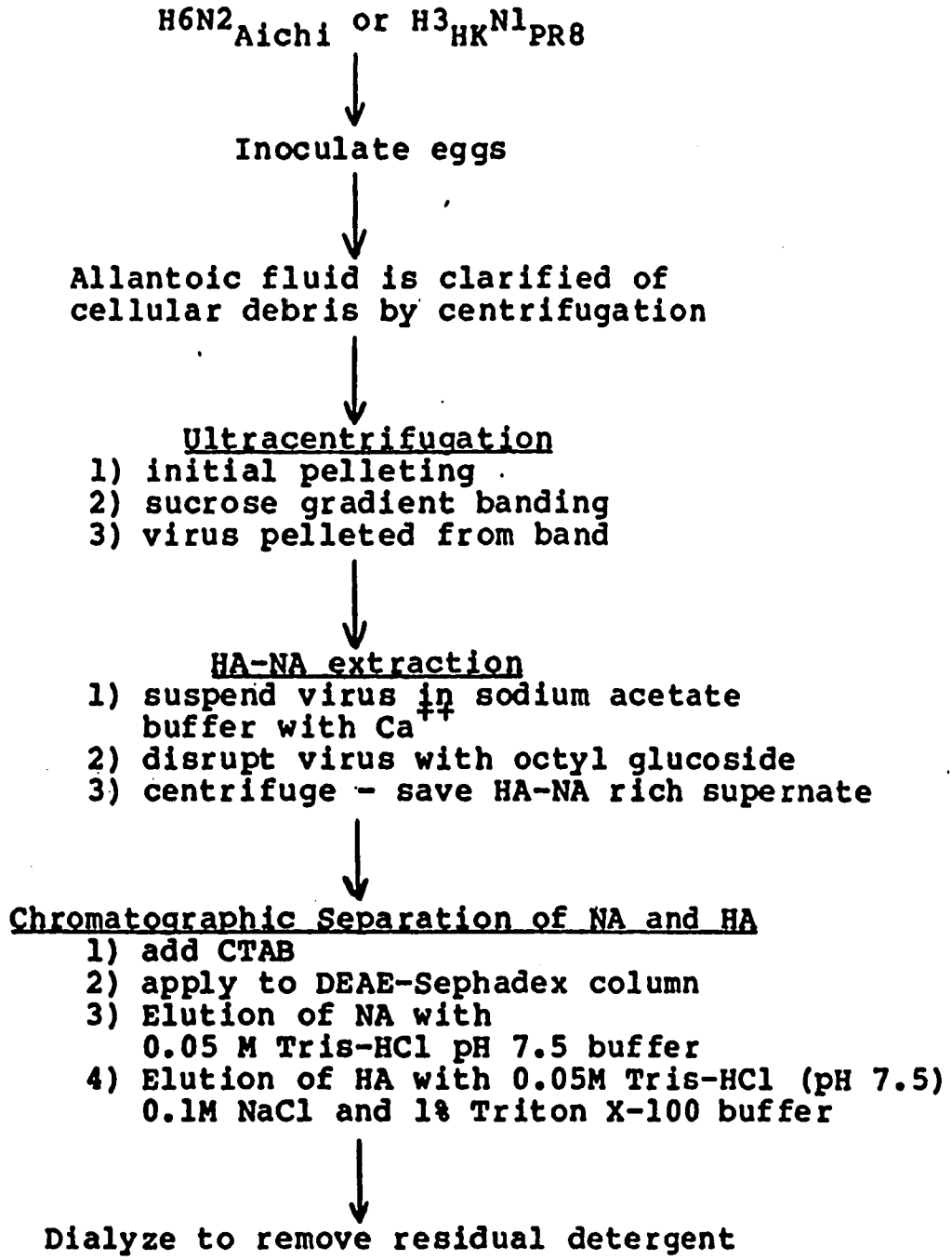

Figure 7. Protocol for isolation of influenza virus HA or NA

(yield 20-40 mg) was resuspended in 1 ml sodium acetate buffer (0.05 M sodium acetate, 2mM $CaCl_2$, 0.2 mM EDTA [pH 7.0]). The resuspended viral preparation was refluxed through a 19 gauge needle to assure a homogeneous suspension. An equal volume of 15% octylglucoside (octyl-β-D-thioglucoside, Calbiochem Co.) in sodium acetate buffer was added with vigorous vortexing. This suspension was centrifuged at 15,000 rpm (Beckman Ti50.1 rotor) for 60 min. The supernatant was carefully removed from the pellet and reserved as the HA-NA rich fraction. 2% aqueous cetyltrimethylammonium bromide (CTAB, Sigma Chemical Co.) was added to the HA-NA rich fraction to a final concentration of 0.1% CTAB (100 µl 2% CTAB/2ml solution). This solution was applied to a DEAE-Sephadex (A-50; Pharmacia Fine Chemicals) ion exchange column (bed, 0.7 cm x 6.0 cm) previously swollen and equilibrated with 0.05 M Tris-hydrochloride (pH 7.5) containing 0.1% octylglucoside (NA eluting buffer). 10-15 fractions (2 ml/fraction) were collected using the NA-eluting buffer, then the elution buffer was changed to low salt HA eluting buffer (0.05M Tris-hydrochloride [pH 7.5], 0.1 M NaCl and 0.1% Triton X-100). After 10-15 fractions (2ml/fraction) were obtained the elution buffer was changed to high salt HA eluting buffer (0.05 M Tris-hydrochloride [pH 7.5], 0.2M NaCl, and 0.1% Triton X-100). After chromatography, individual fractions were dialyzed against sodium acetate buffer with 2mM $CaCl_2$ for 96 hrs to remove any residual detergent.

Ca^{++} in buffers stabilizes viral NA enzymatic activity (Boschman and Jacobs, 1965; Dimmock, 1971) and immunogenicity (E.D. Kilbourne, unpublished results). Each fraction was tested for neuraminidase activity with fetuin substrate (Aminoff, 1961) and hemagglutinating activity with chicken red blood cells (cRBC) (Palmer et al, 1975). Fractions showing optimal activity were analyzed by SDS-polyacrylamide gel electrophoresis under non-reducing conditions by the method of O'Farrell (1975). Gels were silver stained by the method of Ohsawa and Ibata (1983).

NA isolated by this procedure co-elutes with viral lipids and spontaneously forms liposomes subsequent to dialysis (Gallagher et al, 1984). Protein was quantitated by the Lowry assay (Lowry et al, 1951).

Serologic Methods: Sera were obtained from mice by retroorbital bleeding. Mice were bled five times at seven day intervals subsequent to boosting. Non-specific inhibitors were destroyed with V. cholerae NA before use of sera in HI tests. HI tests were performed by the microtiter method (Sever, 1962) employing the reassortant viruses H3N2 (A/Aichi/2/68[R] [X-31]) and H6N1 (A/Turkey/Mass/75 - India/1/80[R]). Tests for neuraminidase inhibition (NI), employing the reassortant viruses H1N2 (A/PR/8/54-Hong Kong/1/68 [R]) and H1N1 (A/PR/8/34) were performed as described previously (Kilbourne, 1976). ELISA were done (Khan, Gallagher, Bucher et al, 1982) on individual serum specimens from every animal and every bleeding. Purified

N2 NA or purified H3, in carbonate buffer (0.15M/Na_2CO_3-0.035 M $NaCO_3$ pH 9.6), were used to sensitize the plates. 400 ng/ml of NA or HA were used in a volume of 100 µl per well. Antibody binding was detected by alkaline phosphatase conjugated sheep anti-mouse Ig (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM) used at the manufacturer's recommended dilution (ICN Biomedical, Inc., Costa Mesa, CA).

Immunizations: The study design and immunization protocol are outlined in Table XXII. 180 BALB/c mice were randomly divided into two groups of 90 animals, each group received injections of either purified H3 hemagglutinin or purified N2 neuraminidase. These two groups were further subdivided into nine subgroups of 10 animals, each member of these subgroups received injections of antigen as indicated. Injections were given either with Freund's Complete Adjuvant (FCA) into the hind foot pads or intravenously (i.v.) without adjuvant. Day 42 boosting injections of 1 µg of HA or NA were given intraperitoneally (i.p.) without adjuvant.

RESULTS

Characterization of purified antigens

Fractions from the N2 NA purification from H6N2 virus that had high NA activity ($\geq$ 3.5 M N-acetyl neuraminic acid released/100 µl/hr) and did not show any hemagglutination of chicken RBCs, were pooled and analyzed by silver staining of polyacrylamide-SDS gels following electrophoresis. As shown in Figure 8, no viral protein bands were detected except for

Table XXII: Study design and immunization protocol for NA/HA immunogenicity studies

Group #	Amount of antigen(μg)[1]	Freund's Complete Adjuvant[2]	Day 42 Boost(μg)[3]
A	0.2	-	1.0
B	1.0	-	1.0
C	5.0	-	1.0
D	25.0	-	1.0
1	0.2	+	1.0
2	1.0	+	1.0
3	5.0	+	1.0
4	25.0	+	1.0
X	0.0	-	1.0

1 Mice received either purified H3 hemagglutinin or N2 neuraminidase.

2 Animals given antigen in Freund's Complete Adjuvant were injected in the hind footpads; animals given antigen alone were injected intravenously by the tail vein.

3 All 1.0 μg booster injections were given intraperitoneally

a single band in the position of N2 NA. Fractions eluted from chromatography of disrupted H3N1 virus were pooled based on high HA activity (> 1280 HAU/50 µl) and minimal NA activity (<0.032 µM N-acetyl neuraminic acid released/100 l/hr). Analysis on SDS gel revealed only a single band in a position corresponding to H3 HA (Fig. 8). Furthermore, at the highest dosage of antigen there was no antibody detectable by ELISA (i.e., threshold 6.2 $\log_2$) against H6 HA in mice immunized with N2 NA purified from H6N2 virus or antibody specific for N1 NA in mice immunized with H3 HA purified from H3N1 virus (data not shown).

Primary Immune Response to Purified HA or NA.

In both the H3 HA and N2 NA immunization protocols, 1 µg of purified antigen without adjuvant was administered i.p. 42 days following a control i.v. injection of sterile PBS (Table XXII, group X). 1 µg doses of H3 HA and N2 NA induced equivalent but low levels of specific ELISA antibody which peaked 14 days after injection (Fig. 9). There were no significant differences in HA and NA antigen-specific primary immune responses measured by ELISA with respect to the maximum antibody titers induced or response kinetics. Measurements of antibody by HI or NI proved less sensitive. No NI antibody against N2 NA was found in any of the 10 mice injected with 1 g of NA antigen (Fig. 10a). Of the 10 mice injected with purified H3 HA only 1 mouse had detectable levels of HI antibody against H3 (Fig. 10c).

Fig. 8: 1 μg of each detergent disrupted whole virus was run in lanes marked PR8, H6N2 and HKPR8. 100 ng of purified antigens were run in lanes marked N2 and H3. Influenza virus proteins, identified on the left, indicate migration of proteins from PR8 virus, in this 10% SDS-PAGE under non-reducing conditions.

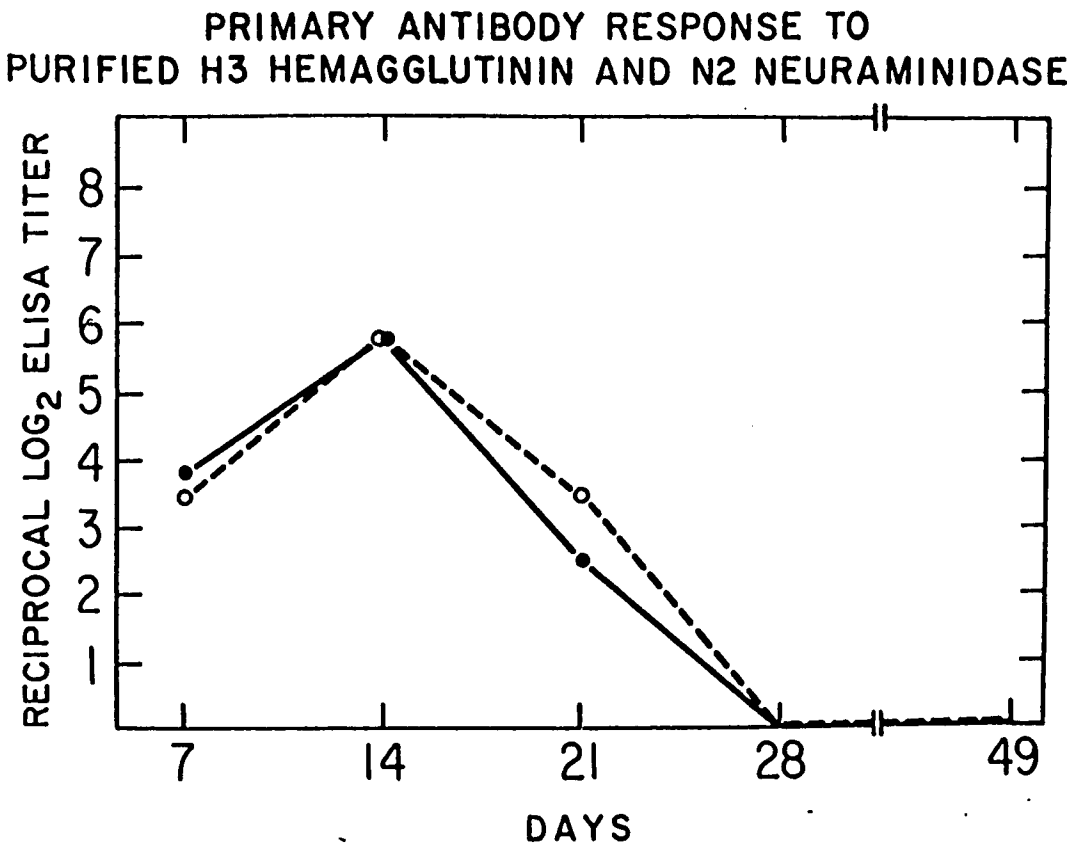

Fig. 9: Mice were injected with sterile PBS on day 0, then 42 days later were immunized with 1 μg of N2 NA (●---●), or 1 μg of H3 HA (+——+). Sera taken on the days specified were tested in ELISA.

Secondary immune response to purified HA and NA.

Data on secondary response are summarized graphically in Figures 10 and 11. Purified N2 NA and purified H3 HA were immunogenic at all dosages (0.2 - 25.0 ug) whether injected with or without FCA. As expected, there were significant differences in antibody response between groups receiving injections containing FCA and those that did not have adjuvant (analysis of variance (ANOVA): $p > 0.0001$). Antibody response to any given dose was always greater in the presence of adjuvant. Specific antibody titers in mice immunized with either purified H3 HA or N2 NA peaked 14 days after administration of a 1 μg boost. This 14 day peak was observed in HI (H3 primed mice), NI tests (N2 primed mice) and in ELISA (both antigen groups) regardless of antigen dosage.

Overall, slightly higher antibody titers measured by ELISA were found in mice injected with N2 NA. This difference was not statistically significant. There were significant differences among most dosage groups ($p > 0.0001$), but tests subsequent to ANOVA found no statistically significant differences in NI or ELISA antibody titers in animals injected with 5 μg or 25 μg of N2 NA (Fig. 11). Both of these groups had a significantly greater antibody response than the 0.2 or 1 μg groups. However, when purified N2 NA was given with FCA, significant differences were found between 5 μg and 25 μg groups as well as among groups given lesser doses.

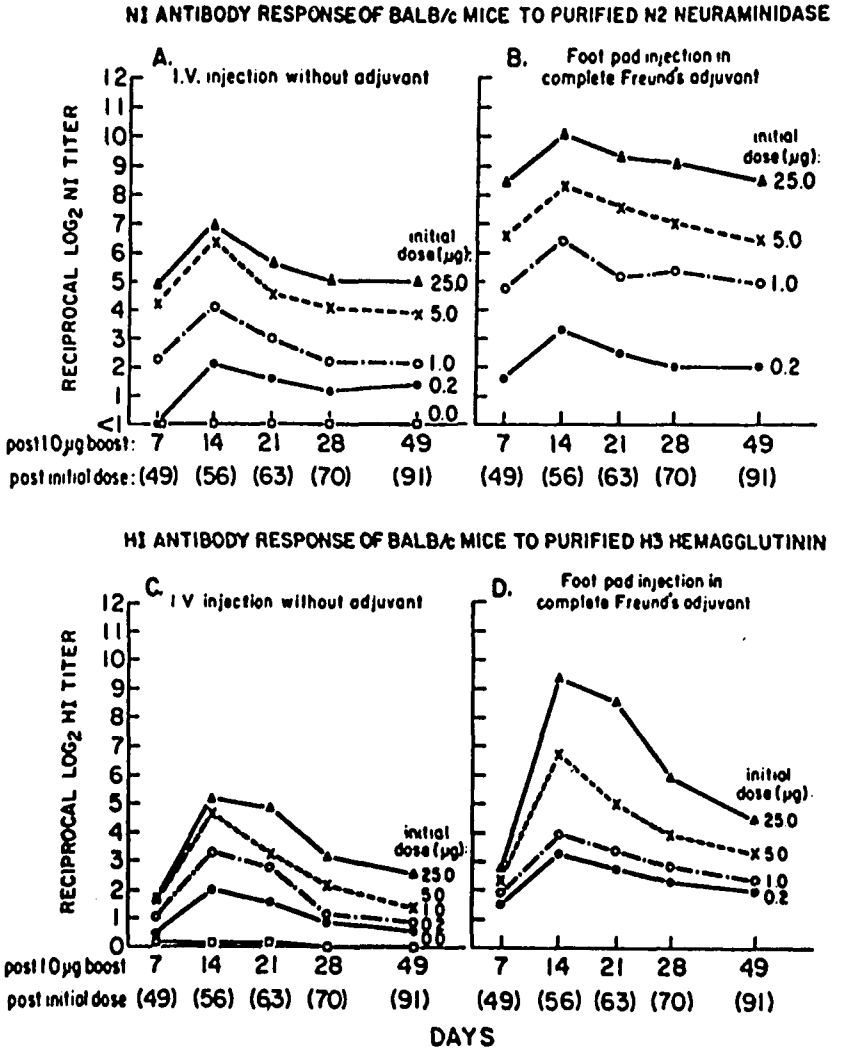

Fig. 10: Antibody to N2 from mice injected with purified N2 alone (panel A) or N2-FCA (Panel B) was measured by NI using whole H1N2 virus. Antibody to H3 from mice injected with purified H3 alone (panel C) or H3-FCA (Panel D) was measured by HI using whole H3N2 virus.

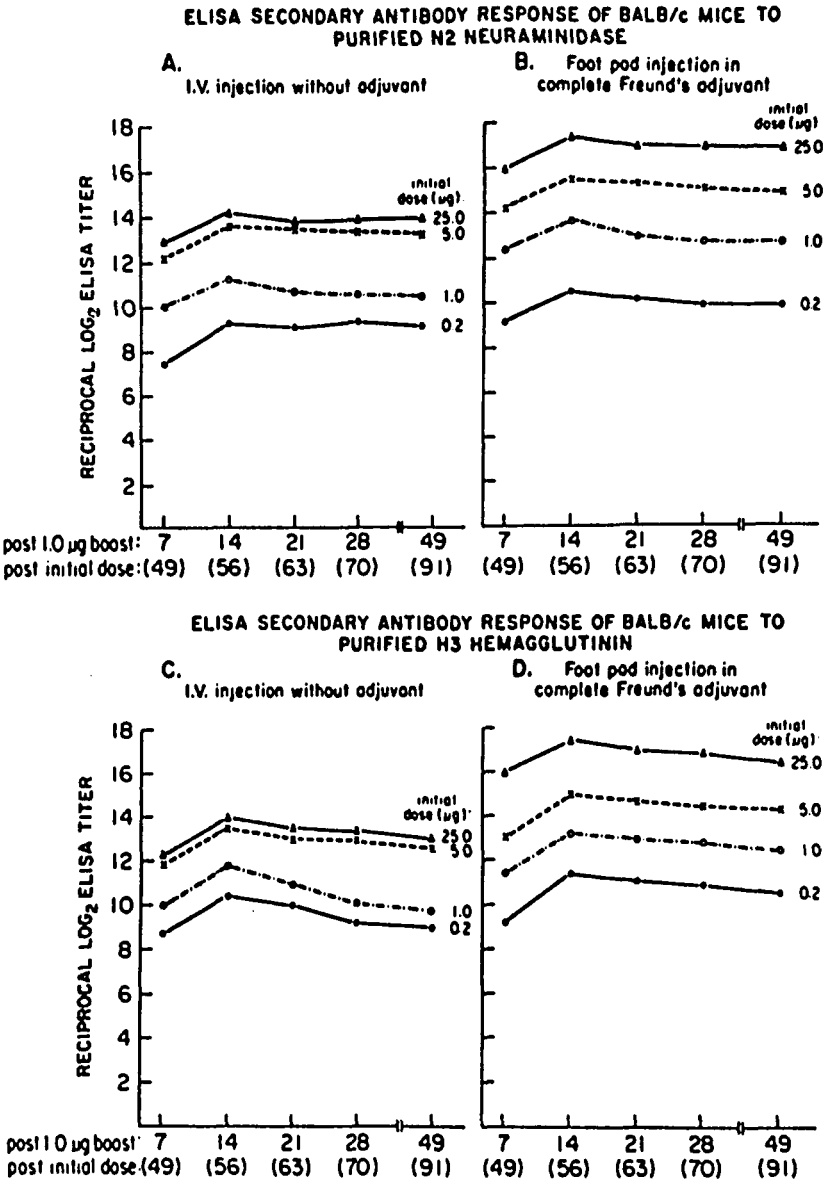

Fig. 11: ELISA antibody to N2 from mice injected with purified N2 alone (panel A) or N2-FCA (panel B). ELISA antibody to H3 from mice injected with purified H3 alone (panel C) or H3-FCA (panel D). Purified N2 (panels A and B) or H3 (panels C and D) were used to coat ELISA plates.

The pattern of differences in H3 antibody measured by ELISA and HI tests in animals that received purified H3 was similar to that found with N2 immunized animals. Injections of 5 g or 25 g of H3 antigen given without FCA, were not significantly different from each other, but were significantly different from the 1.0 µg and 0.2 µg groups. When FCA was included in the inoculum, significant differences ($p > 0.0003$) in ELISA (Figure 11) and HI tests (Figure 10) were found among all groups, with response proportional to antigen dose. The kinetics of HI and NI antibody responses differed. Although both glycoproteins induced significant rises in antibody titer from day 7 post-boost to maximum titer levels on day 14, mice injected with N2 NA had experienced a 15-39% decline in antibody titer by day 49 (Figure 10), whereas the decline from maximal HI titer in H3 immunized mice by that time ranged from 53-77%. However, these apparent differences in the rate of decline of HA and NA antibody were not as pronounced when antibodies were measured by ELISA. In N2 immunized mice there was no significant difference between NA antibody measured by ELISA on days 14 and 49; (69-83% of maximum titer was found on day 49). In comparison, H3 antibody titers were 53-87% of maximum titers by day 49, which represents a slightly significant difference ($p > 0.076$) between day 14 and day 49 ELISA antibody to HA. Furthermore, although NI antibody titers correlated highly with N2 antibody titers measured by ELISA ($r = 0.94$), the intensity of association between HI and H3

ELISA antibody was not as great (r = 0.63).

DISCUSSION

In this chapter we have shown that the influenza A virus surface glycoproteins, HA and NA, are equivalent immunogens in primary and secondary immune responses when equal amounts of these antigens are administered as purified proteins separated from other viral proteins. Injection of purified HA or NA induced identical maximum levels of specific antibody in ELISA tests (Fig. 11A-D). Because there are apparently no intrinsic differences between NA and HA in immunogenicity, the antigenic competition between HA and NA that results in greater B and T cell priming to HA antigen (Johansson et al, 1987c) is most likely a manifestation of the greater molar amount of HA found on the virion's surface and hence its presentation in greater amount to the antigen recognition elements of the immune system. This is consistent with one of the features of antigenic competition between mixed antigens, i.e., a marked dependence on the relative proportions of the antigens in the mixture. The direction and extent of antigen competition are influenced by the dose of the antigens (Brody and Siskind, 1969) and their relative molar ratios (Taussig et al, 1972). A typical example of this was found in competition between diptheria and tetanus toxoids in a mixed vaccine (Barr and Llewellyn-Jones, 1953a, 1953b). One way, therefore, to avoid competition when mixed antigens are injected is to "balance" the mixture, i.e., adjust the proportions of the

antigens so that competition does not occur. Such adjustment may assume particular importance when influenza vaccines are prepared for human use. Because there appear to be no intrinsic differences in the immunogenicity of HA and NA, balanced formulations of the two antigens might induce a more "balanced" anti-influenza virus response, avoiding the HA-skewed response to present inactivated influenza virus vaccines (Kilbourne et al, 1987).

Although HA and NA induced equivalent levels of specific antibody and similar response kinetics in ELISA, interesting differences in antibody response curves are apparent if antibody responses to the two antigens are measured by NI or HI tests. Measured by any method (ELISA, HI or NI) antibody response to both HA and NA peaked 14 days after secondary antigenic stimulation. Thereafter, the degree of antibody decline varied in relation to the immunizing antigen used (HA or NA) and the test employed in the measurement of antibody. The trends observed both in increase and decline of antibody were essentially uninfluenced by the use of adjuvant except for the greater magnitude of response in the adjuvant groups (See Fig. 10).

The sustained levels of ELISA antibodies seen with both antigens (Fig. 11) probably reflects both the greater accessibility of epitopes in that assay system and its lack of dependence on the biologic activity of the antigens to which antibody is measured. Thus, changes in antibody avidity might go undetected in a system that scores multi-

valent antibody to multiple antigenic sites. In contrast, both HI and NI tests depend on competition between antibody and either red blood cell receptors or enzyme substrate for combination with virion antigens, and, understandably, might be more susceptible to decline of high avidity antibodies. In fact, the rate of decline was greatest (down to 20% of peak) for HI antibody, while the difference between NI and NA ELISA titers at 49 days after boost was minimal. Even though the NI test measures the blocking of biologic (i.e., enzymatic) function it may be more comparable to the ELISA in that it may measure a broad range of antibodies to different sites, any of which can act by steric hindrance to block enzymatic activity.

XI. Comparative immunogenicity of influenza A virus surface glycoproteins. II. Contrasting influence on influenza virus infection of specific immunity induced by purified hemagglutinin or neuraminidase

We have shown that purified hemagglutinin (HA) and neuraminidase (NA) (major and minor influenza virus antigens, respectively) are immunogenically equivalent in the induction of antibody measured by enzyme linked immunosorbent assay (ELISA). However, past evidence indicates critical differences in the nature of immunity induced by HA or NA. Earlier studies in which HA and NA were segregated in antigenically hybrid reassortant viruses (Kilbourne, 1976; Schulman, 1969) or that employed chemically isolated NA antigen (Kilbourne et al, 1968; Schulman et al, 1968) established the different roles of the two viral proteins in the induction of immunity to influenza virus infection. Anti-NA antibody is infection-permissive, in that antibody against NA does not prevent acquisition of infection (Kilbourne et al, 1975; Schulman et al, 1968). However, NA immunization can reduce viral replication below a pathogenic threshold so that infection can occur without apparent disease (Beutner et al, 1979; Couch et al, 1974; Kilbourne et al, 1973; Ogra et al, 1977). Antibody against HA is generally regarded as neutralizing, presumably preventing infection by interfering with viral attachment to host cells (Yoden et al, 1986) or by possibly interfering with the fusion event subsequent to endocytosis (Kida et al, 1983; Yoden et al, 1986).

Preliminary vaccine studies in humans (Beutner et al, 1979; Couch et al, 1974; Kilbourne, 1976) and experiments in mice (Schulman, 1969; Schulman et al, 1968) have shown that anti-NA immunity, induced either by a reassortant antigenically hybrid virus vaccine or by a purified NA preparation, protected against disease from a virus containing antigenically homologous NA. Viral challenge resulted in the development of fully protective immunity capable of preventing subsequent reinfection.

Vaccination with conventional inactivated influenza vaccines, containing both HA and NA, stimulates immunity against both antigens (Murphy et al, 1982) although immunologic response to NA is severely suppressed in primed subjects through HA-NA antigenic competition (Johansson et al, 1987a; Kilbourne, 1976). Therefore, with biantigen vaccines the effect of anti-HA immunity is overriding and infection usually is prevented.

Having established that isolated HA and NA are equivalent immunogens on a weight basis we undertook the present study to investigate their relative immunizing capacity without the confounding effects of the high HA to NA ratio per virion characteristic of whole virus vaccines, or of intravirionic/intermolecular HA-NA antigenic competition.

Materials and Methods

Animals: BALB/c female mice immunized with graded doses of purified H3 HA or N2 NA as described in the previous chapter

were used in the present study. The HA and NA antibody titers of these animals on the day of challenge infection are summarized in Table XXIII.

Viruses: The strains of influenza A virus used in this study have been described in the previous chapter). Wild-type H3N2 (A/Hong Kong/1/68) virus was used for infecting mice. All viruses were grown in the allantoic sac of 10 day old chick embryos and stored in multiple aliquots at -70°C.

Infection protocols: 50 days after an intraperitoneal booster injection of 1 µg of purified H3 HA or N2 NA, 10 mice from each primary dosage group were inoculated intranasally under light ether anesthesia with 100 50% mouse infective doses (MID_{50}) of H3N2 virus. The surface glycoproteins contained in this virus are identical to the purified H3 HA and N2 NA used to immunize the animals.

Titration of pulmonary virus:

Three days after inoculation of virus, five mice from each group were killed and 10^{-2} screening dilutions of homogenized lung suspensions were injected into 10 day old chick embryos. The presence of hemagglutinin in harvested allantoic fluids identified virus-positive lungs. Individual virus-positive lungs were titrated for plaque-forming infective virus by inoculation of decimal dilutions (10^{-1} - 10^{-8}) of lung suspensions in Madin-Darby canine kidney (MDCK) cell cultures (3 60 mm plastic plates/dilution). After incubation at 37°C for 72 hours (Jahiel and

Table XXIII. Specific antibody titers of mice on the day of H3N2 virus challenge

	Immunizations			H3 antibody		N2 Antibody	
#	Antigen[1]	Initial dose[2] (ug)	FCA[3]	HI[4]	ELISA[5]	NI[6]	ELISA
1	None	-	-	0	< 4.0	< 1.0	< 4.0
2	H3	0.2	-	0.8	9.0	--	--
3	H3	1.0	-	1.4	9.7	--	--
4	H3	5.0	-	2.1	12.7	--	--
5	H3	25.0	-	2.6	13.3	--	--
6	H3	0.2	+	2.3	11.1	--	--
7	H3	1.0	+	3.0	12.9	--	--
8	H3	5.0	+	4.2	14.7	--	--
9	H3	25.0	+	6.3	17.1	< 1.0	< 4.0
10	None	-	-	0	< 4.0	< 1.0	< 4.0
11	N2	1.0	-		-	2.10	10.5
12	N2	5.0	-	-	-	3.90	13.3
13	N2	1.0	+	-	-	4.03	12.8
14	N2	5.0	+		-	6.37	15.1
15	N2	2.5	+	<1.0	< 4.0	8.54	17.0

1 Purified $H3_{HK}$ or purified $N2_{HK}$

2 All animals were injected with an initial dose and boosted with 1 g of antigen 42 days later

3 Freund's Complete Adjuvant was given in conjunction with antigen where indicated

4 Hemagglutinin inhibition test: geometric mean reciprocal $\log_2$ endpoint titer

5 Neuraminidase inhibition test: mean reciprocal $\log_2$ endpoint titer

6 Enzyme linked immunosorbent assays: geometric mean reciprocal $\log_2$ endpoint titers

Kilbourne, 1966), cell monolayers were stained with 0.1% crystal violet and plaques counted.

RESULTS

Infection in unimmunized mice: Infective virus was recovered from all 10 mice that had not been immunized with H3 HA or N2 NA antigens. Titration of pulmonary virus in each of these animals in MDCK cells gave a geometric mean endpoint titer of 1.53×10^{-8} PFU/0.2 ml of a 1.0% lung (v/v) suspension.

H3N2 virus infection in N2 immunized mice: Consistent with past evidence that NA antibody is infection permissive (Jahiel and Kilbourne, 1966; Kilbourne et al, 1975), we found that all mice immunized with purified N2 NA were infected when challenged with H3N2 virus containing homologous NA. The occurrence of infection in these N2-primed mice was independent of N2 antibody concentrations; i.e., all mice were infected over a broad range of antibody levels (Figure 12). However, the concentration of infective virus decreased in direct relation to pre-infection levels of anti-N2 antibody (Fig. 13). At the lowest N2 antibody level, reduction in pulmonary virus less than 2-fold (80%) was observed, but a further increase in N2 antibody level of approximately 200-fold was associated with a 10-fold reduction of virus.

H3N2 virus infection in H3 immunized mice. Mice immunized with purified H3 HA had comparable antibody titers to those of N2 immunized animals (Table XXIII). However,

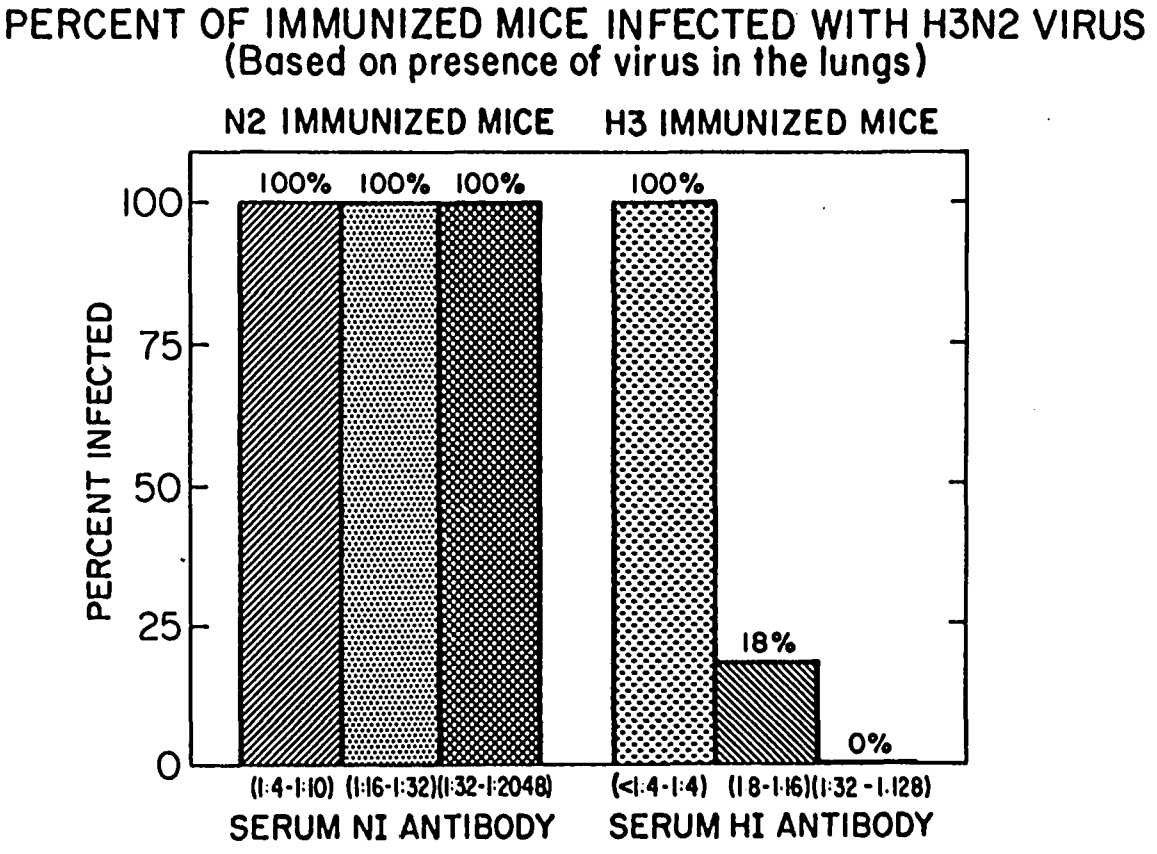

Figure 12. Incidence of infection in mice immunized with N2 (on left) or H3 antigen (on right) infected with H3N2 virus containing homologous antigen. Infection is defined by the detection of egg-infective virus in normal lungs in a concentration of 10^{-2}/0.2 ml of a 1% lung suspension (v/v).

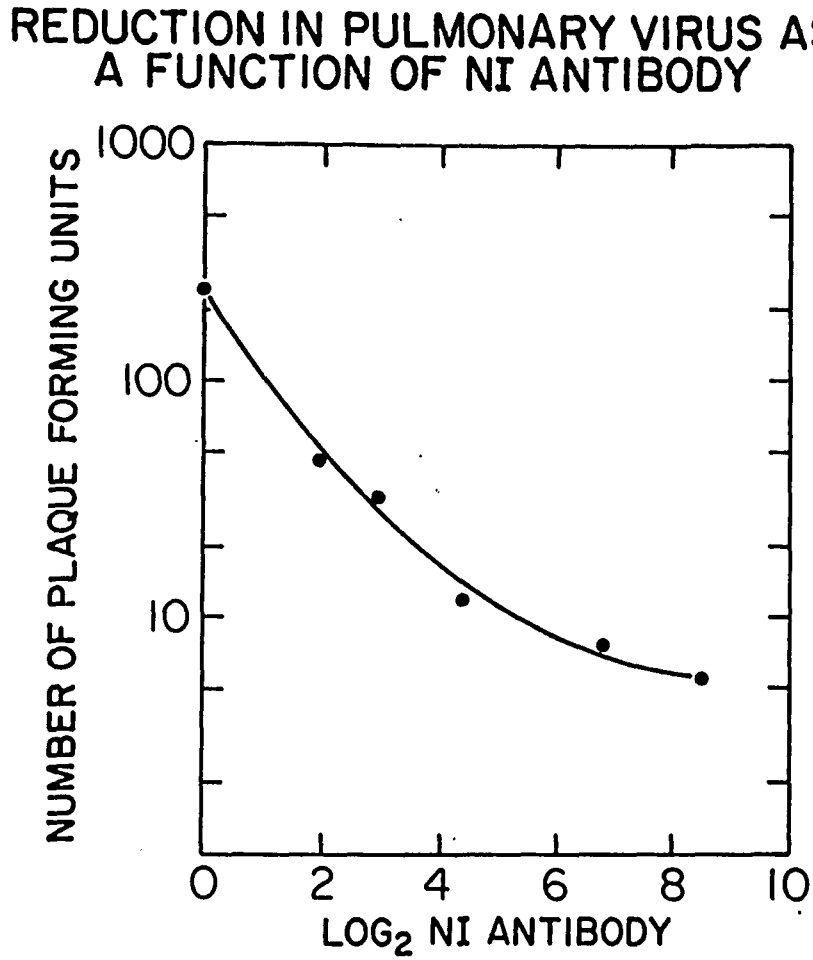

Figure 13. Plot of PFU as a function of the amount of NI antibody in N2-immunized mice challenged with 100 MID_{50} of H3N2 virus.

unlike N2 NA primed animals, H3 immunized mice were susceptible to infection only at relatively low HA antibody levels (HI $\leq$ 1:4). Suppression of infection was evident at HI titers of only 1:8. In animals with HI antibody titers greater than 1:16, infection was ablated. (Figure 12).

As in the case of NI antibody, different levels of HI antibody were associated with proportional and reciprocal changes of pulmonary virus concentration (Fig. 14). At the lowest HI antibody level, an approximately 2-fold reduction in pulmonary virus was observed but a further two-fold increase in HI titer was associated with an approximately 100-fold reduction of virus (Fig. 14). These curves (Figs. 13 and 14) demonstrate that HA immunization was infection permissive only in association with a low level of immunization reflected in a very narrow antibody titer range. At most antibody levels infection was suppressed; in contrast, NA immunization, while associated with decreased virus, was infection-permissive over a broad range of antibody concentrations.

DISCUSSION

The present study of isolated purified antigens confirms earlier evidence that NA-specific immunity is infection permissive over a broad range of antibody levels (Schulman et al, 1968) and that anti-HA immunity can prevent infection at relatively low antibody concentrations. That the effect of NA immunization may be mediated directly by circulating N2 antibody, is suggested by previous evidence

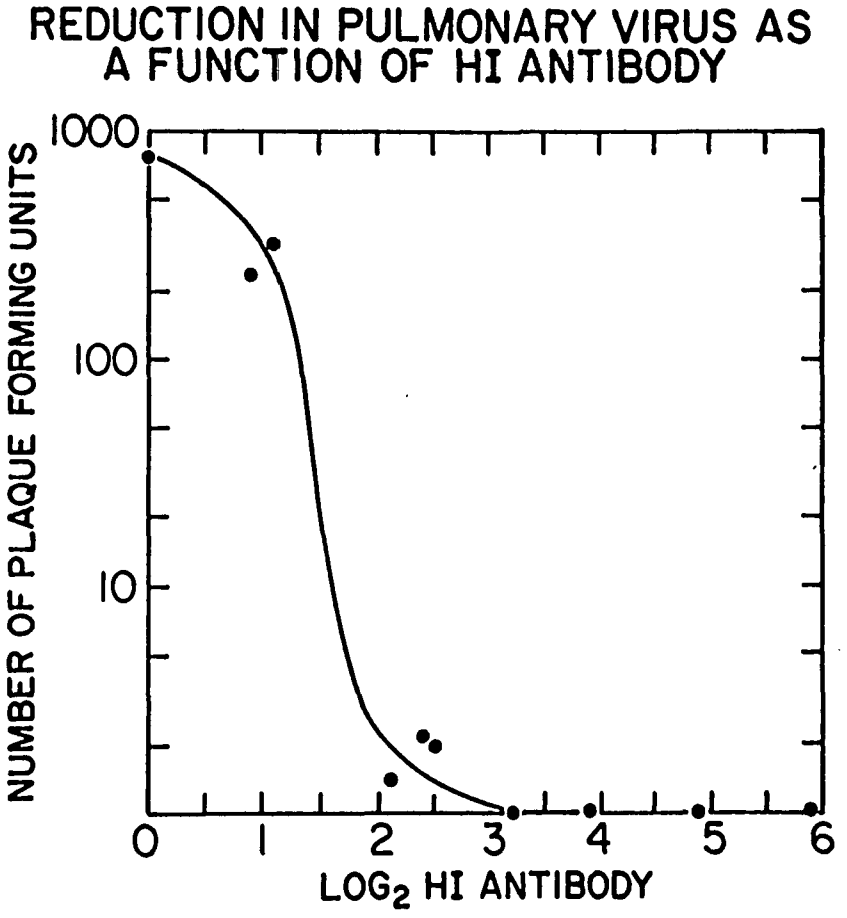

Figure 14. Plot of PFU as a function of HI antibody in mice infected with H3N2 virus. PFU were calculated from lungs of mice primed to H3 HA then infected with 100 MID_{50} of H3N2 virus.

that passive administration of NA-specific antibody can ameliorate infection (Schulman et al, 1968).

Precise measurement of infectious virus present in immunized animals revealed critical differences between immunity induced by HA and NA. Immunization with purified HA in a wide range of doses prevented infection by virus bearing an antigenically homologous HA, a situation analogous to the immunity established by vaccination with conventional influenza vaccines. Purified N2 NA induced immunity similar to that seen subsequent to vaccination by antigenically hybrid "NA-specific" vaccine viruses (Beutner et al, 1979; Couch et al, 1979; Kilbourne, 1976; Ogra et al, 1977). Although challenge virus contained N2 antigenically identical to the priming N2 NA, evidence of infection was found even in mice given the highest doses of NA. The present study of graded dose response is in agreement with previous studies that demonstrated the induction of infection permissive immunity by a single dose of NA purified by electrophoretic separation (Kilbourne et al, 1968; Schulman et al, 1968).

Although immunity is seldom absolute, the non-permissive, anti-infective immunity induced by the influenza virus HA appears to be operative over a range of antibody concentrations, with infection occurring only at the lowest antibody levels. The wide window of permissiveness afforded by even the highest concentration of NA antibody was anticipated from earlier in vitro studies. Investigations by

Jahiel and Kilbourne (1966) on the influence of antisera to H1N1 and H2N2 influenza viruses on the plaquing characteristics of an H1N2 antigenically hybrid virus, showed kinetics in the reduction of virus replication remarkably similar to those found in intact animals in the present study. A plot of viral plaque size against antibody concentration demonstrated reduction in plaque size to be principally a function of NA-specific antibody, while HA specific antibody completely inhibited plaque formation except for a narrow zone of plaque size reduction near the titration endpoint (Fig. 15).

The contrasting effects both <u>in vitro</u> and <u>in vivo</u> of antibody to HA and NA proteins are most likely a reflection of the different biological roles HA and NA play in the virus replication cycle, the HA being essential in viral attachment and initiation of infection and the NA inhibitory to virus release (Kilbourne et al, 1975). The present study of purified surface antigens demonstrates that a cardinal element in establishing immunity to influenza is recognition of the surface glycoproteins of the virus. However, these findings do not negate the importance of cognate help in B/T cell interactions, in which humoral immune responses to HA or NA are supported by T cells reactive with internal components of influenza virus (Johansson et al, 1987c; Russell and Liew, 1979, 1980; Scherle and Gerhard, 1986). After sequential H3N2 virus infection of mice, T cells from these animals proliferated equally in response to hetero-

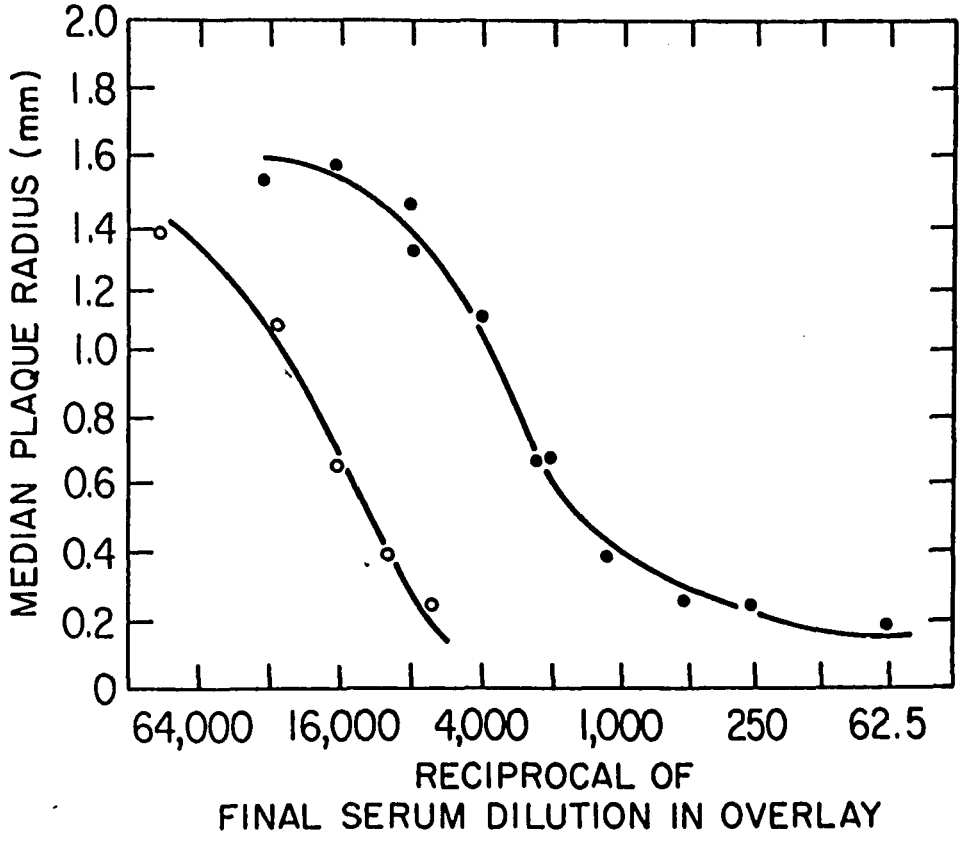

Figure 15. Plot of median plaque size radius versus decreasing amount of specific antibody. Cells were infected with reassortant H1N2 virus; agar overlay contained either antibody to H1N1 virus (HA H1 specific) (O—O) or to H2N2 virus (N2 specific) anti-N2 antibody (●—●). (Data replotted from Jahiel and Kilbourne, 1960).

subtypic (H1N1) influenza virus internal proteins, but varied in their response to HA and NA (Johansson et al, 1987c).

In conjunction with basic data on the primary immunogenicity of the purified NA and HA antigens of influenza virus, the present study shows clearly that immunization with either antigen alone can alter the course of infection. Most important, we have shown unequivocally that in its immunizing effect on virus replication, NA is not merely a weaker HA but that the partial immunity it induces permits subsequent (further immunizing) infection to occur even at the highest level of immunity induced. Thus, immunization with purified NA offers a truly unique approach to the prevention of influenza. It is likely that as a single viral protein NA will have less toxicity than whole virus vaccines, live or inactivated. As isolated from detergent disrupted virus (Kilbourne et al, 1968), NA is highly antigenic, and adjuvants, although demonstrably enhancing, should not be required for its use in man. Finally, the slower rate of antigenic drift of NA in both H1N1 (E.D. Kilbourne, unpublished data) and H3N2 (Johansson et al, 1987a) subtypes offers the prospect of more lasting immunity than that induced by the HA antigen or by conventional vaccines.

VIII. Epilogue

The series of experiments comprising this thesis have shown in a murine model system that antigenic competition between influenza virus HA and NA, which is manifest as a suppression of anti-NA response, results from greater B cell and T_h cell priming to viral HA than to NA. This greater priming to HA reflects the high HA/NA molar ratio found on intact virions. When isolated from the virion and injected as purified antigens, HA and NA are equivalent immunogens. Moreover, isolated glycoproteins induced an immunity paralleling that seen with whole viruses in which HA and NA have been segregated by reassortment, in that NA-specific immunization was infection-permissive and HA-specific immunization suppressed infection. The results of these experiments highlight the potential of using purified NA for vaccination against influenza in humans. What is now needed are experiments designed to ascertain:

1) the minimal immunogenic dose of purified NA in humans;

2) reactogenicity of the purified NA-vaccines;

3) the duration of immunity engendered by this vaccination;

4) the toxicity of purified NA vaccine in young children.

Additional experiments should investigate the use of purified NA as a "companion-vaccine" to conventional inactivated vaccines in order to increase anti-NA response. Also, purified NA could be injected simultaneously with or before a live influenza virus vaccine to increase anti-NA response that would serve as an additional guard against viral escape from attenuation.

XIII. APPENDIX: ABBREVIATIONS

APC	antigen presenting cell
C'	complement
CTL	cytotoxic T lymphocyte
EID_{50}	50% egg infectious dose
ELISA	enzyme-linked immunosorbent assay
HA	hemagglutinin glycoprotein
HA titer	hemagglutination titer
HI	hemagglutination inhibition
MDCK cells	Madin-Darby Canine Kidney cell line
MID_{50}	50% mouse infectious dose
NA	neuraminidase glycoprotein
NI	neuraminidase inhibition
NP	nucleoprotein
PBS	phosphate buffered saline
T_h	T helper cell
U.V.	ultraviolet light

XIV. BIBLIOGRAPHY

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