Animal Pneumoviruses: Molecular Genetics and Pathogenesis

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INTRODUCTION

Pneumoviruses (family *Paramyxoviridae*, subfamily *Pneumovirinae*) are enveloped viruses with negative-sense, nonsegmented RNA genomes. The human pathogen, respiratory syncytial virus (hRSV) remains the best characterized of this group. Despite considerable efforts toward the development of effective treatments for hRSV disease, acute infection remains associated with significant morbidity and mortality, particularly among immunocompromised patients and infants born prematurely. Supportive therapy alone remains the standard of care for the treatment of severe cases of hRSV infection, although prophylactic treatments are now available and encouraging progress has been made toward formulating a vaccine. The recently discovered human pathogen human metapneumovirus (hMPV) also belongs to this virus subfamily, as do the veterinary pathogens avian metapneumovirus (APV), bovine respiratory syncytial virus (bRSV), ovine and caprine RSVs, and pneumonia virus of mice (PVM). The text begins with a review of pneumovirus biology and molecular genetics, continues with a discussion of current concepts related to the pathogenesis of pneumovirus disease as it occurs in vivo and as it is modeled in vitro, and concludes with a discussion of recent therapeutic options for the treatment of hRSV in human subjects.

MOLECULAR GENETICS

Virion Structure

Electron microscopic analysis of the virions of all four members of the subfamily *Pneumovirinae* demonstrates that they are structurally similar to one another and similar to other members of the family *Paramyxoviridae*. Virions, while pleomorphic, are typically spherical (diameters of 150 to 200 nm), although filamentous particles of up to 400 nm in length have been described (9, 22, 192). PVM virions are predominantly filamentous, 100 to 120 nm in diameter, and up to 3 μ m in length (71). The functional differences (if any) based on virion structure are not known, and the differences observed may be a consequence of the cell type used for infection. A diagrammatic representation of a typical pneumovirus is shown in Fig. 1. The pneumovirus virion is surrounded by a lipid envelope derived from the plasma membrane of the host cell into which the three virus glycoproteins, the attachment (G), fusion (F), and small hydrophobic (SH) proteins, are inserted (59, 176). The F protein spans the lipid membrane, with the amino terminus located outside the virion and a short cytoplasmic carboxy-terminal "tail" located inside. In contrast, the G protein is a type 2 membrane protein, with internal amino and external carboxy-terminal components. The F and G proteins comprise the 10- to 14-nm spikes on the virion surface (71). The precise localization of the SH glycoprotein has not yet been established.

The F and G glycoproteins interact with the virus matrix (M) protein layer on the internal surface of the membrane. The details of the molecular interaction(s) between the M protein and the surface F and G glycoprotein(s) are not clear.

The helical nucleocapsid is located within the M-protein layer, and includes the 13- to 15-kb single-stranded, nonsegmented RNA genome in association with the virus nucleoprotein (N) and large (L) proteins and the phosphoprotein (P). The pneumovirus nucleocapsids (diameter, 13 to 14 nm) are significantly smaller than those that have been described for other paramyxoviruses (18 nm) (22). Electron microscopic studies indicate that hRSV nucleocapsids are less rigid and narrower than those of measles virus and Simian virus 5 (SV5), further confirming differences in nucleocapsid morphology between the *Pneumovirinae* and the *Paramyxovirinae* (23). The L protein is often referred to as the RNA polymerase since it is thought to contain all of the catalytic activities necessary for virus RNA synthesis, although it does not function as such without the N and P proteins. The M2-1 transcriptional enhancer protein is also thought to be associated with the nucleocapsid, although this has not yet been determined directly.

Virus Attachment and Entry

The conventional model for pneumovirus attachment involves the virus G protein interacting with a molecule or molecules on the target cell surface (232). While the precise nature of the cellular receptor has not been identified, current evidence suggests that one or more cellular glycosaminoglycans or heparin-like molecules are involved (116, 163, 164, 218, 342). Following G-protein-mediated attachment, the model proposes that the F glycoprotein then promotes pH-independent fusion between the cell membrane and the virus envelope via a mechanism involving the hydrophobic amino-terminal region of the F1 component. The fusion process introduces the internal components of the virion into the cytoplasm of the host cell, where the remainder of the infectious cycle takes place. While pneumovirus infection results in alteration of host cell gene expression, there is no absolute requirement for a functional nucleus, since virus development can proceed in enucleated cells (122).

Recent analyses of deletion mutants of RSV have raised questions about the conventional model of attachment. Karron et al. (205) have characterized a mutant hRSV strain, cp-52, that grows in tissue culture but is attenuated in animals and humans. Sequencing of the genome of this mutant showed that it was lacking both the G and SH coding regions but retained the F-protein gene. Using a recombinant vaccinia virus system. Heminway et al. (172) suggested that the hRSV fusion event required the presence of the F, G, and SH proteins acting in concert. However, Kahn et al. (199) demonstrated that heterologous expression of the hRSV F protein alone promoted infection and cell fusion. Several mutants which lack either the G gene or the SH gene or both have been generated using a reverse genetics approach (341, 342; see below). All of these mutants are viable in tissue culture; their growth rates vary depending on the target cell and are significantly attenuated in vivo compared to that of the wild-type parental strain. There are also data indicating a binding interaction between the RSV

FIG. 1. Schematic of the pneumovirus particle. The RNA genome associates with viral proteins to form the helical nucleocapsid structure (represented on the right and in the center of the virion on the left). The proteins consist of the nucleocapsid protein (N), the phosphoprotein (P), and the large polymerase (L) protein. The M2-1 protein is also thought to be present in this complex (not shown). The nucleocapsid structure is surrounded by the matrix (M) protein, which forms a link between the nucleocapsid and the lipid membrane of the virus particle. Embedded in the lipid membrane are the attachment (G) glycoprotein, the fusion (F) protein, and the small hydrophobic (SH) protein (not shown).

G protein and the fractalkine receptor, CX3CR1 (353), although the latter is not expressed on human epithelial cells. These data suggest that the attachment and fusion steps may be more complex than the conventional model proposes.

Genome Organization of the *Pneumovirinae*

The genomes of the pneumoviruses are ~15,000 nucleotides in length (262, 332; L. Thorpe and A. Easton, unpublished data). Metapneumovirus genomes are smaller (~13,000 kb) than those of the pneumoviruses. While the precise sizes of the genomes are strain dependent, the number of nucleotides does not follow the “rule of six” (42, 43, 312), a feature that differentiates the *Pneumovirinae* from the *Paramyxovirinae*. The reasons underlying the discrepancy have not been determined, but it has been proposed that length restriction among the *Paramyxovirinae* may be due to specific N-protein to RNA interactions (e.g., binding units of six nucleotides) and/or the need to ensure the integrity of the genome in viruses susceptible to non-template-dependent nucleotide insertion.

Figure 2 summarizes the genome organization of the pneumoviruses and metapneumoviruses together with representatives of the *Paramyxovirinae*. While the general strategy for genome organization is similar for all of the viruses, there are differences in specific detail. The genomes of the pneumoviruses and metapneumoviruses encode a greater number of mRNAs (10 and 8, respectively) than the 6 or 7 mRNAs encoded by genomes of the *Paramyxovirinae*. While all members of the family *Paramyxoviridae* encode a standard set of structural proteins (293), one feature which differentiates the *Paramyxovirinae* from the *Pneumovirinae* are different coding potentials of P genes and the use of an RNA editing mechanism to express multiple proteins from this gene (82). Furthermore, the *Pneumovirinae* lack counterparts of the C and V proteins found in *Paramyxovirinae*, although the PVM P gene does direct the synthesis of a polypeptide by using a second open reading frame in a manner analogous to the expression of

the paramyxovirus C proteins (14). Another unique feature of the *Pneumovirinae* is the gene encoding the M2 proteins which have not been identified in any other virus genome.

The genomes of the *Pneumovirinae* contain regulatory sequences at each terminus. The 3' end of the genome sense RNA directs both replication and transcription, while the 5' end of the genome RNA (i.e., the 3' end of the antigenome) contains signals that direct replication of the antigenome for synthesis of progeny virions. The metapneumovirus genome encodes eight distinct transcription units in a linear array, with each unit separated by a short segment of untranscribed sequence. In contrast, the pneumovirus genome encodes 10 transcription units, including nonstructural proteins NS1 and NS2 (106). There is one notable difference in gene order: the F-protein gene follows the G-protein gene in the pneumovirus genomes, the reverse order from that observed among the metapneumoviruses (293). In evolutionary terms, this difference is very striking since recombination among these virus genomes has been observed only in the laboratory setting (329).

While the PVM genome is organized with a tandem array of distinct genes similar to that of the metapneumoviruses, the hRSV genome includes a 68-nucleotide overlap of the gene encoding the M2 proteins with the start of the L gene, discussed further in “Current model for genome transcription” (below).

Pneumovirus Gene Products

As indicated in Fig. 2, the nucleocapsid, phosphoprotein, matrix, fusion, and polymerase proteins are common to all members of the family *Paramyxoviridae*. Much of our understanding of the functions of these proteins has been derived by extrapolation from experiments performed with one or more specific viruses, particularly Sendai virus. More recently, it has been possible to confirm these functions directly by targeted mutagenesis and reverse genetics.

Nonstructural (NS1 and NS2) proteins. The NS1 and NS2 proteins are unique to the pneumoviruses. The genes are lo-

FIG. 2. Genomic structures of viruses of the family *Paramyxoviridae*. The subfamilies, genera, and representative viruses are as indicated. *, pneumonia virus of mice is similar, without the M2-L gene overlap.

cated at the 3'-most positions of the genome and, as a consequence, are the most abundantly transcribed genes. The NS1 and NS2 proteins are relatively small, with the NS1 proteins of hRSV and PVM being 139 and 113 amino acids in length, respectively, and the NS2 proteins being 124 and 156 amino acids, respectively (50, 57, 58).

The hRSV NS1 protein interacts with the M protein in infected cells, and two-hybrid analysis identified an additional interaction between NS1 and the P protein (106, 173). In reverse genetics studies, expression of the NS proteins inhibited RNA synthesis, suggesting a regulatory role for the NS proteins in vivo (8, 343). While the finding of hRSV mutants lacking the NS1 or NS2 genes confirmed that the NS proteins are dispensable for virus replication in vitro, recombinant viruses grew more poorly than the parental virus and the mutants were attenuated in chimpanzees (188, 344, 347, 378).

Other studies suggest that the pneumovirus NS proteins play a role in inhibiting responses to interferon (30, 31, 315). Recombinant rabies viruses each expressing one of the bovine RSV NS genes were resistant to interferon treatment if cells were dually infected with the virus but not if they were infected alone (315), suggesting that the NS1 and NS2 proteins function together to inhibit responses to interferon in this setting. Most recently, Bossert et al. (31) have demonstrated that

NS1 and NS2 proteins inhibit the activation of interferon regulatory factor 3 and thus the production of beta interferon in bRSV-infected MDBK cells. Valarcher et al. (358) likewise demonstrated that recombinant, NS-2 lacking, bRSV are potent inducers of interferon in bovine nasal fibroblasts and bronchoalveolar macrophages. Studies using recombinant rabies viruses that express the PVM or hRSV NS1 and NS2 genes have yielded similar results (30, B. Bossert, A. J. Easton, and K. K. Conzelmann, unpublished data). The mechanism by which the interferon inhibition is achieved is not yet understood.

Nucleocapsid (N) protein. The N protein forms an integral part of the nucleocapsid complex of the virion and is an essential component of the polymerase complex. The N protein is thought to be responsible for giving the RNA genome its helical structure through a tight association with the virus genome RNA; interestingly, hRSV N protein expressed in insect cells spontaneously formed nucleocapsid structures containing RNA (24). While the RSV N protein (391 amino acids) is smaller than its counterparts among the *Paramyxovirinae* (489 to 553 amino acids) and shows little sequence similarity, alignments with the amino-terminal 400 amino acids of the N proteins of various nonsegmented negative-sense RNA viruses suggested similarities among predicted secondary structures (13). The hRSV N protein has a relatively high level of amino

acid identity (60%) to the N protein of PVM (13) and has regions of specific sequence homology to the N proteins of APV and hMPV (234, 361).

Deletion mutant analysis of N protein expressed in cells suggests that a large segment of this protein is required for interacting with the P protein (128). Barr and Easton (12) demonstrated that the amino-terminal half of the PVM N protein was unable to bind to immobilized P protein while the carboxy-terminal half of the N protein bound with only 17.6% of the affinity seen with the full-length protein, results suggesting that the complete N protein is important for this interaction.

Phosphoprotein (P). The P protein is an essential component of the replication and transcription complexes of the pneumoviruses (14, 225, 234, 314, 361). Analysis of the interaction between the P and N proteins has shown that the carboxy terminus of the P protein contains most of the elements necessary to bind to the N protein (128, 173, 207, 242, 247, 326). The P-protein genes of the *Pneumovirinae* encode several different proteins using alternative start codons. In addition to the full-length sequence, the P gene of hRSV encodes an mRNA for an additional, short polypeptide of unknown function (45). While the PVM and hRSV P proteins have significant amino acid identity, the two proteins align well only at the extreme amino terminus and within the carboxy-terminal half of the protein, and there are large regions near the amino terminus with limited to nonexistent sequence identity (14), including the second open reading frame of the PVM P gene. The protein product of this second open reading frame has been detected in infected cells, as have four additional proteins generated by initiation at internal AUG translation initiation codons within the P-protein gene (14). No additional proteins have been identified from the P genes of the metapneumoviruses.

The P protein is phosphorylated at specific serine residues (98). Villanueva et al. (366) demonstrated that most of the phosphorylation, occurring at residues 116, 117, 119, and 232, is not essential for transcription or replication.

Matrix (M) protein. The M protein is associated with the inner face of the lipid membrane of infected target cells (284, 382). All M proteins include a carboxy-terminal hydrophobic domain. The M proteins of nonsegmented minus-strand viruses are thought to function by rendering the nucleocapsid transcriptionally inactive before packaging and also by promoting the association of the nucleocapsid complex with the nascent envelope (140, 343).

Small hydrophobic (SH) protein. The SH proteins of pneumoviruses are small integral membrane proteins. The sizes of the proteins vary considerably, with hRSV having the smallest SH protein (64 amino acids) and hMPV having the largest (183 amino acids) (58, 100, 361). The RSV SH protein is anchored in the membrane by a hydrophobic signal-anchor sequence (275) and is a type 2 membrane protein (59, 117). The precise function of the SH protein is unknown, and analysis of hRSV mutants lacking the SH and/or G genes suggests that the SH protein is not absolutely necessary for attachment, infectivity, or virion assembly. However, the hRSV G and SH proteins have also been implicated in impairing the Th1-mediated host antiviral responses (352).

Attachment (G) protein. The hRSV G protein was identified as the viral attachment protein based on the observation that

G-specific polyclonal antibody blocked absorption of the virus to the surface of target cells (232). Similarly, a monoclonal antibody that inhibited the hemagglutinating activity of PVM likewise bound to the G protein (235). The pneumovirus G proteins do not show any sequence or structural homology to the HN or H attachment proteins of other viruses of the family *Paramyxoviridae*, although all are type 2 glycoproteins (373). The G proteins of pneumoviruses are heavily glycosylated with both N- and O-glycosylated sites, adding approximately 55 kDa to the mass of the hRSV polypeptide. While the roles of these glycosyl groups have not been completely clarified, extensive glycosylation of the extracellular domain of the G protein could reduce its antigenicity by shielding the virus protein with host-specified sugars. Alternatively, partial utilization of glycosylation sites can result in antigenic heterogeneity, representing a source of diversity within a genetically homogenous virus population. The immune response to hRSV correlates with and provides selection pressure for G-protein variation (127, 261).

The hRSV G protein in infected cells is present in two forms. The first is translated from the entire open reading frame and contains an amino-terminal sequence prior to the putative signal/membrane anchor region. A second, secreted form of the protein has been observed (304) which is generated by the initiation of translation at an internal AUG initiation codon that is in the same reading frame as that used for the full-length G protein. The function of the secreted protein is not known, but it may alter the immune response of the host, possibly by acting as a "decoy" or by altering specific aspects of the host inflammatory response (190, 191).

While the G protein of PVM shares structural features with those of hRSV and the metapneumoviruses (295), the G-protein genes of two strains of PVM have been sequenced and show intriguing differences. The G protein of the nonpathogenic PVM strain 15 does not have an amino-terminal sequence prior to the putative membrane anchor, while the G gene of a pathogenic PVM strain contains a single base deletion and several mutations (295).

A final interesting feature of the G genes of hRSV and PVM is the presence of a small open reading frame just 5' to the main coding region, of function unknown (295, 373).

Fusion (F) protein. The F protein of hRSV was first identified by immunoprecipitation using monoclonal antibodies which inhibit the formation of multicell syncytia in cell culture. Although the pneumovirus F proteins show only limited amino acid sequence identity to the F proteins of other viruses of the family *Paramyxoviridae*, they show significant structural similarities to members of this group (15, 18, 51, 64, 361, 386). The F protein of hRSV is synthesized as an inactive precursor (F0) that assembles into a homo-oligomer in the rough endoplasmic reticulum (ER), forming a structure that is presumed to represent a single virion spike (62). When the F0 protein reaches the *trans* Golgi network, it is activated by cleavage into two disulfide-linked subunits, F1 and F2, at two furin consensus sites (142, 392, 393). The short (27-amino-acid) peptide between the two furin cleavage sites does not remain associated with the F-protein subunits (19) and a novel biological activity for this protein has recently been described (314; see "Virus evasion of innate immunity" below).

M2 proteins. The genes encoding the M2 proteins of pneumoviruses and metapneumoviruses contain two overlapping

FIG. 3. Diagrammatic representation of the organization of the pneumovirus M2-1 and M2-2 genes. The grey bar represents the M2-1 open reading frame, and the white bar represents the M2-2 open reading frame. The positions of the translation initiation and termination codons of are identified. aa, amino acids.

open reading frames (Fig. 3) (4, 58, 165, 237, 361, 387). The first open reading frame, initiated by the 5'-proximal AUG initiation codon, encodes the M2-1 protein, which is involved in virus RNA synthesis as described below. Immunocytochemical analysis of cytoplasmic inclusions showed that genome RNA, N, P, L, and M2-1 proteins were colocalized in hRSV-infected cells (126). Reverse genetics studies have shown that while the minimal *trans*-acting requirements for hRSV (i.e., proteins that must be added for efficient first-round virus replication) are the N, P, and L proteins, the presence of the M2 gene significantly enhanced the production of full length virus mRNA (62, 63, 112, 152, 388). In cells expressing the N, P, and L proteins, virus-specific transcription resulted in the production of prematurely terminated mRNA (62) and expression of the M2-1 protein served to promote the production of full-length mRNA without altering genome RNA replication (63). These data suggest that the M2-1 protein functions as a transcription elongation factor.

The product of the second open reading frame, the M2-2 protein, has been detected in cells infected with hRSV and PVM (4). No M2-2 protein was detected in APV-infected cells, and to date there is no information about the putative M2-2 protein of hMPV. Expression of the M2-2 open reading frame resulted in the inhibition of virus gene expression from synthetic genomes. The RSV M2-2 protein accumulates during infection, and it has been proposed that the role of this protein is to switch the virus from replicative to assembly mode prior to virion release (4, 22).

Large (L) polymerase protein. By analogy to other viruses, the pneumovirus L protein is thought to be the major component of the viral RNA-dependent RNA polymerase complex, which is responsible for the synthesis of all viral RNA, including mRNA, replicative intermediates, and the progeny RNA genomes. The L protein is also thought to be responsible for mRNA methylation and capping, although this has not been shown directly. As the name indicates, the L protein is very large—the hRSV L protein contains 2,165 amino acids, and the hMPV L protein contains 2,000 amino acids (297, 361).

Poch et al. (289, 290) described six conserved domains for polymerase proteins of minus-strand RNA viruses, including three functional domains and distinct interdomain regions acting as hinges to assemble the functional domains into an appropriate conformation. Domain III contains the polymerase GDNQ motif, and domain IV contains a putative ATP binding site (200). There have been few direct studies of the specifics of the L proteins of the *Pneumovirinae*. Most recently, Cartee et al. (47) demonstrated that a single amino acid change (N1049D) resulted in aberrant transcriptional termination without altering the rate of virus replication.

Current Model for Transcription

The details of the current model of transcription and replication of the pneumovirus genomes are consistent with those proposed for all nonsegmented negative-sense RNA viruses. The virus genome contains a single polymerase entry site, termed the leader or promoter sequence, at the 3' end of the RNA. The polymerase complex initiates transcription at the conserved transcriptional start sequence that is present at the beginning of all transcribed genes. The polymerase then travels along the genome until it reaches a conserved signal that marks the point at which transcription terminates. At some point during the transcription process, the nascent mRNA is methylated to produce a cap structure that ensures translation on host cell ribosomes; there are no reports directly addressing this process. The signal marking the end of transcription among the pneumoviruses contains a short run of U residues, and this marks the position at which the polyadenylated tail is added to the mRNA. When the polyadenylation process is complete, the mRNA is released for translation on either free or membrane-bound ribosomes, depending on the nature of the protein product. At this point, the polymerase does not automatically progress along the genome. Rather, only a proportion of the active polymerase molecules do so, with estimates suggesting that approximately 50% of the polymerase molecules are able to continue. The dissociated polymerases

can reinitiate transcription only by translocation to the 3'-terminal leader region to begin transcription at the first gene. The remaining polymerases traverse the genome without transcribing until they encounter the conserved transcriptional start sequence of the second gene. At the end of this gene, the remaining polymerases repeat the dissociation-versus-continuation process. The result is that genes proximal to the 3' leader region are represented in mRNA more abundantly than are those closer to the 5' end in a gradient of transcription (215). The intergenic regions of the pneumoviruses are variable in length and tolerate mutation and insertion of additional nucleotides (18, 42, 49, 361).

The process of termination and reinitiation is poorly understood, but the M2-1 protein of RSV has been shown to be essential for production of full-length virus mRNA (62, 63, 112, 166). The antitermination activity during transcription permits the viral polymerase to remain associated with the template, thereby increasing the production of full-length mRNA and enhancing the production of products of genes that are distal to the 3' leader region. The eight RSV gene junctions, which vary in their ability to terminate transcription, all direct the production of more readthrough mRNA in the presence of M2-1 protein, although they vary in their sensitivity to the presence of the M2-1 protein (167).

The genome structure of hRSV contains an exception to the process described above. The transcriptional stop signal for the M2 gene is located 68 nucleotides downstream of the transcriptional start sequence for the L gene (65). Thus, a large proportion of the mRNA initiated at the L-gene start sequence terminates at the M2 transcriptional stop sequence (65, 111).

Elements within intergenic regions play a vital role in the regulation of transcription (114). The intergenic regions of the *Pneumovirinae* show considerable sequence diversity and vary from 2 to 56 nucleotides in length. The transcriptional start sequences are thought to be 10 nucleotides in length, and the consensus sequences, while conserved, are slightly different for each pneumovirus (61, 221). The PVM transcriptional start signals are more variable than those of RSV or APV, which are absolutely conserved with the exception of the L gene (49, 296, 332).

The transcriptional stop signals of hRSV consist of a conserved pentanucleotide sequence together with a 1- to 4-nucleotide AU-rich region and a 4- to 7-nucleotide poly(U) tract. Saturation mutagenesis of the hRSV gene transcriptional start sequences showed that residues 1, 3, 6, 7, and 9 were critical in directing transcription initiation, although there was some variability in efficiency, particularly with the NS1 and NS2 gene start sequences, which were approximately 40% less efficient than the others (219, 220, 221).

Deletion of a transcriptional stop signal from an upstream gene resulted in transcriptional readthrough to the next gene without termination. Harmon et al. (168) analyzed the hRSV M-gene transcriptional stop sequence and determined that the integrity of the pentanucleotide and AU-rich regions was essential for efficient termination. The residue following the poly(U) tract might also be important for efficient transcription termination (335).

No significant difference in transcriptional activity or efficiency was seen with different sized RSV intergenic sequences (167, 219). Bukreyev et al. (42) described the recovery of a

recombinant hRSV containing an intergenic region of 160 nucleotides. This very long intergenic region had little effect on sequential transcription.

The genome structure of hRSV contains an exception to the process described above. The gene end signal for the M2 gene is located 68 nucleotides downstream of the gene start sequence for the L gene (65), and much of the mRNA that initiates at the L transcriptional start site terminates at the M2 gene end sequence (65, 111). The role (if any) of this truncated L transcript remains to be elucidated.

Current Model for Genome Replication

The current model, based on that determined for other nonsegmented negative-sense RNA viruses, proposes that the nucleocapsid complex is the functional unit for both replication and transcription and that the L, N, and P proteins are involved in both processes. Transcription and replication promoters of hRSV overlap but are not identical (114). In the replicative mode, the polymerase binds to the 3' end of the genome RNA and initiates RNA synthesis *de novo*. Once initiated, the polymerase is committed to continue to the end of the template to produce an antigenome. The polymerase complex then uses the antigenome as a template, binding to and initiating RNA synthesis at the 3' end of the antigenome RNA. In the genome sense RNA, the trailer region contains the antisense copy of the sequence necessary for initiating replication to produce more genome RNA from the antigenome.

It is not yet clear precisely how the polymerase complex alters its activity and switches from the transcription to the replication mode. Fearn et al. (113) showed that increased expression of the N protein stimulated hRSV replication. This is consistent with the proposal made for other negative-sense RNA viruses, suggesting that replication is dependent on RNA encapsidation (224). However, there are some conflicting data suggesting that high levels of N protein may also stimulate transcription, and, more recently, a role for M2-2 protein has been proposed (21).

The 3' and 5' termini of the pneumovirus genome contain the sequences that direct replication. Reverse genetics studies showed that the 3'-terminal 44-nucleotide leader region and the 5'-terminal 40 nucleotides of the trailer region of hRSV were necessary for replication, encapsidation, and assembly (66). The immediate termini of the pneumovirus genomes have complementary sequences, as has been described for other negative-sense RNA viruses (262, 296, 361). The trailer sequences are more efficient than the leader sequences at directing replication, as would be expected from their role in replication and the requirement for the leader region to direct both replication and transcription (114).

Analysis of the leader and trailer sequences suggested that nucleotide positions 1, 2, 3, 5, 6, and 7 were particularly important for replication, while position 4 was found to be tolerant of alteration (285). Using synthetic chimeric minigenomes with terminal sequences from both APV and RSV in virus infected cells, Marriott et al. (250) showed that paired termini from the same virus were required for replication to occur. It is not clear whether the critical step is replication or encapsidation, but the data suggest that an association between the leader and trailer is necessary for productive infection (250).

Packaging and Assembly

Reverse genetics experiments suggest that the accumulation of the M protein may be a component of the trigger to initiate the assembly of virions (343). It is anticipated that the nucleocapsid complex/M-protein structure associates with the internal tails of the major glycoproteins that are inserted into the membrane of the infected cell after processing through the Golgi complex. In parallel with studies performed with other viruses, a progressive series of interactions between the glycoproteins and the M protein, which acts as a bridge with the nucleocapsids, results in the progeny virions budding from the surface of the cell in the form of filament structures (280). In polarized cells, such as those of the respiratory tract, hRSV buds from the apical surface and is shed into the airways, from which it can be transmitted to new hosts (303).

PATHOGENESIS OF DISEASE

This section is divided into four parts. APV, hMPV, and BRSV infections are discussed individually. In the fourth part, we consider the human pneumovirus pathogen hRSV. The current state of understanding of the human disease as assessed by direct studies of patients and patient-derived materials is reviewed, followed by the results obtained from cell culture and animal models of hRSV. Consideration of the mouse pneumovirus pathogen mPVM has been interwoven with the information about hRSV, since it has been developed specifically as a surrogate for severe hRSV infection in rodent models.

Avian Metapneumovirus Infection

APV, a metapneumovirus previously known as turkey rhinotracheitis virus, is the cause of severe respiratory infection in turkeys (reviewed in references 73, 244 and 274) and may also be the etiologic agent of swollen head syndrome in chickens (73). Originally identified in South Africa in the late 1970s, APV was later found in Europe, Asia, and South America and has only recently emerged among flocks of domestic birds in the United States. Sequence divergence among the genes encoding the APV viral G (attachment) proteins suggested division of the European isolates into two subtypes, APV/A (in the United Kingdom) and APV/B (in continental Europe). A new subtype, APV/C, first emerged in the United States in 1996 in Colorado and was followed by a 1997 outbreak in Minnesota. Sequence analysis of APV/C isolates indicates that it is the most closely related to the newly discovered human metapneumovirus pathogen, hMPV (5, 274, 360). The U.S. outbreaks of APV infection have occurred in a seasonal pattern (spring and autumn), and several studies have implicated wild migratory birds in the spread of the disease (321). This disease has had devastating consequences for the turkey industry in Minnesota, resulting in much recent attention to disease detection, pathogenesis, and prevention (74, 322).

In the wild, APV leads to severe upper respiratory infection, often resulting in death of the infected bird. The clinical syndrome and the pathology observed in response to experimental infection with APV/A, APV/B, and APV/C (the last of these from the Colorado outbreak) have been described in several

reports (75, 76, 193, 194, 277), in addition to a recent experimental study describing the natural history of APV/C from Minnesota (189). In the natural history study, respiratory symptoms were detected as early as 2 days postinoculation and included open mouth breathing, sneezing, swollen sinuses, and nasal discharge. Microscopically, inflammatory infiltrates (lymphocytes, macrophages, plasma cells, and heterophils) were detected in nasal turbinate and sinus tissues, although, interestingly, the ciliated epithelium, which was subjected to significant disruption in the earlier studies (including that describing APV/C from Colorado), remained intact. Anti-APV antibody responses emerged at 7 days postinoculation, although it is unclear whether this antibody response is protective (271). While the events promoting disease and tissue destruction are not yet understood at the molecular level, Munir and Kapur (266) have recently presented the results of a subtractive hybridization and gene microarray approach and have reported increased expression of transcripts encoding interferon response proteins and other elements of the inflammatory response and ubiquitination pathways in cultured chicken embryo cells infected with APV/C.

As might be anticipated, significant research has gone into vaccine studies. In various trials, several groups have demonstrated protection in response to immunization with high-passage virus (154, 283, 363, 381), with one report using high-passage virus from the Minnesota outbreak (APV/MN/turkey/1-a/97) together with the immunomodulatory agent S-28828 (299), an imidazoquinolinamine agent that induces interferon production and macrophage activation in avian species (203).

Human Metapneumovirus Infection

The very recently discovered hMPV is the first human pathogen of this family to be identified and is most closely related to the APVs described above; evolutionary analysis suggests that hMPV has existed in the human population for at least 50 years. This virus was first identified in the Netherlands by van den Hoogen et al. (360), and evidence of infection has since been collected worldwide (26, 105, 110, 273, 286), with one reported fatality in a stem cell transplant recipient (44). The clinical syndrome is currently indistinguishable from that resulting from hRSV infection, with some cases characterized by upper respiratory tract infection and others characterized by severe bronchiolitis and pneumonia (123). While several reports have associated hMPV infection with asthma exacerbation (123, 187, 247, 337), one report suggests that the association is less strong than that of asthma and rhinovirus infection (300). The current diagnostic approach involves reverse transcriptase PCR (RT-PCR) (245), facilitated by the substantial amount of virus gene sequence that is available (18, 245, 360, 361). Similar to what has been reported for hRSV infection, one group has reported acute wheezing and elevated concentrations of interleukin-8 (IL-8) in nasal secretions from patients infected with hMPV (187). For more detail, the reader is referred to two excellent recent reviews on this subject (197, 198).

Why was this virus invisible prior to 2001? The authors of the original study (360) discuss some possibilities, including very slow replication of hMPV in the standard cell lines used to isolate respiratory virus pathogens. Among the other possibil-

ities, most clinical cases of hMPV disease are not life-threatening, and, as such, the impetus to identify novel pathogens among the pool of hRSV-negative respiratory infections has not been overwhelming. One can only wonder what other pathogens have been overlooked.

Bovine Pneumovirus Infection

Research on bRSV has focused primarily on issues related to its prevalence as a respiratory virus pathogen in dairy and beef cattle worldwide (reviewed in references 10, 103, 227, and 355). There are also distinct ovine and caprine RSV pathogens, infecting sheep and goats, respectively (reviewed in references 34 and 97). bRSV was first isolated and identified as a unique pathogen in the 1970s, and it is currently the closest known phylogenetic relative of hRSV (360), although there is no evidence for antigenic grouping into A/B strains (104). Similar to hRSV, bRSV results in seasonal infections among domestic cattle herds, and transmission is thought to occur via contact with respiratory secretions, with a short incubation period followed by signs and symptoms of upper and lower respiratory tract infection. Interestingly, and unlike hRSV infection, natural bRSV infection is often accompanied by concomitant bacterial infection (*Mannheimia haemolytica*, *Pasteurella multocida*, and *Haemophilus somnus*), resulting in what has been defined as the bovine respiratory disease complex. One study has documented sequence clustering based on geography (357), while yet another reports extensive sequence divergence in outbreaks distributed over time within a single herd (228).

There are several vaccine formulations in existence—modified live and inactivated, in both adjuvanted and nonadjuvanted forms (102, 374, 375)—and there is significant debate about the relative efficacy and modes of appropriate response. Modified live vaccines preferentially stimulate the production of neutralizing antibodies that detect the virus F (fusion) protein and stimulate lymphocyte proliferation and cytokine responses. Inactivated vaccines also stimulate lymphocyte proliferation and production of virus neutralizing antibodies, but the latter are produced together with nonneutralizing antibodies. There is some information relating to a potential immunopotentialization in response to challenge after formalin-inactivated vaccine administration (138, 318), analogous to what was observed in the human trials. While much of the focus has been on the development of humoral immunity to bRSV, recent results suggest that vaccine efficacy may relate to the development of cellular immunity, specifically Th1 (gamma interferon) responses (102, 121, 125, 374).

Experimental models of bRSV infection in calves involve aerosol administration of virions, resulting in early-onset cough and nasal discharge and progressing to signs and symptoms of lower respiratory tract infection (287, 350, 367, 380). In the study performed by Philippou et al. (287), lungs from animals sacrificed at 12 weeks postinoculation were atelectatic with evidence of bronchiolar obstruction and profound inflammatory infiltration. Disease progression has been associated with increased expression of IL-2, IL-4, and/or gamma interferon in cells drained from pulmonary lymph (139, 214) and in peripheral blood mononuclear cells (257), detection of tumor necrosis factor alpha and leukotriene B₄ in respiratory secretions (302, 308), and elevations in levels of serum acute-phase

response proteins (171). Aside from the challenging aspects of working with large experimental animals, research in this field is hampered by the fact that there are comparatively few bovine cytokine gene sequences available in the public domain and even fewer commercially available reagents.

Buchholz et al. (35) described the creation of recombinant bRSV virions in vitro utilizing the complete sequence genome of bRSV (389) by methods analogous to those developed for the study of hRSV. Among the results as they relate to disease pathogenesis are the finding that the bRSV NS1 and NS2 proteins function in synchrony to antagonize alpha/beta interferon-mediated antiviral activities in a species-specific fashion (30, 315) and that the SH and G genes are dispensable for bRSV replication in vitro (204). This technology has already been applied to the development of the next generation of bRSV and hRSV vaccines (36, 317).

Human Respiratory Syncytial Virus Infection

Human RSV is a seasonal infection, which in North America is observed most frequently in winter and spring. Transmission between individuals occurs via contact with respiratory secretions, either directly or via handled surfaces and objects or via large aerosolized droplets. The incubation period is typically 2 to 8 days, with virus replication spreading from the upper respiratory tract epithelial cells to the lower respiratory tract. The clinical signs and symptoms of acute hRSV infection have been described in significant detail in several recent reviews (86, 141, 155, 161, 162, 216, 372). The complex interplay of factors involved in acquired immunity to hRSV is discussed in the section on vaccines. The focus here is on several specific topics of recent interest.

Genetic susceptibility to severe infection. While hRSV infection appears to be universal, there is great interest in understanding why and how some children proceed from initial infection to severe, life-threatening forms of lower respiratory tract disease. There are several straightforward and clearly understood conditions that lead to increased susceptibility to severe infection, including premature birth, congenital dysplasias, and immunodeficiency disorders (reviewed in references 52, 96, 260, 340, and 371). Less well understood are the conditions under which otherwise “normal” children succumb to severe hRSV disease. Several groups have approached this question via genotyping and have focused on questions relating to normal variation within the human population. Among the results, Hallman and colleagues (223, 239) have documented correlations between specific alleles of the genes encoding surfactant A and D with increased disease severity and Kwiatkowski and colleagues (177, 178) have provided evidence supporting a relationship between disease and a susceptibility locus related to the gene encoding IL-8. Two groups, Choi et al. (56) and Hoebee et al. (175), have identified alleles of IL-4, and IL-4 and its receptor, respectively, that are also associated with more severe sequelae of RSV infection in vivo. At present, it is not clear how these genetic factors influence responses to hRSV infection at the molecular level.

Inflammatory responses to infection. To understand the concepts underlying inflammatory responses to hRSV, it is important to recognize that there are two different forms of hRSV-related disease discussed in the literature. Primary

hRSV infection, or natural hRSV infection, occurs in a naive (i.e., unvaccinated) host, and the inflammatory responses are those of the innate immune system responding to virus replication *in situ*. This is to be contrasted with what has been termed “enhanced” or “augmented” disease, which describes the aberrant inflammatory responses observed in children who had been vaccinated with formalin-fixed virus preparations and later challenged “in the wild” with natural virus infection. The postvaccination response has been characterized extensively, and the profound lymphocytic and eosinophilic inflammation observed has been attributed to an induced Th1-Th2 imbalance, with dominance of Th2 lymphocyte responses in this setting (32, 148, 180).

With respect to primary hRSV infection, the earliest histologic studies were, of necessity, examinations of postmortem specimens. Severe infection progressed to include necrosis of airway epithelium, interstitial inflammation, and mucus secretion leading to airway obstruction and respiratory compromise. While lymphocytes were predominant in postmortem specimens, more recent studies have pointed to neutrophil influx as an earlier acute cellular response and to neutrophil-mediated activities as a major cause of respiratory symptoms (107, 209, 328, 370). Neutrophils are proinflammatory leukocytes of the granulocyte series that develop from pluripotent stem cells in the bone marrow, released into the circulation, and recruited into tissues via the coordinated actions of various cytokines and chemokines. Activated neutrophils participate in phagocytosis and degranulation and release reactive oxygen metabolites into the surrounding tissue. While the role of the neutrophil is to promote host defense—very clearly established for bacteria and fungi, less clearly for viruses—neutrophils at the same time can promote tissue damage and destruction, representing the two sides of their classically defined “double-edged sword” (see reference 309 for a general review of the role of neutrophils in innate immunity). Eosinophils are also recruited to the lungs in response to primary hRSV bronchiolitis (133, 169), and both neutrophils and eosinophils are recruited to lung tissue in the PVM model of acute pneumovirus infection *in vivo* (88). Virus-specific cytotoxic T lymphocytes have been detected within 10 days of infection in human subjects (54, 181), and roles for CD4⁺ and CD8⁺ T lymphocytes in virus clearance have been inferred from rodent studies (81, 146, 147).

Proinflammatory cytokines have been detected in respiratory secretions and in bronchoalveolar lavage samples from individuals infected with hRSV (1, 169, 182, 263). Three of the major chemokines detected—IL-8, RANTES, and macrophage inflammatory protein 1 α (MIP-1 α)—have each been implicated in neutrophil and eosinophil chemotaxis and recruitment. MIP-1 α has been of particular interest, since its levels have been shown to correlate with levels of eosinophil degranulation products (169) and with severity of disease (136).

Several recent studies have described a role for the pattern recognition receptors CD14 and/or toll-like receptor 4 (TL4) in innate host defense against hRSV *in vivo* (158, 170, 222). Among the findings, these studies documented persistence of hRSV virions in TLR-4-deficient mouse lungs, impaired NK cell trafficking and function, and reduced early NF- κ B-dependent inflammatory responses. However, analogous experi-

ments performed with TLR-4 deficient mice and the natural rodent paramyxovirus pathogen Sendai virus (mouse parainfluenza 1) yielded completely contradictory results (362). The use of natural host-pathogen pairs for the study of acute respiratory virus infection is discussed further in “Biochemical responses in mouse models” (below).

Modeling hRSV Infection in Cell Lines and in Experimental Animals

PVM. The human pathogen, hRSV, and the mouse pathogen, PVM, can induce similar disorders in their natural hosts. As such, the mouse data that are available are compared and contrasted to what is currently understood about pneumovirus infection in a human host.

Target cells. The primary targets of hRSV *in vivo* are respiratory epithelial cells, although infection of alveolar macrophages has been reported (278). This has been modeled in tissue culture, and the epithelial cell lines HEP-2 and A549 and primary human epithelial cultures have been used to study the cellular and molecular responses to hRSV infection. Other cells are susceptible to hRSV infection in culture, including the fibroblast cell line MRC-5, macrophage and macrophage-like cell lines (301, 313), and, for uptake if not outright infection, eosinophils (211). Mouse PVM has similar target affinity and infects respiratory epithelial cells *in vivo* and the mouse epithelial cell line LA4 (265).

Cellular receptors. The receptor for PVM has not been characterized. While no one has yet identified a single specific protein, lipid, or carbohydrate entity that serves as a unique, virus-specific receptor for the hRSV pathogen, the ongoing story is an interesting one, with multiple virus and cell determinants coming into play. Among the earliest findings, Krusat and Streckert (218) demonstrated that the glycosaminoglycan (GAG) heparin, when included in an *in vitro* culture system, could inhibit the infection of target cells with hRSV. This finding has withstood the test of time, and several groups have followed through with significant elucidations and refinements. Taking the whole-virus approach, Hallak et al. (163, 164) reported that only N-sulfated moieties of GAGs (and not C6-O or C2-O sulfation) are important mediators of RSV infection and later documented the specific role of iduronic acid-substituted forms. This is consistent with the current overall thinking on GAGs as heterogeneous structures that promote specificity to a greater degree than initially appreciated. The reader is referred to several excellent reviews of GAG biology for a larger sense of the role of these carbohydrates in pathogen invasion (174, 238, 270, 330).

From the perspective of the virus, initial studies focused on the RSV G protein. Feldman et al. (115, 116) confirmed the interaction of the RSV G protein with heparin, identified a linear heparin binding domain within the G protein, and documented an interaction of heparin with the RSV F protein as well. However, Teng et al. (347) later demonstrated that deletion of the G-protein binding domain did not alter sensitivity to heparin, and this was followed by a study by Techaarpornkul et al. (341) demonstrating that recombinant RSV with deleted G and SH proteins not only was able to infect epithelial cells in culture but also was able to do so in a partially GAG-independent fashion. Teng et al. (347) also demonstrated that the

membrane-bound form of the G protein was not absolutely required for replication *in vitro* and that the secreted form, without cytoplasmic and transmembrane domains, served adequately in its stead; a subsequent study (345) demonstrated that the conserved "cystine noose" region separating the two extracellular glycosylated domains of the hRSV G protein were likewise dispensable.

In summary, the issue of cellular receptors for hRSV remains one with many unanswered questions. GAGs clearly play a significant role in the infectivity of wild-type virus, and the recent identification of protein binding domains with high affinity for specific GAGs may be of some help in this endeavor (174). One of the most important questions that remains unanswered is that of species specificity; on that topic, a very recent and intriguing observation by Schlender et al. (316) documents the role of the F2 subunit domain in modulating the species specificity observed for human versus bovine RSV infection. Also of note is the report by Tripp et al. (353) on chemokine mimicry by the hRSV G protein, leading some to conclude that the chemokine receptor for fractalkine, CX3CR1, might serve as a coreceptor for hRSV infection. However, while epithelial cells synthesize and secrete fractalkine, there is no evidence for CX3CR1 expression in these major target cells for hRSV infection. Finally, the most recent report by Malhotra et al. (246) suggests a role for annexin II, which itself is a heparin binding protein and as such may interact with the glycosylated virus proteins and glycosylated target cell proteins in unexplored and unexpected ways.

Cellular and biochemical responses. (i) Syncytium formation. The classic multicellular cytopathic syncytia formed in epithelial cell culture in response to infection with hRSV have been described in significant detail (195). While several lines of evidence suggest a role for the hRSV F protein in inducing syncytium formation, the molecular details of this morphologic response remain largely unknown. Recent evidence has demonstrated increased expression of the cytoskeleton filament protein cytokeratin-17 and its localization at sites of syncytium formation (91) as well as involvement of the small GTPase RhoA (281, 282).

(ii) Biochemical responses in cell culture. The biochemical responses of epithelial cell cultures infected with hRSV have been reviewed recently (78, 92, 131); this will permit us to cover the highlights and focus here on the most recent studies. Among the most prominent biochemical responses of epithelial cells is the synthesis and secretion of the potent neutrophil chemoattractant IL-8. The transcriptional control of IL-8 relies on the activation of the master transcriptional activator, NF- κ B (119, 135, 253, 255), with the involvement of NF-IL-6 (185, 254), ERK-2 (53), and unique hRSV-responding enhancer elements in the IL-8 gene promoter (48). hRSV infection of epithelial cells *in vitro* induces the production of other chemokines, including RANTES, monocyte chemotactic protein 1 (MCP-1), and MIP-1 α , the last chemokine being of particular note because it is directly related to the inflammatory responses to both hRSV and PVM observed *in vivo* (90, 136, 169). Other prominent biochemical responses to hRSV infection *in vitro* include the production of inducible nitric oxide synthase and nitric oxide (155, 201, 356) beta interferon, IL-1 α , IL-6, and IL-11 and increased expression of cell surface markers intercellular cell adhesion molecule 1 (ICAM-1;

CD54), CD18, vascular cell adhesion molecule (VCAM), major histocompatibility complex classes I and II, CD14, and CD15 (reviewed in reference 92). See also the section on gene microarray approaches in "Gene microarray expression studies" below.

(iii) Biochemical responses in mouse models. A number of groups have studied the pathogenesis of hRSV inoculation in various strains of inbred mice (primarily BALB/c) and cotton rats. All describe the moderate influx of inflammatory cells, with reports including lymphocytes and eosinophils as components of the respiratory infiltrates (120, 145, 147, 319, 320). Blanco et al. (25) documented the increased expression of transcripts encoding several cytokines, chemokines, alpha and gamma interferons, and the interferon response gene, IP-10, in response to inoculation of cotton rats with 10⁵ PFU of hRSV-A Long strain. These results were similar to those reported by Haeberle et al. (157), who evaluated the responses of inoculated BALB/c mice, although the latter group also reported the inducible expression of MIP-1 α . However, others have shown that while MIP-1 α is produced in this model, the levels detected do not correlate with the extent of the inflammatory response (89), as has been observed in both human disease (136, 169) and the PVM mouse model (90).

This latter point leads us to an important caveat. It is important to understand that, just as there are two distinct disease entities studied in humans (discussed in "Inflammatory responses to infection" above), there are two distinct rodent models involving hRSV inoculation. The immunopotential model, in which mice are inoculated with live, attenuated, or fixed hRSV or hRSV components and then challenged with hRSV, is a model of acquired immunity and has been developed in conjunction with studies related to vaccine development. This application of hRSV needs to be distinguished from primary infection models, in which one is attempting to evaluate innate responses and innate immunity to infection. In many studies of the pathogenesis of primary infection in these nonnatural rat and mouse hosts, it is difficult to determine whether the responses observed are to the very limited virus replication observed or simply to large quantities of inoculated foreign antigens that happen to be viral in origin. The responses to infection and the responses to antigen bolus are not interchangeable, since they reflect different capacities and different reactivities of the innate inflammatory response. The remarkable evolutionary divergence of proteins involved in innate host defense (269) suggests that experiments relating to disease pathogenesis and innate host response need to be performed using appropriate species-pathogen pairs. Studies using natural host-pathogen pairs may be limited and limiting in other ways, but they ultimately provide better representations of the pathogenesis of pneumovirus infection *in vivo*.

PVM has recently been developed as an alternative model for the study of the pathogenesis of severe pneumovirus disease (27, 77, 88, 90, 93). Intranasal inoculation of fewer than 30 PFU of virus results in clear-cut replication in mouse lung tissue, yielding titers as high as 10⁸ PFU/g. Virus replication is accompanied by a profound inflammatory response, mucus production, and airway obstruction, leading to significant morbidity and mortality. As noted above, the inflammatory response is promoted by local production of the CC chemokine MIP-1 α and its signaling through the receptor, CCR1, a path-

way under exploration for combined antiviral-immunomodulatory therapy, as discussed in "Combination therapy" (below).

(iv) Gene microarray expression studies. The gene microarray is a more global approach to the study of transcriptional responses to defined physiologic or pathophysiologic stimuli. As of this writing, three published studies have used this approach to evaluate the transcriptional responses of epithelial cells to hRSV in vitro and a fourth study has focused on the responses of whole mouse lung to infection with PVM in vivo. The first of these systematic studies, reported by Zhang et al. (390), presents an evaluation of the responses of the human epithelial cell line A549 and primary human SAE cells to infection with the A2 strain of RSV. The A549 responses included the anticipated increases in the levels of transcripts encoding the chemokines IL-8, RANTES, MCP-1, and MIP-1 α , and have extended the list to include, among others, I-309 and fractalkine. Most interesting was the fact that the pattern observed in the primary SAE cells was not precisely identical, demonstrating the existence of distinct responses in what are often thought to be completely interchangeable model systems. The gene array study of the transcriptional responses in whole-mouse lung mRNA to infection with the pathogenic PVM strain J3666 likewise demonstrates increased expression of a specific array of proinflammatory mediators, including transcripts encoding murine MCP-1, RANTES, MCP-3, beta interferon, and 10 characterized interferon response and interferon-related genes (93). The second publication focused on the in vitro response (349) has provided us with a more general look at the responses directly related to the activation of the proinflammatory transcriptional master switch, NF- κ B. Using a clever, dominant-negative mutant approach, the authors compared the responses of hRSV-A2-infected HeLa cells designed to express the I κ B α -dominant negative mutant under doxycycline control and established that in addition to what we already know are NF- κ B regulated transcripts (the I κ B inhibitors and IL-8), several interferon response factors (IRF-1 and IRF-7B) and the transcription factor STAT-1 are also under NF- κ B control. The third publication from this group (391) focuses on the antiviral agent ribavirin and its interference with hRSV-A2-mediated transcriptional activation of the A549 cell line. Among the conclusions, the authors determined that ribavirin, while an effective antiviral agent, actually does not interfere in a very substantial way with the inflammatory cascade initiated by hRSV replication, a result that has been echoed by the findings of Bonville et al. (27) in their study of the cellular inflammatory responses to PVM in vivo. Another important conclusion from the gene array study was that ribavirin administration resulted in enhanced expression of several components of the interferon-signaling pathway via as yet undefined interactions with the interferon-stimulated response enhancer elements common among these up-regulated transcripts (391), adding to our understanding of the more subtle immunomodulatory properties of ribavirin.

Variants and Mutants with Altered Pathogenicity

Natural variation. Naturally occurring variants of hRSV fall into two diagnostic subgroups, hRSV-A and hRSV-B, initially identified on the basis of differential reactivity with monoclonal antibodies and later confirmed on the basis of gene sequence,

with particular divergence noted within the sequence of the virus G gene (129). While the evolutionary significance of this divergence remains largely unknown, Woelk and Holmes (379) have identified six sites within the sequence of the hRSV G protein that have been subjected to positive selection, all within the extracellular domain and all associated to some degree with specific anti-hRSV immunoglobulin epitopes and/or glycosylation, suggesting immune-driven natural selection. The question of the relative pathogenicity of hRSV-A and hRSV-B variants has been addressed in a recent study by Martinello et al. (251) in which they found no association of disease severity with either the hRSV-A or hRSV-B subgroup (137 and 84 isolates, respectively), but went on to identify a specific A subgroup clade (GA3) with this characteristic. In contrast, a study of 28 isolates by Brandenburg et al. (33) found no relationship between hRSV-A lineages and disease severity. Further study is necessary to distinguish between these sets of results.

Tissue culture attenuation and random mutagenesis. Prior to the development of reverse genetics, studies of the contributions of virus sequence to disease severity were dependent on the identification, isolation, and sequence analysis of naturally occurring virus variants, typically the result of the relaxation of functional constraints provided by replication in tissue culture. A good example of this comes from the PVM literature, specifically the study by Randhawa et al. (295) in which G-protein sequences of the mouse-passaged PVM strain J3666 and the tissue culture-passaged, attenuated PVM strain 15 were compared; the authors demonstrated different translational start sites leading to the absence of a putative amino-terminal cytoplasmic domain in the latter. The pace of discovery was enhanced by the addition of random mutagenesis methods, which led to the identification of important mutations in the P (phosphoprotein) (242) and L (polymerase) (351) proteins of hRSV.

Antibody escape mutants. Escape mutants are essentially a technological form of immune-drive natural selection (see reference 261 for an excellent review and details of earlier studies). Escape mutants have been created from hRSV grown in tissue culture in the presence of neutralizing monoclonal antibodies directed against the virus G or F proteins (241, 252, 253, 368). This work has generated detailed antigenic maps of these proteins, has provided a virtual "snapshot" of the way in which hRSV virions respond to immunoglobulins in vivo, and has provided significant guidance on the development of passive immunoprophylaxis for hRSV (80).

Recombinant viruses. Collins et al. (67) were the first to report the successful production of infectious hRSV from cloned cDNA in tissue culture. Cotransfection of HEp-2 cells with a plasmid containing the 15,223-nucleotide antigenome together with expression plasmids encoding the N, P, L, and M2 proteins permitted the recovery of infectious virions and led the way to the important next step, the creation of point, replacement, addition, and deletion mutations in the virus backbone. The use of this technology, known as reverse genetics, in studies of pneumovirus biology in general (68, 249) and its application to the vaccine effort (60, 69, 268) have recently been reviewed. With respect to the vaccine studies, among the more intriguing applications of this technology has been the inclusion of immunomodulatory cytokines IL-2 (40) and gran-

ulocyte-macrophage colony-stimulating factor (41) within the virus backbone in an attempt to alter the host response as part of the overall vaccination strategy. Deletion and substitution mutational analyses using this or alternative genome-based methods have led to a greater understanding of the role of various virus-encoded proteins (46, 188, 242, 339, 341), particularly that of F and G proteins in hRSV infectivity and species specificity (130, 316, 347, 392), described in detail in "Cellular receptors" (above).

Virus Evasion of Innate Immunity

RNA viruses use a large number and variety of mechanisms to subvert innate antiviral host defenses; many of those mechanisms involve evasion of interferon and interferon-regulated responses. Comparatively little is known about such strategies among the pneumoviruses (see references 143 and 385 for reviews discussing strategies used by other viruses of the family *Paramyxoviridae*). However, Atreya and Kulkarni (7) demonstrated that hRSV replication was not diminished in response to treatment with either alpha or beta interferon in several standard tissue culture model systems. Given that there is ample evidence for induced production of beta interferon by hRSV-infected epithelial cells (7, 134, 186, 356), the question of resistance remains acute. The most clear-cut evidence for resistance strategies to date comes from Conzelmann and colleagues (315), who demonstrated that the NS1 and NS2 proteins of bRSV and hRSV function coordinately and in a species-restricted fashion to antagonize the antiviral activities of alpha and beta interferons. Also, as noted above, bRSV NS-1 and NS-2 proteins inhibit the production of beta interferon by reducing the activation of interferon-regulatory factor 3 (28) and bRSV NS-2 specifically is involved in interferon production in bovine nasal fibroblasts and bronchoalveolar macrophages (358). Teng, Whitehead, and colleagues (346, 378) have demonstrated the attenuation of hRSV NS-1 and M2-2 or NS-1 and SII deletion mutants in the chimpanzee model of infection, although without specific reference to interferon-related phenomena. Interestingly, hRSV is sensitive to inhibition by gamma interferon (11, 20, 39, 99, 276, 365), and potential hRSV-mediated modulation pathways have been identified (6, 17).

Chemokine mimicry is another means by which viruses are known to subvert innate antiviral host defense. The potential role of the hRSV G protein in chemokine mimicry is an intriguing lead to be explored further (353). In another recent development, Zimmer et al. (394) describe virokinin, a post-translationally modified cleavage product of bRSV F protein that contains a classical motif identifying it as a member of the conserved tachykinin family of peptide hormones. The role of this virus-encoded protein in binding to tachykinin receptors in the respiratory tract and potentially altering the course of bRSV infection remains to be clarified.

Regarding virus evasion in vivo, the responses are complex and often difficult to evaluate or pinpoint with any assurance at the molecular level. One interesting interpretation of viral evasion in vivo comes from the work of Sakurai et al., (311), who isolated and evaluated naturally occurring antibodies reactive with the immunodominant F glycoprotein and found a subset of nonneutralizing antibodies with a high affinity for the

immature, viral lysate forms of F, together with neutralizing subset with high affinity for mature F on infected cells and on virions. The authors consider the intriguing possibility that by evolving and maintaining two developmental forms of the F protein with distinct antigenicity, the virus can evade and subvert humoral immunity more effectively.

CLINICAL STRATEGIES

Passive Immunoprophylaxis for hRSV Disease

There are two formulations of anti-RSV immunoglobulins in use for prophylaxis against hRSV infection in high-risk patient groups. The earliest studies with experimental animals suggested that immunoglobulin preparations containing high-titer antibodies to hRSV (hRSV-Ig) protected against development of severe disease (150, 291, 323). While passive prophylaxis could attenuate the severity of hRSV disease, it did not prevent infection, and an increased number of unexplained deaths occurred in hRSV-Ig recipients with congenital heart disease, leading to the recommendation that RSV-Ig not be used in patients with this underlying condition (324); considerable controversy ensued regarding specific indications due primarily to the high cost of administering this agent (294). Other problems associated with the use of hRSV-Ig included the potential for transmission of blood-borne pathogens, the need to administer a large volume of product (15 ml/kg of body weight), and the associated fever and desaturation that can accompany the administration of any blood product.

In response to the need for a better prophylactic agent, a humanized monoclonal antibody against the hRSV F protein was developed for clinical use (reviewed in references 151 and 325). A randomized trial of humanized anti-F monoclonal antibody, palivizumab, was performed at 139 centers in North America and the United Kingdom, with intramuscular administration of this agent to high-risk infants once per month during hRSV season. This approach was shown to be highly effective in reducing hRSV-related hospitalizations (180a). Palivizumab was licensed by the Food and Drug Administration in June 1998. Emerging data demonstrating the safety of this monoclonal antibody product in patients with congenital heart disease will very probably lead to a Food and Drug Administration-approved indication for use in this group of high-risk individuals.

While hRSV-Ig and monoclonal anti-F antibody already play an important role in the prevention of serious hRSV infection, neither appears to be effective in the treatment of this disease. In a study of previously healthy infants randomized to receive RSV-Ig or placebo, no significant differences were observed in the duration of hospitalization, intensive care unit stay, mechanical ventilation, supplemental oxygen use, or adverse events between the two groups (306). One explanation for the lack of clinical efficacy during acute hRSV infection is that by the time respiratory symptoms are present, the virus is sequestered from host humoral immunity (294). Furthermore, gene microarray (391) and mouse model (27, 29) studies suggest that the inflammatory responses to pneumovirus infection are initiated very early on and that antiviral therapy alone is not effective in controlling the negative sequelae. Despite these observations, there is growing evidence that using palivizumab

in transplant patients with documented hRSV upper respiratory tract infection may prevent the spread of infection to the lower lung when used in combination with ribavirin (85). This strategy is currently under investigation by the Collaborative Antiviral Study Group and may be of particular importance in this very high-risk patient population.

Active Immunoprophylaxis for hRSV Disease

The morbidity and mortality associated with hRSV bronchiolitis, coupled with its frequency and worldwide distribution, make it a prime target for the development of a vaccine that can be used early in life. There have been several forces at work that have hampered progress on a vaccine against hRSV. Most important is the possibility that vaccination will result in adverse reactions to naturally occurring hRSV disease, as observed with the formalin-inactivated vaccine used in the 1960s (55, 124, 202, 210). Furthermore, newborns and very young infants may not mount a protective immune response because of relative immunologic immaturity or suppression of their immune response due to circulating maternally derived anti-hRSV antibodies (68, 79, 108, 267). Natural hRSV infection itself elicits only partial immunity, and reinfection in later life is commonplace. Several strategies have been implemented recently in an attempt to generate safe and effective subunit, inactivated, and live-attenuated hRSV vaccines (60, 109). Currently, two promising candidate vaccines under study in clinical trials are an hRSV F subunit vaccine for immunization of patients who have already experienced primary RSV infection, such as the elderly and older hRSV-seropositive children with conditions predisposing them to severe hRSV disease (108, 279, 288, 354), and cold-passaged, temperature sensitive (cpts) attenuated hRSV strains (196, 206). cpts mutant virus strains are difficult, if not impossible, to “fine tune” by serial passage in tissue culture, emphasizing the importance of using newer recombinant (reverse genetics) technologies to advance the field. Recombinant hRSV vaccines under evaluation in clinical trials are largely based on the cpts 248/404 “backbone” virus, with molecular deletions and/or point mutations designed to enhance several desirable features. Another promising area involves hRSV vaccine strains that lack the NS1 and/or NS2 genes since deletion of these sequences augments the host cell interferon response pathways (31, 315).

Therapy for hRSV Disease

Antiviral agents. While significant advances in our knowledge of hRSV biology, immunology, pathophysiology, and epidemiology have been made in the past 50 years, there continues to be a fair amount of controversy regarding the optimum management of infants and children with hRSV infection (369). Supportive therapy, including hospitalization, supplemental oxygen delivery, nasal and pulmonary toilet, and, in the most severe cases, intubation and mechanical ventilation, is the mainstay of clinical management.

(i) Ribavirin. Lack of consensus regarding the use of ribavirin for moderate to severe forms of hRSV-induced bronchiolitis cannot be attributed to a paucity of clinical trials. Results from at least 14 trials of bronchodilators, 3 trials of adjunctive systemic glucocorticoid treatment, and 11 randomized trials of

the antiviral synthetic nucleoside ribavirin have been published (16, 149, 159, 160, 229, 233, 259, 264, 294, 305, 327, 336, 369, 376). In six controlled, randomized studies, ribavirin treatment was found to reduce the severity of hRSV illness by decreasing viral shedding, improving oxygen saturation, and improving clinical scores (16, 159, 160, 207, 327, 336), but in 1996, a systematic overview of these studies highlighted potential flaws in study design and interpretation (296). The results of this meta-analysis were discouraging but were consistent with the clinical experience of pediatricians, specifically that ribavirin treatment alone did not lead to significant improvement in clinically important outcomes. The growing concern regarding the efficacy of ribavirin and the high cost of its use (118) led to a wording change in the American Academy of Pediatrics recommendation for its usage from “should be used” to “may be considered” for selected infants and young children at high risk for serious hRSV disease (70).

Severe hRSV infection during infancy is a known risk factor for the development of subsequent wheezing illnesses during the first decade of life. Studies of the outcome of bronchiolitis during infancy have included studies that evaluated the effects of ribavirin therapy during acute illness on the long-term sequelae of recurrent wheezing during childhood (101, 132, 208, 240, 256, 310), with promising results. In contrast, Krilov et al. (217) found no differences between ribavirin-treated and untreated infants in wheezing or other pulmonary function measures 6 to 8 years beyond their infection. Interestingly, new attention has also been raised regarding potential long-term benefits of using the leukotriene receptor antagonist montelukast during acute infection for the prevention of post-hRSV infection wheezing (24). A large multicenter clinical trial to test this hypothesis is under way.

(ii) New antiviral strategies: fusion inhibitors, RNases, and others. New strategies for therapeutic intervention during moderate to severe hRSV disease are clearly needed. A variety of antiviral compounds have been tested for their ability to impair hRSV replication in vitro. Speculation that inhibiting cell-cell fusion (i.e., syncytium formation) might lessen the severity of clinical disease has led to attempts to develop hRSV-specific fusion inhibitors. Peptides derived from epitopes of the hRSV fusion or F protein blocked hRSV-induced syncytium formation at concentrations as low as 0.015 μ M (226). Small-molecule hRSV-specific fusion inhibitors have neutralizing activity against A and B group hRSV isolates in vitro and in vivo via blockade of cell-cell fusion (179, 258, 272), while a benzodithiin compound has demonstrated ability to interfere with intracellular processing of the hRSV fusion protein, thereby resulting in decreased infectivity (333). Other agents reported to have anti-hRSV activity have included crude plant protein extracts (3), some forms of superoxide dismutase (384), an L-aspartate transcarbamoylase inhibitor (383), eosinophil-derived neurotoxin, a potent RNase (87), and lovastatin (144).

While nucleoside derivatives other than ribavirin have generated some interest by investigators in this field (359), enthusiasm for the development of antisense technology has been more robust. Specific cleavage of hRSV genome RNA has been detected at antisense oligonucleotide binding sites (184). Because of the pattern of RNA cleavage observed, it was suggested that endogenous RNase H participated in hRSV

genomic degradation. Other RNases have been studied for their antiviral properties. For example, recruitment and activation of RNase L to an hRSV mRNA using 2-5A antisense oligonucleotide technology showed potent inhibition of hRSV replication in vitro (2) and, more recently, in vivo using an African green monkey model of hRSV infection (230). As of this writing, there are no publications describing antisense therapy in human clinical trials.

Combination therapy: antivirals with antibody, anti-inflammatory, and immunomodulatory strategies. Clinically, the negative sequelae of pneumovirus infection involve active viral replication in conjunction with the host cell inflammatory response. Combination therapy with the antiviral ribavirin and systemically administered immunoglobulin has been tried with some success in the cotton rat model of hRSV infection (153), and one report of combined aerosolized ribavirin and systemic immunoglobulin treatment in bone marrow transplant recipients who developed hRSV disease showed a 22% mortality among patients treated before the onset of respiratory failure and 100% mortality in patients who went untreated or in whom treatment was initiated within 24 h of respiratory failure (377). Parenteral therapy with a monoclonal anti-F antibody in the cotton rat model of human hRSV infection resulted in clearance of hRSV within 24 h but had no effect on early pulmonary pathology, while glucocorticoid treatment reduced the inflammatory component of the pneumonitis but delayed virus clearance; when the two interventions were combined, an antiviral and anti-inflammatory effect was appreciated (292).

Increasing attention is being paid to the need for concurrent anti-inflammatory therapy for moderate to severe hRSV disease. To this end, glucocorticoids are generally considered to have broad-reaching anti-inflammatory properties and in fact have been the focus of several clinical hRSV trials. Unfortunately, these trials suggest that when used alone in the absence of antivirals, glucocorticoid treatment has only marginal, if any, benefit in the treatment of hRSV-infection and its associated inflammatory response (72, 83, 84, 212, 213, 231, 307, 331, 334, 338, 365). A meta-analysis of several of these studies demonstrated that systemic glucocorticoids reduced the total length of hospital stay by only 0.43 day per patient, an effect reduced to only 0.29 day per patient when patients with previous episodes of wheezing were excluded from the analysis (137), a statistically significant difference with overall minor clinical impact. Interestingly, several recent studies showed that glucocorticoid treatment during hRSV bronchiolitis in vivo had no effect on hRSV titers measured in upper or lower respiratory secretions or on the release of the proinflammatory chemokine IL-8 (37; H. S. Jafri, C. M. Carubella, S. C. Buckingham, P. Sheeran, J. Hatfield, R. D. Hardy, J. P. DeVincenzo and O. Ramilo, *Pediatr. Acad. Soc. Joint Meet*, abstr. 1564, 2000) or on the release of IL-8 from hRSV-infected epithelial cells (28). The observation that glucocorticoids failed to blunt the virus-directed IL-8 response suggests the possibility that hRSV-infected epithelial cells may be resistant to the anti-inflammatory effects usually seen with steroid administration, an observation that was reproduced in mice infected with the natural pneumovirus pathogen PVM (94).

The approach involving a combined strategy that includes antiviral and agents directed at specific virus-induced proinflammatory events has been best characterized using the PVM

mouse model (92, 94), described in detail in "Pathogenesis of disease" (above). The proinflammatory chemokine MIP-1 α has been identified as an essential link between virus infection and granulocyte recruitment to infected lung tissue (90). However, once the virus infection is established, the continued production of MIP-1 α and the cellular inflammatory response that ensues are not tightly linked to virus replication, and, while ribavirin has significant efficacy in reducing virus replication, it does nothing to ameliorate the virus-induced proinflammatory cascade (27). Dramatic clinical improvement was observed in response to systemic administration of ribavirin in combination with either neutralizing anti-MIP-1 α antibody (27) or by pharmacologic blockade of the primary MIP-1 α receptor CCR1 (29, 95). Translation of these laboratory findings may result in a clinically achievable therapeutic strategy for the management of severe hRSV infection in human infants.

CONCLUSIONS

Pneumovirus infection remains a significant problem for practitioners of both human and veterinary medicine. Advances in our understanding of pneumovirus biology have provided pathways toward new vaccine formulations. At the same time, advances in our understanding of the pathogenesis of pneumovirus disease in specific natural hosts have provided strategies for combined antiviral and anti-inflammatory/immunomodulatory therapies.

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