JOURNAL OF VIROLOGY, Oct. 2000, p. 9317–9321 Vol. 74, No. 19
0022-538X/00/$04.00+0

Recombinant Respiratory Syncytial Virus That Does Not Express the NS1 or M2-2 Protein Is Highly Attenuated and Immunogenic in Chimpanzees

MICHAEL N. TENG,[1] STEPHEN S. WHITEHEAD,[1] ALISON BERMINGHAM,[1] MARISA ST. CLAIRE,[2] WILLIAM R. ELKINS,[3] BRIAN R. MURPHY,[1] AND PETER L. COLLINS[1]*

Respiratory Viruses Section[1] and Experimental Primate Virology Section,[3] Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, 20892, and Bioqual, Inc., Rockville, Maryland, 20850[2]

Received 11 May 2000/Accepted 28 June 2000

Mutant recombinant respiratory syncytial viruses (RSV) which cannot express the NS1 and M2-2 proteins, designated rA2ΔNS1 and rA2ΔM2-2, respectively, were evaluated as live-attenuated RSV vaccines. The rA2ΔNS1 virus contains a large deletion that should have the advantageous property of genetic stability during replication in vitro and in vivo. In vitro, rA2ΔNS1 replicated approximately 10-fold less well than wild-type recombinant RSV (rA2), while rA2ΔM2-2 had delayed growth kinetics but reached a final titer similar to that of rA2. Each virus was administered to the respiratory tracts of RSV-seronegative chimpanzees to assess replication, immunogenicity, and protective efficacy. The rA2ΔNS1 and rA2ΔM2-2 viruses were 2,200- to 55,000-fold restricted in replication in the upper and lower respiratory tracts but induced a level of RSV-neutralizing antibody in serum that was only slightly reduced compared to the level induced by wild-type RSV. The replication of wild-type RSV in immunized chimpanzees after challenge was reduced more than 10,000-fold at each site. Importantly, rA2ΔNS1 and rA2ΔM2-2 were 10-fold more restricted in replication in the upper respiratory tract than was the *cpts*248/404 virus, a vaccine candidate that retained mild reactogenicity in the upper respiratory tracts of 1-month-old infants. Thus, either rA2ΔNS1 or rA2ΔM2-2 might be appropriately attenuated for this age group, which is the major target population for an RSV vaccine. In addition, these results show that neither NS1 nor M2-2 is essential for RSV replication in vivo, although each is important for efficient replication.

Respiratory syncytial virus (RSV) is the leading etiologic agent of serious pediatric viral bronchiolitis and pneumonia worldwide and is responsible for approximately 100,000 hospitalizations and 4,500 deaths among infants and children in the United States per annum (7, 14, 25). In addition, RSV infection can cause severe respiratory illness in the elderly (23) and in immunocompromised individuals (28). To date, an effective licensed vaccine for RSV is not available despite the pressing need for such an agent.

Since 1967, our laboratory has focused on developing a live-attenuated RSV vaccine for intranasal administration. By mimicking a natural infection, such a vaccine should stimulate both cellular and humoral immunity and would obviate the potentiated disease that was observed with certain nonreplicating or subunit vaccines (7, 16, 24, 27). The intranasal route also partially abrogates the immunosuppressive effects of maternal antibodies present in the sera of young infants and stimulates both local and systemic immunity (10).

A number of live-attenuated RSV vaccine candidates have been developed by biological or recombinant methods and evaluated in animals and humans (8, 15, 16, 29, 30, 32). The most promising biologically derived candidate, a cold-passaged (*cp*) temperature-sensitive (*ts*) virus called *cpts*248/404, was evaluated in RSV-naive 1- to 2-month-old infants and was found to be infectious, immunogenic, and protective against a second vaccine dose (33). However, some vaccinees experienced mild upper respiratory tract congestion, indicating that further attenuation is necessary. In addition, virus isolated late during the course of infection from a single vaccinee showed partial phenotypic reversion and loss of an attenuating mutation. Thus, our strategy to develop improved live-attenuated vaccine candidates has been (i) to use recombinant methods to combine attenuating mutations identified in a panel of biologically derived attenuated viruses including *cpts*248/404 and (ii) to develop new types of attenuating mutations by focusing on gene deletions which should be refractory to genetic reversion.

RSV is the prototype member of the *Pneumovirus* genus of the family *Paramyxoviridae*. Its genome is a single-stranded, negative-sense RNA of 15.2 kb that encodes 10 subgenomic mRNAs from which 11 proteins are translated. These proteins include the nucleocapsid N protein, phosphoprotein P, and large polymerase subunit L, which together comprise the minimal viral polymerase; fully processive transcription by the RSV polymerase requires the presence of the transcription antitermination factor M2-1 (6, 18, 19, 34). There are four envelope-associated proteins: the internal matrix (M) protein and three transmembrane surface proteins, namely, the attachment (G), fusion (F), and small hydrophobic (SH) proteins (7). Finally, RSV encodes two nonstructural proteins, NS1 and NS2, and also the M2-2 protein, whose status as structural or nonstructural is unknown. NS1 and M2-2 appear to have roles in RNA synthesis.

We previously described a reverse-genetics system for producing recombinant subgroup A RSV (rRSV) by coexpression of antigenomic RNA and the N, P, L, and M2-1 proteins from cotransfected plasmids (5). One application of this system has been to identify viral genes that can be deleted or silenced without ablating replication in vitro but are still necessary for

* Corresponding author. Mailing address: LID, NIAID, 7 Center Dr., MSC 0720, Bethesda, MD 20892-0720. Phone: (301) 496-4205. Fax: (301) 496-8312. E-mail: pcollins@niaid.nih.gov.

virus replication in vivo (4, 26). Deletion of the SH gene resulted in a virus, designated rA2ΔSH, that replicated in vitro with an efficiency equal to or slightly better than that of wild-type rRSV (rA2) and which was moderately attenuated in mice and chimpanzees (4, 29). rRSV from which the NS2 gene was deleted, designated rA2ΔNS2, exhibited reduced growth kinetics and a reduced yield of infectious virus in vitro and was markedly attenuated in mice and chimpanzees (26, 29). Similar in vitro properties were noted for a recombinant bovine RSV from which the NS2 gene was deleted (2). These two deletion mutations are now being incorporated into recombinant live-attenuated vaccine candidates for clinical evaluation.

More recently, the M2-2 open reading frame was silenced in rRSV (rA2ΔM2-2, previously designated rA2-K5) by mutating each of the three potential translational initiation codons and inserting a translation termination codon in each of the three reading frames (1). A second research group made a comparable virus in which M2-2 was silenced by deletion of most of its open reading frame, which resulted in a virus that appeared to be phenotypically similar to rA2ΔM2-2 (20). The rA2ΔM2-2 virus exhibited increased plaque size, reduced growth kinetics (though the final titer was similar to that of the wild type), and a partial shift in RNA synthesis from RNA replication to transcription (1). Thus, the M2-2 protein appears to be a regulatory protein that negatively regulates transcription and positively regulates RNA replication. In addition, an rRSV was constructed from which the NS1 gene was deleted by the removal of nucleotides 122 to 630 in the antigenomic cDNA, resulting in the joining of the upstream nontranslated region of NS1 to the translational initiation codon of NS2. This virus, designated rA2ΔNS1, exhibited reduced RNA replication, plaque size, and growth kinetics and an approximately 10-fold lower yield of infectious virus in vitro (M. N. Teng and P. L. Collins, submitted for publication). Other paramyxoviruses encode proteins, such as the V protein of Sendai virus, that are not essential for replication in vitro. However, ablation of expression of V by recombinant Sendai virus results in attenuation in vivo (22). It was suggested that this protein functioned to antagonize some aspect of the mouse's innate immune system. More recently, the V protein of simian virus 5 was shown to block signalling for both type I and type II interferon responses (13). Any of the RSV "accessory" proteins, including the NS1, NS2, M2-2, SH, and G proteins, are candidates for antagonizing host immune mechanisms.

In the present study, we evaluated the rA2ΔM2-2 and rA2ΔNS1 viruses for replication, immunogenicity, and protective efficacy in the upper and lower respiratory tracts of chimpanzees, the only experimental animal in which RSV replication and virulence approaches that observed in humans. The rA2ΔM2-2 and rA2ΔNS1 viruses described above were constructed in the original version of the antigenomic cDNA described by Collins et al. (5). All recombinant viruses that have been constructed for vaccine purposes in our laboratory contain two types of modification to this background: (i) the introduction of a set of six translationally silent restriction markers in the L gene, called the sites mutations, and (ii) two amino acid substitutions in the F protein, called the HEK mutations, which make the recombinant virus identical at the amino acid level to the wild-type RSV A2 parent from which the *cpts*248/404 series of biological vaccine candidates was derived (21, 30). These mutations were shown to be phenotypically silent in chimpanzees (32). The rA2ΔNS1 virus used in this study was reconstructed in a sites-HEK background, in preparation for clinical evaluation, whereas the rA2ΔM2-2 virus is in the original genetic background, a difference that is not relevant for the present study (1, 30).

The rA2ΔNS1 and rA2ΔM2-2 viruses were administered individually to juvenile RSV-seronegative chimpanzees by combined intranasal and intratracheal inoculation, as described previously (11). Since both viruses were attenuated in vitro, we chose to inoculate the animals with 10^5 PFU per ml per site, which is a 10-fold higher concentration than that typically used to inoculate chimpanzees. To monitor virus replication in the upper and lower respiratory tracts, respectively, nasopharyngeal swabs and tracheal lavage samples were collected at intervals over 10 days postinfection and subsequently were assayed for virus titer. The mean peak virus titer was determined for each group (Table 1). The chimpanzees were monitored daily for rhinorrhea, a symptom of upper respiratory tract illness, and the mean peak score was determined for each group (Table 1). Due to the limited availability of RSV-seronegative chimpanzees, the number of animals per group was small, making it necessary to include controls from previous studies in which we had evaluated biologically derived RSV strain A2 (wild-type RSV A2), rA2, rA2ΔSH, rA2ΔNS2, and a recombinant version of the above-mentioned *cpts*248/404 vaccine candidate (rA2cp248/404) (Table 1).

Levels of replication of rA2ΔNS1 and rA2ΔM2-2 were reduced more than 2,200-fold and more than 2,800-fold, respectively, in the upper respiratory tract compared to that of rA2 (Table 1). Shedding of rA2ΔNS1 or rA2ΔM2-2 was detected sporadically and at a low level beginning 2 to 7 days postinfection, and each animal shed virus over a period of 3 to 8 days (data not shown). Thus, the recovered virus was not carried over from the initial inoculum but represented replication near the level of detection over a period of several days. In the lower respiratory tract, the level of replication of rA2ΔNS1 was reduced more than 17,000-fold compared to that of rA2, while rA2ΔM2-2 was undetectable at all time points (greater than 55,000-fold reduction). It is important to note that the dose of rA2ΔNS1 and rA2ΔM2-2 used was 10-fold greater than that of rA2. Furthermore, both viruses were more attenuated than rA2cp248/404, which was given at the same dose, particularly in the case of rA2ΔM2-2, which was not recovered from the lungs of infected chimps. In addition, both rA2ΔNS1 and rA2ΔM2-2 were unusual in being equally restricted in the upper and lower respiratory tracts. In the upper respiratory tract, each virus was approximately 10-fold more restricted than *cpts*248/404 and 175-fold more restricted than rA2ΔNS2. Since upper respiratory tract congestion was observed during clinical evaluation of the *cpts*248/404 virus in 1- to 2-month-old infants (33) and since infants of that age are obligate nose breathers, mutations that confer a level of restriction of replication in the upper respiratory tract greater than that of *cpts*248/404 would be desirable for inclusion in a live-attenuated vaccine virus. Animals receiving rA2ΔNS1 or rA2ΔM2-2 had slightly more rhinorrhea than those infected with rA2cp248/404, though still less than that of animals infected with a 10-fold smaller dose of rA2. While it is possible that the absence of NS1 or M2-2 resulted in a virus that retained a moderate level of virulence but replicated poorly, we think that this possibility is unlikely. Our experience is that quantitation of rhinorrhea and the comparison of such values from different studies performed at different times can be somewhat subjective and hence not completely reproducible. We anticipate that further evaluation, including clinical studies, will show that the amount of residual virulence associated with rA2ΔNS1 and rA2ΔM2-2 will reflect their greatly reduced replication.

Despite the highly restricted replication of these viruses, immunization with either rA2ΔNS1 or rA2ΔM2-2 induced a level of RSV-neutralizing antibody in serum that was within threefold of that induced by rA2cp248/404 (Table 1). Further-

TABLE 1. rA2ΔNS1 and rA2ΔM2-2 are highly attenuated in both the upper and lower respiratory tracts of chimpanzees but are highly immunogenic

Virus used to infect chimpanzees[a]	No. of animals	Dose[b] (per site, $\log_{10}$ PFU)	Mean peak virus titer[c] ($\log_{10}$ PFU/ml) ± SE (Duncan grouping)		Mean peak rhinorrhea score[d] (range, 0–4)	Mean neutralizing antibody titer in serum[e] (reciprocal $\log_2$)	
			Nasopharyngeal swab	Tracheal lavage		Day 0	Day 28
Wild-type RSV A2[f]	2	4.0	5.0 ± 0.35 (A)	5.5 ± 0.40 (A)	3.0	<3.3	11.2
rA2[g]	2	4.0	4.9 ± 0.15 (A)	5.4 ± 0.05 (A)	2.5	<3.3	10.5
rA2ΔSH[g]	3	4.0	4.6 ± 0.10 (A)	3.8 ± 0.31 (B)	1.0	<3.3	10.2
rA2ΔNS2[g]	4	4.0	3.8 ± 0.41 (B)	1.4 ± 0.29 (C)	1.0	3.4	10.6
rA2cp248/404[g]	4	5.0	2.5 ± 0.25 (C)	1.4 ± 0.37 (C)	0.8	3.4	10.6
rA2ΔNS1	4	5.0	1.6 ± 0.12 (D)	1.2 ± 0.43 (C)	2.0	<3.3	9.8
rA2ΔM2-2	4	5.0	1.5 ± 0.09 (D)	<0.7	1.8	<3.3	9.1

[a] All recombinant-derived viruses contain the sites and HEK mutations (see the text), except for rA2ΔM2-2.
[b] Chimpanzees were inoculated by the intranasal and intratracheal routes with the indicated amount of virus in a 1-ml inoculum per site.
[c] Nasopharyngeal swab samples were collected daily for 10 days, and tracheal lavage samples were collected on days 2, 5, 6, 8, and 10. Mean peak titers were calculated and assigned to statistically similar groups by Duncan's multiple-range test ($\alpha = 0.05$). Means in each column with different letters are significantly different.
[d] The amount of rhinorrhea was estimated daily and assigned a score (0 to 4) that indicated extent and severity. Scores indicate severe (4), moderate (3), mild (2), trace (1), or no (0) rhinorrhea. Shown are the mean peak scores.
[e] Serum RSV-neutralizing antibody titers were determined by a complement-enhanced 60% plaque reduction assay using wild-type RSV A2 and HEp-2 cell monolayer cultures incubated at 37°C. RSV-seronegative chimpanzee serum used as a negative control had a neutralizing antibody titer of <3.3 $\log_2$ reciprocal. Adult human serum used as a positive control had a neutralizing antibody titer of 11.4 $\log_2$ reciprocal.
[f] Historic controls from the study of Crowe et al. (10).
[g] Data from the study of Whitehead et al. (29).

more, animals previously infected with either rA2ΔNS1 or rA2ΔM2-2 were highly resistant to the replication of wild-type RSV administered intranasally and intratracheally 56 days postimmunization (Table 2). The levels of protection in both cases were similar in the upper respiratory tract and somewhat lower in the lower respiratory tract than that seen with *cpts*248/404, both in mean peak titer and in mean days of shedding.

The challenge in developing a live-attenuated RSV vaccine is to eliminate residual virulence without compromising immunogenicity. Observations to date indicate that the severity of RSV disease is closely related to the level of RSV replication in the respiratory tract. It is possible that one or more attenuating mutations that reduce virulence through another mechanism will be identified; indeed, it was hoped that deletion of one or more of the nonessential RSV proteins, such as those described in the present paper, might reveal such a virulence factor. However, a factor of this nature has not yet been identified for RSV. Thus, the present method for attenuating RSV is to reduce its level of replication, which unfortunately can reduce its immunogenicity due to the reduced production of antigen. The attenuating mutations that we have identified to date include (i) a set of five amino acid substitutions in the N, F, and L proteins that were identified in *cp*RSV and that confer attenuation in chimpanzees and humans (9, 16, 32); (ii) a series of amino acid substitutions in the L protein and a nucleotide substitution in the gene-start signal of the M2 gene, which were identified in biologically derived *ts* derivatives of *cp* RSV and which each confer the *ts* and attenuation phenotypes (12, 15, 21, 30); and (iii) deletion of individual or combinations of RSV genes such as the SH and NS2 genes (4, 26). Bovine RSV genes have also been used to confer attenuation based on host range restriction (3). Here, we add two additional knock-

TABLE 2. Infection of chimpanzees with rA2ΔNS1 or rA2ΔM2-2 induced significant protection against subsequent challenge with wild-type RSV A2 in the upper and lower respiratory tracts

Immunizing virus	Inoculum dose[a] ($\log_{10}$ PFU/ml)	No. of animals	Replication of RSV challenge virus at the indicated site[b]				Mean peak rhinorrhea score
			Nasopharynx		Trachea		
			Mean no. of days of shedding ± SE	Mean peak titer[c] ± SE	Mean no. of days of shedding ± SE	Mean peak titer ± SE	
rA2ΔNS1	5.0	4	2.8 ± 0.75	1.7 ± 0.46	1.0 ± 0.41	1.8 ± 0.73	1.0
rA2ΔM2-2	5.0	4	3.5 ± 0.87	2.3 ± 0.71	1.0 ± 0.71	1.7 ± 0.63	1.0
rA2ΔNS2[d]	4.0	4	ND	1.9 ± 0.30	ND	2.2 ± 0.77	1.0
*cpts*248/404[e]	4.7	2	3.5 ± 0.50	2.3 ± 0.25	0	<0.7	1.0
None[e]		2	8.5 ± 0.50	5.0 ± 0.35	6.0 ± 1.0	4.8 ± 0.30	3.0

[a] Each virus was initially administered at the indicated dose in a 1.0-ml inoculum given intranasally and intratracheally.
[b] On day 56, chimpanzees were challenged with wild-type RSV A2 administered at a dose of 10^4 PFU/ml in a 1.0-ml inoculum given intranasally and intratracheally. Nasopharyngeal swab samples were collected daily for 12 days, and tracheal lavage samples were collected on days 2, 5, 6, 8, and 12. ND, not determined.
[c] Mean peak titers ($\log_{10}$ PFU/ml) were calculated by using the peak virus titer achieved in each animal.
[d] Data from the study of Whitehead et al. (29).
[e] Historic control animals from the study of Crowe et al. (10) were used.

out mutations to the list, namely, the deletion of NS1 and the silencing of the M2-2 open reading frame.

Among the mutant viruses shown in Table 1, the order of increasing attenuation in seronegative juvenile chimpanzees was rA2ΔSH < rA2ΔNS2 < rA2cp248/404 < rA2ΔNS1 < rA2ΔM2-2. All viruses provided similar, high levels of protection against challenge with wild-type RSV (Table 2). Thus, rA2ΔNS1 and rA2ΔM2-2 each have the desired property of being slightly more attenuated than rA2cp248/404, the recombinant version of *cpts*248/404, which was slightly too reactogenic in RSV-naive 1- to 2-month-old infants, as mentioned above (33). The finding that rA2ΔM2-2 is slightly more attenuated than rA2ΔNS1 increases the chances that one of these viruses will have an optimal level of attenuation. The seronegative juvenile chimpanzee is somewhat less permissive to RSV replication and disease than is the RSV-naive human infant. Thus, whether rA2ΔNS1, rA2ΔM2-2, or both have an appropriate level of attenuation can be determined only by clinical trials with the target vaccine population, 1- to 2-month-old infants.

Deletion mutants should be extremely stable both in vitro and in vivo, thus making them attractive candidates for vaccine development. This property might be important in light of the finding that one infant who had been vaccinated with *cpts*248/404 shed virus that exhibited a partial reversion (33). A low level of genetic instability in an RSV vaccine likely would not be a problem in normal individuals, particularly considering the high prevalence of fully virulent wild-type RSV. However, vaccine virus might have prolonged replication in immunocompromised individuals. Thus, it would be desirable to engineer a recombinant vaccine virus to contain attenuating mutations that cannot revert.

Although the major target for an RSV vaccine is the 1- to 2-month-old infant, a second target is the elderly. The *cpts*248/404 vaccine candidate, which was insufficiently attenuated in the RSV-naive infant, was found to be overattenuated in the RSV-experienced adult (17). Thus, a live-attenuated vaccine for RSV-naive infants will need to be more attenuated than one for use in adults. Since the rA2ΔNS1 and rA2ΔM2-2 viruses are similar to *cpts*248/404 in their levels of replication, they likely will be too attenuated to be useful as an adult vaccine. However, each virus is appropriate for further evaluation as a pediatric RSV vaccine, either as currently constructed or with the inclusion of a single or a combination of additional attenuating mutations. It should be noted that if either candidate vaccine proves satisfactory, a partner subgroup B candidate can be rapidly generated by replacing the F and G glycoproteins (31).

We thank Robert Chanock for critical review.

This work is part of a continuing program of research and development with Wyeth Lederle Vaccines through CRADA no. AI-000087 and AI-000099.

REFERENCES

1. **Bermingham, A., and P. L. Collins.** 1999. The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. Proc. Natl. Acad. Sci. USA **96:**11259–11264.
2. **Buchholz, U. J., S. Finke, and K. K. Conzelmann.** 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. J. Virol. **73:**251–259.
3. **Buchholz, U. J., H. Granzow, K. Schuldt, S. S. Whitehead, B. R. Murphy, and P. L. Collins.** 2000. Chimeric bovine respiratory syncytial virus with glycoprotein gene substitutions from human respiratory syncytial virus (HRSV): effects on host range and evaluation as a live-attenuated HRSV vaccine. J. Virol. **74:**1187–1199.
4. **Bukreyev, A., S. S. Whitehead, B. R. Murphy, and P. L. Collins.** 1997. Recombinant respiratory syncytial virus from which the entire SH gene has been deleted grows efficiently in cell culture and exhibits site-specific attenuation in the respiratory tract of the mouse. J. Virol. **71:**8973–8982.
5. **Collins, P. L., M. G. Hill, E. Camargo, H. Grosfeld, R. M. Chanock, and B. R. Murphy.** 1995. Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5′ proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. Proc. Natl. Acad. Sci. USA **92:**11563–11567.
6. **Collins, P. L., M. G. Hill, J. Cristina, and H. Grosfeld.** 1996. Transcription elongation factor of respiratory syncytial virus, a nonsegmented negative-strand RNA virus. Proc. Natl. Acad. Sci. USA **93:**81–85.
7. **Collins, P. L., K. McIntosh, and R. M. Chanock.** 1996. Respiratory syncytial virus, p. 1313–1352. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Fields virology, 3rd ed., vol. 2. Lippincott-Raven, Philadelphia, Pa.
8. **Collins, P. L., S. S. Whitehead, A. Bukreyev, R. Fearns, M. N. Teng, K. Juhasz, R. M. Chanock, and B. R. Murphy.** 1999. Rational design of live-attenuated recombinant vaccine virus for human respiratory syncytial virus by reverse genetics. Adv. Virus Res. **54:**423–451.
9. **Connors, M., J. E. Crowe, Jr., C.-Y. Firestone, B. R. Murphy, and P. L. Collins.** 1995. A cold-passaged, attenuated strain of human respiratory syncytial virus contains mutations in the F and L genes. Virology **208:**478–484.
10. **Crowe, J. E., Jr., P. T. Bui, G. R. Siber, W. R. Elkins, R. M. Chanock, and B. R. Murphy.** 1995. Cold-passaged, temperature-sensitive mutants of human respiratory syncytial virus (RSV) are highly attenuated, immunogenic, and protective in seronegative chimpanzees, even when RSV antibodies are infused shortly before immunization. Vaccine **13:**847–855.
11. **Crowe, J. E., Jr., P. T. Bui, A. R. Davis, R. M. Chanock, and B. R. Murphy.** 1994. A further attenuated derivative of a cold-passaged temperature-sensitive mutant of human respiratory syncytial virus retains immunogenicity and protective efficacy against wild-type challenge in seronegative chimpanzees. Vaccine **12:**783–790.
12. **Crowe, J. E., Jr., P. T. Bui, W. T. London, A. R. Davis, P. P. Hung, R. M. Chanock, and B. R. Murphy.** 1994. Satisfactorily attenuated and protective mutants derived from a partially attenuated cold-passaged respiratory syncytial virus mutant by introduction of additional attenuating mutations during chemical mutagenesis. Vaccine **12:**691–699.
13. **Didcock, L., D. F. Young, S. Goodbourn, and R. E. Randall.** 1999. The V protein of simian virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. J. Virol. **73:**9928–9933.
14. **Dudas, R. A., and R. A. Karron.** 1998. Respiratory syncytial virus vaccines. Clin. Microbiol. Rev. **11:**430–439.
15. **Firestone, C. Y., S. S. Whitehead, P. L. Collins, B. R. Murphy, and J. E. Crowe, Jr.** 1996. Nucleotide sequence analysis of the respiratory syncytial virus subgroup A cold-passaged (cp) temperature sensitive (ts) cpts-248/404 live attenuated virus vaccine candidate. Virology **225:**419–422.
16. **Friedewald, W. T., B. R. Forsyth, C. B. Smith, M. A. Gharpure, and R. M. Chanock.** 1968. Low-temperature-grown RS virus in adult volunteers. JAMA **203:**690–694.
17. **Gonzalez, I. M., R. A. Karron, M. Eichelberger, E. E. Walsh, V. W. Delagarza, R. Bennett, R. M. Chanock, B. R. Murphy, M. L. Clements-Mann, and A. R. Falsey.** 2000. Evaluation of the live attenuated cpts 248/404 RSV vaccine in combination with a subunit RSV vaccine (PFP-2) in healthy young and older adults. Vaccine **18:**1763–1772.
18. **Grosfeld, H., M. G. Hill, and P. L. Collins.** 1995. RNA replication by respiratory syncytial virus (RSV) is directed by the N, P, and L proteins; transcription also occurs under these conditions but requires RSV superinfection for efficient synthesis of full-length mRNA. J. Virol. **69:**5677–5686.
19. **Hardy, R. W., and G. W. Wertz.** 1998. The product of the respiratory syncytial virus M2 gene ORF1 enhances readthrough of intergenic junctions during viral transcription. J. Virol. **72:**520–526.
20. **Jin, H., X. Cheng, H. Z. Zhou, S. Li, and A. Seddiqui.** 2000. Respiratory syncytial virus that lacks open reading frame 2 of the M2 gene (M2-2) has altered growth characteristics and is attenuated in rodents. J. Virol. **74:**74–82.
21. **Juhasz, K., S. S. Whitehead, P. T. Bui, J. M. Biggs, C. A. Boulanger, P. L. Collins, and B. R. Murphy.** 1997. The temperature-sensitive (*ts*) phenotype of a cold-passaged (*cp*) live attenuated respiratory syncytial virus vaccine candidate, designated *cpts*530, results from a single amino acid substitution in the L protein. J. Virol. **71:**5814–5819.
22. **Kato, A., K. Kiyotani, Y. Sakai, T. Yoshida, and Y. Nagai.** 1997. The paramyxovirus, Sendai virus, V protein encodes a luxury function required for viral pathogenesis. EMBO J. **16:**578–587.
23. **Mlinaric-Galinovic, G., A. R. Falsey, and E. E. Walsh.** 1996. Respiratory syncytial virus infection in the elderly. Eur. J. Clin. Microbiol. Infect. Dis. **15:**777–781.
24. **Murphy, B. R., S. L. Hall, A. B. Kulkarni, J. E. Crowe, Jr., P. L. Collins, M. Connors, R. A. Karron, and R. M. Chanock.** 1994. An update on approaches to the development of respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) vaccines. Virus Res. **32:**13–36.
25. **Shay, D. K., R. C. Holman, R. D. Newman, L. L. Liu, J. W. Stout, and L. J. Anderson.** 1999. Bronchiolitis-associated hospitalizations among US chil-

dren, 1980–1996. JAMA **282:**1440–1446.
26. **Teng, M. N., and P. L. Collins.** 1999. Altered growth characteristics of recombinant respiratory syncytial viruses which do not produce NS2 protein. J. Virol. **73:**466–473.
27. **Waris, M. E., C. Tsou, D. D. Erdman, D. B. Day, and L. J. Anderson.** 1997. Priming with live respiratory syncytial virus (RSV) prevents the enhanced pulmonary inflammatory response seen after RSV challenge in BALB/c mice immunized with formalin-inactivated RSV. J. Virol. **71:**6935–6939.
28. **Wendt, C. H., and M. I. Hertz.** 1995. Respiratory syncytial virus and parainfluenza virus infections in the immunocompromised host. Semin. Respir. Infect. **10:**224–231.
29. **Whitehead, S. S., A. Bukreyev, M. N. Teng, C. Y. Firestone, M. St. Claire, W. R. Elkins, P. L. Collins, and B. R. Murphy.** 1999. Recombinant respiratory syncytial virus bearing a deletion of either the NS2 or SH gene is attenuated in chimpanzees. J. Virol. **73:**3438–3442.
30. **Whitehead, S. S., C. Y. Firestone, P. L. Collins, and B. R. Murphy.** 1998. A single nucleotide substitution in the transcription start signal of the M2 gene of respiratory syncytial virus vaccine candidate cpts248/404 is the major determinant of the temperature-sensitive and attenuation phenotypes. Virology **247:**232–239.
31. **Whitehead, S. S., M. G. Hill, C. Y. Firestone, M. St. Claire, W. R. Elkins, B. R. Murphy, and P. L. Collins.** 1999. Replacement of the F and G proteins of respiratory syncytial virus (RSV) subgroup A with those of subgroup B generates chimeric live attenuated RSV subgroup B vaccine candidates. J. Virol. **73:**9773–9780.
32. **Whitehead, S. S., K. Juhasz, C. Y. Firestone, P. L. Collins, and B. R. Murphy.** 1998. Recombinant respiratory syncytial virus (RSV) bearing a set of mutations from cold-passaged RSV is attenuated in chimpanzees. J. Virol. **72:**4467–4471.
33. **Wright, P. F., R. A. Karron, R. B. Belshe, J. Thompson, J. E. Crowe, Jr., T. G. Boyce, L. L. Halburnt, G. W. Reed, S. S. Whitehead, E. L. Anderson, A. E. Wittek, R. Casey, M. Eichelberger, B. Thumar, V. B. Randolph, S. A. Udem, R. M. Chanock, and B. R. Murphy.** Evaluation of a live, cold-passaged, temperature-sensitive, respiratory syncytial virus (RSV) vaccine candidate in infancy. J. Infect. Dis., in press.
34. **Yu, Q., R. W. Hardy, and G. W. Wertz.** 1995. Functional cDNA clones of the human respiratory syncytial (RS) virus N, P, and L proteins support replication of RS virus genomic RNA analogs and define minimal *trans*-acting requirements for RNA replication. J. Virol. **69:**2412–2419.