Memoranda/Mémoires

Current status of respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) vaccine development: Memorandum from a Joint WHO/NIAID meeting*

A Joint WHO/National Institute of Allergy and Infectious Diseases (NIAID) meeting on the current status of respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) vaccine development was held in Bethesda, MD, from 30 September to 1 October 1996. The meeting summarized the worldwide impact of RSV and PIV3; presented the current status of development of RSV and PIV3 vaccines; and examined the applications of recombinant DNA technology to the development and characterization of vaccines and to the understanding of viral pathogenesis.

Worldwide impact of RSV and PIV3

Published information and ongoing epidemiological studies on the impact of respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) in South America, Africa, South-east Asia and the Western Pacific Region were reviewed. RSV is the major viral respiratory tract pathogen responsible for infants and children coming to hospital in these regions, with annual epidemics occurring during the winter months. It is also regularly responsible for over 20% of hospitalizations for serious lower respiratory tract disease — bronchiolitis and pneumonia being the major presenting illnesses. In contrast, PIV3 is an endemic virus infection and can be isolated during most months of the year. PIV3 has a more variable impact, being responsible for 3–10% of hospitalizations — bronchiolitis, pneumonia, and croup being the major presenting illnesses. Both RSV and PIV3 cause serious disease during the first 6 months of life, with the incidence of RSV illness peaking early in the first 3 months and steadily declining from a high level thereafter. PIV3 illness is more evenly distributed with a broad peak at 4–12 months of age.

Vaccines for PIV3

Bovine PIV3

Bovine PIV3 (BPIV3) was chosen as a candidate live-virus vaccine to protect against infection with human PIV3 (HPIV3) because it is closely related antigenically to the latter, induces resistance to HPIV3 challenge, and is attenuated in nonhuman primates. In humans, replication of BPIV3 is restricted, and it is poorly infectious and totally avirulent in both seropositive children and adults. In contrast, among seronegative vaccinees BPIV3 is highly infectious but nonreactogenic. Despite replicating at a level about 100-fold lower than HPIV3 in seronegative children, BPIV3 induced an immune response to HPIV3 in a majority of infants. Addi-
Live, attenuated, cold-adapted human PIV3

The results obtained with the cold-adapted JS strain of human PIV3 were summarized. Cold-passage (cp) PIV3 mutants were selected by serial passage of the JS strain of wild-type human PIV3 in cell culture at successively lower temperatures down to 20°C or 22°C. Three mutants that were selected at passage 12, 18, or 45 (cp12, cp18, and cp45, resp.) possessed the desired properties of cold adaptation (ca), temperature sensitivity (ts), and attenuation (att) for rodents and nonhuman primates. The cp45 mutant was the most promising candidate vaccine strain, as indicated by its high level of attenuation in the upper and lower respiratory tract of chimpanzees and rhesus monkeys; also, it maintained its ts and att phenotypes following replication in the respiratory tract of these nonhuman primates. The cp45 candidate vaccine virus, grown in FRhL-2 (fetal Rhesus lung-2) cells, was highly infectious, satisfactorily attenuated, immunogenic, poorly transmissible, and genetically stable during replication in seronegative infants and young children. It was safe for the five seronegative young infants (<6 months of age) studied. The virus replicates in Vero cells (a continuous cell line of simian origin that is licensed for use in human vaccines) maintained as microcarrier cultures without losing its ts, ca, and att phenotypes. The Vero cell-grown cp45 was highly attenuated in the lower respiratory tract of rhesus monkeys and was similar to its FRhL-cell-grown counterpart. Each of the viral isolates that was recovered retained the ts and ca phenotypes. Studies in humans have been initiated with Vero-cell-grown cp45.

Vaccinia recombinants

The results of evaluating the immunogenicity and efficacy of a new generation of vaccinia-PIV3 recombinant viruses were summarized. The highly attenuated, host-range restricted, modified vaccinia virus Ankara (MVA) strain was used to express the fusion (F) or the haemagglutinin–neuraminidase (HN) glycoprotein of PIV3. This vector was chosen because it grows efficiently in a licensed cell substrate (chick embryo fibroblasts obtained from specific-pathogen-free embryonated eggs) but produces very little infectious virus in most mammalian cells. Therefore, it should, unlike conventional vaccinia virus, be completely safe in humans, especially for the expanding population of immunocompromised individuals infected with human immunodeficiency virus (HIV). Cotton rats and rhesus monkeys inoculated by the intramuscular, intratracheal, or intranasal route with recombinant MVA expressing F or HN developed high levels of PIV3 antibodies and were highly resistant to challenge with wild-type PIV3.

Subunit vaccines

A trivalent PIV1-, PIV2-, and PIV3-purified HN and F glycoprotein subunit vaccine has been developed using proteins purified from virus replicated to high titre in Vero cells grown in microcarrier culture. The monovalent PIV-1 and PIV-2 HN and F glycoprotein preparations efficiently induced haemagglutination inhibition (HAI) and neutralizing antibodies in rodents, with amounts of protein in the range 0.1–10μg. A trivalent PIV1–3 subunit vaccine was comparably immunogenic, inducing levels of antibodies for each component that were equivalent to those in rodents immunized with the individual components alone. The PIV3 subunit vaccine has previously been shown to be efficacious in rodents, but this was not evaluated for the PIV1 and PIV2 components. The monovalent PIV3 vaccine is safe in adult seropositive human volunteers.

Vaccines for RSV

Cold-adapted mutants of RSV antigenic subgroup B

A summary was given of the development and characterization of a live, attenuated subgroup B RSV candidate vaccine. The virus was derived by low temperature passage of a wild-type subgroup B strain of RSV (termed 2B) and identification of clonal populations that had acquired the ts phenotype. A mutant (clone 2B33F) was identified that was ts at 39°C, moderately cold-adapted, and highly attenuated in the upper and lower respiratory tract of cotton rats and African green monkeys. Evidence of loss of ts phenotype occurred in vivo and in vitro, indicating that this phenotype was somewhat unstable. It was concluded that the 2B33F mutant would serve as a useful substrate for the introduction of additional ts or other attenuating mutations.

Live attenuated, subgroup A and B mutants of RSV

A series of subgroup A candidate vaccines has been produced using one or two cycles of chemical
mutagenesis and plaque isolation to introduce one or more attenuating ts mutations into the incompletely attenuated cold-passaged RSV (cpRSV) mutant. Three cpts mutants of subgroup A RSV (cpts248/955, cpts530/1009, and cpts248/404) are attenuated and protective in chimpanzees and have been evaluated in adults, seropositive children, or seronegative infants and children. Subgroup B RSV candidate vaccines have similarly been developed by passaging the RSV B-1 wild-type strain in tissue culture at low temperature followed by chemical mutagenesis; three subgroup B RSV vaccine candidates were isolated (cp52, cpts176 and cpts176/427) that were attenuated in cotton rats and were subsequently evaluated in humans.

The cpRSV mutant of the A2 strain RSV subgroup A is attenuated for adult volunteers, as indicated by decreased infectivity, decreased frequency of virus shedding, decreased level of virus replication, and decreased frequency of illness. This suggests that cpRSV contains non-ts-attenuating mutations operative in the upper respiratory tract of humans. The cpts248/955 mutant is incompletely attenuated for seronegative infants. The cpts530/1009 virus exhibits a greater degree of restriction of replication than cpts248/955 in seropositive and seronegative children. Full analysis of the efficiency of plaque formation at three restrictive temperatures of virus shed by cpts530/1009- or cpts248/955-seronegative vaccinees did not detect a significant drift in the level of temperature sensitivity of the mutant. Both cpts530/1009 and cpts248/955 spread to a contact control from whom ts virus was isolated. The cpts248/404 mutant has been evaluated in over 25 seronegative infants and young children and found to be safe, phenotypically stable, poorly transmissible, and reasonably immunogenic. The level and rate of viral replication of the cpts248/404 vaccine candidate are lower than those of the other two cpts mutants. These findings in the aggregate are encouraging and demonstrate for the first time that it is possible to develop a live, attenuated subgroup A RSV vaccine that is stable phenotypically and that appears, in the limited number of seronegative subjects tested, to exhibit the proper balance between attenuation and immunogenicity.

The RSV B1 wild-type virus has been confirmed to be virulent for human adults and the attenuating effect of mutations introduced by cold passage or mutagenesis has been evaluated. The RSV B1 cp52 virus was unable to infect adults, indicating that it had acquired host-range attenuating mutations for humans. The more attenuated derivatives of cp52 virus (i.e. cpts176 and cpts176/427) were over-attenuated for seronegative subjects; cp52 infected 2 of 5 seronegative vaccinees at a dose of 10^5 and was shed at a relatively low level indicating that it is indeed attenuated for this population.

The nucleotide sequences were described for three ts mutants (ts1A, ts1B, and ts1C), which represent successive steps of chemical mutagenesis and plaque isolation of an RSV subgroup A wild-type virus. The three mutants, which had been previously evaluated for safety and immunogenicity in adults, possess multiple nucleotide and amino acid changes that could have contributed to the ts or at phenotype. However, the ts1A and ts1B mutants differ by only one amino acid (in the L protein), suggesting that it is responsible for the phenotypic differences between the two viruses.

**Subunit vaccines**

Studies were summarized on the purified RSV F glycoprotein, alum-adjuvanted, vaccine candidate (PEP-2), which is being developed to protect the newborn via maternal immunization and to augment immunity in seropositive individuals who are at high risk for severe RSV disease, i.e. elderly adults and persons of all ages with compromised cardiopulmonary function. The majority of a study group of elderly adults, children with cystic fibrosis, and young women of child-bearing age immunized with 50µg of F glycoprotein experienced an increase in neutralizing antibody titre. Surveillance of vaccinees during the ensuing RSV season suggested that potentiated disease did not accompany subsequent RSV infection.

The development of a chimeric protein that contains a bacterial albumin-binding protein fused to amino acid residues 130–230 of the RSV attachment (G) glycoprotein was described. This chimeric protein, purified from Escherichia coli on an albumin column, was immunogenic and efficacious in BALB/c mice.

**Vaccinia recombinants**

The poxvirus-RSV recombinant viruses ALVAC (an avian poxvirus) and NYVAC (a vaccinia virus derivative), which express the F glycoprotein of RSV, are immunogenic and efficacious in mice. Infection of mice with the ALVAC–RSV recombinant also resulted in the induction of an RSV-specific cellular (CD8+ CTLs) response.

**Infectious virus from cDNA**

Recently, several laboratories have independently described the successful recovery of infectious virus from cDNA for the following non-segmented, negative strand RNA viruses — *Rhabdoviridae* (rabies
and vesicular stomatitis viruses), Paramyxoviridae (Sendai and measles viruses), and RSV. This provides the first available method for direct genetic manipulation of this type of virus.

**Basic systems used for “rescue”**

Progress was described for four “rescue” systems: vesicular stomatitis virus (VSV), Sendai virus (SeV), measles virus (MeV), and RSV. In each case, cDNA has been constructed to express a full length, positive-sense copy (representing a replicative intermediate RNA or antigenome) of the viral genome. Synthesis of the antigenome is controlled by a promoter for T7 RNA polymerase, and the downstream end of the antigenome is produced by a self-cleaving ribozyme. A common strategy is used to recover VSV, SeV, and RSV: the antigenome plasmid is transfected into tissue culture cells together with T7 expression plasmids encoding the subset of viral proteins required for transcription and RNA replication. For VSV and SeV, the required proteins are the nucleocapsid (N) protein, the phosphoprotein (P) and the large polymerase subunit (L). In addition to these proteins, transcription by RSV requires the 22kDa M2 protein (a transcription processivity factor) expression of which is necessary for recovery of RSV. In these systems, T7 RNA polymerase was supplied by a vaccinia virus recombinant. The strategy for recovery of MeV was somewhat different: a cell line was established that constitutively expresses the T7 RNA polymerase and the MeV N and P proteins — this line was transfected with plasmids encoding the MeV antigenome and the MeV L protein. This approach obviates the need for the vaccinia-T7 recombinant virus and probably would be advantageous for the recovery of debilitated viruses. For all of these viruses, the basic concept was that coexpression of these components would generate antigenome nucleocapsid; which would then be replicated into genomic nucleocapsid and in turn initiate a productive infection. Each of these systems produces recombinant virus reasonably reliably. SeV and MeV recombinants were recovered only when the nucleotide length of the genome was an even multiple of six, which confirms the “rule of six” described previously by Roux et al. using defective interfering (DI) genome replicons. This rule is thought to arise because of a nucleocapsid structure in which each N protein monomer associates with six nucleotides; however, no comparable rule appears to exist for Rhabdoviridae or for RSV.

**Expression of foreign genes**

One application of this “rescue” technology is to use negative-strand RNA viruses as expression vectors for foreign genes. Different viruses might have different advantages; for example, VSV has a broad host range and extremely high levels of replication and gene expression, whereas SeV or RSV would be appropriate for targeting expression to the respiratory tract. VSV, MeV, and RSV are each capable of expressing a foreign translational open reading frame (ORF), which was placed under the control of viral transcription signals, inserted into the genome, and expressed as a separate mRNA. Initial studies have involved marker proteins such as chloramphenicol acetyl transferase (CAT), luciferase, or green fluorescent protein. VSV also has been used successfully to express the MeV H protein, MeV F protein, CD4, or CD4 containing the cytoplasmic and transmembrane domains of the VSV G protein.

The foreign proteins have been expressed at levels comparable to those of the other viral proteins; the level of expression depends on the position of insertion into the transcriptional map, consistent with the polar nature of transcription. Studies with VSV produced the surprising finding that each of the expressed foreign transmembrane proteins was packaged in the virion at levels up to 62% of the coexpressed VSV G protein; the foreign proteins appeared to be filling free space in the virion envelope rather than displacing the G protein. The level of cell surface expression of the heterologous protein also influences its level of incorporation into virions. The upper limit for insert size remains to be determined; the 11.2kbp VSV genome can accommodate at least an additional 3.2kbp. Interestingly, increases in VSV genome length are associated with proportional increases in virion length. The foreign inserts have little or no effect on the replication of VSV or MeV. In contrast, insertion of the small CAT gene into RSV significantly retards virus growth and reduces production of infectious virus, and thus constitutes an attenuating mutation.

Remarkably, the foreign genes are usually maintained with a high degree of stability. For example, when the VSV-CAT virus was passaged ten times and plaques were assayed by CAT-specific immunofluorescence, every plaque expressed CAT. With MeV, a similar passage series resulted in some isolates in which expression was lost or reduced. None the less, the level of stability of the foreign genes in these recombinants, as well as in the RSV recombinants, seems to be much greater than observed with positive-sense RNA viruses. Sequence analysis of VSV, MeV, and RSV recombinants indicates that the foreign gene slowly accumulates point mutations, but the virus does not appear to delete the foreign sequence. In one case (VSV bearing the MeV F protein), the foreign protein appears to inter-
fere with viral replication, and mutations in which expression is silenced are quickly selected.

**Viral molecular biology**

The "rescue" systems also have been used to investigate basic features of viral molecular biology. For example, MeV recombinants have been constructed in which the V or C protein ORFs are silenced individually. Remarkably, knockout of either gene did not affect virus growth in tissue culture. Effects in vivo remain to be investigated. The recombinant virus is based on the tissue-culture-adapted Edmonston vaccine strain, and such knockouts may lack effect for this reason. Interestingly, MeV lacking the M gene also is successfully produced from cDNA, although it appears to be debilitated in virion formation and is mostly cell associated. In contrast, the MeV mutants, which lacked most of the cytoplasmic tails of F or HN or carried a heterologous tail, grew to relatively high titres. MeV mutants were constructed in which the F and H coding regions were mostly or completely replaced by those of the VSV G protein, which presumably changes the method of entry from surface fusion to endocytosis followed by pH-activated entry; these chimeric viruses grew efficiently in a variety of human and rodent cell lines. Such studies indicate that negative-sense RNA viruses can tolerate considerable genetic manipulation, including drastic reduction or exchange of envelope components.

In the case of SeV, the 3' leader region of the genome has been deleted and replaced with a comparably sized piece from the 3'-end of the antigenome. This generates a full-length SeV genome (called rSeV-GP42), whose termini are exactly complementary and thus mimic those of the "copy-back" type of DI particle genome. Whereas the natural SeV genome has terminal complementarity only for approximately 12 nucleotides, the GP42 genome has exact complementarity for 42 nucleotides. This virus is non-cytopathic in tissue culture, perhaps because of a failure to induce apoptosis; it does not form plaques, but instead establishes a persistent infection that can be maintained indefinitely. Despite its apparent growth restriction and reduced levels of gene expression, the GP42 virus was dominant over the wild-type virus and eliminated it during mixed infection. These data demonstrate the capacity of genetic engineering to alter profoundly the growth properties of a non-segmented negative strand RNA virus.

The function of the SeV C protein has been investigated with cDNA-encoded DI genomes whose replication is complemented by viral protein expressed from co-transfected plasmids. One type of DI genome was of the copy-back variety described above and the other was an internal-deletion type that retained the normal 3' genome end. The C protein mediates promoter-specific, negative regulation of RNA replication, inhibiting the genomic promoter (as in the internal-deletion DI RNA) but not the antigenomic promoter (as in the copy-back DI). This was confirmed with the wild type and GP42 recombinant viruses: the former, but not the latter, being inhibited by C protein. This explains the dominance of the GP42 virus over the wild type since it is free of such negative regulation. The same explanation accounts for the dominance of the copy-back type DI genome: it too, is free of such negative regulation. Other studies with SeV have focused on mapping the sequences at the 3'-end of the genome responsible for RNA replication. Essential sequence elements therefore lie within the upstream non-translated region of the N gene in addition to the leader region. Thus, the genomic promoter and accessory sequence elements are much more extensive than had been expected.

**Development of RSV and PIV3 vaccines**

The ability to introduce predetermined changes into infectious virus provides an important new capability for the development of live, attenuated vaccine virus and is of particular importance for RSV and PIV3. In this connection, the results of sequence analyses of the cpRSV, cpts248, cpts248/404, cpts530, and cpts530/1009 viruses were reported. The cpRSV contains five point mutations (each encoding an amino acid difference) relative to the wild-type parent, and each of the subsequent derivatives contains one or two point mutations. Each result in an amino acid change, mostly in the L protein, except for a single change in the transcription start signal of the M2 gene.

Studies are being carried out to insert systematically these mutations into wild-type recombinant RSV to identify the phenotype associated with each mutation and thereby prepare a list of RSV attenuation mutations. For example, a point mutation that was identified in the L protein of cpts530 RSV has been found to confer temperature sensitivity to the wild-type recombinant virus. The cpts248/404 virus is currently being reconstructed as a recombinant by inserting one or more mutations from the 530 lineage to further attenuate it. Thus, this methodology offers the capability of combining desirable mutations from different viruses.

The "rescue" methodology should have other uses in vaccine development. DNA represents a stable vaccine seed. In many cases, mutations can be stabilized against reversion by choosing codons that
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differ from the wild-type assignment at more than one nucleotide. Vaccine virus can be modified to accommodate antigenic drift. It might also be possible to replace the surface antigens of an attenuated subgroup A virus with those of a subgroup B virus, thereby creating an attenuated subgroup B vaccine virus. In addition, the expression of protective antigens by a vaccine virus might be increased by genetic engineering; for example, by removing alternative reading frames or changing gene order. Vaccine viruses might be improved by coexpression of one or more cytokine genes, such as IL-2 (to broadly stimulate immunity), or IL-12 (to enhance a Th1-type response).

PIV3 has not yet been recovered from cDNA, although a complete cDNA has been assembled, and a minireplicon system established and used to confirm the functionality of the N, P, and L cDNAs. When the method of recovery has been established for PIV3, it will be used to characterize the mutations of the cp45 virus and to construct PIV1 and PIV2 vaccines by replacing the cp45 HN and F genes with those of PIV1 and PIV2.

In summary, the recovery of infectious engineered virus from cDNA will be an important part of future studies of molecular biology, pathogenesis, and vaccine development for the nonsegmented negative-strand RNA viruses. These viruses appear to be surprisingly tolerant of genetic engineering and the insertion and expression of foreign sequences, perhaps because of the modular nature of the viral genome.

Résumé
Etat d'avancement des vaccins contre le virus respiratoire syncytial (RSV) et le virus parainfluenza type 3 (PIV3): Mémorandum d'une réunion conjointe OMS/NIAID

L'OMS et le National Institute of Allergy and Infectious Diseases (NIAID) ont parrainé une réunion conjointe sur l'état d'avancement des vaccins contre le virus respiratoire syncytial (RSV) et le virus parainfluenza type 3 (PIV3), qui s'est tenue à Bethesda, MD, les 30 septembre et 1er octobre 1996. La réunion avait pour principaux objectifs de faire le point sur l'impact mondial du RSV et du PIV3, décrire l'état actuel du développement des vaccins dirigés contre ces virus, et examiner les applications du génie génétique au développement et à la caractérisation des vaccins ainsi qu'à l'étude des mécanismes de la pathogénicité des virus. Le RSV et le PIV3 sont, à l'échelle mondiale, les deux principaux agents pathogènes des voies respiratoires. Deux vaccins vivants atténués — l'un à base de PIV3 bovin antigéniquement apparenté et l'autre à base de PIV3 humain adapté au froid — ont satisfait aux essais de phase I chez des sujets sérénégatifs. De plus, un vaccin candidat à base de virus de la vaccine recombinant, fondé sur la souche modifiée Ankara de virus de la vaccine, restreinte par la gamme d'hôtes, qui exprime les antigènes protecteurs du PIV3, a induit une résistance à une infection d'épreuve chez des rongeurs et des primates. Un vaccin sous-unité trivalent — PIV1, PIV2, PIV3 — consistant en hémagglutinine-neuraminidase (HN) et en glycoprotéine de fusion (F) purifiées de chaque PIV, s'est montré efficace lors d'essais sur l'animal.

Un mutant thermosensible vivant atténué, adapté au froid par passages successifs, d'un RSV humain de sous-groupe A a également satisfait aux essais de phase I chez des sujets sérénégatifs. Le phénotype de ce mutant était stable après réplication chez des sujets sérénégatifs, et les sujets vaccinés présentaient des titres modérés d'anticorps neutralisants. On n'a pas encore trouvé d'équivalent vivant atténué pour le RSV humain du sous-groupe B. Huit vaccins vivants atténués contre le RSV ont été séquencés et un menu de mutations atténuantes est en cours d'assemblage. Une ou plusieurs mutations issues de ce menu sont introduites dans le virus infectieux grâce à une technique innovante de génie génétique. Une préparation vaccinale consistant en glycoprotéine F purifiée de RSV, adjugée par l'alun (PEP-2), en cours de mise au point pour protéger le nouveau-né par vaccination de la mère et pour renforcer l'immunité chez les sujets séropositifs à haut risque d'infection grave par le RSV, à savoir les personnes âgées et les sujets de tous âges dont la fonction pulmonaire est affaiblie, est immunogène chez l'homme. Un autre vaccin candidat à base d'une protéine chimère composée d'une protéine de liaison à l'albumine bactérienne fusionnée avec les restes 130–230 d'acides aminés de la glycoprotéine d'attachement (G) du RSV, a induit une résistance à une infection d'épreuve chez la souris.

Grâce aux techniques de génétique inverse, il est maintenant possible d'obtenir des RSV et d'autres paramyxovirus infectieux à partir de constructeurs d'ADNc par co-expression du génome complet ou de l'antigénome d'ARN et du sous-ensemble de protéines virales nécessaires pour la transcription et la réplication. Il est possible d'introduire des mutations dans ces constructeurs et d'obtenir des virus porteurs de ces mutations en vue de la caractérisation ultérieure de leur phéno-
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type. On a également réussi à insérer et exprimer des gènes étrangers, et à retirer des gènes accessoires de virus infectieux. Outre les études sur la virologie moléculaire et sur la pathogénie des para-
myxoviroses, cette nouvelle technique est utilisée pour identifier les mutations atténuantes dans les vaccins candidats anti-RSV vivants atténués et pour développer de nouveaux vaccins expérimentaux.