JOURNAL OF CLINICAL MICROBIOLOGY, June 1991, p. 1175–1182
0095-1137/91/061175-08$02.00/0

Vol. 29, No. 6

Evaluation of Bovine, Cold-Adapted Human, and Wild-Type Human Parainfluenza Type 3 Viruses in Adult Volunteers and in Chimpanzees

MARY LOU CLEMENTS,[1,2]* ROBERT B. BELSHE,[3] JAMES KING,[1] FRANCES NEWMAN,[3] T. ULF WESTBLOM,[3] EVELINE L. TIERNEY,[4] WILLIAM T. LONDON,[5] AND BRIAN R. MURPHY[4]

Center for Immunization Research, Department of International Health, Johns Hopkins University School of Hygiene and Public Health,[1] and Division of Infectious Diseases, Department of Medicine, Johns Hopkins University School of Medicine,[2] Baltimore, Maryland 21208; Division of Infectious Diseases, Department of Internal Medicine, St. Louis University School of Medicine, St. Louis, Missouri 63104[3]; Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892[4]; and Division of Molecular Virology and Immunology, Department of Microbiology, Georgetown University, Rockville, Maryland 20852[5]

Received 6 December 1990/Accepted 11 March 1991

In an attempt to evaluate the level of attenuation of live parainfluenza type 3 virus (PIV3) vaccine candidates, we compared the responses of partially immune adult volunteers inoculated intranasally with 10^6 to 10^7 50% tissue culture infective dose ($TCID_{50}$) of bovine PIV3 ($n = 18$) or cold-adapted (*ca*) PIV3 ($n = 37$) with those of 28 adults administered 10^6 to 10^7 $TCID_{50}$ of wild-type PIV3. The candidate vaccine viruses and the wild-type virus were avirulent and poorly infectious for these adults even though all of them had a low level of nasal antibodies to PIV3. To determine whether the *ca* PIV3 was attenuated, we then administered 10^4 $TCID_{50}$ of *ca* PIV3 (cold-passage 12) or wild-type PIV3 intranasally and intratracheally to two fully susceptible chimpanzees, respectively, and challenged the four primates with wild-type virus 1 month later. Compared with wild-type virus, which caused upper respiratory tract illness, the *ca* PIV3 was highly attenuated and manifested a 500-fold reduction in virus replication in both the upper and lower respiratory tracts of the two immunized animals. Despite restriction of virus replication, infection with *ca* PIV3 conferred a high level of protective immunity against challenge with wild-type virus. The *ca* PIV3 which had been passaged 12 times at 20°C did not retain its *ts* phenotype. These findings indicate that *ca* PIV3 may be a promising vaccine candidate for human beings if a passage level can be identified that is genetically stable, satisfactorily attenuated, and immunogenic.

Type 3 parainfluenza virus (PIV3) causes pneumonia and bronchiolitis in infants and young children throughout the world (3, 7, 21, 25, 36, 37). PIV3 is second to respiratory syncytial virus (RSV) as the most important viral cause of acute lower respiratory disease requiring hospitalization in infants and young children in the United States (5). Primary infection with PIV3 is associated with serious illness, whereas subsequent infections tend to be less severe (20). A vaccine is needed to reduce the significant morbidity in infants resulting from primary infection.

In the past, attempts to produce effective inactivated vaccines against paramyxoviruses have been unsuccessful. For example, a formalin-inactivated PIV3 vaccine failed to induce resistance to disease following natural exposure to the virus (9, 19). One explanation for this failure may have been the inability of an inactivated vaccine to stimulate local secretory immunoglobulin (Ig) A antibody, which is known to play an important role in restriction of virus replication and illness (6, 8, 12, 14, 22, 28, 41, 45, 47). Of further concern were the findings that inactivated RSV and measles virus vaccines induced immune responses that resulted in a more severe or an atypical disease following natural infection with the homologous virus (24, 33, 40). In contrast, live attenuated paramyxovirus vaccines are safe and highly effective for prevention of measles and mumps (4, 38). Parenteral administration of live RSV was poorly immunogenic in young infants and failed to confer protection against naturally acquired infection or illness (2). It is thought that the low levels of maternal antibody in these young infants neutralized the infectivity of the vaccine virus and prevented effective immunization. Importantly, disease potentiation was not observed in these infants. For these reasons, attention has been directed to the development of a live attenuated, intranasally administered PIV3 vaccine for immunoprophylaxis of infants and children (5, 6, 33).

Two approaches have been taken to develop live attenuated PIV3 vaccines: (i) the production of cold-adapted (*ca*), temperature-sensitive (*ts*) mutants of wild-type human PIV3 (1, 17, 18), and (ii) the use of wild-type bovine parainfluenza virus (a host-range variant) which is closely related antigenically to human PIV3 (5, 16, 33). Both viruses have been shown to be attenuated in experimental animal hosts, as evidenced by restriction of replication in the lower respiratory tract (5, 16, 18). Moreover, each vaccine candidate has induced resistance to human PIV3, as demonstrated by a reduction in virus replication in the respiratory tract of hamsters immunized with *ca* PIV3 vaccines (18) and in the respiratory tract of monkeys immunized with bovine PIV3 vaccine (16, 35).

In the present study, we evaluated the safety and immunogenicity of the bovine PIV3 vaccine and two passage levels of the *ca* PIV3 vaccine in adult volunteers. These *ca* PIV3s are designated cp12 and cp18 for cold-passage levels 12 and 18, respectively. We conducted studies in adult volunteers in an attempt to characterize the virulence of the

* Corresponding author.

human wild-type virus parent (JS strain) of *ca* PIV3 and to demonstrate the level of attenuation of the *ca* and bovine PIV3 vaccine candidates. We also conducted a study with cp12 *ca* PIV3 and the wild-type JS strain in fully susceptible chimpanzees (35) and characterized isolates recovered from animals infected with the *ca* vaccine to determine the genetic stability of the *ca* and *ts* phenotypes of this *ca* mutant after replication in vivo.

MATERIALS AND METHODS

Viruses. The JS strain of human wild-type PIV3 was used both as the parent strain for developing *ca* mutants and as the challenge virus in these studies. This virus was initially isolated in primary bovine embryonic kidney (BEK) cells from a specimen collected from a 1-year-old child with febrile respiratory disease. As described previously (1), this wild-type isolate was passaged 14 times in African green monkey kidney (AGMK) or rhesus monkey kidney cells at 37°C and then was plaque purified. The plaque-purified virus was used to produce *ca* mutants. To satisfy the safety requirements of the Bureau of Biologics of the Federal Drug Administration, the serially passaged wild-type virus was passaged again 5 times in AGMK cells and 10 times in fetal rhesus lung (FRhL) cells, including three passages at terminal dilution. Clone 127, pool 282 of the JS strain, was used for all challenge studies.

To prepare *ca* mutants, the plaque-purified wild-type virus was passaged 3 times in AGMK cells at 33.5°C and then was serially passaged in AGMK cells at temperatures suboptimal for PIV3 replication: 10 times at 22°C followed by 35 times at 20°C (1). After 12 (10 passages at 22°C, 2 at 20°C), 18 (10 passages at 22°C, 8 at 20°C), or 45 (10 passages at 22°C, 35 at 20°C) cold passages, mutants were biologically cloned. They exhibited three properties: (i) cold adaptation (*ca*), i.e., the ability to replicate efficiently at the suboptimal temperature of 20°C; (ii) temperature sensitivity (*ts*), i.e., decreased ability to form plaques at 39°C; and (iii) small-plaque morphology. The mutant clones at cp12, cp18, and cp45 exhibited three different levels of restriction of replication in the respiratory tract of hamsters, with the cp12 being the least and the cp45 being the most restricted (17). To produce vaccine virus, the cp12 and cp18 *ca* mutants were further passaged three (cp12) or four (cp18) times in AGMK cells and nine times (including three passages at terminal dilution) in FRhL cells, a cell line suitable for production of vaccines for administration to humans. We first studied cp18 (clone 1146, pool 284) in volunteers, and, when it was shown to be safe, cp12 (clone 1150, pool 283) was then evaluated in volunteers.

The Kansas/15626/84 bovine PIV3 strain (kindly provided by Robert Phillips, Kansas State University, Manhattan) was recovered from a calf with pneumonia and propagated in primary BEK cells (16). This virus was passaged nine times in FRhL cells, including three passages at terminal dilution. The identity of bovine PIV3 was confirmed by testing the virus against a panel of monoclonal antibodies to human PIV3 hemagglutinin-neuraminidase protein, and a pattern of reactivity similar to that of the prototype bovine PIV3 was obtained (data not shown). Clone 5-2-4 of the bovine PIV3 was evaluated in volunteers.

Suspensions of virus used in all volunteer studies were grown in FRhL cells. Neomycin, erythromycin, and amphotericin were added to the virus suspensions to maintain sterility. All virus suspensions were prepared, characterized, and tested for the presence of adventitious agents by Louis Potash (Flow Laboratories, McLean, Va.); none were found. In addition, Michael Hendry (Center for Biologics, Food and Drug Administration, Bethesda, Md.) and Phillip Johnson (Georgetown University, Rockville, Md.) tested the human wild-type and *ca* mutants for simian retroviruses, simian immunodeficiency virus and simian T-lymphotrophic virus (SIV and STLV), since these viruses had a passage history in AGMK cells; none were found. Appropriate dilutions of the cp18 (lot HPI3-2; $10^{6.5}$ 50% tissue culture infective dose [$TCID_{50}$] per ml), the cp12 (lot HPI3-3; $10^{6.7}$ $TCID_{50}$ per ml), the bovine PIV3 (lot BPI3; $10^{7.6}$ $TCID_{50}$ per ml), and the wild-type virus, JS strain (lot HPI3-6; $10^{7.2}$ $TCID_{50}$ per ml), were administered intranasally in a 0.5-ml inoculum to volunteers.

Clinical studies in volunteers. Study protocols were approved by the Clinical Research Subpanel of the National Institute of Allergy and Infectious Diseases, the Joint Committee on Clinical Investigations of the Johns Hopkins Medical Institutions, the Marshall University Institutional Review Board, and the St. Louis University Institutional Review Board.

Healthy adults between the ages of 18 and 40 years were recruited from Baltimore, Md., and Huntington, W. Va. Volunteers were not eligible if they had a history of allergy to bovine products or the antibiotics used in the virus suspension or if they lived with immunosuppressed individuals or children less than 3 years of age. On the first visit, a screening nasal wash specimen was collected from each volunteer and tested for PIV3-specific IgA antibody by kinetic enzyme-linked immunosorbent assay (KELISA) as described below. Only those subjects who had a low level of antibody to PIV3 in their nasal wash specimens were selected for participation in the studies. The good health of the volunteers was confirmed by a normal history, physical examination, complete blood count (including blood urea nitrogen, plasma glucose, and serum alanine aminotransferase), and urinalysis, and by negative tests for human immunodeficiency virus antibody, hepatitis B surface antigen, and pregnancy (females). Those who participated in the studies gave written, informed consent.

All volunteer studies to evaluate the safety, infectivity, and antigenicity of the *ca* and bovine PIV3 vaccines and to determine the virulence of the wild-type PIV3 strain were conducted in an isolation facility. Briefly, volunteers were admitted to an isolation unit at Francis Scott Key Medical Center or at the Marshall University School of Medicine isolation unit. Volunteers were observed for 3 days before and 10 days after inoculation with vaccine or wild-type virus. Methods for the clinical observations, intranasal administration of virus, and collection of nasal washes for isolating virus have been described elsewhere (11, 13, 29, 30). Temperatures were taken four times daily, and volunteers were examined twice a day by investigators. A volunteer was considered ill if symptoms or physical findings consistent with parainfluenza illness developed within 10 days after inoculation. The definition of parainfluenza illness was based on any of the following criteria: fever, i.e., an oral temperature $\geq$37.7°C; systemic illness, i.e., occurrence of myalgia alone or with chills or sweats; upper respiratory tract illness, i.e., the presence of rhinorrhea, pharyngitis, or both on 2 or more consecutive days; and lower respiratory tract illness, i.e., the presence of a persistent cough on 2 or more consecutive days. An illness was attributed to PIV3 when there was laboratory confirmation of PIV3 infection as documented by recovery of virus from nasal washes, development of a fourfold rise in antibody titer, or both.

The safety, infectivity, and antigenicity of the bovine and cp18 *ca* PIV3 vaccines were evaluated in a dose-escalating fashion by administering a 10^6 $TCID_{50}$ of each vaccine candidate intranasally to volunteers in Baltimore. After demonstrating the safety of these doses of the vaccines, a 10-fold-higher dose of each vaccine was evaluated in another group of subjects. Subsequently, at Marshall University, volunteers were inoculated intranasally with $10^{6.5}$ $TCID_{50}$ of cp12 *ca* PIV3 vaccine to evaluate the safety and infectivity of the lower-passage level of *ca* virus.

To assess the virulence of the wild-type parent virus of *ca* PIV3, we administered 10^6 $TCID_{50}$ of the JS strain of PIV3 to volunteers intranasally. After this dose failed to cause illness, we gave another group of volunteers a 10-fold-higher dose (10^7 $TCID_{50}$) of wild-type PIV3. Since this virus proved to be avirulent, even at the 10^7 $TCID_{50}$, we did not pursue additional studies with higher dosages.

Immunologic studies. A nasal-wash specimen (10 ml) was collected initially from each volunteer recruited for the studies. IgA antibody to PIV3 in nasal wash specimens was measured by a KELISA described elsewhere (42). The antigen used in the KELISA was the prototype human strain of PIV3 isolated in 1957 (referred to as PIV3-57). Briefly, the reagents consisted of a purified preparation of PIV3-57 diluted to contain 4 hemagglutinin units of virus which was adsorbed to the plate in carbonate buffer, followed by undiluted nasal wash specimen, rabbit anti-human IgA antibody, goat anti-rabbit antibody conjugated with alkaline phosphatase, and substrate. After the addition of substrate, each well of the 96-well plate was read by using a V_{max} kinetic microplate reader (V_{max} Molecular Devices, Palo Alto, Calif.). The rate of color development (optical density [OD] milliunits per minute) in each well was calculated as the slope of the regression line defined by the OD readings for that well. We selected the most immunologically susceptible subjects for the study, i.e., those volunteers whose levels of nasal wash IgA PIV3 antibodies were in the lowest quartile of the samples tested.

For determination of antibody responses, serum and nasal wash specimens were collected before vaccination or challenge and 4 weeks afterwards. The methods for collecting, pooling, and concentrating the nasal wash specimens were described elsewhere (30, 34). Serum samples were assayed for antibodies to PIV3 by hemagglutination inhibition assay (HAI) at 25°C by using 0.5% guinea pig erythrocytes and, as antigen, human wild-type PIV3-57. The antibody titer was defined as the endpoint serum dilution that inhibited hemagglutination.

A previously described ELISA (31, 32) was used to measure serum IgG and nasal wash IgA antibodies. The antigen used in the ELISA was the purified human PIV3-57 described above. The sequence of reagents from solid phase outward consisted of the following: (i) PIV3-57; (ii) serum (initial dilution, 1:40) or nasal wash (initial dilution, 1:2) specimen; (iii) rabbit anti-human IgG or IgA; (iv) goat anti-rabbit IgG conjugated with alkaline phosphatase; and (v) *p*-nitrophenyl phosphate disodium substrate. The ELISA titer was expressed as the highest dilution in which the OD of the antigen-containing well was at least 0.2 OD units and greater than twice the OD of the respective control well lacking antigen. The ELISA nasal wash anti-PIV3 IgA titers were corrected to a total concentration of 100 mg/ml.

Isolation and quantitation of virus. In studies with volunteers and chimpanzees, 0.1 ml of fresh, undiluted nasal wash, nasopharyngeal, or tracheal lavage fluid was inoculated onto LLCMK2 (for isolation of wild-type or *ca* PIV3) or Madin-Darby bovine kidney (MDBK) (for isolation of bovine PIV3) monolayers in 24-well plates. Monolayers were observed for 14 days for cytopathic effect, which was confirmed by hemadsorption of the monolayers with 0.1% guinea pig erythrocytes. The quantity of virus replication was determined by inoculating 0.02 ml of decimal dilutions of the positive nasopharyngeal and tracheal lavage specimens onto cell monolayers in 96- or 24-well plates. The lowest titer (log_{10}) of virus detected in our tissue culture assays was 0.75 $TCID_{50}$ per ml; an undetectable level was assigned a titer (log_{10}) of 0.5 $TCID_{50}$.

Studies in chimpanzees. Replication of *ca* cp12 virus and that of the wild-type parental PIV3 and the levels of protection conferred by infection with *ca* virus and wild-type virus were compared in chimpanzees (35). Two seronegative (HAI titer, $\leq$1:4) chimpanzees were inoculated with 10^4 $TCID_{50}$ of the JS strain of wild-type PIV3 intratracheally and intranasally, and two seronegative chimpanzees received the same dose of the cp12 *ca* mutant. One month after inoculation, all four chimpanzees were challenged by the administration of 10^4 $TCID_{50}$ of the JS strain of wild-type PIV3 intratracheally and intranasally. For the isolation and quantitation of virus, nasopharyngeal swab specimens were obtained daily for 12 days and tracheal lavage specimens were collected on days 2, 4, 6, 8, and 10, as previously described (16). For determination of HAI antibody responses, serum was obtained from each chimpanzee before inoculation of *ca* or wild-type PIV3, 1 month later, and 2 months later, i.e., 1 month postchallenge.

Phenotypic characterization of isolates from chimpanzees. Tissue-passage isolates from nasopharyngeal washes and tracheal lavage washes were first characterized for the temperature-sensitive (*ts*) property by plaque assay in L-132 cells at 32 and 39°C. Briefly, monolayers of L-132 cells were inoculated with serial 10-fold dilutions of an isolate which was allowed to adsorb at room temperature for 90 min and overlaid with L-15 containing 10% agamma calf serum and 0.9% methyl cellulose. Plates were fixed in formalin and stained with hematoxylin and eosin after 5 days of incubation at 25°C or after 3 days of incubation at 32 or 39°C.

As shown previously (1), the wild-type virus produces plaques efficiently at 39 and 32°C but not at 25°C. It does not replicate in roller tubes of tissue culture cells when incubated at 20°C. The *ca* vaccine viruses exhibit a *ts* phenotype that is defined as a $\geq$100-fold reduction in plaque formation at 39°C compared with plaque formation at 32°C. Virus that increases in titer during 14 days of incubation at 20°C is considered to have the *ca* phenotype.

Data analysis. Laboratory evidence of PIV3 infection was defined as isolation of the test virus or a fourfold or greater rise in titer of serum ELISA IgG antibody, serum HAI antibody, or nasal wash IgA PIV3 antibody. The HAI and ELISA titers were expressed as reciprocal mean log_2. Data were analyzed for statistical significance by using the two-tailed Fisher's exact test, the chi-square test, or the Student's *t* test, where appropriate. Differences that were statistically significant are indicated.

RESULTS

Reactogenicity, infectivity, and antigenicity of *ca*, bovine, and wild-type PIV3 in human beings. Despite our attempts to select the most susceptible volunteers, i.e., individuals with a low level of PIV3 nasal secretory antibodies, we observed that the *ca* and bovine vaccine viruses as well as the wild-type PIV3 were poorly infectious for adults (Tables 1

TABLE 1. Virological and clinical responses of adult human volunteers to *ca*, bovine, or wild-type PIV3[a]

PIV3 administered	$TCID_{50}$ administered	No. of volunteers (% infected)[b]	Shedding of virus (nasal wash)			Volunteers with indicated illness (%)[c]		
			% of volunteers	Duration (days) (mean ± SD)[c]	Peak titer (log_{10} $TCID_{50}$) (mean ± SD)[c,d]	Febrile, systemic, or both	Upper or lower respiratory or both	Any illness
ca								
cp18	$10^{6.0}$	9 (11)	0	0	≤0.5	0	11	11
cp18	$10^{7.0}$	8 (38)	0	0	≤0.5	0	0	0
cp12	$10^{6.5}$	20 (30)	0	0	≤0.5	5	5	10
Bovine	$10^{6.0}$	9 (44)	11	0.8 ± 1.3	0.6 ± 1.3	0	0	0
	$10^{7.0}$	9 (56)	0	0	≤0.5	0	0	0
Wild type	$10^{6.0}$	20 (60)	5	0.6 ± 2.0	0.6 ± 0.4	0	0	0
	$10^{7.0}$	8 (38)	0	0	≤0.5	0	12	12

[a] Only those volunteers who had low levels of antibody to wild-type PIV3 in their initial nasal washes were enrolled in the study. Volunteers received 0.5 ml of *ca*, bovine, or wild-type virus intranasally.
[b] Virus isolation, antibody response, or both signified infection.
[c] Data from infected volunteers were used for calculations.
[d] Culture-negative samples were assigned a value of $10^{0.5}$ $TCID_{50}$ per ml for purposes of calculation.

and 2). PIV3 was recovered from only one individual who had received 10^6 $TCID_{50}$ of bovine PIV3 and one who had received wild-type PIV3. Each of these volunteers shed a small quantity of virus ($10^{0.75}$ or $10^{1.75}$ $TCID_{50}$ per ml, respectively) for only 1 or 2 days. The highest dose ($10^{6.5}$ to 10^7 $TCID_{50}$) of cp12 PIV3, cp18 *ca* PIV3, bovine PIV3, or wild-type PIV3 infected only 30 to 56% of the volunteers.

All volunteers had relatively low levels of PIV3-specific antibody in their initial (screening) nasal wash specimens measured by KELISA. In contrast, the level of PIV3 specific antibody measured in preinoculation nasal wash specimens by ELISA was higher in the volunteers who received $10^{6.5}$ $TCID_{50}$ of cp12 or 10^6 $TCID_{50}$ of wild-type PIV3 than in the other volunteers. Each group had a comparable level of ELISA IgG and HAI antibody in their preinoculation sera, with one exception, namely, the group that received 10^7 $TCID_{50}$ of wild-type PIV3 had a higher preinoculation HAI titer. Despite these differences, all groups mounted similar antibody responses in serum or nasal wash specimens postinoculation (Table 2). Less than half of the recipients of *ca*, bovine, or wild-type virus developed a fourfold or greater rise in titers of antibodies in serum samples detected by HAI or ELISA IgG or in titers of antibodies in nasal wash samples detected by ELISA IgG.

We could not reliably assess the level of attenuation of the cp12 or cp18 *ca* PIV3 vaccine candidate in adults (Table 1). The 10^6 $TCID_{50}$ of wild-type virus parent of the *ca* PIV3 was avirulent, and the 10-fold-higher dose caused only mild illness (rhinorrhea for 2 days) in one of eight volunteers. The few illnesses attributable to infection with PIV3 included a low-grade fever for 1 day (a cp12 vaccinee) and pharyngitis for 2 days (a cp12 vaccinee) or 4 days (a cp18 recipient). No lower respiratory symptoms were observed in any of the volunteers infected with vaccine or wild-type virus.

Level of attenuation, efficacy, and genetic stability of cp12 PIV3 in chimpanzees. Since the bovine PIV3 was previously shown to be attenuated in chimpanzees (16), we used chimpanzees to determine whether the *ca* PIV3 was also attenuated. Since only four chimpanzees were available for study, we initiated our study with the cp12 PIV3 assuming that the cp18 or cp45 virus would manifest the same or a greater level of attenuation. To assess whether the cp12 *ca* PIV3 vaccine candidate was attenuated, we compared the level of virus replication and illness in two susceptible chimpanzees inoculated intranasally and intratracheally with 10^4 $TCID_{50}$ of cp12 *ca* PIV3 or wild-type strain JS PIV3. The wild-type PIV3 replicated to high titer ($10^{6.5}$ $TCID_{50}$) in the upper and lower respiratory tracts of the two seronegative animals inoculated with this virus (Table 3). Compared with the wild-type virus, the replication of cp12 *ca* PIV3 was restricted approximately 500-fold in the upper and lower respiratory tracts. This decreased level of cp12 *ca* PIV3 replication was reflected in a delayed onset (day 7) of rhinorrhea and in a reduction in the duration of rhinorrhea.

TABLE 2. Antibody responses of adult volunteers to *ca* (cp12 or cp18), bovine, or wild-type PIV3[a]

PIV3 administered	$TCID_{50}$	No. of volunteers	Serum HAI antibody response			Serum ELISA IgG response			Nasal wash ELISA IgA[b] response		
			Titer (reciprocal mean log_2 ± SD)		% of volunteers with fourfold rise in titer	Titer (reciprocal mean log_2 ± SD)		% of volunteers with fourfold rise in titer	Titer (reciprocal mean log_2 ± SD)		% of volunteers with fourfold rise in titer
			Pre	Post		Pre	Post		Pre	Post	
ca											
cp18	$10^{6.0}$	9	6.0 ± 0.8	6.3 ± 0.7	11	14.9 ± 1.3	14.9 ± 1.3	0	5.4 ± 1.4	5.2 ± 1.3	0
cp18	$10^{7.0}$	7	7.9 ± 2.1	8.3 ± 1.9	14	14.7 ± 1.4	15.0 ± 1.3	28	6.7 ± 2.0	7.1 ± 1.0	14
cp12	$10^{6.5}$	20	5.6 ± 0.9	5.6 ± 0.9	0	14.5 ± 1.6	14.6 ± 1.6	10	9.2 ± 1.3	8.8 ± 1.7	25
Bovine	$10^{6.0}$	9	6.6 ± 1.6	6.8 ± 1.5	0	14.0 ± 0.9	14.0 ± 1.3	11	5.0 ± 0.9	5.3 ± 1.0	0
	$10^{7.0}$	9	7.2 ± 1.3	8.1 ± 2.1	44	13.7 ± 1.3	14.6 ± 1.3	44	6.7 ± 1.6	7.2 ± 1.7	0
Wild type	$10^{6.0}$	20	6.7 ± 1.0	7.2 ± 1.0	15	14.4 ± 1.0	15.1 ± 1.1	35	9.2 ± 2.7	10.1 ± 2.0	21
	$10^{7.0}$	8	9.5 ± 1.3	10.0 ± 1.2	12	14.8 ± 0.9	15.3 ± 1.7	12	7.3 ± 1.5	8.6 ± 1.2	29

[a] Antibody values shown are those of paired sera and nasal washes. Pre, preinoculation; post, postinoculation.
[b] Nasal wash specimens from one volunteer in each group that was administered 10^6 or 10^7 $TCID_{50}$ of wild-type virus were not available for testing.

TABLE 3. Response of seronegative chimpanzees to intranasal and intratracheal infection with 10^4 $TCID_{50}$ of *ca* or wild-type PIV3 and to challenge with wild-type PIV3[a]

PIV3 administered	Chimpanzee no.	Response to initial infection						Response to challenge with wild-type PIV3[b]			
		No. of days with rhinorrhea	Duration (days) of virus shedding in:		Peak titer (log_{10} PFU/ml) of virus in:		Postinfection HAI antibody titer	Duration (days) of virus shedding in:		Peak titer (log_{10} PFU/ml) of virus in:	
			Nose	Trachea	Nose	Trachea		Nasopharynx	Trachea	Nose	Trachea
ca	1406	0	12	10	4.0	3.0	1:128	0	6	≤0.5	1.0
	1417	2	10	8	3.7	4.0	1:128	0	0	≤0.5	≤0.5
Wild type	1420	8	12	8	6.5	6.5	1:128	0	0	≤0.5	≤0.5
	1422	6	11	10	6.5	5.5	1:64	0	0	≤0.5	≤0.5

[a] Chimpanzees were seronegative (HAI titer, ≤1:4) before inoculation with *ca* or wild-type virus.
[b] Challenge virus ($10^{4.0}$ $TCID_{50}$) was administered intranasally and intratracheally 1 month after inoculation with *ca* or wild-type PIV3.

The vaccine virus also induced a high level (1:128) of HAI antibody in the serum samples of the chimpanzees.

Infection with cp12 *ca* PIV3 induced resistance against challenge with wild-type virus (Table 3). The replication of the JS wild-type challenge virus was almost completely restricted in the upper and lower respiratory tracts of animals previously immunized with live attenuated cp12 *ca* PIV3 or infected with wild-type PIV3. Thus, prior infection with the cp12 *ca* PIV3 induced a high level of resistance to a virulent wild-type PIV3 which is capable of replicating to high titer in the respiratory tract of susceptible chimpanzees.

The genetic stability of the *ca* PIV3 isolated from chimpanzees was then examined. Viral isolates recovered from the upper and lower respiratory tracts of the chimpanzees infected with cp12 *ca* PIV3 were evaluated for the *ts* and *ca* phenotypes to determine the stability of these properties following replication in vivo. Chimpanzees 1406 and 1417 each shed a mixture of attenuated viruses (Table 4). Chimpanzee 1406 shed virus that was identical to the vaccine virus in *ts* and *ca* phenotypes on day 5 (nasopharyngeal sample) and on day 4 (tracheal lavage sample). The virus isolated from the nasopharyngeal washes on days 1, 4, 7, 8, and 9 postinoculation and from the tracheal lavage specimens on days 6 and 10 had lost the *ts* phenotype. Seven of the isolates did retain the *ca* phenotype (Table 4). Virus recovered from chimpanzee 1417 had lost the *ts* phenotype

TABLE 4. Phenotype of cp12 PIV3 isolated from chimpanzees following vaccination compared with wild-type virus and cp12 PIV3 viruses

Virus	Chimpanzee no.	Specimen[a]	Days postvaccination	*ts* phenotype			*ca* phenotype			
				Titer of virus (log_{10} PFU/ml) at temp of:		Fold reduction (log_{10})	Titer of virus (log_{10} PFU/ml) at temp of:		Phenotype	
				32°C	39°C		32°C	39°C	*ts*[b]	*ca*[c]
Wild-type PIV3, JS strain				6.8	6.5	0.3	6.8	<1.0	No	No
PIV3, cp12				5.8	2.5	2.3	4.4	2.0	Yes	Yes
PIV3, cp12	1406	NPW	1	2.6	<1.0	1.6	5.7	<1.0	No	No
			4	4.8	4.5	0.3	7.4	<1.0	No	No
			5	7.0	3.0	4.0	7.0	5.8	Yes	Yes
			7	5.3	4.8	0.5	7.4	3.6	No	Yes
			8	5.2	4.2	1.0	7.6	4.7	No	Yes
			9	6.6	6.0	0.6	6.6	4.4	No	Yes
	1406	TL	4	6.9	3.0	3.9	6.9	5.3	Yes	Yes
			6	7.0	6.3	0.7	7.2	5.9	No	Yes
			10	7.0	6.2	0.8	6.9	5.9	No	Yes
	1417	NPW	2	6.2	4.8	1.4	6.2	3.6	No	Yes
			3	6.5	5.6	0.9	6.5	2.0	No	Yes
			4	6.6	6.2	0.4	6.6	3.5	No	Yes
			5	6.6	6.1	0.5	6.6	2.2	No	Yes
			6	6.7	5.7	1.0	6.7	2.2	No	Yes
			7	6.6	6.2	0.4	6.6	2.5	No	Yes
			8	5.6	5.5	0.1	5.6	2.3	No	Yes
			9	6.5	6.2	0.3	6.5	2.5	No	Yes
			10	6.6	5.8	0.8	6.6	2.3	No	Yes
	1417	TL	4	6.9	6.0	0.9	6.9	3.7	No	Yes
			6	6.9	6.0	0.9	6.9	2.2	No	Yes
			8	6.5	6.0	0.5	7.3	3.1	No	Yes

[a] Specimens tested included viruses isolated in LLCMK2 tissue culture inoculated with nasopharyngeal wash (NPW) and tracheal lavage (TL) fluids.
[b] *ts* viruses exhibit ≥100-fold less plaque formation at 39°C than at 32°C.
[c] *ca* viruses by growth assay exhibit ≥100 PFU/ml growth at 20°C after 14 days of incubation.

in nasopharyngeal wash specimens collected on days 2 through 10 postinoculation and in tracheal lavage specimens collected on days 4, 6, and 8. All of the isolates from this chimpanzee that were tested exhibited the *ca* phenotype.

DISCUSSION

Current strategies for the development of live attenuated virus vaccines against PIV3 include the use of a cold-adapted mutant human PIV3 or a wild-type bovine strain of PIV3 for immunization (1, 5, 33). Human and bovine PIV3 are closely related antigenically and share several neutralization epitopes on the F and HN glycoproteins, each of which are major protective antigens (10, 15, 26, 27, 39). The bovine PIV3 was previously shown to be attenuated in rhesus monkeys and chimpanzees (16) and able to induce significant resistance to human wild-type PIV3, as documented by a reduction in virus replication in challenged cotton rats or squirrel monkeys (15, 16). Three different cold-passage levels of *ca* PIV3 which possess attenuation, *ts*, and *ca* phenotypes have been studied in hamsters (1, 17). The attenuation phenotype was manifest by restriction of virus replication in weanling hamsters (17). Also, the PIV3 *ca* mutants have induced resistance to challenge with wild-type PIV3 in immunized hamsters (18). Furthermore, virus recovered from the respiratory tract of hamsters inoculated with *ca* virus passaged 12 or more times retained its *ca* phenotype, indicating stability of this phenotype in vivo in this species (17).

We conducted a series of clinical studies in adult volunteers and chimpanzees to determine whether the *ca* and bovine PIV3 vaccine candidates possess the following desirable properties of a live attenuated virus vaccine candidate: (i) satisfactory level of attenuation, (ii) reduced level of replication compared with that of wild-type human PIV3, (iii) maintenance of laboratory markers associated with the attenuation phenotype, and (iv) induction of resistance to wild-type virus challenge. Previous studies had shown that 10^5 to $10^{5.7}$ $TCID_{50}$ of wild-type PIV3, given both by aerosol and intranasally, infected 50 to 75% of individuals tested and caused coldlike illnesses in 35 to 75% of adult volunteers who had a low level of neutralizing antibody in their initial nasal wash specimens (23, 46). To increase the likelihood that the adults in our studies would be susceptible to infection with PIV3, we enrolled only those volunteers who had a low level of PIV3-specific IgA antibody in their initial nasal wash specimens. Nevertheless, we were not able to detect differences in the clinical, virological, or immunological responses between those who were inoculated intranasally with attenuated PIV3 and those inoculated with wild-type PIV3. Each of the viruses tested was avirulent, poorly to moderately infectious, and only moderately antigenic for adults. The two cold-passage levels of *ca* PIV3 induced local or systemic antibody slightly less often than the bovine PIV3 or wild-type PIV3. Among the 83 volunteers inoculated, PIV3 virus was recovered from only one volunteer who received wild-type virus and from one given the bovine PIV3. Therefore, we could not be certain whether the bovine and *ca* strains were attenuated satisfactorily or overly attenuated. One explanation for our failure to demonstrate the virulence of the wild-type PIV3 in these adult volunteers was that, despite their low level of nasal wash antibodies to PIV3, they were partially immune as a result of repeated previous infections with PIV3. Alternatively, it is possible that the JS strain was attenuated since, in two previous experimental challenge studies, two wild-type parainfluenza strains and a RSV each caused upper respiratory tract illness in adults (21a, 46). The fact that the JS strain replicated to a high titer in chimpanzees like other human PIV3s suggests that it is at least moderately virulent and that the cp12 PIV3 is indeed attenuated.

Previous studies demonstrated that replication of respiratory viruses bearing only *ts* mutations was significantly more restricted in the lower respiratory tract than in the upper respiratory tract (6). Our findings from studies in fully susceptible (seronegative) chimpanzees indicated that, compared with the wild-type JS parent PIV3, cp12 *ca* PIV3 was equally restricted in replication in the upper and lower respiratory tracts (35). This suggests that one or more mutations, in addition to those responsible for the *ts* phenotype, contribute to the restriction of replication of the cp12 PIV3 in the chimpanzee. It is possible that the mutations that confer the attenuation phenotype differ from those that confer the *ts* phenotype, but this remains to be established. Infection of the chimpanzee with the cp12 *ca* PIV3 induced a high level of resistance to challenge with wild-type JS strain PIV3, indicating that, despite the restricted level of replication of the cp12 *ca* PIV3, virus replication was sufficient to induce a high level of resistance to wild-type virus (35).

The cp12 PIV3 generally lost the *ts* phenotype after replication in chimpanzees, whereas all but two of the isolates that were tested retained the *ca* phenotype. The loss of the *ts* phenotype would suggest that the cp12 virus resembles several of the influenza A and RSV *ts* vaccines previously studied (6, 43, 44) and that the cp12 is not an acceptable candidate vaccine for use in human beings. It is hoped that the more extensively passaged cp18 and cp45 viruses are more stable after replication in the fully susceptible host.

It is encouraging that the *ca* mutant (cp12) that was least attenuated in hamsters appears to be satisfactorily attenuated and immunogenic for chimpanzees. Equally encouraging was the high level of resistance to wild-type virus challenge induced by the cp12 *ca* PIV3. Because the cp18 and cp45 viruses are more restricted than the cp12 virus in hamsters, it is anticipated that they will be even more attenuated for humans and possibly will be more genetically stable than cp12. To determine whether the *ca* and bovine PIV3s will be satisfactorily attenuated and sufficiently immunogenic for use as vaccine candidates, both strains are being evaluated in young children and infants. Because of the inability to predict the level of attenuation of these viruses for susceptible young children, these vaccine candidates will be evaluated cautiously in seropositive young children first and then, if they prove to be safe, in seronegative, fully susceptible children and infants. These studies will determine which PIV3 vaccine candidate is most acceptable, in terms of level of attenuation and immunogenicity, for immunoprophylaxis of fully susceptible infants and children.

ACKNOWLEDGMENTS

This research was supported by Public Health Service contracts NO1 AI 62515, NO1 AI 52575, and NO1 AI 05051 from the National Institute of Allergy and Infectious Diseases.

We thank Karen Christina, Bhavin Thumar, Stephanie Boddie, Paul Kilman, and Lorena Downs from the Center for Immunization Research, Betty Burk and Carol Berry from Marshall University, Becky Hood and Lisa Henseler from St. Louis University, and Clarence Banks from the National Institutes of Health for special assistance with the study. We also thank Alan Leach for preparation of the manuscript.

REFERENCES

1. **Belshe, R. B., and F. K. Hissom.** 1982. Cold adaptation of parainfluenza virus type 3: induction of three phenotypic markers. J. Med. Virol. **10**:235–242.
2. **Belshe, R. B., L. P. Van Voris, and M. A. Mufson.** 1982. Parenterally administered live respiratory syncytial virus vaccine: results of a field trial. J. Infect. Dis. **145**:311–319.
3. **Brandt, C. D., H. W. Kim, Arrobio, B. C. Jeffries, B. C. Wood, R. M. Chanock, and R. H. Parrott.** 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. III. Composite analysis of eleven consecutive yearly epidemics. Am. J. Epidemiol. **98**:355–364.
4. **Brunell, P. A., A. Brickman, and S. Steinberg.** 1969. Evaluation of a live attenuated mumps vaccine Jeryl Lynn: with observations on the optimal tissue for testing serologic responses. Am. J. Dis. Child. **118**:435–440.
5. **Chanock, R. M., and K. McIntosh.** 1990. Parainfluenza viruses, p. 947–972. *In* B. N. Fields (ed.), Virology. Raven Press, New York.
6. **Chanock, R. M., and B. R. Murphy.** 1980. Use of temperature-sensitive and cold-adapted mutant viruses in immunoprophylaxis of acute respiratory tract disease. Rev. Infect. Dis. **2**:421–432.
7. **Chanock, R. M., and R. H. Parrott.** 1965. Acute respiratory disease in infancy and childhood: present understanding and prospects for prevention. Pediatrics **36**:21–39.
8. **Chanock, R. M., R. H. Parrott, K. M. Johnson, A. Z. Kapikian, and J. A. Bell.** 1963. Myxoviruses: parainfluenza. Am. Rev. Respir. Dis. **88**:152–166.
9. **Chin, J., R. L. Magoffin, L. A. Shearer, J. H. Schieble, and E. H. Lennette.** 1969. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am. J. Epidemiol. **89**:449–463.
10. **Choppin, P. W., and A. Scheid.** 1980. The role of viral glycoproteins in adsorption, penetration, and pathogenicity of viruses. Rev. Infect. Dis. **2**:40–61.
11. **Clements, M. L., R. F. Betts, and B. R. Murphy.** 1984. Advantages of live attenuated cold-adapted influenza A virus over inactivated vaccine for A/Washington/80 (H3N2) wild-type virus infection. Lancet **i**:705–708.
12. **Clements, M. L., R. F. Betts, E. L. Tierney, and B. R. Murphy.** 1986. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. J. Clin. Microbiol. **24**:157–160.
13. **Clements, M. L., R. F. Betts, E. L. Tierney, and B. R. Murphy.** 1986. Resistance of adults to challenge with influenza A wild-type virus after receiving live or inactivated virus vaccine. J. Clin. Microbiol. **23**:73–76.
14. **Clements, M. L., S. O'Donnell, M. M. Levine, R. M. Chanock, and B. R. Murphy.** 1983. Dose response of A/Alaska/6/77 (H3N2) cold-adapted reassortant vaccine virus in adult volunteers: role of local antibody in resistance to infection with vaccine virus. Infect. Immun. **40**:1044–1051.
15. **Coelingh, K. L. V. W., B. R. Murphy, J. M. Rice, R. C. Kimball, C. C. Winter, and P. L. Collins.** 1987. Hemagglutinin-neuraminidase protein epitopes shared by human and bovine parainfluenza type 3 viruses: nucleotide sequence analysis of variants selected with monoclonal antibodies, p. 391–396. *In* B. Mahy and D. Kolapofsky (ed.), The biology of negative strand viruses. Elsevier Science Publications, Amsterdam.
16. **Coelingh, K. L. V. W., C. C. Winter, E. L. Tierney, W. T. London, and B. R. Murphy.** 1988. Attenuation of bovine parainfluenza virus type 3 in nonhuman primates and its ability to confer immunity to human parainfluenza virus type 3 challenge. J. Infect. Dis. **157**:655–662.
17. **Crookshanks, F. K., and R. B. Belshe.** 1984. Evaluation of cold-adapted and temperature-sensitive mutants of parainfluenza virus type 3 in weanling hamsters. J. Med. Virol. **13**:243–249.
18. **Crookshanks-Newman, F. K., and R. B. Belshe.** 1986. Protection of weanling hamsters from experimental infection with wild-type parainfluenza virus type 3 (para 3) and cold-adapted mutants of para 3. J. Med. Virol. **18**:131–137.
19. **Fulginiti, V. A., J. J. Eller, O. F. Sieber, J. W. Joyner, M. Minamitani, and G. Meiklejohn.** 1969. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines: an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am. J. Epidemiol. **89**:435–448.
20. **Glezen, W. P., A. L. Frank, L. H. Taber, and J. A. Kasel.** 1984. Parainfluenza virus type 3: seasonality and risk of infection and reinfection in young children. J. Infect. Dis. **150**:851–857.
21. **Glezen, W. P., F. A. Loda, W. A. Clyde, Jr., R. J. Senior, C. I. Sheaffer, W. G. Conley, and F. W. Denny.** 1971. Epidemiologic patterns of acute lower respiratory disease of children in a pediatric group practice. J. Pediatr. **78**:397–406.

21a. **Hall, C. B., E. E. Walsh, C. E. Long, and K. C. Schnabel.** 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. J. Infect. Dis. **163**:693–698.

22. **Johnson, P. R., S. Feldman, J. M. Thompson, J. D. Mahoney, and P. F. Wright.** 1986. Immunity to influenza A virus infection in young children: a comparison of natural infection, live cold-adapted vaccine, and inactivated vaccine. J. Infect. Dis. **154**:121–127.
23. **Kapikian, A. Z., R. M. Chanock, T. E. Reichelderfer, T. G. Ward, R. J. Huebner, and J. A. Bell.** 1961. Inoculation of human volunteers with parainfluenza virus 3. JAMA **18**:537–541.
24. **Kapikian, A. Z., R. H. Mitchell, R. M. Chanock, R. A. Shvedoff, and C. E. Stewart.** 1969. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am. J. Epidemiol. **89**:405–421.
25. **Kim, H. W., J. O. Arrobio, C. D. Brandt, B. C. Jeffries, G. Pyles, J. L. Reid, R. M. Chanock, and R. H. Parrott.** 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. I. Importance of the virus in different respiratory tract disease syndromes and temporal distribution of infection. Am. J. Epidemiol. **98**:216–225.
26. **Merz, D. C., A. Scheid, and P. W. Choppin.** 1981. Immunological studies of the functions of paramyxovirus glycoproteins. Virology **109**:94–105.
27. **Merz, D. C., A. Scheid, and P. W. Choppin.** 1980. Importance of antibodies to the fusion glycoprotein of paramyxoviruses in the prevention of spread of infection. J. Exp. Med. **151**:275–288.
28. **Murphy, B. R., E. G. Chalhub, S. R. Nusinoff, J. Kasel, and R. M. Chanock.** 1973. Temperature-sensitive mutants of influenza virus. III. Further characterization of the *ts*-1 [E] influenza A recombinant (H3N2) virus in man. J. Infect. Dis. **128**:479–487.
29. **Murphy, B. R., R. M. Chanock, M. L. Clements, W. C. Anthony, A. J. Sear, L. A. Cisneros, M. B. Rennels, E. H. Miller, R. E. Black, M. M. Levine, R. F. Betts, R. G. Douglas, Jr., H. F. Maassab, N. J. Cox, and A. P. Kendal.** 1981. Evaluation of A/Alaska/6/77 (H3N2) cold-adapted recombinant viruses derived from A/Ann Arbor/6/60 cold-adapted donor virus in adult seronegative volunteers. Infect. Immun. **32**:693–697.
30. **Murphy, B. R., M. L. Clements, E. L. Tierney, R. E. Black, J. Steinberg, and R. M. Chanock.** 1985. Dose response of influenza A/Washington/897/80 (H3N2) avian-human reassortant virus in adult volunteers. J. Infect. Dis. **152**:225–229.
31. **Murphy, B. R., D. L. Nelson, P. F. Wright, E. L. Tierney, M. A. Phelan, and R. M. Chanock.** 1982. Secretory and systemic immunological response in children infected with live attenuated influenza A virus vaccines. Infect. Immun. **36**:1102–1108.
32. **Murphy, B. R., M. A. Phelan, D. L. Nelson, R. Yarchoan, E. L. Tierney, D. W. Alling, and R. M. Chanock.** 1981. Hemagglutinin-specific enzyme-linked immunosorbent assay for antibodies to influenza A and B viruses. J. Clin. Microbiol. **13**:554–560.
33. **Murphy, B. R., G. A. Prince, P. L. Collins, K. L. V. W. Coelingh, R. A. Olmsted, M. K. Spriggs, R. H. Parrott, H.-W. Kim, C. D. Brandt, and R. M. Chanock.** 1988. Current approaches to the development of vaccines effective against parainfluenza and respiratory syncytial viruses. Virus Res. **11**:1–15.
34. **Murphy, B. R., M. B. Rennels, R. G. Douglas, Jr., R. F. Betts, R. B. Couch, T. R. Cate, Jr., R. M. Chanock, A. P. Kendal, H. F. Maassab, S. Suwanagool, S. B. Sotman, L. A. Cisneros,**

W. C. Anthony, D. Nalin, and M. M. Levine. 1980. Evaluation of influenza A/Hong Kong/123/77 (H1N1) *ts*-1A2 and cold-adapted recombinant viruses in seronegative adult volunteers. Infect. Immun. **29**:348–355.

35. **Murphy, B. R., E. L. Tierney, W. T. London, and R. B. Belshe.** 1989. A cold-adapted mutant of human parainfluenza virus type 3 is attenuated and protective in chimpanzees, p. 91–95. *In* F. Brown, R. N. Chanock, H. S. Ginsberg, and R. A. Lerner (ed.), Vaccines 90: modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Parrott, R. H., H. W. Kim, J. O. Arrobio, D. S. Hodes, B. R. Murphy, C. D. Brandt, E. Camargo, and R. M. Chanock.** 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am. J. Epidemiol. **98**:289–300.
37. **Parrott, R. H., A. Vargoski, A. Luckey, H. W. Kim, C. Cumming, and R. Chanock.** 1959. Clinical features of infection with hemadsorption viruses. N. Engl. J. Med. **260**:731–738.
38. **Preblud, S. R., and S. L. Katz.** 1988. Measles vaccine, p. 182–222. *In* S. A. Plotkin and E. A. Mortimer (ed.), Vaccines. The W. B. Saunders Co., Philadelphia.
39. **Ray, R., V. E. Brown, and R. W. Compans.** 1985. Glycoproteins of human parainfluenza virus type 3: characterization and evaluation as a subunit vaccine. J. Infect. Dis. **152**:1219–1230.
40. **Scott, F. M. T., and D. E. Bonanno.** 1967. Reactions to live-measles-virus vaccine in children previously inoculated with killed-virus vaccine. N. Engl. J. Med. **277**:248–250.
41. **Smith, C. B., R. H. Purcell, J. A. Bellanti, and R. M. Chanock.** 1960. Protective effect of antibody to parainfluenza type 1 virus. N. Engl. J. Med. **275**:1145–1152.
42. **Snyder, M. H., S. Banks, and B. R. Murphy.** 1988. Determination of antibody response to influenza virus surface glycoproteins by kinetic enzyme-linked immunosorbent assay. J. Clin. Microbiol. **26**:2034–2040.
43. **Tolpin, M. D., M. L. Clements, M. M. Levine, R. E. Black, A. J. Saah, W. C. Anthony, L. Cisneros, R. M. Chanock, and B. R. Murphy.** 1982. Evaluation of a phenotypic revertant of the A/Alaska/77-*ts*-1A2 reassortant virus in hamsters and in seronegative adult volunteers: further evidence that the temperature-sensitive phenotype is responsible for attenuation of *ts*-1A2 reassortment viruses. Infect. Immun. **36**:645–650.
44. **Tolpin, M. D., J. G. Massicot, M. G. Mullinix, H. W. Kim, R. H. Parrott, R. M. Chanock, and B. R. Murphy.** 1981. Genetic factors associated with loss of the temperature-sensitive phenotype of the influenza A/Alaska/77-*ts*-1A2 recombinant during growth *in vivo*. Virology **112**:505–517.
45. **Tremonti, L. P., J. S. L. Lin, and G. C. Jackson.** 1968. Neutralizing activity in nasal secretions and serum in resistance of volunteers to parainfluenza virus type 2. J. Immunol. **101**: 572–577.
46. **Tyrrell, D. A. J., M. L. Bynoe, K. Birkum, S. Petersen, and M. S. Pereira.** 1959. Inoculation of human volunteers with parainfluenza viruses 1 and 3 (HA_2 and HA_1). Br. Med. J. **2**:909–911.
47. **Yanagihara, R., and K. McIntosh.** 1980. Secretory immunological response in infants and children to parainfluenza virus types 1 and 2. Infect. Immun. **30**:23–28.