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Current approaches to the development of vaccines effective against parainfluenza viruses

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Infection of infants and children with parainfluenza viruses can lead to serious lower respiratory tract disease. In order to review new information on the genetic organization, epidemiology, and immunobiology of these viruses and of respiratory syncytial virus, WHO, in conjunction with the National Institute of Allergy and Infectious Diseases, held a workshop in Bethesda, MD, USA, 4–5 May 1987. New data on the immunobiology and epidemiology of human parainfluenza 3 virus were presented that should facilitate the development of a vaccine against this pathogen.

Parainfluenza viruses can cause serious lower respiratory tract infections in infants and children (*1*). In particular, parainfluenza viruses 1 and 2 are primarily associated with croup, whereas parainfluenza virus 3 (PIV3) is responsible for pneumonia and bronchiolitis as well as croup (*1*). PIV3 is second only to respiratory syncytial virus as a cause of cases of serious acute respiratory infections that require hospitalization of infants and children (*1*). The WHO programme for vaccine development has therefore designated parainfluenza viruses as targets for intensive study, with the aim of developing new strategies of immunization to bring these important viruses under control. In order to review new information on the genetic organization, epidemiology, and immunobiology of these pathogens, WHO, in conjunction with the National Institute of Allergy and Infectious Diseases, USA, held a Workshop on Respiratory Syncytial and Parainfluenza Viruses at the National Institutes of Health in Bethesda, MD, 4–5 May 1987. Here are outlined various strategies for immunization against parainfluenza viruses, focusing primarily on information presented and discussed at the Workshop.

New data on the immunobiology and epidemiology of human PIV3 were presented that provide a framework for the development of vaccines against this virus. The two major protective antigens of PIV3 are the haemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins (*2–4*). Antibody to either the HN or F glycoprotein can neutralize the infectivity of the virus, and immunity to either glycoprotein affords protection against a challenge with PIV3. Importantly, extensive work on the HN glycoprotein of human PIV3 has revealed that there is only one antigenic type. Also, characterization of field strains of human PIV3 has established that there has been extensive conservation of the major neutralization epitopes of the virus over a 25-year period, while nucleotide sequence analysis has demonstrated only limited sequence diversity (*5*). Thus, unlike influenza virus, the antigenic composition of PIV3 appears to

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be monotypic and stable—findings that are encouraging for the development of effective vaccines against this pathogen.

LIVE VIRUS VACCINES

The four approaches outlined below are being explored to develop a safe and effective live virus vaccine against PIV3.

Cold-adapted mutants

The conventional cold-adaptation method to produce mutants of PIV3 was described, in which a human wild-type PIV3 strain was serially passaged in simian tissue culture at temperatures that were suboptimal for its replication (*6–8*). The virus was serially passaged 10 times in primary African green monkey tissue at 22 °C and subsequently passaged an additional 35 times at 20 °C. After 12, 18, or 45 passages, viruses were biologically cloned and mutants were identified that exhibited the following three properties: cold-adaptation (*ca*), i.e., the ability to replicate efficiently at the suboptimal temperature of 20 °C; temperature sensitivity (*ts*), i.e., decreased ability to form plaques at 39 °C; and small-plaque morphology. Each of these *in vitro* phenotypes has been associated with restriction of *in vivo* virus replication and is therefore considered an *in vitro* marker of attenuation. The mutant clones at cold passage levels 12 and 18 (cp12 and cp18, respectively) exhibit the following properties that are desirable for a vaccine strain: stable cold-adapted, temperature-sensitive, and small-plaque phenotypes after *in vitro* and *in vivo* replication; restricted viral replication in the upper and lower respiratory tract of hamsters; and induction of the resistance of hamsters to wild-type PIV3 challenge. The cp12, cp18, and cp45 cold-adapted mutants exhibited different levels of restriction of *in vivo* replication, with the cp12 being the least and the cp45 the most restricted. In particular, one or more of these cold-adapted viruses may have the desired balance between attenuation and immunogenicity for use as a vaccine in humans. The cp12 and cp18 cold-adapted mutants have been serially passaged and biologically cloned in a cell line suitable for use in human volunteers, and studies with these viruses in humans are planned.

Bovine parainfluenza virus 3

The idea of using a virus strain of animal or avian origin as a vaccine against an antigenically-related human virus was first suggested by Jenner nearly 200 years ago. Attenuation of such viruses in human hosts arises because during evolution of the virus in its natural host numerous genetic changes occur, some of which restrict replication in humans. Examples of prior successes of this approach include: vaccinia virus, which successfully immunizes humans against smallpox; bovine and simian rotaviruses, which offer protection against human rotavirus disease (*9, 10*); and avian-human influenza A reassortant viruses, which are attenuated by their avian influenza A virus genes and protect humans against experimental challenge with wild-type human influenza A virus (*11*).

Bovine PIV3 is a promising candidate vaccine because it is closely related antigenically to human PIV3. Archetti-Horsfall analysis of the antibody responses of squirrel monkeys to human or bovine PIV3 indicates that these viruses are 35%-related and 25%-related, according to the results of haemagglutination-inhibition and cross-neutralization tests, respectively. This is a high level of antigenic relatedness for viruses whose F and HN surface glycoproteins exhibit only 80% and 77% conservation of amino acid sequences, respectively (*13*). Both bovine and human PIV3 also exhibit significant sequence divergence (62–86%) of their NP, P, C, and M proteins (*14*).

Although bovine PIV3 clearly differs antigenically from the human strain of the virus, it induces a high level of resistance to human PIV3 in cotton rats (*15*) and has therefore been evaluated further in primates to determine its potential as a live virus vaccine for preventing the human PIV3 disease. Infection of squirrel monkeys with bovine PIV3 induced a moderate level of serum antibodies that cross-react with human PIV3. Monkeys immunized with bovine PIV3 exhibited significant resistance to replication of human PIV3 in the upper and lower respiratory tracts. The level of replication of both the human and bovine strains of the virus was similar in both squirrel and owl monkeys, while that of bovine PIV3 was moderately restricted in the lower respiratory tract of rhesus monkeys and chimpanzees. Although strains of bovine PIV3 were restricted in rhesus monkeys, the virus grew sufficiently well to induce a neutralizing antibody response that, on the basis of previous findings, should provide resistance to human PIV3 infection. These encouraging observations provide a basis for the clinical evaluation of bovine PIV3 as a live vaccine for the prevention of infections caused by human PIV3.

Protease activation mutants

Proteolytic cleavage of the F glycoprotein of paramyxoviruses (as well as of the haemagglutinin of orthomyxoviruses) is required to activate infectivity and this process may play an important role in the

pathogenicity of such viruses. Trypsin-like enzymes in host tissues cleave the fusion protein precursor F0 into its disulfide-linked F1 and F2 subunits. Tashiro & Homma have previously reported the isolation of a protease activation (*pa*) mutant (TR-2) of murine parainfluenza virus 1 (Sendai virus) that is resistant to cleavage by trypsin but is cleaved by chymotrypsin (*16, 17*). This virus was less virulent in mice presumably because these animals lack a protease capable of efficiently cleaving the F0 glycoprotein of the mutant virus. Infection with this *pa* mutant was restricted but it was still able to induce resistance to the wild-type virus. The TR-2 *pa* mutant was genetically stable after replication in the murine respiratory tract. At the Workshop a parallel series of experiments were outlined that involve *pa* mutants with known mutations around the cleavage site (*18*). For example, the *pa*-c1 mutant, which requires either chymotrypsin or elastase for cleavage activation, has amino acid substitutions at positions 109 (Asn→Asp) and 116 (Arg→Ile), while the *pa*-e2 mutant, which requires elastase for activation, is substituted at positions 116 (Arg→Ile) and 119 (Gly→Asp). For each mutant, the substitution Arg→Ile at position 116 removed the cleavage site for trypsin and a new *N*-terminus of F1 was generated by cleavage with chymotrypsin or elastase. The *pa* mutants were attenuated in mice, as indicated by an increase in the LD_{50} value, and also by a decrease in the pulmonary histopathological changes in mutant-infected mice compared with those infected with the wild-type virus. Histological examination indicated that the *pa*-e2 was less virulent than the *pa*-c1 mutant. The *pa* mutants were immunogenic and induced resistance to challenge with wild-type murine parainfluenza virus. These results clearly indicate that *pa* mutants of human parainfluenza viruses should be sought and evaluated for their suitability as vaccine viruses. However, a critical step in the production of the Sendai virus *pa* mutants was the identification of a cell line that requires exogenous protease to generate infectious virus, and for human parainfluenza virus a similar cell line will have to be identified that is acceptable to regulatory agencies. Unfortunately, this requirement might present a considerable obstacle to adapting such an approach to generate *pa* mutants of human parainfluenza viruses.

Recombinant vaccinia virus

A further approach to the development of a safe, effective live virus vaccine against PIV3 involves the use of a stable attenuated vaccine virus strain to construct viable recombinants that express the protective antigen of respiratory viruses. To date, the greatest success has been achieved using vaccinia virus as the recombinant vector. Vaccinia virus is highly suitable for this purpose since it has a large genome with a variety of sites into which foreign viral genes can be inserted and expressed without seriously compromising the capacity of the recombinant to replicate. Equally important, vaccinia virus has the longest history of successful use in humans as an effective, attenuated live virus vaccine.

The production and evaluation of the vaccinia-PIV3 HN and vaccinia-PIV3 F recombinants, which express the HN and F surface glycoprotein of PIV3, respectively, were described (*4*). The glycoproteins produced in this way were authentic biochemically and immunologically, and each was expressed on the infected cell surface and was functionally active. Immunization of cotton rats with either vaccinia recombinant induced a high level of PIV3-specific serum-neutralizing antibodies, and immunized animals exhibited significant resistance to challenge. These findings indicate that each of the glycoproteins is a major protective antigen, although the HN glycoprotein appears to play a greater role in providing protective immunity. Similar results were described for a vaccinia-SV5 HN or vaccinia-SV5 F recombinant, which was used as an immunogen in hamsters (*3*) (SV-5 is a parainfluenza virus 2 that infects and causes a croup-like disease in dogs and is a model for infection with human parainfluenza virus 2).

The evaluation was then described of vaccinia PIV3 recombinants in patas monkeys that had been immunized intradermally with a mixture of two vaccinia virus recombinants that individually express the HN or F glycoprotein of human PIV3. A single immunization with both recombinants induced a high titre of PIV3 serum-neutralizing antibodies. One month after immunization, monkeys were challenged intratracheally with PIV3, and subsequent replication of the virus was greatly reduced in the upper and lower respiratory tracts of these monkeys compared with that of control animals. These findings demonstrate that a single intradermal dose of live recombinant vaccinia virus can significantly restrict the replication of a virus that primarily infects the epithelial cells of the respiratory tract. Furthermore, vaccine strategies that involve use of live attenuated recombinant vaccinia viruses present a feasible approach to immunization against important respiratory tract pathogens such as PIV3.

SUBUNIT VACCINES

Administration of inactivated vaccines against two paramyxoviruses — respiratory syncytial virus (RSV) and measles virus — can result in unexpected potentiation or alteration of natural disease (*19, 20*). For

example, with RSV the frequency and severity of the natural infection was increased, whereas with measles the infection took the form of a severe pneumonia with atypical rashes. These two results have led to a halt in the further development of inactivated parainfluenza virus vaccines. Inactivation of the vaccines with formalin produced alterations in one or both of the surface glycoproteins (*21–23*); with measles virus, the immunogenicity of the F glycoprotein was reduced, and with RSV the immunogenicity of the GP-90 (G) and GP-70 (F) glycoproteins was altered in such a way that although enzyme-linked immunosorbent assay (ELISA) binding antibody was efficiently induced it did not efficiently neutralize virus infectivity (*23*). The decreased ability of the formalin-inactivated vaccine to induce functional, i.e., neutralizing, antibodies against surface glycoproteins probably contributed to the immunopotentiation of the disease. Biopsy of the skin lesions induced in recipients of formalin-inactivated measles vaccine (*24*) produced findings that were consistent with an Arthus reaction, with deposition of complement, immunoglobulin, and measles antigen in an area of polymorphonuclear cell infiltration.

There followed a description of the cotton-rat model of formalin-inactivated RSV-vaccine-induced immunopathology (*22*). Upon live RSV challenge, cotton rats that had previously been vaccinated with formalin-inactivated RSV vaccine develop an intense polymorphonuclear cell infiltration of the alveoli, which contain deposits of IgG and complement, and an infiltration of the peribronchiolar region with lymphocytes. The response is dose dependent—high or low doses of vaccine failing to potentiate the pulmonary lesions upon challenge, whereas such lesions are potentiated by intermediate doses. High doses of formalin-inactivated vaccine may induce sufficient resistance to prevent infection with RSV. Such protected cotton rats would not display RSV antigens and an immunopathological reaction would not be observed. Similar histopathological changes have been observed in cotton rats that previously had been given formalin-inactivated PIV3 vaccine after being challenged with PIV3.

The viral antigens involved in these immunohistopathological reactions have not yet been identified, and the relative contributions of the humoral and cellular components of the immune response have not been quantified. Because of these findings with inactivated whole virus vaccines, efforts to develop vaccines other than live virus vaccines have focused on purified viral subunits.

A subunit PIV3 vaccine

Two approaches to producing a subunit vaccine that is effective against PIV3 were described (*25, 26*). In the first of these, the HN and F glycoproteins of PIV3 were solubilized with octyl glucopyranoside and, after removal of the detergent, the solubilized glycoproteins were used to immunize hamsters. The antibody response of these hamsters was then compared with that of others that had been immunized with formalin-inactivated PIV3. A single subcutaneous immunization with the PIV3 subunit vaccine that contained predominantly HN and F glycoproteins induced higher titres of haemagglutination-inhibiting (HAI), fusion-inhibiting, and neutralizing antibodies than did a comparable dose of formalin-inactivated vaccine. In addition, hamsters immunized with the highest dose of envelope glycoproteins showed complete protection from challenge infection, whereas those immunized with inactivated vaccine exhibited only incomplete protection.

In the second approach, immunoaffinity purification of HN and F glycoproteins using monoclonal antibodies specific for either of these proteins was outlined. Affinity-purified HN glycoprotein induced HAI and neutralizing antibodies in rabbits (but no fusion-inhibiting antibodies), whereas the F glycoprotein induced fusion-inhibiting antibodies and neutralizing antibodies (but no HAI antibodies). Passive transfer of both HN and F antibodies protected baby hamsters from PIV3 challenge, and a vaccine incorporating the PIV3 F and HN glycoproteins and reconstituted into lipid vesicles is being prepared for immunization by parenteral and local routes. Preliminary evidence suggests that both glycoproteins are necessary as vaccine antigens to give complete protection against PIV3 virus infection.

Baculovirus-expressed antigens as subunit vaccines

Baculoviruses have been used as vectors for a large array of proteins to produce large quantities of such antigens for biochemical and immunological studies. Studies were described in which the HN gene of human PIV3 was inserted into the genome of a baculovirus vector (Autographa Californica nuclear polyhedrosis virus) under the control of the polyhedrin promoter (*27*). Biologically active HN protein that was produced in insect cells by the recombinant baculovirus was glycosylated and transported to the cell surface. All of the HN epitopes that had previously been mapped to the region of the HN glycoprotein involved in neuraminidase and/or haemagglutination activities were conformationally unaltered on the baculovirus-expressed HN protein, and the HN produced stimulated HAI and neutralizing antibodies in cotton rats. Immunized cotton rats were protected against PIV3 challenge, and it is therefore reasonable to conclude that the HN expressed in insect cells

represents a source of authentic HN glycoprotein suitable for use in immunization studies. Investigations are in progress to purify the HN protein obtained from baculovirus-infected cells in order to determine its suitability for use as an immunogen, and similar studies are planned for the F glycoprotein of human PIV3.

It is important to emphasize that, for immunization procedures involving purified proteins or subunit vaccines, studies should be carried out to determine whether there is immunopotentiation in vaccinated animals that have been exposed to wild-type virus. In the design of such studies attention should be given to the findings for formalin-inactivated RSV that immunopotentiation is dependent on the vaccine dose. Furthermore, studies to estimate the ratio of functional (neutralizing) antibody to binding antibodies will be helpful in evaluating the degree of denaturation of the protein that has occurred during purification. An additional concern for immunization against PIV3 is that the vaccine will have to be administered within the first 2 months of life, because serious disease caused by this virus can, like RSV, occur in infants aged between 2 and 6 months of age. Also, for RSV, the antibody response to the G and F glycoproteins is significantly decreased in young infants because of immunological immaturity or immunosuppression by maternal antibodies (*28*). Each immunization procedure must therefore be able to induce an immune response in the young infant in the presence of maternal antibodies, a task that may be difficult to accomplish in practice.

Current approaches to immunization against human parainfluenza virus are encouraging in their diversity. Within the next few years, it is likely that safety and immunogenicity studies will be initiated of cold-adapted mutants of human PIV3 and bovine PIV3. Also, studies with other live viruses as well as purified subunits may proceed to trials in humans within the next 5 years.

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RÉSUMÉ

MISE AU POINT DE VACCINS CONTRE LES VIRUS PARA-INFLUENZA: APPROCHES ACTUELLES

Les paramyxovirus sont responsables de maladies graves des voies respiratoires inférieures chez les nourrissons et les enfants. C'est la raison pour laquelle le programme OMS de mise au point des vaccins a décidé que ces virus devraient faire l'objet d'études intensives. L'OMS et le National Institute of Allergy and Infectious Diseases des Etats-Unis d'Amérique ont donc organisé conjointement un atelier sur le virus respiratoire syncytial et les virus para-influenza au National Institute of Health, à Bethesda (Maryland), les 4 et 5 mai 1987. Le présent article expose les différentes stratégies envisagées pour la mise au point de vaccins contre les virus para-influenza, en insistant sur les aspects qui ont été abordés à cet atelier.

Les deux principaux antigènes protecteurs du virus para-influenza 3 (PIV3) sont les glycoprotéines F (fusion) et HN (hémagglutinine-neuraminidase), qui toutes deux stimulent la production d'anticorps neutralisants. La caractérisation des isolements de PIV3 par les anticorps monoclonaux et l'analyse des séquences de nucléotides montrent qu'il n'existe qu'un seul type antigénique.

On a décrit quatre méthodes de production de vaccins vivants.

Dans la première, le PIV3 a été adapté au froid par passages en série sur des tissus simiens et les mutants PIV3 atténués ont été identifiés (mutants *ca*). Ces mutants *ca* possédaient les phénotypes suivants: sensibilité à la température, adaptation au froid stabilisée, formation de plages de dimensions restreintes, et atténuation chez le hamster. Les hamsters immunisés avec les mutants *ca* se sont montrés résistants à une infection d'épreuve par le PIV3.

Dans la deuxième méthode, on a constaté que le PIV3 bovin avait une parenté antigénique de 25% avec le PIV3 humain, d'après les résultats des essais de neutralisation, et on a considéré qu'il pourrait servir de vaccin vivant contre la maladie provoquée par le PIV3 humain. Des singes infectés avec le PIV3 bovin ont manifesté un degré élevé de résistance aux infections des voies respiratoires supérieures et inférieures par le PIV3 humain. La réplication du virus PIV3 bovin dans les voies respiratoires inférieures de singes rhésus et de chimpanzés a été moindre que celle du PIV3 humain, ce qui pourrait signifier que le PIV3 bovin est atténué chez l'homme.

Dans la troisième méthode, on a isolé un virus Sendai ayant subi une mutation pour l'activation par les protéases

(mutants *pa*). Ces mutants se répliquent en présence d'élastase ou de chymotrypsine, mais résistent au clivage par la trypsine. Chez deux d'entre eux la mutation, qui touchait le résidu d'acide aminé 116, avait supprimé le site de clivage par la trypsine sur la protéine F, tandis que le clivage par les protéases utilisées pour l'activation (chymotrypsine ou élastase) avait créé une nouvelle terminaison N sur la sous-unité F1. Ces mutants *pa* étaient atténués pour la souris et ils ont induit une résistance à une infection d'épreuve par le virus Sendai virulent sauvage. A partir de ces constatations, des expériences devraient être entreprises en vue d'identifier des mutants similaires chez les virus para-influenza humains.

Dans la quatrième méthode, on a créé par recombinaison génétique des virus hybrides vaccine-PIV3 HN et vaccine-PIV3 F qui expriment la glycoprotéine de surface du PIV3. On a trouvé des titres élevés d'anticorps neutralisants chez le rat des cotonniers et le singe pleureur infectés avec le virus recombinant vaccine-PIV3, et les animaux ainsi vaccinés se sont révélés très résistants à l'infection des voies respiratoires supérieures et inférieures par le PIV3. Cela montre qu'une dose intradermique unique de virus vivant obtenu par recombinaison du virus de la vaccine peut réduire de façon importante la réplication d'un virus infectant les cellules épithéliales des voies respiratoires. On peut donc envisager des stratégies de vaccination comportant l'utilisation de produits de recombinaison vivants et atténués du virus de la vaccine pour lutter contre des agents pathogènes tels que le PIV3, qui sont responsables de graves maladies des voies respiratoires.

Des vaccins à base de sous-unités constituées par les glycoprotéines de surface du PIV3 font également l'objet d'évaluations. On a obtenu les glycoprotéines HN et G purifiées du PIV3 à partir de virions, par extraction avec un détergent, ou à partir de cellules infectées, par chromatographie d'immuno-affinité. Ces protéines se sont révélées immunogènes et efficaces chez l'animal. Ainsi, la glycoprotéine HN du PIV3 exprimée sur baculovirus est fonctionnellement et antigéniquement authentique. Des rats des cotonniers immunisés par des cellules d'insectes exprimant la glycoprotéine HN ont produit des anticorps neutralisants et ont résisté à une infection d'épreuve par le PIV3. Ces résultats montrent que la glycoprotéine HN exprimée dans les cellules d'insectes est identique à la glycoprotéine naturelle et peut être utilisée dans les études de vaccination. Il faut souligner que, lorsqu'on envisage des procédés de vaccination comportant l'utilisation de vaccins à base de protéines ou de sous-unités purifiées, des études doivent être entreprises pour déterminer s'il y a immunopotentialisation chez les animaux vaccinés, après exposition au virus sauvage.

Les approches actuellement suivies pour mettre au point un vaccin contre le virus para-influenza humain sont encourageantes dans leur diversité. Il est probable qu'au cours des prochaines années, des études d'innocuité et d'immunogénicité seront entreprises sur des mutants de PIV3 humain et bovin adaptés au froid. D'autre part, les études portant sur d'autres virus para-influenza vivants ainsi que sur des vaccins à base de sous-unités purifiées pourraient atteindre le stade des essais chez l'homme au cours des cinq prochaines années.

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