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New Aspects of Influenza Viruses

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INTRODUCTION AND HISTORICAL BACKGROUND

Characterization of the causal agents of human influenza began when Smith et al. (183) used ferrets to isolate a virus from patients with influenza. Those researchers also noted that convalescent-stage sera of the patients contained neutralizing antibodies to the virus. Isolation of virus was promptly confirmed by other investigators, and it was demonstrated that specific antibodies develop during the course of the disease (53). The virus was adapted to mice, serologic tests were developed, and an opportunity to study the nature and distribution of the virus was thus provided. In human volunteers, the virus induced a disease clinically characteristic of influenza. This original virus isolate and later isolates of similar viruses are now designated type A and have been continually monitored during the regular seasonal epidemics they cause (55, 152).

In 1940, a second major serotype, type B, was identified during an epidemic and was retrospectively associated with earlier outbreaks (54). It, too, has been shown to cause regional outbreaks and epidemics, although less frequently than type A influenza viruses (57, 140).

A virus isolated from a patient in 1949 was found to be antigenically distinct from influenza virus types A and B with the antibody response of the patient specific for the new virus (201). The next year, a similar virus was isolated during an institutional outbreak of influenza. This new serotype of influenza virus was designated type C (56). Later, similar viruses from numerous localized outbreaks were characterized in various countries. The almost universal distribution of the agent is shown by the high frequency of antibody in the general population. Type C influenza virus has not been associated with as severe a disease as types A and B and does not exhibit the same pattern of regular seasonal epidemic activity (94). Because of the seasonality and antigenic variation of influenza virus types A and B, their epidemiology is distinctive when compared with other less variable viruses causing epidemic respiratory diseases of humans.

Within influenza virus types A and B, different degrees of variation have been observed since the original descriptions of the types. Both virus types undergo gradual antigenic variation, referred to as antigenic drift. Type A viruses also undergo less frequent, major changes referred to as antigenic

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shift. These major changes result in the emergence of new influenza virus type A subtypes and are associated with pandemics of varying severity. The most recent antigenic shifts occurred in 1957 when type A(H2N2) viruses replaced the previously circulating A(H1N1) strains and in 1968 when type A(H3N2) strains emerged and displaced the H2N2 viruses. Until 1977 it was believed that the appearance of a new subtype must be associated with the disappearance of the previous subtype. However, when H1N1 viruses reemerged in 1977, H3N2 viruses continued to circulate and the two subtypes have cocirculated up to the present time (140). The periods of circulation of human influenza virus type A subtypes since the original isolation in 1933 are as follows: H1N1, 1918 (the presence of H1N1 in the 1918 pandemic has been inferred on the basis of retrospective seroepidemiology [154]) to 1957; H2N2, 1957 to 1968; H3N2, 1968 to present; and H1N1, 1977 to present.

The chief distinction between pandemic influenza and interpandemic influenza is that the former, associated with antigenic shift, is disseminated rapidly through an immunologically naive population over the entire globe, while the latter, associated with antigenic drift, often spreads more slowly. Nonetheless, the cumulative mortality during interpandemic periods is substantial (61, 140). The excess mortality attributed to influenza has exceeded 10,000 in each of 19 epidemics occurring from 1957 to 1986 in the United States, with three epidemics causing more than 40,000 excess deaths each (29).

The continual antigenic drift of influenza virus types A and B and the occasional antigenic shift of type A viruses necessitate regular reformulation of influenza virus vaccines. The current commercial inactivated influenza virus vaccines are trivalent and include a representative strain of each of the H1N1, H3N2, and B viruses. A major problem facing the biomedical researcher is finding a way to anticipate the antigenic changes of the influenza viruses. The fact that influenza virus infection recurs in the population at regular intervals demonstrates that immunity developed after infection is not necessarily protective against antigenic variants encountered later. Wide variations in virulence also occur along with antigenic differences (55, 94, 199). With advances in biotechnology, studies of the molecular biology of the virus are becoming increasingly relevant to clinical strategies as the mechanisms of antigenic change are examined from new perspectives. Antigenic changes have been attributed to (i) a high rate of mutation, either spontaneous or because of immunologic pressure, and (ii) genetic reassortment among and between human and animal strains. By far the most closely studied variations affecting the epidemiologic behavior of the virus are those related to antigenicity.

MOLECULAR BIOLOGY OF INFLUENZA VIRUSES

Genome Organization

Influenza virus types A and B each encode at least 10 polypeptides on their eight negative-strand RNA genome segments (Table 1). Influenza virus type C has only seven genome segments because it possesses a single surface glycoprotein (referred to as HEF because it contains the viral hemagglutinating, esterase, and fusion activities) instead of the two (hemagglutinin [HA] and neuraminidase [NA]) found on type A and B influenza viruses. The similar arthropod-borne viruses Dhori and Thogoto also each have a segmented negative-strand genome and a single surface glycoprotein (31, 58, 190). For this reason, the International Committee on Nomenclature of Viruses has placed type C influenza, Dhori, and Thogoto viruses in a separate genus from the "true" orthomyxoviruses, influenza virus types A and B. For the purposes of this review, influenza virus type A will be used as the example except when noted.

TABLE 1. Influenza virus-coded proteins

Approx RNA segment length (bases)	Gene product(s)	Approx mol wt	Function
2,340	PB1	96,000	RNA transcription and replication
2,340	PB2	87,000	RNA transcription
2,230	PA	85,000	RNA replication
1,775	HA	75,000	Attachment and fusion glycoprotein
1,565	NP	60,000	Major nucleocapsid structural component
1,410	NA	55,000	Receptor-destroying enzyme
	NB	18,000	Membrane glycoprotein found only in type B of unknown function
1,025	M1	28,000	Membrane matrix protein
	M2	11,000	Membrane ion channel (?) only found in type A
	BM2	12,000	Nonstructural protein of unknown function found only in type B
890	NS1	25,000	Nonstructural protein of unknown function
	NS2	12,000	Cellular and virion protein of unknown function

Transcription and Replication

The ribonucleoprotein (RNP) complexes of the influenza virion consist of the genome RNA in association with the three polymerase (P) proteins, designated PA, PB1, and PB2 on the basis of their overall acid or basic amino acid composition, and the nucleocapsid protein, NP. The three P proteins supply the enzymatic activities and with NP make up the minimum subset of proteins necessary for expression and replication of the viral genome (83).

The proteins in the RNP complexes all contain karyophilic amino acid sequences that cause them to be transported to the nucleus, where all RNA transcription and replication take place (173). The RNPs of the infecting virus are transported into the nucleus via the nuclear pores with high efficiency soon after the M1 protein is removed from the viral cores (124). The virion transcriptase complex is unable to initiate viral mRNA synthesis de novo or to cap and methylate the 5′ termini of the mRNAs, yet those mRNAs found in the infected cell are capped and methylated. This is possible because the viral transcriptase synthesizes mRNA by cleavage of 10- to 14-nucleotide 5′-terminal fragments from capped and methylated host cell mRNAs and uses them as primers for the initiation of transcription (155).

The molecules responsible for the initiation and transcription activities were deduced by UV-induced cross-linking (20). PB2 binds the cap 1 structure of host cell mRNAs, which are then cleaved to generate capped and methylated primer fragments. PB1 adds nucleotides to the 3′ terminus of the nascent viral mRNA, probably completing transcription. The PA protein has not been assigned a role in viral mRNA synthesis and appears more likely to be involved in replication (97).

The selection of host mRNAs to serve as primers is not completely random; host RNA primers that contain a 3′-terminal Py-G-C-A sequence before the presumed endonuclease cleavage site are preferred (178). Transcripts terminate at sites 15 to 25 bases from the ends of the templates, where oligo(U) sequences are used to add 3′-terminal poly(A) tails, probably by transcriptase "chatter" due to template secondary structure (see below).

The use of host-derived mRNA fragments as primers results in a heterogeneous sequence at the 5′ end of the viral mRNAs before the virus-specific sequences. Bunyaviruses utilize a similar transcription strategy in which host mRNAs are "cannibalized" for the production of primers yielding heterogeneous 5′ mRNA termini (17). However, unlike influenza viruses, bunyaviruses replicate in the cytoplasm; thus, it is likely that the obligate nuclear phase in influenza virus replication is due to viral use of host mRNA splicing mechanisms for the processing of certain of the viral mRNA transcripts (described below) rather than the requirement for host-derived mRNA primers.

In addition to the four proteins directly involved in RNP formation and RNA transcription and replication, the virion membrane protein M1 may also play a role in transcription (229). M1 inhibits transcription probably through its interaction with NP, which causes immobilization of the P protein complex (69). This effect would cause polymerase activity to cease as virion assembly occurs, thus stabilizing the RNP complex during encapsidation. RNP complexes associated with M1 synthesized in the infected cell are transported out of the nucleus, making the newly synthesized genomic RNPs available for virion assembly (124).

The transcription of viral mRNAs is temporally regulated to yield a biphasic shift in the relative abundances of various gene products (72). NP and NS1 are predominant early, with the remaining structural proteins more prominent later.

In addition to the polypeptides translated from the longest open reading frames found on the uninterrupted colinear mRNA transcripts, the influenza viruses express additional polypeptides by utilizing other reading frames in several of the genes. The coding strategies used exploit both spliced transcripts and true bicistronic mRNAs.

In all influenza viruses, transcripts of the smallest segment of genomic RNA are found as both colinear and spliced mRNAs, which are translated to yield the nonstructural (NS) proteins NS1 and NS2, respectively (23, 100, 138). In the case of influenza virus type A, which was characterized first and serves as an example, 473 nucleotides are spliced out of the colinear (NS1) transcript to yield the less abundant NS2 mRNA. As a result, the first nine amino acids are common to both proteins, after which the NS2 protein is translated from the +1 reading frame (100).

Only about 10% of the mRNA transcripts of the NS gene are spliced, which in turn determines the relative amounts of the NS1 and NS2 proteins present in the infected cell. This is apparently caused by transport of the unspliced (NS1) mRNA out of the nucleus since the degree of splicing (and, therefore, the relative amount of NS2 mRNA) rises when nucleocytoplasmic transport is blocked (3).

The expression of the polypeptides encoded by the viral RNA (vRNA) encoding the viral membrane protein, M1, is different in all three types of influenza viruses. In influenza virus type A, the transcripts are analogous to those synthesized from the NS gene: an uninterrupted, colinear mRNA encodes the M1 protein, while a spliced transcript encodes the M2 polypeptide (101). As with the type A virus NS proteins, the first nine amino acids are common to both M1 and M2, with the remainder of M2 translated from the +1 reading frame. A second spliced transcript, designated mRNA3, is predicted to yield an as yet undetected nine-amino-acid peptide equivalent to the carboxy terminus of M1.

Influenza virus type B also possesses an open reading frame on this genome segment in addition to that encoding M1, although, unlike influenza virus type A it apparently produces no spliced transcripts (24). Instead, the second open reading frame is translated via a termination-reinitiation scheme of tandem cistrons; the initiation codon of this protein, designated BM2, overlaps the termination codon for the M1 protein in a UAAUG pentanucleotide sequence (81). However, this translation mechanism appears to be relatively inefficient since little of the BM2 protein is seen in infected cells.

Influenza virus type C presents a different coding strategy for the M1 protein: M1 is translated from a spliced transcript shorter than the much less abundant colinear transcript (225). In this case, the splicing event introduces a stop codon that truncates the protein to a predicted 242 amino acids from the potential 374-amino-acid product of the unspliced transcript.

In addition to the mRNA encoding M1 and BM2, influenza virus type B produces a second bicistronic mRNA that allows the translation of two polypeptides from the transcript of genome segment 6. The virion NA glycoprotein is translated from the longest open reading frame, while a second integral membrane glycoprotein, designated NB, is translated from a shorter, overlapping reading frame. In this case, the NB initiation codon is 5′ proximal on the mRNA, with NA initiated from the second AUG in the sequence AUGAACAAUG (176).

Unlike viral mRNA transcription, genome replication requires no primers. The production of full-length viral complementary RNA (vcRNA) begins only after viral protein synthesis has occurred. Antibody depletion and *ts* mutant experiments indicate that newly synthesized NP (i.e., NP not associated with RNPs) is required for the antitermination activity permitting readthrough of the mRNA polyadenylation site, thus allowing synthesis of full-length vcRNA and vRNA (13, 174).

NS1, because of its ability to bind single-stranded RNA (181, 231), is also a potential candidate for transcription or replication modulation. However, neither NP nor NS1 has detectable antitermination activity when added to preformed RNPs in vitro (181). This suggests that assembled RNP complexes are "precommitted" to either transcription or replication and interaction with any regulatory proteins must occur as the RNPs are formed.

There is evidence that the biphasic regulation of transcription occurs during the template synthesis steps rather than during mRNA transcription; the NS vRNA (and, therefore, the NS mRNA and proteins) is preferentially synthesized first, while M-gene vRNA synthesis is delayed. During the second phase, synthesis of all vRNAs is at or near maximum levels, while mRNA synthesis declines drastically (173). Viral mRNAs and new genomic vRNAs are transported to the cytoplasm, while the vcRNA templates remain in the nucleus (78).

The synthesis of full-length vcRNA and vRNA involves both PB1 and PA (98, 123). While PB2 may possibly play a role in replication, its primer-generating endonuclease activity is exclusively used in transcription of mRNAs.

The predicted secondary structure of the vRNA genome segments suggests a possible basis for the differential repli-

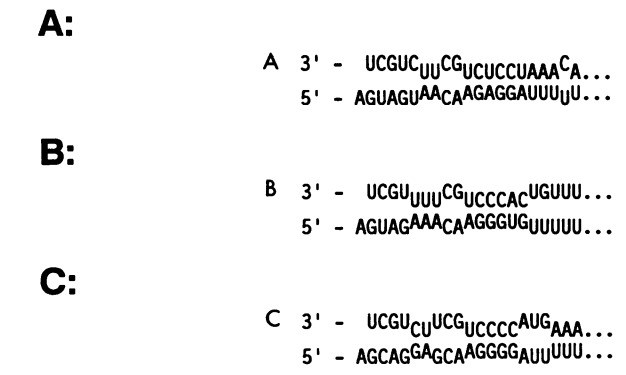

FIG. 1. Predicted panhandle structures of the NS vRNA from type A, B, and C influenza viruses. Bases which would not pair in this scheme are displaced relative to the predicted duplex regions. The remainder of the vRNA sequences not participating directly in panhandle formation would continue to the right of the nucleotides shown. Adapted from Stoeckle et al. (195).

cation. The 15 3'-terminal and 16 5'-terminal nucleotides of the vRNAs are capable of forming panhandle structures that are seen in both virions and infected cells (82). The nucleotides participating in panhandle formation are within the terminal base sequences known to be sufficient to allow polymerase recognition of the RNA molecule. When a chloramphenicol acetyltransferase gene was modified to contain the 22 5'-terminal and 26 3'-terminal nucleotides from the influenza virus NS gene, it was transcribed, replicated, and packaged when reacted with purified viral NP and P proteins before transfection into cells infected with helper virus (115). This technique holds much promise for future research into viral gene functions since it allows site-directed mutagenesis of a DNA intermediate before the intermediate is used as template for the production of a synthetic RNA transcript which will be introduced into the virus (50). This procedure has been used to introduce specific mutations into the viral NA gene (49) and to demonstrate the requirement for a stretch of uninterrupted uridines adjacent to the panhandle structure of the vRNA corresponding to the mRNA polyadenylation site (114). Mutations that progressively open up the panhandle structure increasingly diminish the expression of a chloramphenicol acetyltransferase gene flanked by the modified termini.

Differences in the predicted base pairing on the vRNA panhandle structures of A, B, and C influenza viruses (Fig. 1) could conceivably explain the lack of intertypic gene segment reassortment since this region contains the transcription promoter site. Presumably, the transcriptases of the different influenza virus types are specific for their own promoter sequence conformations and would therefore be unable to recognize the panhandle structures of another virus type. As described above, the predicted panhandle structures put the genome oligo(U) site immediately upstream, possibly explaining the transcriptase chatter yielding the poly(A) tails (114). Also, there is a segment-specific, three- to four-nucleotide sequence found immediately at the interior terminus of the panhandle structure that could conceivably allow the viral polymerase complex to "identify" a template and thus regulate the relative abundance of its complement (195).

Viral Glycoproteins

HA. The viral HA has been the subject of considerable study because of its role as the major antigen against which a protective immune response is directed. (For a comprehensive review of the antigenic structure of HA, see Wilson and Cox [219].) Approximately 25% of the influenza virion protein is in the HA spikes which are distributed evenly on the surface of the virus particles (134). It is this glycoprotein that allows the virus to attach to specific receptors on the cell surface and causes fusion of the virion and cell membranes during entry (214).

Bromelain treatment of the virion releases the water-soluble external domains of the HA spikes which were crystallized for the X-ray crystallographic determination of the three-dimensional structures (220). The HA monomer is synthesized as a single polypeptide chain that is cleaved twice posttranslationally. The removal of an amino-terminal signal sequence results in membrane attachment through a carboxy-terminal hydrophobic amino acid sequence. An additional cleavage is necessary for infectivity (96, 108); the resultant HA1 and HA2 polypeptide chains remain associated through a single disulfide bond. The surface HA spikes are trimers of these HA1-HA2 units (220).

The HA1 monomer has a globular membrane-distal domain of antiparallel β sheets which contains the receptor-binding site. The regions of antigenic variation are clustered within five regions of this distal portion of the molecule (180, 214, 219, 220).

Cleavage of HA into HA1 and HA2 is not necessary for the molecule's receptor-binding activity but must occur for the virus to become fusion competent (95). Because of this, the relative ease with which the HA of a given virus can be cleaved in a particular host is one of the determinants of pathogenicity. The dramatic increase in pathogenicity seen in the 1983 outbreak of avian influenza in Pennsylvania was traced to a point mutation that eliminated a carbohydrate side chain that had masked the HA cleavage site in the nonpathogenic strains (42, 87, 88). Interaction between influenza viruses and bacteria such as *Staphylococcus aureus* can greatly increase pathogenicity probably because of protease activities furnished by the bacterium (95).

Cleavage of the HA monomer frees the hydrophobic fusion peptide at the amino terminus of the HA2 subunit. At physiologic pH, the fusion peptide is hidden between the monomers in the HA trimer structure (220). When the pH is lowered to the 5.0 to 5.5 range optimal for membrane fusion (215), a conformational change exposes the HA2 fusion peptide (180). During the entry of virus into the target cell, this pH change presumably occurs after the viral particle has been taken into the cell by receptor-mediated endocytosis (215).

Because of their removal prior to crystallization for structural analysis, much less is known about the transmembrane and intracellular domains of HA. Nonetheless, recent experimental results have yielded some insights into the potential functions of these regions.

A synthetic decapeptide corresponding to the predicted cytoplasmic domain of HA2 specifically inhibits the release of virus particles from the infected cell without affecting protein synthesis (32). This suggests that the intracellular domain of the molecule is somehow involved in virus assembly, possibly via interaction with M1.

The cytoplasmic and transmembrane regions of HA2 are acylated through phosphodiester bonds at three Cys residues conserved in all type A HAs (209). The significance of the

ester-linked fatty acids is uncertain; HA mutants lacking any or all of the three sites are still membrane inserted and posttranslationally processed to yield biologically active molecules (191, 209). Also, one of the three acylation sites conserved in the type A HAs is missing in the type B HA (99).

The complete amino acid sequences of many different HA subtypes have been deduced. Conservation of cysteine residues and certain other critical amino acids suggests that the 14 currently recognized influenza virus type A HA subtypes and the influenza virus type B HA all evolved from a common ancestor (89, 99). The HA has been shown to be one of the determinants of virus host range because of both changes in the HA1–HA2 cleavage site and altered receptor-binding specificity (163). Amino acid changes associated with adaptation of the virus to different cells tend to cluster around the receptor-binding site (159, 160, 166). This receptor adaptation appears to be a potential determinant of both tissue tropism and virus host range.

NA. Viral NA is an exoglycosidase that hydrolyzes terminal sialic acid residues from any glycoconjugate, including the viral glycoproteins themselves (63). The virion NA spikes are tetramers of the NA molecules that are anchored in the lipid bilayer by an amino-terminal hydrophobic amino acid sequence (208). Unlike HA, NA does not undergo posttranslational proteolytic processing (18), and the NA spikes are distributed asymmetrically on the surfaces of the progeny virions (134).

The NA spikes can be eluted by pronase or trypsin treatment, yielding crystallizable, enzymatically-active, and antigenically equivalent tetramers, which has allowed their three-dimensional structure to be determined (208). The box-shaped apex of the molecule is formed by six *B*-pleated sheets arranged like the blades of a propeller. The fourfold symmetry is stabilized by metal ions and an inward-facing carbohydrate side chain that would be hidden and therefore protected from immunologic pressure (33).

Because of the NA receptor-destroying enzyme activity, the sites of viral proteins on the cell surface are devoid of terminal sialic acid residues. Desialation is necessary to prevent aggregation of the progeny virions, which greatly decreases infectivity (146). It has also been proposed that the enzyme allows the virus to be transported through respiratory mucin secretions, thus avoiding nonspecific HA inhibitors (95).

The enzymatic specificities of different NAs allow the grouping of virus isolates into a receptor gradient on the basis of their relative receptor-destroying enzyme activity (197). Both HA and NA contribute to this phenomenon, with NA activity complementary to HA receptor specificity (12). Because of this, NA is also a potential determinant of pathogenicity. An optimal combination of HA and NA specificities would more efficiently elute the progeny virions from the host cell.

The receptor-destroying enzyme activity of the influenza virus type C HEF glycoprotein is not that of a neuraminidase as is the NA of types A and B. The cell surface receptor is 9-*O*-acetyl-*N*-acetylneuraminic acid (162). Consequently, the receptor-destroying enzyme is a neuraminate-*O*-acetylesterase (77).

The N9 subtype of influenza virus type A NAs is apparently unique in its ability to agglutinate erythrocytes (105). However, unlike the type C HEF (which structurally is more closely related to the HA of types A and B influenza viruses [153]), the N9 NA has no membrane fusion activity. The site having HA activity has been determined to be associated with a small depression on the surface of the membrane-distal heads of the tetramers that is separate from the enzyme active site (141).

NB. As described in the preceding section, Transcription and Replication, the influenza virus type B NA gene encodes a second glycoprotein, NB, that is not found in influenza virus type A (176). NB is a dimeric integral membrane protein anchored in the lipid bilayer with the glycosylated amino terminus exposed on the cell surface (216). Both carbohydrate chains of NB are largely modified to contain polylactosaminoglycan moieties (NBp), although a small amount remains in the unmodified, high-mannose form (217). NB and NA are synthesized in approximately equimolar amounts in the infected cell, although NB does not appear to be incorporated into progeny virions (175, 218). The function of NB is unknown, but its conservation in all influenza virus type B isolates examined (2) and its structural similarities to the influenza virus type A M2 protein (see below) suggest that the two proteins may have similar functions.

Nonglycosylated Viral Proteins

P proteins. The proposed enzymatic activities of the one acidic (PA) and two basic (PB1 and PB2) influenza virus P proteins were described above. When synthesized independently of the other viral proteins, they associate to form immunoprecipitable PB1-PB2 or PA-PB1-PB2 complexes, depending on the expression system (43, 189). Immunogold-labeling studies indicate that the P protein complex is associated with a single polymerase binding site at or near the end of each RNP (135).

In the live attenuated cold-adapted (*ca*) influenza virus type A vaccine strain, all three P genes contribute to the attenuated phenotype (185). The PA gene also contributes significantly to the attenuation of the less well-characterized *ca* influenza B/Ann Arbor/1/66 vaccine strain (46).

Because of predicted nucleotide sequence differences, PA genes from different host species are thought to have arisen from multiple lineages, a single one of which was represented by the human strains (142). These observations are consistent with the hypothesis discussed below (Evolution and Variation) that swine serve as intermediaries in the reassortment between avian and mammalian influenza viruses (168).

NP. NP is the most abundant protein in the viral RNP complexes and is one of the type-specific antigens that distinguish among type A, B, and C influenza viruses. It plays a structural role in RNP formation and, as described above, is involved in viral genome replication (13, 174). The predominant phosphorylated form of NP has been shown to have a kinase activity capable of phosphorylating NS1; unphosphorylated NP lacks this activity (181).

NP apparently plays an important role in host range determination, since the replacement of human virus NP gene with an avian NP confers attenuation of the virus in monkeys (186, 202). As with the PA genes, sequence analyses of influenza virus type A NP genes from multiple species allow us to group them into human and nonhuman lineages (26, 59, 62). Swine were the only nonhuman hosts found to yield isolates having both NP subtypes, further supporting the swine "mixing vessel" hypothesis (59, 62).

NP is one of the major antigens targeted by cytotoxic T lymphocytes (CTLs) which are able to recognize conserved epitopes (11, 19, 203). However, passive immunization with monoclonal anti-NP serum or direct immunization with NP alone induces very limited resistance to challenge (6, 7, 222).

Viral membrane proteins M1 and M2. The membrane or matrix protein M1, the most abundant protein in the virion, is found underneath the lipid envelope. Like NP, it is one of the type-specific antigens of the virion. Its deduced amino acid sequence reveals an internal hydrophobic area that could participate in interactions with either lipid or the hydrophobic regions of other proteins (100). Its exact interactions are unclear.

Studies of virus-infected cells indicate that M1 specifically cofractionates with plasma membranes containing HA but is not associated with membranes that lack HA (71). Since influenza viruses mature by budding through the plasma membrane (35), M1 may cause assembly of the internal viral components by interacting with the cytoplasmic tails of HA and NA and with NP in the RNP complexes. The observation that a synthetic peptide corresponding to the cytoplasmic tail of HA inhibits virion formation lends support to this model for assembly (32). M1 association with NP may therefore serve two purposes: (i) bringing together the internal and external virion components at the site of budding and (ii) immobilization of the virion P protein complexes on the assembled RNPs (69). M1 can also be found in association with NP in the cell nucleus and cytoplasm (151).

M1 appears to perform different functions depending on whether it is supplied as a structural component of the infecting virus or is newly synthesized in the infected cell. Soon after HA-mediated membrane fusion occurs, allowing entry of the viral cores from late endocytic vesicles, M1 dissociates from the RNP complexes, allowing them to be transported into the nucleus. In contrast, newly synthesized M1, which appears only after viral transcription has occurred in the nucleus, enters the nucleus by diffusion and binds the newly assembled viral RNPs, causing them to be transported into the cytoplasm where they participate in virion assembly (124).

On the basis of these observations, it has been postulated that the M1 found in virions is somehow different from newly synthesized M1 since the former releases the RNPs as the virus uncoats while the latter actively binds the RNPs, masking their nuclear localization signal sequences and allowing transport out of the nucleus (124). Presumably, the change resulting in M1 function alteration occurs as the progeny virions mature at the plasma membrane. The nature of the change is unknown.

The 97-amino-acid M2 protein of influenza virus type A is translated from a spliced mRNA transcript of genome segment 7 that also encodes M1 (101). As an integral membrane protein, it is oriented with an amino-terminal, 18- to 23-amino-acid extracellular domain (102). Ten of these external residues are conserved in all sequences determined from human virus isolates.

Although most M2 is cell associated, between 14 and 68 molecules of the protein have been estimated to be incorporated into virions (233). In the infected cell, M2 is found as a homotetramer formed by the noncovalent association of two disulfide-linked dimers (200). The predicted amphiphilic helix formed by the transmembrane regions could therefore form a bilayer-spanning tunnel suggestive of a selective ion channel.

M2 has become the subject of increasing research because of its clear association with resistance to the drug amantadine (73). Since drug-resistant strains show substitutions in the membrane-spanning portion of M2 (15, 73), it has been proposed that amantadine and its derivatives exert their effects by interfering with the putative membrane ion channel activity of M2 (200).

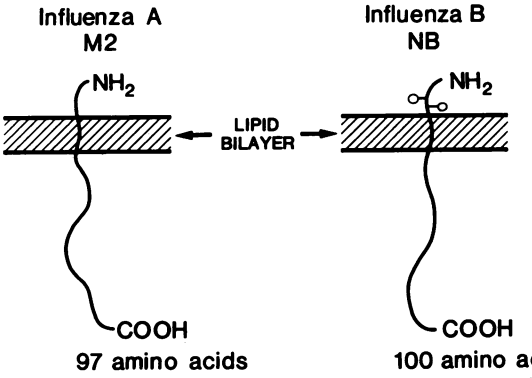

FIG. 2. Diagrammatic representation of the transmembrane orientation of the M2 protein of type A influenza virus and the NB glycoprotein of type B influenza virus. The monomers are depicted with the short external amino-terminal domains toward the top and the longer cytoplasmic tails below the lipid bilayer. The circles on NB correspond to the carbohydrate side chains. Adapted from Lamb et al. (102) and Williams and Lamb (216) with permission of the publishers.

Unlike influenza virus type A M2, BM2 is not translated from a spliced mRNA but from a bicistronic mRNA transcript from genome segment 7 (81). Also, the BM2 protein has no obvious structural similarities with the influenza virus type A M2 protein (24), making it unlikely to be functionally equivalent. The influenza virus type B NB glycoprotein, because of its similar transmembrane orientation, size, and location in the infected cell (216), appears more likely to be the influenza virus type B counterpart of influenza virus type A M2. The structural similarities of the M2 and NB integral membrane proteins are shown diagrammatically in Fig. 2.

No obvious candidate for an M2 equivalent in influenza virus type C has been found. However, it has been observed that the translation product of the relatively rare unspliced transcript of the gene encoding M1, which is translated from a spliced mRNA, would be translated into a protein essentially consisting of M1 with an additional 132 amino acids at the carboxy terminus, which would include a potential membrane-spanning region (225).

NS proteins NS1 and NS2. The smallest genomic RNA segment in any influenza virus encodes two NS proteins, designated NS1 and NS2, which are translated from the uninterrupted and spliced mRNA transcripts, respectively (23, 100, 138). NS1 is synthesized in large amounts early in infection and accumulates in the nucleus (109). As described above, NS1 is phosphorylated by NP in the infected cell (181). Influenza virus type A NS1 protein has two nuclear localization signals, one of which is conserved in the influenza virus type B NS1 protein (65). NS2, which is present in much smaller amounts, appears later in infection and also has been shown to accumulate in the nucleus of the infected cell (64). Like NP and NS1, NS2 is phosphorylated in the infected cell (157). NS2 has also been detected in purified virions in a trypsin-resistant form, suggesting an internal location (157). Thus, NS2 may not be a true "nonstructural" protein.

The amounts of NS1 seen in infected cells are rivaled only by the amounts of NP, which also appears early in the replication cycle (129). In addition to nucleus-associated NS1, the protein is also found in large amounts in the cytoplasm, where it cofractionates with infected cell polysomes (34). NS1 is often found nonspecifically bound to

single-stranded RNA (181, 231). Late in infection, cytoplasmic NS1 can often be found in electron-dense paracrystalline inclusions in association with RNA (131, 177, 231). The significance of inclusion formation is uncertain; the inclusions may be by-products resulting from aggregation of excess NS1 with RNA in a manner unrelated to the protein's actual function. Large deletions in the NS1 carboxy terminus cause little or no observable effect (148).

Like the P and NP genes, the NS genes of influenza viruses can be grouped on the basis of their nucleotide sequence differences. Group A contains both avian and mammalian isolates, while group B is represented only by NS genes of avian origin (111). On the basis of these studies, 13 amino acid positions in NS1 can be used to further define human and nonhuman subgroups within group B.

EVOLUTION AND VARIATION

The segmented nature of the influenza virus genome is responsible for the dramatic variations possible in both genotype and phenotype. When a cell is infected with more than one strain of influenza virus, reassortment of gene segments can yield progeny virions with new gene constellations. As would be expected, eight recombination groups can be defined for the type A viruses (122). Reassortment has been demonstrated in vivo between animal and human type A influenza viruses and between human strains (38, 212).

New pandemic strains of influenza virus type A are thought to arise from the reassortment of the genes of human and animal strains during dual infection of an intermediate host, most probably swine (168). The human and nonhuman groupings of the P, NP, and NS genes described above are all consistent with the swine mixing-vessel hypothesis since influenza isolates from humans and swine can infect either host (79) and swine have also been shown to serve as hosts for avian influenza strains (93, 167). Since at least 14 HA subtypes and 9 NA subtypes are found in various mammalian and avian species (80, 89), there are 126 possible combinations of these two genes alone.

Since the first isolation of influenza virus in 1933, two major antigenic shifts have occurred in humans, three if the 1977 re-emergence of H1N1 is included (94). Characterization of contemporary strains and retrospective seroepidemiology have provided indications of the subtypes of pandemic strains that circulated prior to 1933. The 1889–1890 pandemic has been attributed to an H2N8 virus, and the 1900 pandemic has been attributed to an H3N8 virus (126, 132). The devastating pandemic of 1918 was almost certainly caused by a swinelike H1N1 strain (40, 154).

The observation of associations between the predicted amino acid sequences of certain viral proteins and host species suggests that probably only a limited number of the almost infinite number of possible gene constellations are viable in humans (26, 59, 106, 142, 168). Consequently, the apparently unique ability of porcine species to host both human and animal strains is hypothesized to furnish the circumstances that allow human and nonhuman strains to exchange surface antigens (59, 168).

On the basis of phylogenetic analyses of available NP gene sequences, it has been proposed that current human and swine influenza virus strains arose from a common ancestor in 1912 or 1913 (62). Since that time, the human and swine NP gene pools have apparently evolved independently at a steady rate. In the case of the NS gene, however, nucleotide sequence analysis has revealed a significantly increased rate of change since the 1957 antigenic shift (111). It was postulated that this increased evolutionary rate was the result of a new selective pressure on NS1 encountered when the virus acquired an avian PB1 gene in addition to the new HA and NA surface antigens.

Type B and C influenza viruses have not been shown to undergo antigenic shifts, probably because they lack the extensive animal reservoir of the type A viruses. However, like the type A viruses, they can undergo less drastic antigenic drift due to point mutations in the relevant genes. The observed patterns of variation with time suggest that different mechanisms may be at work for the three virus types (27, 147, 224).

Unexpected insight into the mechanisms of antigenic drift has been gained by the analysis of virus shed over a 10-month period by a child with severe combined immunodeficiency syndrome who was persistently infected with influenza virus type A (161). Though the first isolates obtained closely resembled circulating H1N1 strains, the virus isolates obtained as the study proceeded revealed a continued antigenic and genetic variation in the absence of any immune pressure except possibly from null killer (NK) cells. Multiple nucleotide and amino acid changes were observed in the HA and NP genes, although a progressive accumulation of mutations was not seen. All amino acid changes in HA1 were in or near the major antigenic sites where alterations can occur without affecting the structure or function of the molecule (219). This suggests that the immune status of the host does not drive the variation seen in viral surface antigens during antigenic drift but rather serves as a selective pressure favoring strains having mutations resulting in novel surface epitopes.

Variation in HA can also occur because of differences in the cell surface ligands to which it binds during virus attachment. Consequently, adaptation of influenza viruses to different host cell types causes alterations in the receptor-binding site that result in antigenic variation (159, 160, 166). Characterization of these host range HA variants demonstrated amino acid changes near or within the receptor-binding pocket at the distal tip of HA1. Since influenza virus used as vaccine, whether inactivated or live attenuated, is grown in embryonated hens' eggs, some concerns have arisen as to whether this adaptation to growth in a nonmammalian host alters the antigenicity sufficiently to make them less protective against the circulating human strains.

In one of the better characterized examples, a single clinical specimen of influenza virus type B yielded two distinct variants selected by cultivation in either embryonated eggs or canine kidney (MDCK) cells (166). Molecular analysis of these subpopulations revealed that the egg-adapted population had lost an N-linked glycosylation site present in the MDCK-adapted strain HA caused by a single amino acid change (residue 198) near the receptor-binding site. This change also caused a change in antigenicity discernible with rabbit antisera.

These host-selected influenza virus type B HA variants both elicited a protective immune response in mice when used for immunization in the form of clonally pure vaccinia virus recombinant vaccines (164). The data indicated that the egg-adapted HA presented a greater array of potential antibody-binding sites, probably because of the loss of the carbohydrate side chain that could have covered epitopes in the MDCK-selected variant. These results are consistent with studies of influenza virus type A variants which showed that egg- and MDCK-derived H3N2 vaccine strains both protected against subsequent challenge (86). Thus, available

experimental edivence indicates that mammalian-grown influenza virus vaccines offer no inherent advantage over the egg-grown preparations currently used. The additional expense associated with the mammalian cell lines acceptable for vaccine production puts them at a disadvantage when compared with eggs.

In addition to variation in the character of the viral surface antigens, genetic reassortment can alter other properties of the virus, including virulence. As described above, several genes, especially those for HA and NA and the components of the viral RNP complex, have effects on the host range and relative virulence of a given strain. Even reassortment between two pathogenic strains can yield both pathogenic and nonpathogenic progeny (165). Similarly, reassortment between nonpathogenic strains can yield pathogenic strains with unexpected tropisms (169). In such cases, changes in pathogenicity correlate with changes in host range; a pathogenic virus strain is well suited for growth in the host. Host range and pathogenicity are clearly multigene phenomena; the sum of the effects of the genes is often not obvious from their individual characters.

IMMUNOLOGIC DETERMINANTS IN INFLUENZA VIRUS INFECTION

Two different but interrelated problems are encountered when the determinants of immunity to influenza virus infection in humans are characterized. The first involves resistance or relative susceptibility of the population to the virus, and the second concerns the type of immune response offered by the individual. Evidence shows that antibody levels in serum, as determined by hemagglutination-inhibiting and virus-neutralizing antibodies and anti-NA activity, are good predictors of an individual's relative resistance to infection (1, 36). The duration of an individual's immunity to influenza has not been adequately determined because of multiple antigenic variants of the infectious agent and a complex antibody response.

Epidemiologic studies have shown a clear relationship between the level of circulating antibodies to the current virus and resistance to natural infection; the severity of the clinical response to induced infection in humans is inversely related to specific antibody levels, including those elicited by vaccination. Because influenza virus infection is essentially an attack on a superficial extravascular tissue, the ability of antibody to prevent injury to the involved respiratory epithelium is believed to result primarily from the secretory immunoglobulin A (sIgA) generated by the local epithelium. Studies designed to characterize cell-mediated and humoral immune responses to influenza virus infection and vaccination have offered some insight into the roles that differing immunologic determinants play in recovery from and prevention of the disease (1).

The cellular immune response as well as the humoral immune response are intimately involved in recovery from influenza virus infection. Since viral antigens are often expressed on infected cell membranes before detectable virus production, cell lysis by immune cells could occur early enough to interrupt the replication cycle in the individual cells (before virus assembly has occurred) to significantly reduce spread in affected organs.

The types of cell-mediated immunity active in the response to influenza virus infection can be divided into four basic categories: NK cells (51), CTLs (230) and T-helper (T_H) lymphocytes (1), antibody-dependent cell-mediated cytotoxicity (ADCC) (70), and macrophage "killer" cells (36). All appear to play a role in defense against infection by influenza viruses, and all are operative early in the course of disease to provide the body with an effective means of eliminating the viral infection.

The most involved cell-mediated immune response is orchestrated by the T lymphocytes, which can be subdivided on the basis of their roles in the response: the regulatory T_H cells required for both CTL and B-cell antibody-secreting activities; T suppressor cells, which control and subdue T- and B-cell responses; the T cells responsible for delayed-type hypersensitivity reactions (T_{DTH} cells); and CTLs that specifically lyse virus-infected cells. The effect of cell-mediated immunity on the course of influenza virus infection has received attention since about 1975, with most of the studies being done with influenza virus type A and with mice as the model (170, 230).

The studies of Burlington, Djeu, Stein-Streilein, and Ennis and their collaborators concentrated on NK cells and their responses to influenza virus infection and interferon production (28, 44, 51, 194). According to one interpretation (44), the response to influenza virus-infected cells by human NK cells is twofold: rapid induction of interferon and a resultant enhancement of NK activity. Peak levels of interferon production by NK cells were induced within 24 h of exposure to virus and were not dependent on T_H cells. Burlington et al. (28) showed that alpha interferon was produced by NK cells in response to influenza virus independently of CTL activity. In contrast, the production of gamma interferon was dependent on the presence of both T and NK cells. NK cells were required for optimum CTL activity and stimulated such activity when the two cell populations were separated by a permeable membrane in Marbrook vessels, which implies that a soluble factor is supplied by the NK cells. Thus, NK cells are cytotoxic in themselves and also provide an accessory function in the development of the CTL response.

Stein-Streilein and coworkers studied the cytotoxic activity of mouse spleen and lung NK cells and found that, 48 h after intranasal infection of mice with influenza virus, NK cells from the lungs showed increased cytotoxic activity but splenic N cells were not stimulated by the infection (194). These findings were interpreted as evidence of early local cellular immunity in the affected organ.

ADCC activity against a *ca* recombinant strain of influenza virus, A/Alaska/6/77, was studied by Hashimoto et al. and shown to be mediated by T cells, null cells, and macrophages, all bearing receptors for the Fc portion of IgG molecules (70). ADCC-active antibody, which appeared earlier and lasted longer than hemagglutination-inhibiting antibody, was obtained from the sera of children after natural infection. The ADCC antibody response as assayed was directed solely toward the virion HA or NA, or both, but not toward the internal virion proteins (NP, M1, etc.). Target cells could be lysed in as little as 5 h after infection, before the release of infectious virus. Both adherent and nonadherent cells were shown to have cytotoxic activity in this system.

Studies on cell-mediated immunity attributable to CTL activity are the most numerous (230), with the work of Ada and colleagues being representative (52, 110, 136, 137). Mice infected intravenously with the A/WSN strain of influenza virus were used to obtain immune spleen cells for cytotoxicity assays against influenza virus-infected target cells. The effector cells were CTLs that had a peak activity in the spleen at day 6 after infection. CTLs specifically lysed influenza virus-infected target cells and needed to share

genes in the major histocompatibility complex (notably at the K and D loci) with the target cells. The frequency of CTL memory clones is deduced to be 1 in 8,000 spleen cells in young mice and 1 in 16,000 in older mice.

Yap and Ada transferred immune splenic CTLs to naive mice that earlier had been challenged intranasally with a lethal dose of A/WSN virus (227, 228). The mice that received immune cells intravenously 1 day after challenge were protected and showed a significant reduction of infectious virus in the lungs. Protection depended on the transfer of such cells into hosts that were homologous at the K and D major histocompatibility complex loci. Allogeneic mice (mice with a different major histocompatibility complex haplotype) were not protected.

Similar studies of the adoptive transfer of immunity against viruses with different HA and NA antigens have also demonstrated cross-protection between type A influenza viruses (145). This was particularly evident when the CTLs were primed with one virus serotype before stimulating the memory cells with cells infected with a different serotype. Such stimulation increased protection against the second virus. It was postulated that the common antigen recognized by the CTLs may be the viral membrane protein M1.

There remains considerable controversy concerning the nature and potential value of cross-protection by CTLs (230). Askonas and colleagues have cloned cytotoxic T cells and agree with Yap and Ada that some clones can exhibit cross-protection among A strains but not between type A and B influenza viruses (6, 7). The clones elicited exhibit differing biological properties in that some produce interferon and others do not. Also, some are protective in vivo, while others are cytotoxic only in vitro.

Braciale and colleagues have produced CTL clones that are highly specific for the HA and NA antigens with which they were primed and that will protect only against those viruses (21, 113). In fact, if mice are dually infected after passive transfer of cloned CTLs, the titer of only the virus with antigens appropriate to the specific CTLs will be reduced; the mouse will die from the other virus. However, other clones showed some cross-protection. Thus, CTL clone specificity and activity can vary.

Yap and Ada demonstrated the presence of CTLs in the lungs of mice infected with influenza virus type A and could recover them from bronchial washings (226). As the number of cytotoxic T cells in the lungs rises, the virus titer decreases proportionally. CTL activity could be detected only when live virus was used; if inactivated virus was used, CTL activity was absent, but T_{DTH} could be detected. When T_{DTH} cells were passively transferred to mice before challenge with a lethal dose of virus, the mice were not protected and, in fact, died more rapidly than the controls.

In vivo protection by the adoptive transfer of immune spleen cells was demonstrated by Wells et al., who protected both nude and immunocompetent mice from influenza viral pneumonia by injecting immune spleen cells 24 h after challenge with virus (213). The spleen cells had to be restimulated with virus-infected cells for 5 days in vitro to be protective. The spleen cells were influenza virus type A cross reactive and major histocompatibility complex restricted.

McMichael et al. inoculated 63 volunteers with live unattenuated influenza A/Munich/1/79 virus and then measured their serum antibody and their T-cell response (127). All volunteers with demonstrable CTL-mediated immunity cleared virus effectively. The CTLs showed cross-reactivity in their recognition of different subtypes of influenza virus type A, while antibody response was type specific, thus demonstrating that the cross-reactive CTL response occurs in humans as well as in mice.

The identity and nature of the antigens being recognized by the cross-reactive CTLs remain controversial, since several candidates appear to play measurable roles, including HA and NA (230). Many of the more recent studies have dealt with antigens expressed on the surface of the target cell in an attempt to determine those responsible for cross-reactive recognition by cytotoxic T cells. Candidate antigens include NP (207), M1 (156), the integral membrane protein M2 (102), the P proteins (230), and NS1 (179), all of which have been detected on the surface of infected cells by various means.

Reiss and Schulman used monoclonal antiserum to M1 to abrogate T-cell-mediated cytotoxicity and concluded that cross-reactive CTL responses are due at least in part to recognition of a common M1 protein antigen expressed on the surface of virus-infected target cells (156). However, Townsend and Skehel used target cells infected with genotyped reassortant viruses and concluded that the viral genes that controlled the expression of most antigens recognized by the cross-reactive T-cell clones mapped to the gene for the viral NP (207). When two viruses that differed only in their NP antigens were exposed to CTL clones, cytotoxicity against the virus-infected cells differed. The two NP genes tested differed by only 30 of 498 amino acids, yet CTL clones were specific for one genotype over the other.

Lamb et al. showed that M2 is an infected-cell surface antigen and could therefore possibly be recognized by cytotoxic T cells (102). At least 18 amino acids are exposed at the cell surface, 10 of which are conserved in all strains of human type A influenza viruses. The nine conserved amino-terminal residues shared with M1 may explain at least part of the anti-M1 CTL activity.

The search for cross-protective influenza virus type A antigens will continue because their use as immunogens might provide protection against multiple, serotypically distinct variants. Clearly, all viral gene products must be considered to be potential T-cell targets. In addition to intact proteins expressed during viral infection, peptides produced during intracellular processing also appear to play a role in CTL and T_H recognition (230). Certain peptide fragments of HA and NP have been shown to be recognized by T cells (66, 205, 206), suggesting that many of the epitopes recognized by T_H cells and CTLs can be supplied by synthetic peptides.

The available data suggest that a live influenza virus vaccine should provide better cell-mediated immunity, including cross-reactive CTL responses, than nonreplicating vaccines because of the expression of various gene products on the cell surface. Webster and Askonas compared infectious virus with inactivated whole virus and subunit vaccines by giving A/X31 (H3N2) and A/USSR (H1N1) intranasally to mice (211). The live virus vaccine produced significant CTL activity which showed cross-protection when the mice were later challenged. The inactivated virus vaccine induced three to four times less CTL activity than did live virus vaccine. The subunit vaccine induced very low levels of CTL activity. Neither of the inactivated virus vaccines proved effective at inducing cross-protection, but the inactivated whole virus vaccine provided better protection from a homologous virus challenge than did the subunit vaccine.

DIAGNOSIS

Differential Diagnosis

Symptoms of influenza virus infection can vary widely, ranging from a minor upper respiratory illness to the classic febrile respiratory disease of abrupt onset accompanied by systemic symptoms such as headache, myalgias, extreme weakness, and malaise. Because the symptoms of influenza are not readily distinguishable from those caused by other respiratory pathogens, influenza cannot be diagnosed on clinical grounds alone, although during a well-defined outbreak or epidemic, influenza is responsible for a high proportion of acute respiratory illnesses (61, 139). There is also no consistent clinical basis on which to differentiate between type A, B, and C influenza virus infections, although the symptoms of type C virus are almost always milder than those caused by type A or B (140).

Since the clinical presentation of numerous illnesses may resemble influenza, diagnosis can be confirmed only by laboratory tests. Methods for rapid, accurate diagnosis on an individual basis are the subject of continuing research, including work on rapid, economical tests to distinguish between type A and B influenza virus, since this differentiation can determine the potential treatments available. Amantadine, the only chemotherapeutic agent licensed for specific use against influenza virus infection, is effective only against type A.

Laboratory Diagnosis

The procedures of greatest diagnostic worth are generally chosen because of their abilities to isolate virus rapidly, detect viral antigen in clinical specimens, or demonstrate a specific rise in serum antibody to the influenza virus. Successful isolation of the virus depends on the procurement of appropriate swabs or washings from the patient's throat or nasopharynx. The likelihood of successful isolation will also depend on the interval between the onset of symptoms and the procurement of the specimen and the temperature and duration of specimen storage. Specimens from infected individuals are most likely to yield virus when they are obtained within 3 days of symptom onset and stored at 4°C for less than 48 h (or at −70°C for prolonged storage). The composition and other characteristics of the collecting medium, including pH (7.0), the presence of broad-spectrum antibiotics, and the absence of serum (which contains nonspecific HA inhibitors) also influence the success of virus isolation (91).

A variety of cell culture systems have proven useful for the isolation of influenza viruses from clinical specimens. Table 2 lists those systems most commonly used and the method of detecting influenza viruses growing in them. In all cases, the isolated viruses must be characterized serologically to confirm the diagnosis, since several potential respiratory virus pathogens, especially the paramyxoviruses, can also cause hemadsorption or hemagglutination or both. In the application of these techniques, the quality of reagents is crucial to obtain the sensitivity and specificity required, and suitably trained personnel should be available for evaluation of the results. The most commonly used techniques offer the advantages of speed or cost-effectiveness compared with more laborious, albeit more productive, techniques (55, 188).

Many rapid diagnostic techniques are based on direct detection of the viral antigen(s) in the clinical specimen (221). The techniques involve (i) direct immunofluorescence to examine cells in the patient aspirate (128, 196), (ii) solid-phase immunoassay (172, 184, 210), and (iii) antigen capture and staining of cells with monoclonal antibodies. Newer molecular techniques can also be applied for rapid detection and amplification of the viral genome or antigens, although these techniques are largely beyond the capabilities of individual clinical laboratories. Some of these techniques, such as nucleic acid hybridization and the polymerase chain reaction, are still considered primarily research tools, and their routine practical application for influenza diagnosis requires further refinement (22, 158, 184).

TABLE 2. Cell systems suitable for isolation of influenza virus

System	Type of cell[a]	Cytopathic effect	Method of detection
Monkey kidney	Primary	+	Hemadsorption and/or hemagglutination
Human embryonic kidney	Primary	+	Hemadsorption and/or hemagglutination
Chick kidney	Primary	+	Hemadsorption and/or hemagglutination
MDCK	Continuous	+	Hemadsorption and/or hemagglutination
Embryonated hens' eggs (10-day amniotic route)	Primary	−	Hemagglutination

[a] The maintenance media of all cell types should not have any serum additives. For the MDCK line, trypsin (1 to 3 μg/ml) should be included to ensure cleavage of the viral HA.

Serodiagnosis

Serologic diagnosis is based on the fact that recovery from influenza virus infection is accompanied by the development of demonstrable antibodies to the virus. The antibodies may be detected as early as 4 to 7 days after symptom onset and reach their peak after 14 to 21 days. Since a significant proportion of the population, especially adults, may already possess strain-specific antibodies as a result of previous exposure to related strains, it is essential that two serum specimens be obtained, one in the acute phase and another in convalescence, for a comparative titration of antibody levels (91).

Little or no antibody may be present in the initial specimen, especially when strains are encountered that differ sharply from those prevalent earlier. Under other circumstances, antibody is evaluated in relation to the initial titer: a significant rise in antibody must exceed the margin of error of the technical procedure. The methods in general use are reasonably accurate to within a twofold variation; therefore, a fourfold rise in antibody titer against a specific type or strain of influenza virus can be considered diagnostic. High titers in convalescent-stage sera to a recently isolated strain that is present only at low titer in the population in general can sometimes be of presumptive diagnostic value (91, 188).

Definitive identification of the isolates and characterization of the specific antibody generated during infection are based on the qualitative and quantitative results of the following tests: hemagglutination inhibition, virus neutralization (e.g., plaque reduction assay), hemadsorption inhibition, enzyme-linked immunoassay, complement fixation, and single radial hemolysis. The choice of the appropriate test(s) and the use of the data generated will determine the accuracy of and confidence in potential interpretations re-

garding the isolates and the antigenic nature of the circulating strains (25, 90, 92, 143, 232).

PREVENTION AND CONTROL

Antiviral Agents

Amantadine and rimantadine. Amantadine (1-aminoadamantane HCl) is currently licensed in the United States for influenza virus type A prophylaxis and therapy. Rimantadine (α-methyl-1-adamantane methylamine HCl) is equally effective and causes fewer side effects (45, 204); licensure for use in this country is currently under consideration. These cyclic compounds are effective against all strains of type A influenza virus and may be used in both adults and children (204). They have no antiviral activity against type B or C influenza viruses at clinically tolerable levels (47, 103).

According to in vitro experiments, spontaneous mutations causing drug resistance occur at an estimated frequency of 10^{-3} to 10^{-4} (4), and in the laboratory resistant viruses readily emerge in the presence of amantadine or rimantadine (144). Resistant viruses have been isolated from patients, especially children, during treatment with the drugs (68, 76).

Recent studies have suggested that resistant viruses shed during treatment may be transmitted to other individuals within households or institutional settings (76, 125). However, in spite of increasing use of amantadine for treatment and prophylaxis of influenza virus type A in the United States, resistant strains have rarely been found among isolates obtained in clinical settings or from population-based surveillance (14), suggesting that such strains have no growth advantage over susceptible strains. Such observations are consistent with the notion that resistant strains may actually be at a selective disadvantage when not in the presence of drug. There is also no evidence suggesting that drug-resistant strains are more virulent than susceptible ones. There have been no clinical significant differences observed between treated patients from whom resistant viruses have been isolated and those in whom such viruses have not been detected (68, 76). However, further studies are needed to better characterize the dynamics and potential effects of the emergence and possible transmission of resistant viruses during clinical application of amantadine or rimantadine.

Characterizations of drug-resistant mutants indicate that the compounds interact with the membrane-spanning domain of the M2 polypeptide (15, 73), although HA also appears to play a role in some cases (75). The fact that type B and C influenza viruses have no definite analog to the type A influenza virus M2 protein (see above) explains their lack of susceptibility to the drugs.

The observation that in order to be effective the drugs must be present before viral protein synthesis occurs suggests that they somehow interfere with protein-protein interaction and thus inhibit virus assembly (74). As described above, the deduced structure of M2 tetramers in the lipid bilayer suggests a transmembrane selective ion channel similar to the nicotinic acetylcholine receptor (200). Amantadine is postulated to interfere with channel activity by interacting with residues lining the transmembrane tunnel and thus block ion transfer. This blockage could conceivably affect local pH and cause an alteration in the conformation of HA, as has been observed in antibody-binding experiments (75).

Ribavirin. Ribavirin (1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a synthetic purine nucleoside analog licensed for use against respiratory syncytial virus, also has antiviral activity against type A and B influenza viruses. Aerosol administration is therapeutic (60), but oral administration is of uncertain value (182). As a nucleoside analog, ribavirin inhibits both transcription and replication by causing nucleotide incorporation errors. Resistant mutants are rare.

Vaccines

Pandemics and epidemics consistent with those caused by influenza virus have been regular occurrences since the earliest recorded medical commentary (140). In recent times, this recurrence has necessitated a frequent reexamination of the available vaccines and the policies for their use. Most attention has been directed toward the type A influenza viruses because they alone have the potential for major antigenic shift and resultant pandemic spread through an immunologically naive population (85). However, epidemics of type B influenza virus occur regularly, and there is ample evidence of antigenic drift among the circulating strains of type B viruses. Although type A virus epidemics are more often associated with excess mortality, it has also been observed during type B influenza epidemics; both types cause considerable morbidity (8, 10, 37, 188). Vaccination is the single most effective and most widely used measure now available for the prevention of influenza, and inactivated whole or split virus vaccines administered parenterally are the only vaccines currently licensed in the United States.

Inactivated virus vaccines. Inactivated influenza virus vaccine is effective in reducing the incidence of influenza, although there have been wide variations in efficacy attributed to age, other underlying host factors, and the closeness of the match between the vaccine and the circulating strains (37, 48, 199).

The effectiveness of vaccination in reducing the impact of influenza may be greater than is indicated by simply comparing vaccinated and unvaccinated subjects in a single homogeneous population (130). Although inactivated virus vaccine is generally more effective in preventing illness in children and younger adults than in the elderly, it is substantially more effective in preventing influenza-related complications and deaths in the elderly than in preventing uncomplicated upper respiratory illness. Among the elderly, influenza vaccine efficacy has been shown to increase as the severity of the outcome increases: vaccine is more effective in preventing pneumonia and other complications than in preventing upper respiratory illness and is even more effective in preventing death (9, 149, 198). Efficacy does vary within the older age group, and vaccination appears to provide least protection to those who are most debilitated (5, 149). Attaining high rates of vaccination among individuals in closed environments such as nursing homes can control the spread of infection and lower the risk of institutional outbreaks, thus indirectly providing protection to residents of these facilities who may not be well protected by an immune response to the vaccine (5, 150). Recommendations and options for the control of influenza through vaccination and antiviral prophylaxis and treatment are published regularly ("Recommendations of the Immunization Practices Advisory Committee," published annually in *Morbidity and Mortality Weekly Report*).

Live attenuated virus vaccines. Postinfection respiratory secretions contain IgA antibodies to influenza virus that represent the most efficient first line of defense against subsequent infection. In the mouse system, postinfection immunity can be abrogated by neutralization of the nasal

sIgA response, indicating that the major, if not sole, mediator of nasal immunity to influenza virus infection is sIgA (16). The protective ability of nasal secretions can be enhanced by the local replication of an attenuated live virus to establish a subclinical infection. For this reason, the search for an effective, safe, live influenza virus vaccine for use in humans continues.

Unlike inactivated virus vaccines, live attenuated virus preparations potentiate all relevant immune responses by establishing a limited infection via the natural route. Because all viral antigens, including internal and nonstructural antigens, are presented as they would be encountered during natural infection, the immune response elicited by live virus vaccines is broader and includes a greater CTL and sIgA response than is seen with inactivated virus vaccines.

The major problem faced during the development of any live virus vaccine is that of ensuring stable attenuation so as to minimize reversion to virulence. The continual antigenic variation of the influenza viruses presents an additional problem in that vaccines need regular reformulation. However, the segmented nature of the viruses allows the surface HA and NA antigens from circulating strains to be expressed against an attenuated background of the six internal genes (6/2 reassortants). Thus, live virus vaccines have no inherent disadvantage in this respect compared with inactivated virus vaccines because both require regular reformulation to match circulating strains.

For the development of live attenuated influenza virus vaccines, two approaches have shown sufficient promise to justify extensive clinical trials in humans. Both techniques rely on the development of an attenuated strain by altering internal gene configuration and into which contemporary surface glycoprotein genes can be transferred. The two approaches, host range incompatibility and cold adaptation, exploit very different characteristics of influenza viruses.

(i) Host range variant virus vaccines. The observation that type A influenza virus isolates can be grouped as human or nonhuman strains on the basis of the nucleotide sequences of certain internal genes (see above) suggested that internal genes not found in human viruses might confer attenuation on them. Several avian influenza virus strains are restricted in replication in primates (133), and one of these, A/Mallard/NY/6750/78, confers attenuation on human strains when its six internal genes are present in reassortant vaccine strains (187).

Attenuation of these 6/2 reassortant vaccines (referred to as *ah* or avian-human reassortants) requires that certain viral proteins act in concert and, therefore, must be compatible for most efficient function. The three P proteins are obvious candidates since they function as a unit during viral genome transcription and replication. NP, because of its interaction with the P protein complex, is also closely associated with host range attenuation. As described above, the products of the M and NS genes also show host range variation, although their interactions are less clear. The assumption is that a virus adapted through centuries of evolution for growth in a particular host, in this case, avian species, will be less efficient for growth in a different host (humans).

Extensive clinical trials of *ah* reassortant vaccines conducted in adult volunteers demonstrated consistent attenuation, immunogenicity, and genetic stability (30, 171, 187). However, adults of increasing age are more likely to have an anamnestic response from prior infections which can inhibit the replication of a live attenuated influenza virus vaccine. Consequently, efficacy as well as attenuation in infants and children are crucial requirements for any live attenuated influenza virus vaccine since those age groups are least likely to have had prior exposure to the virus and are most likely to develop protective antibodies in response to the vaccine.

Initial studies comparing *ah* and *ca* live attenuated influenza virus vaccines indicated comparable attenuation and immunogenicity in infants and children (193). However, more recent studies have shown a residual virulence of the *ah* reassortant vaccines in infants and children that was not shown by *ca* reassortants (192). This reactogenicity makes the *ah* live virus influenza virus vaccines unlikely candidates for licensure as currently configured.

(ii) Cold-adapted influenza virus vaccines. Studies in several laboratories have provided evidence that the incubation temperature of virus-infected cells can alter the virulence of mammalian viruses grown in cell culture (119). A correlation was seen between an increase in the virulence of certain viruses and their abilities to grow at a temperature above the normal physiological range (38 to 39°C). In contrast, adaptation of the viruses to growth at suboptimal temperature (25°C) resulted in a decrease of their virulence (116). Thus, virulence can be quantified and can be added to or lost from a virus strain, an observation exploited in our development of the parent strains for the vaccine that uses live attenuated *ca* influenza virus.

A *ca* attenuated live influenza virus vaccine was developed by adapting influenza virus types A and B for growth to maximum titer at 25°C in cell culture (SPAFAS-derived primary chick kidney cells) and deriving *ca* mutants for use as the vaccine parent strain in humans. Conceivably, this approach could be applied to other viral pathogens of medical importance, including, most obviously, other respiratory pathogens.

The experimental design was based on techniques meeting the following conditions. (i) The procedures must provide a reliable and reproducible method of attenuation (cold adaptation at 25°C). (ii) An animal model must be available to evaluate pathogenicity prior to use in humans. In this case, the model was the ferret system (67, 120). (iii) Assayable phenotypic markers, i.e., cold adaptation and temperature sensitivity, should be present so the vaccine can be monitored in laboratory and field studies. (iv) The vaccine strain should be nontransmissible. (For the ideal live virus vaccine, nontransmissibility is important to minimize reversion and potential spread in the community.) (v) The vaccine can be administered by the natural route of transmission for the virus (i.e., into the respiratory tract; e.g., intranasally). (vi) The vaccine must have convincing genetic stability, since a live virus vaccine that reverts easily to virulence would be unacceptable and would probably not be licensed for use in humans.

The *ca* viruses developed were found to be attenuated in animals (ferrets) and humans, to be immunogenic, and to possess two easily assayed markers (cold adaption and temperature sensitivity) that clearly correlate with the loss of virulence by the vaccine strain (118). The process of cold adaptation as applied involved a stepwise adaptation of influenza virus until maximum titers at 25°C were seen (119). In ferrets and humans, the type A virus cold variants A/Ann Arbor/6/60 (H2N2) and A/Aichi/2/68 (H3N2) and the type B virus B/Ann Arbor/1/66 were shed at low levels in pharyngeal secretions, with no reversion to virulence. Transmission of the shed virus was not seen (121).

Molecular characterizations of these viruses were based on the electrophoretic migrations of the viral RNA genome segments, which showed that A/Ann Arbor/6/60 (H2N2) and B/Ann Arbor/1/66 both exhibited changes in the migrations

of their genome segments compared with the migrations of the corresponding segments of the original unadapted viruses. This difference suggests that multiple mutagenic lesions had occurred during cold adaptation. These multigenic changes are most likely responsible for the remarkable genetic stability of these strains (39). For this reason, the *ca* type A virus A/Ann Arbor/6/60 (H2N2) was designated the "master" or donor strain for use in the derivation of cold reassortant vaccine lines for use in humans. Similarly, B/Ann Arbor/1/66 was chosen as the donor of attenuated genes for the development of the type B live influenza virus vaccine (41, 117).

This stepwise method for the derivation of *ca* variants for individual circulating strains is not practical because of the length of time necessary to develop, test, and produce such a vaccine. However, the ability of influenza virus to undergo genetic reassortment during mixed infection and the resultant emergence of strains having genes inherited from both parents offer the chance to confer attenuation by the transfer of the appropriate genes from a new, wild-type epidemic strain to the *ca* master strain. Thus, the relevant surface antigens, the HA and NA glycoproteins, are present in the live virus vaccine reassortant and confer the necessary protection against influenza virus infection.

(iii) The 6/2 *ca* live virus vaccine line. The 6/2 *ca* reassortant vaccines have shown consistent attenuation, immunogenicity, and acceptability compared with the donor attenuated virus, A/Ann Arbor/6/60 (171, 192, 193). Thus, laboratory criteria for rapidly developing and monitoring a new live influenza virus vaccine prior to its administration to humans are available. In addition, genetic analyses of these vaccine candidates have allowed the *ca* reassortants to be monitored during all phases of vaccine development, from the laboratory to the manufacturer to their ultimate use in humans, and also have allowed the characterization of isolates obtained during the field trials of the vaccine.

The biophysical characteristics of the 6/2 reassortant vaccine strains are consistent in all cases. For the past 12 years, these live attenuated *ca* virus vaccines have been proven to be predictably attenuated, immunogenic, and incapable of spread to unimmunized contacts. The remarkable genetic stability of the attenuated phenotype is demonstrated by the retention of the *ca* and *ts* phenotypes in all isolates obtained from trials in adult volunteers (119, 121, 171, 192).

Studies of the *ca* influenza virus vaccines in seronegative infants and children indicated that, in contrast to the *ah* vaccines, the transfer of the six internal genes of the parental A/Ann Arbor/6/60 (H2N2) *ca* parent to different wild-type parents (H1N1 and H3N2) reproducibly conferred the capability of efficient infection, an acceptable level of attenuation, and a significant protective immune response to natural or artificial challenge (192). Long-lasting (at least 12 months) immunity was demonstrated when hemagglutination-inhibiting antibodies were assayed (104, 107). The induction of nasal sIgA and cell-mediated immunity was also demonstrated in recipients of the 6/2 *ca* virus vaccine. An additional advantage was demonstrated when Wright and coworkers showed an anamnestic response of sIgA-producing cells in children experiencing a later wild-type infection compared with children with no prior experience with influenza virus infection (84, 223).

(iv) Development of vaccines from live influenza virus type B *ca* reassortants. Because the type B influenza viruses lack an animal reservoir, strains naturally attenuated because of host range are not alternatives for live virus vaccine development. Studies aimed at the development of *ca* strain to provide a vaccine from attenuated live type B influenza virus are still in their infancy. Identification of a master strain to serve as a donor of attenuated genes has been accomplished with the choice of the *ca* B/Ann Arbor/1/66 virus (119). During the past year *ca* reassortants of the type B viruses with different genotypes have been developed. As with the type A virus vaccine, emphasis is on the derivation of 6/2 *ca* reassortants having internal genes derived from the master strain.

Such reassortants are immunogenic yet nonreactogenic in the ferret and have the attenuating phenotypes. The reassortants are cold adapted and temperature sensitive, with a shutoff temperature of 37°C (as opposed to a shutoff temperature of 39°C for the type A virus vaccines). In limited trials in adult volunteers in different geographic areas, the results have been encouraging in terms of a lack of reactogenicity and of the ability to replicate in an attenuated manner in the human host.

FUTURE PROSPECTS

Increasing insight into the structure and function of the various components of the influenza viruses allows the consideration of alternative means to interrupt the viral replication cycle. For example, the X-ray crystallographic determinations of the three-dimensional structures of the virion surface glycoproteins and their ligand-binding sites (33, 214) may allow the design of synthetic compounds tailored to specifically bind HA and/or NA with higher affinity than the natural ligands. The conservation of certain critical amino acids in the glycoprotein binding sites suggests that resistant mutants might be unlikely to appear compared with current antiviral agents.

Virus assembly is also a potential target for chemotherapeutic intervention. The observation that a synthetic peptide mimicking the intracellular domain of HA prevents the release of progeny virions suggests that this approach might be feasible (32). Since viral protein synthesis is unaffected, immune recognition of infected cells would continue enhancing clearance of the virus. This sort of approach is obviously still tentative, and no data as to potential toxicity, efficiency of delivery, etc., are available. Also, differences in the amino acid sequences of the cytoplasmic tails of the glycoproteins from type A, B, and C influenza viruses probably mean that different peptides will be needed for each. The proven variability of influenza virus proteins could conceivably allow resistant mutants to appear.

Characterization of the influenza virus type A M2 protein has given some indication as to its function in the infected cell and yielded insight into its interaction with amantadine and rimantadine (200). It may therefore be possible to design drugs that are more specific in their antiviral activity and less likely to have undesirable side effects. Determination of the proteins in type B and C influenza viruses serving the same function as M2 may make the design of a "universal" anti-influenza virus agent possible.

Probably the most exciting advance has been the development of means to specifically mutagenize the viral genome (50, 114, 115). With this approach, it will be possible to investigate the functions of the viral gene products in ways that were never possible before. Ultimately, it may be possible to introduce selected changes to confer attenuation or enhance the immunogenicity of vaccine strains.

The variability of the influenza viruses makes them unique pathogens necessitating continued research into means for

prevention and treatment of the illness they cause. The fact that regular antigenic changes make the virus a moving target complicates the design and application of vaccines in particular. Our rapidly expanding knowledge of the molecular biology and ecology of influenza viruses will offer new opportunities to exploit the characteristics of these viruses as we devise strategies for the design and application of vaccines and antiviral agents.

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