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Vaccines & Antibodies

The role of reverse genetics in the development of vaccines against respiratory viruses

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Despite their significance, the only available vaccines against respiratory viruses are those for the prevention of influenza. Attempts have been made to produce vaccines against other respiratory viruses using traditional techniques, but have met with little success. Reverse genetics, although still a relatively new tool for the manipulation of negative-strand RNA viruses, has great potential for the preparation of vaccines against many of the common respiratory viruses. In the preparation of live vaccines, reverse genetics systems allow the direct modification of the specific regions in the genomes of negative-stranded RNA viruses concerned with attenuation; the ultimate goal is the introduction of site-specific mutations through a cDNA intermediate in order to develop strains with the requisite attenuation, antigenic and growth properties needed in a vaccine. These techniques can also be used to disarm potentially highly pathogenic viruses, such as emerging H5N1 avian influenza viruses, in order to facilitate large-scale preparation of viruses for use in inactivated vaccines under conditions of manufacturing safety. Before these vaccines become available, residual issues concerned with intellectual property rights to the technology and its application will need to be resolved.

Keywords: respiratory, reverse genetics, RNA, vaccine, virus

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1. Respiratory viruses

Respiratory diseases are the most common cause of human illness. Acute respiratory infections, caused mainly by viruses, are the leading cause of medical consultations in most countries (20%) and are responsible for 30% of work absences [1]. In developing countries, respiratory illnesses are responsible for the deaths of ~ 4 million children per year [2]. On average, preschool children encounter 6 – 10 episodes of respiratory illness per year and experience symptoms during 30 – 40% of the year; adults usually experience 2 – 4 episodes over the same period [3-5]. Approximately 200 viruses are involved and these include, in order of importance, influenza and respiratory syncytial viruses (RSVs) [8,9], rhinoviruses, coronaviruses (including the severe acute respiratory syndrome [SARS] coronavirus), coxsackieviruses, parainfluenza viruses (PIVs), human metapneumoviruses (hMPVs) and adenoviruses [6]. Of these, vaccines are only available against influenza viruses.

In most countries, influenza vaccines are administered as inactivated whole or subunit/split virus preparations. Vaccines contain the haemagglutinin and neuraminidase surface antigens of influenza A H1N1 and H3N2 viruses and those of influenza B viruses [7]. In order for vaccines to be effective, both antigens must be updated regularly to take account of antigenic drift. Seed viruses used to prepare inactivated vaccines consist of strains that grow to very high titre or, in the case of influenza A viruses, reassortants containing the surface antigen genes of existing strains and the

genes for enhanced growth from a high-yielding parental virus, such as A/PR/8/34 [7]. Vaccine viruses are usually grown in the allantoic cavity of embryonated chicken eggs and are then concentrated and purified by zonal centrifugation and inactivated by treatment with β-propiolactone or formaldehyde. Reactogenicity associated with whole viruses is usually removed by disruption with a detergent (split vaccines) or by separation of non-surface antigens from purified whole virus (subunit vaccines) [8].

Many attempts have been made to develop vaccines against other respiratory viruses. It is widely believed that live vaccines administered directly to the respiratory tract offer the prospect of producing a much broader and long-lasting protection than parenterally administered killed vaccines [9,10]. Live vaccines induce the same repertoire of IgM, IgG, local or secretory IgA antibody and cell-mediated responses as those induced by natural infections [11,12]. Vaccine viruses replicate to a limited extent in the respiratory tract and so induce responses to many more epitopes than is possible with non-replicating antigens [13]. A further potential advantage of mucosal compared with parenteral vaccination is that immune responses can be induced in the presence of maternal antibody [14]. Effective live vaccines require the use of viruses that are suitably attenuated and, at the same time, immunogenic and capable of *in vitro* growth to economic titres for vaccine production.

Live attenuated vaccines against influenza have been recently licensed for restricted use in the US, but have been used in Russia for many years [12,15-17]. Concerns about the use of a live vaccine in the face of a new pandemic are related to the possibility that reassortment occurs between the genes of the pandemic virus and those of the live influenza A vaccine to produce virus(es) of unpredictable virulence. These concerns are much diminished during interpandemic periods, where the surface antigen genes of both the epidemic and vaccine reassortants are similar. Inactivated vaccines will be required and particular issues relating to work safety will arise if, for example, avian (H5N1) strains, which have become endemic in avian species in many regions of Asia, replace H1N1 and H3N2 viruses as predominant human influenza A subtypes [18].

Many attempts have been made to produce live virus vaccines against other respiratory viruses using traditional techniques, such as multiple cold-passage [19,20] or chemical mutagenesis [21]. Attempts to develop live vaccines have been pursued because of failures at inactivated vaccine development. The most notable of these was the attempted development of a formalin-inactivated vaccine against RSV, in which vaccinated infants exhibited enhanced immunopathology following natural RSV challenge [22,23]. Similarly, attempts to develop inactivated vaccines against paramyxoviruses and rhinoviruses have been unsuccessful [24,25]. The sheer number of rhinovirus serotypes (> 100) is a significant disincentive to attempts at vaccine development, and most efforts aimed at the prevention of rhinoviruses have been directed towards the development of group-specific antiviral chemotherapeutic agents. Similar considerations apply to adenoviruses, which, unlike other respiratory viruses, possess a double-stranded DNA genome. However, a live vaccine against serotype 4 has been developed for institutional use, which is delivered as an enteric capsule; respiratory immunity is developed by the common mucosal pathway [26]. Non-SARS coronaviruses have an important role in the aetiology of common colds [27], but are technically too difficult to grow in cell culture to be considered for vaccine development. The SARS coronavirus, on the other hand, grows readily in cell culture and inactivated viruses have been shown to induce a high level of neutralising antibody in BALB/c mice [28]. If required, a cell culture-derived inactivated vaccine could be developed as a preventive measure against SARS, but, as with pandemic influenza viruses, the manufacturing process represents a significant biohazard and the need for robust measures of biological containment. As with potential pandemic influenza strains, it would be highly desirable to disarm the virus by specifically modifying genes associated with virulence by reverse genetics.

This review is concerned principally with new approaches to the development of vaccines against influenza viruses, RSV and paramyxoviruses – all negative-stranded RNA viruses – by the application of reverse genetics. Reverse genetics allows for directed manipulation of cDNA copies of the RNA genomes of parental viruses, thereby providing a means of rationally attenuating the viruses for use as live vaccine candidates or, in the case of pandemic influenza, for removing virulence factors and allowing large-scale manufacture of inactivated vaccines under conditions of industrial safety.

2. Reverse genetics

The term 'reverse genetics' is used to define the directed modification of cDNA for functional or phenotypic analysis [29]. Reverse genetics is used in molecular virology to generate infectious viruses possessing genomes derived from cloned cDNA that have been modified in order to study the consequent effects on phenotype. The first reverse genetics systems described were for positive-sense RNA viruses [30,31]. The transfection of full-length genomic RNA from positive-sense RNA viruses into eukaryotic cells resulted in the RNA acting as mRNA(s) for the translation of viral proteins and, in turn, infectious virus. Reverse genetics is widely used in vaccine development, viral protein interaction studies, recombinant protein expression and gene therapy.

By contrast, the genomes of negative-sense RNA viruses have no messenger function and are non-infectious [32]. Initiation of RNA transcription from viral RNA requires the presence of the viral ribonucleoprotein (RNP) complex. RNP is essential for the transcription of the viral RNA into mRNA and the replication of the virus genome. The role of RNP was first shown for vesicular stomatitis virus, where purified polymerase protein was only active in the presence of virion RNP [33-35].

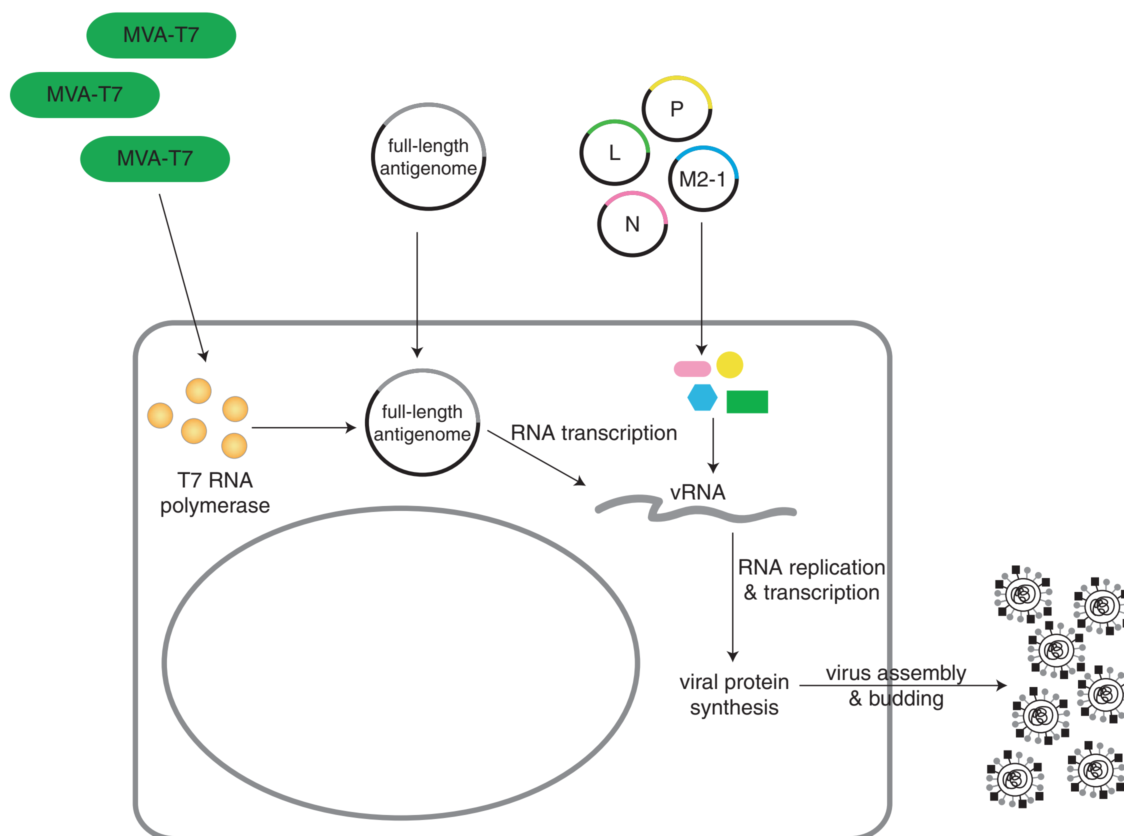

Figure 1. Schematic representation of the rescue of RSV from cDNA. Cells are cotransfected with protein-expressing plasmids encoding N, P, L and M2-1 proteins and a plasmid containing the full-length antigenome, all under the control of T7 RNA polymerase. The T7 RNA polymerase is supplied to the cells by infection of the cells with a recombinant MVA expressing T7 RNA polymerase. The T7 RNA polymerase synthesises the vRNA and virus replication is initiated.

MVA: Modified vaccinia Ankara; RSV: Respiratory syncytial virus.

The first negative-sense RNA virus generated entirely from cDNA was described for the rabies virus [36]. Cells were cotransfected with a cDNA construct encoding the full-length rabies virus antigenome and L, P and N protein-expressing constructs, all under the control of the T7 RNA polymerase [36]. A recombinant vaccinia virus expressing T7 RNA polymerase [37] supplied the RNA polymerase for transcription of mRNA and genomic RNA. The key step in the procedure was expression of the antigenomic RNA, which was unable to hybridise with mRNAs encoding the L, P and N proteins. Other groups have used a cDNA construct that expressed positive-sense cRNA to rescue Sendai [38], human PIV (hPIV)3 [39] and Ebola viruses [40], although at a lower efficiency of rescue than for positive-sense cDNA transfections.

By contrast, rescue of segmented negative-sense RNA viruses is more difficult. The first demonstration of rescue for a segmented negative-sense RNA virus entirely from cDNA was achieved with Bunyamwera virus [41], and shortly thereafter with influenza viruses [42,43].

3. Manipulation of the genomes of respiratory viruses

Reverse genetics systems have been developed for many respiratory viruses, including RSV [44,45], influenza A [42,43,46,47], influenza B [48], hPIV2 [49], hPIV3 [39,50], hMPV [51,52], adenoviruses [53], rhinoviruses [54] and coronaviruses, including SARS [55,56].

3.1 Genetic manipulation of RSV, hMPV and hPIV

Systems that have been described for the rescue of RSV, hMPV, and hPIV2 and 3 are very similar; rescue of infectious recombinant viruses requires the coexpression of the nucleocapsid (N), phosphoprotein (P) and the large polymerase (L) proteins. Rescue of recombinant RSV (**Figure 1**) and hMPV also requires coexpression of the first open reading frame (ORF) of the M2 gene (M2-1) [51,57].

The RSV reverse genetics system described by Collins *et al.* [44] comprised a cDNA copy of the RSV antigenome with a T7 RNA polymerase promoter at the 5′ end,

a hammerhead ribozyme and a T7 RNA polymerase transcription terminator at the 3′ end. The hammerhead ribozyme sequence was included to allow for correct cleavage of the 3′ end of the antigenome.

The strategy for production of recombinant infectious virus from cDNA using these systems involved expression of antigenome, together with coexpression of the N, P and L (M2-1 was included for RSV and hMPV) proteins [44]. These proteins are necessary for the synthesis of antigenomic RNA from a genomic RNA template. Transfected cells are simultaneously infected with a modified vaccinia Ankara virus expressing the T7 RNA polymerase (MVA-T7). The MVA-T7 strain used is a host range-restricted mutant that replicates efficiently only in cells of avian origin; in mammalian cells there is a block at a late stage of virion maturation which significantly reduces yields of infectious virus [37]. Virus yields from these systems are low, although sufficient for the recovery of virus by further passage.

3.2 Genetic manipulation of influenza viruses

The generation of influenza viruses by reverse genetics is far more complex than for other negative-stranded viruses, as eight vRNA segments and four different viral proteins are involved. Furthermore, because viral RNA replication and transcription occur in the nuclei of influenza virus-infected cells, *in vitro* generated vRNP transfected to cells must be transported to the nucleus. The development of methods for the rescue of influenza viruses from cloned cDNA has been refined over the past decade to the point where it is now possible to rescue influenza entirely from cloned cDNA [42,43]. In the system reported by Neumann *et al.* [43], cDNA from each of the eight genome segments was cloned in a negative orientation between the human RNA polymerase I promoter and mouse RNA polymerase I terminator. Transfection of all eight plasmids into a human-derived cell line (the 293T line) resulted in vRNA synthesis by cellular RNA polymerase I. If these eight RNA-producing plasmids, together with nine protein-expressing plasmids (PB2, PB1, PA, HA, NP, NA, M1, M2 and NS2), are cotransfected in 293T cells, yields of $> 10^7$ plaque forming units (pfu)/ml of virus are produced within 48 h [43]. As helper virus is not required for the generation of recombinant virus, the cumbersome selection process described in earlier studies involving RNP was not necessary. Similar titres were also reported following transfection of the eight RNA-producing plasmids and four protein-expressing plasmids (PB2, PB1, PA and NP; **Figure 2**) [43]. A similar approach was taken by Fodor *et al.* [42]. They inserted cDNA copies of the eight genome segments between the human RNA polymerase I promoter and the hepatitis delta virus ribozyme. These plasmids, together with nine protein-expressing plasmids, were then transfected to Vero cells and yields of $10 - 20$ pfu/10^7 cells were obtained within 4 days.

Further improvements to these systems were reported by Hoffmann *et al.* [58,59], in which only eight plasmids were required. The plasmids contained cDNA of genome segments that had been cloned in a positive orientation with a human RNA polymerase I promoter at the 3′ end and the mouse RNA polymerase I terminator at the 5′ end. Upstream of the RNA polymerase I terminator was a cytomegalovirus (CMV) immediate-early promoter, and downstream a polyadenylation sequence. In this system the eight plasmids are transfected to a mixed culture of 293T cells (required for human RNA polymerase I) and MDCK cells. Cellular RNA polymerase I transcribes the cDNA into vRNA and the CMV promoter drives protein expression from each clone. Expressed viral protein and vRNA are then assembled and bud from the cell membrane as progeny virus. These progeny viruses then infect other (MDCK) cells, replicate and produce yields of $10^5 - 10^7$ pfu/ml within 72 h.

4. Potential of reverse genetics in developing improved vaccines

4.1 Improved influenza vaccines

With the development of techniques for the manipulation of influenza virus genes [42,43], it is now possible to prepare live influenza donor strains with specific properties of attenuation. Many examples exist in the literature where small changes to the genomes led to changes in virulence of the virus. One example of a specific change leading to attenuation is the exchange of the promoter region of the NA gene of influenza A virus with that of an influenza B gene. This change produced a virus that was attenuated for mice [60]. Another approach that could be used is insertion of several attenuating mutations from naturally attenuated viruses to a donor strain by reverse genetics, to produce a virus that is sufficiently attenuated and genetically stable [61].

Using reverse genetics it is possible to create viruses that contain deletions in specific genes, which are more stable than the single base changes seen in *ts* and *ca* viruses prepared by growth at reduced temperatures [62]. An example of this is the influenza NS1 protein, which has the ability to inhibit the host antiviral interferon response. Viruses lacking the entire NS1 gene are susceptible to the interferon response [63] and have been shown to be highly attenuated for mice [64]. However, mutants with large deletions may be overattenuated and less immunogenic for humans because they do not replicate sufficiently well in the respiratory tract [62]. An alternative approach may be to use viruses with truncated NS1 proteins. Viruses expressing only the N-terminal 99 or 126 amino acids of NS1 have been shown to possess intermediate interferon-inhibiting properties in mice [62] and may be more suitable for use as vaccine donor strains [62]. Another promising approach is the use of deletion mutants of the M2 gene. Elimination of this gene leads to a replication-defective virus that grows efficiently in cell culture, but poorly in mice [65].

Another approach developed by Brownlee's group at Oxford is the use of alternative base pairs in the conserved vRNA promoter regions [66]. Changing the conserved bases by reverse genetics gives rise to a virus that is attenuated. Attenuation has been demonstrated for the PA, NA and NS

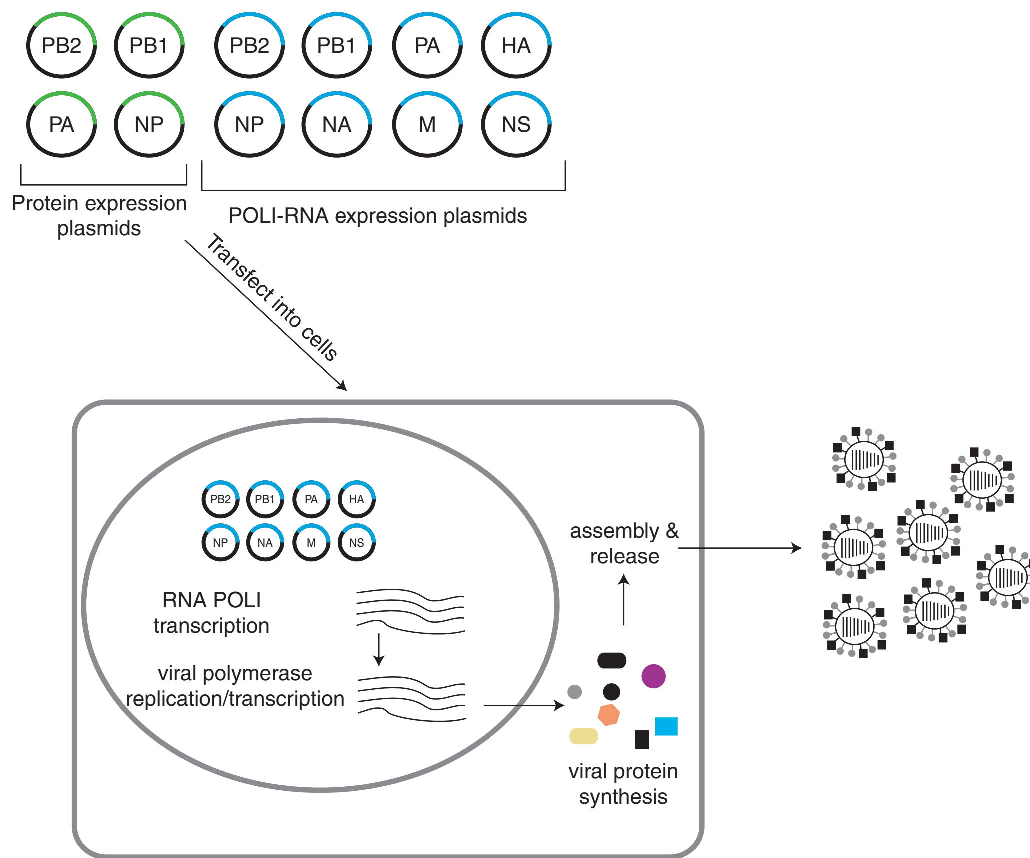

Figure 2. Schematic representation of the generation of influenza virus entirely from cDNA. Cells are cotransfected with plasmids encoding all eight segments of vRNA under the control of RNA polymerase I. Cellular RNA polymerase I synthesises vRNAs that are replicated and transcribed by the viral polymerase and NP proteins, all provided either by the four protein-expressing plasmids (pictured). An alternative approach involves the transfection of eight plasmids under the control of both RNA polymerase I and II for the production of both vRNA and mRNA.
POLI: RNA polymerase I.

genome segments and would be expected to lead to virus attenuation if introduced into any of the eight genome segments [66]. Such mutations may be useful in the development of donor strains for use in live vaccines.

Reverse genetics for influenza also has the potential to simplify the process and shorten the time required for inactivated vaccine preparation [46,67]. The influenza A viruses used consist of reassortants of a high-yielding donor strain and current epidemic strains. Reassortants are usually prepared by coinfection of eggs with an egg-adapted preparation of a current epidemic strain and a donor strain. Progeny virus is screened for the presence of reassortants that contain the HA and NA of the epidemic strain and the internal genes of the high-yielding parent that specify high growth [68]. These procedures are time-consuming and unpredictable; coinfection of eggs with two different viruses could, in theory, generate 2^8, or 256, different types of reassortant. Comprehensive screening procedures must be undertaken to select the desired reassortant for use as a vaccine seed, a process that takes 6 – 8 weeks [69]. Timelines for vaccine preparation are generally tight and are primarily dependent on the number of embryonated eggs required for growth of the vaccine virus. Egg supplies must be anticipated many months in advance. Potential problems that interrupt their supply or problems with vaccine manufacture can have a major effect on the timeline for vaccine delivery [70].

A method using reverse genetics described by Schickli *et al.* [46] involved transfection of eight RNA expression plasmids containing the HA and NA genes from epidemic strains and four protein expression plasmids containing the PB2, PB1, PA and NP genes prepared from the H1N1 virus A/WSN/33. All plasmids were used to transfect co-cultures of the 293T and MDCK cell lines. Progeny viruses possessed the surface antigens of the epidemic strain and the internal genes of the donor (i.e., they were 6:2 constructs), and had similar growth properties to viruses prepared by conventional reassortment in eggs using the PR/8 donor strain. Hoffmann *et al.* [67] described a modified method for virus rescue that required transfection to 293T and MDCK co-cultures by only eight plasmids. All rescued viruses grew to high titre in embryonated eggs and had the surface antigens of the parental epidemic strains [67].

The main disadvantage in the above application of reverse genetics lies in the use of the 293T and MDCK cell lines. 293T is a transformed human cell line and there have been concerns about the suitability of the MDCK line, so it seems unlikely that either would be licensed for use as a substrate for the preparation of human vaccines. Moreover, human RNA polymerase I promoters can only be used in cells of human or primate origin [71]. Ozaki *et al.* [71] investigated the possibility of using cells of the Vero line for transfection. The Vero line is derived from kidney cells of the African green monkey and has been licensed in the preparation of several human virus vaccines, including those used for the prevention of poliomyelitis [72,73] and rabies [74,75]. Cells of the Vero line support the growth of influenza viruses in the presence of trypsin [74,75] and have been used for the preparation of influenza vaccines [76-78]. These vaccines have been shown to elicit comparable antibody responses and higher cytotoxic T lymphocyte responses than those obtained from egg-grown vaccines [78].

A reverse genetics approach to vaccine production has been considered essential in the event of a pandemic caused by a new human influenza virus. Human infections with avian influenza strains have been observed in 1997 (H5N1), 1999 (H9N2), 2003 (H5N1 and H7N7) and 2004 (H5N1 and H7N3). Of these influenza A subtypes, H5 and H7 viruses have been associated with highly pathogenic infections [79]. These highly pathogenic strains are lethal for chickens and chicken embryos [80-82]. Lethality has been associated with the presence of a multiple basic amino acid motif adjacent to the cleavage site of the HA glycoprotein. The presence of this motif increases the range of target organs that can support the growth of these viruses because they contain endogenous proteases [80]. Unless this cleavage site is removed, it would not be possible to grow vaccine viruses in embryonated eggs and alternative methods of cultivation that include cell culture would have to be used. In addition, the large-scale growth of unmodified viruses constitutes a potential public health problem. In an approach to produce suitable vaccine candidates against highly pathogenic influenza strains, Subbarao *et al.* [83] and Webby *et al.* [84] have produced viruses by reverse genetics, in which the multiple basic amino acid motif was removed from the 1997 Hong Kong and 2003 Asian viruses of the H5N1 subtype, respectively. These viruses were prepared with the modified HA and NA derived from the H5N1 avian strain and the internal genes from A/PR/8/34, and were replicated to high titre in embryonated eggs. Viruses containing deleted HAs were shown to be attenuated compared with the wild-type avian parent, which was lethal for chickens and mice [83]. Subbarao *et al.* [83] reported that vaccination of mice with a formalin-inactivated preparation of the mutant 1997 reassortant virus resulted in an immune response that was comparable to that of a formalin-inactivated preparation of the H5N1 avian parent [83].

4.2 RSV vaccines

Although RSV was first isolated in 1956 [85], all attempts so far at producing a suitable vaccine have been unsuccessful. A formalin-inactivated RSV (FI-RSV) vaccine was tested in infants and children in the 1960s and found to be poorly protective [86]. Quite unexpectedly, an increased frequency of infection and enhanced severe RSV illness following subsequent infection was observed in vaccinated individuals compared with unvaccinated children [22,23,87].

Purified native F and G glycoproteins have been studied as potential vaccine candidates. Proteins have been purified from various expression systems using mammalian cells, insect cells and bacteria, as well as from RSV-infected mammalian cell cultures using immuno-affinity and other chromatographic methods. These protein vaccines have been evaluated extensively and studies suggest that purified protein vaccines may also induce an altered T cell response similar to that of FI-RSV [88-91].

Development of live attenuated RSV vaccines began in the 1970s using the conventional techniques of multiple passage and chemical mutagenesis [92]. The most promising of these strains, a cold-passaged RSV A2 strain, is the moderately attenuated strain *cp*-RSV. This strain was further mutated by two rounds of chemical mutagenesis to produce temperature-sensitive (*ts*) mutants. These strains were evaluated in both seropositive and seronegative children, and one, *cpts*-248/404, was shown to be sufficiently attenuated to undergo testing in RSV-negative infants 1 – 2 months of age [19]. When tested in this group, 80% of vaccinated infants shed moderate levels of virus, developed significant rises in RSV-specific IgA and were highly resistant to re-infection with a second dose of vaccine virus given 1 month later [19]. However, one infant shed low amounts of vaccine virus with a partial loss of the *ts* phenotype and some loss of attenuation, indicating that the attenuating mutations were unstable.

The use of reverse genetics has allowed the generation of RSV mutants with defined molecular changes to the viral genome. As a consequence, it is possible to define the genetic basis of attenuation for the existing *cp*-RSV and *cpts*-RSV mutants, and the generation of vaccine candidates with genetically defined mutations [93]. A further approach has been to produce mutants containing deletions of non-essential genes for the development of highly stable attenuation characteristics [93]. Five RSV genes, NS1, NS2, SH, G and M2-2, are regarded as almost non-essential, in that deletion or silencing of any of them has little effect on virus growth in culture [94-102]. Deletion of the G gene, one of the major neutralising antigens of RSV, would be unlikely to produce a suitable vaccine strain, as viruses lacking the G gene have been shown to be overattenuated for mice, with little virus replication occurring in the upper respiratory tract [97]. In addition, a cold-passaged RSV B virus, with a resulting deletion of most of the SH and G ORFs, was tested in primates and clinical trials and was found to be overattenuated in seronegative infants [103].

Other deletion mutants produced different levels of attenuation for chimpanzees. Deletion of either the SH or NS2 gene produced a virus less attenuated than *cpts*-248/404, and deletion of NS1 and M2-2 produced viruses that were more

attenuated [96]. Gene deletion, therefore, appears to be a promising method of attenuation in vaccine development and allows the possibility of combining with other methods of attenuation. Another approach is the expression of cytokine or chemokine genes from within the virus genome [104]. An example is a recombinant RSV expressing granulocyte-macrophage colony-stimulating factor [105]; intranasal inoculation of mice results in a dramatic increase in pulmonary dendritic cells and macrophages [105].

4.3 Human parainfluenza viruses (hPIV1, 2 and 3)

Initial attempts to produce parainfluenza virus vaccines involved inactivated hPIV1, 2 and 3 viruses prepared from infected embryonated eggs or primary monkey kidney cell culture. This vaccine was shown to be immunogenic in children, inducing serum HI and neutralising antibody, but did not provide protection against disease [106].

Subunit vaccines, preparations of the hPIV3 HN and F glycoproteins isolated from purified hPIV3 or from insect cells infected with recombinant baculoviruses expressing the HN or F protein, have also been evaluated [107,108]. Immunisation of cotton rats with these viral glycoproteins induced satisfactory levels of neutralising, HI and FI antibodies, providing protection against intranasal challenge. However, these subunit vaccines have not been tested in humans or non-human primates.

Two different approaches have been used in the development of live hPIV vaccines. The first involved the use of bovine PIV3 (bPIV3), a virus which is naturally attenuated in the respiratory tract of rhesus monkeys and chimpanzees [109]. In clinical trials, bPIV3 was administered intranasally to 50 infants and children 2 – 60 months of age [110]. This candidate vaccine was well-tolerated and appeared to be satisfactorily attenuated, and induced a protective antibody response against hPIV3 infection. The second involved cold passage of a virulent strain of hPIV3 [20]. A promising vaccine candidate, *cp*45, was developed by 45 passages at progressively reduced temperatures. This virus was cold-adapted, temperature-sensitive and attenuated for hamsters, monkeys and humans [111]. Clinical evaluation in infants 1 – 2 months of age demonstrated this candidate strain to be both suitably attenuated and immunogenic [112].

The development and characterisation of live attenuated hPIV vaccines have been greatly facilitated by the application of reverse genetics, which allows attenuating mutations in the vaccines to be differentiated from spontaneous mutations. These approaches also allow the modification of existing strains to produce improved vaccine candidates.

Reverse genetics systems were not available for hPIV1 or 2 until comparatively recently. As a consequence, vaccine candidates consisting of chimeric viruses were prepared, in which the F and HN ORFs of hPIV3 were replaced by the corresponding sequences of hPIV1 or 2 [113-115]. The resulting chimeric viruses were shown to be immunogenic for hamsters. It may, therefore, be possible to develop a polyvalent vaccine against hPIV1, 2 and 3 using antigenic chimaeras prepared from the hPIV3 *cp*45 backbone.

An additional application of reverse genetics to hPIV vaccine development involves the construction of novel mutations for attenuation that do not exist in nature, such as C or V protein knockout mutations that attenuate virus growth in hamsters and monkeys [116]. Another approach is the construction of chimaeras that contain a mixture of internal protein genes of hPIV3 and bPIV3. In one chimaera the NP ORF of hPIV3 was replaced with that of bPIV3; the resulting virus exhibited host-range restriction in monkeys typical of bPIV3 [117].

5. Issues with vaccines prepared by reverse genetics

Issues concerning intellectual property rights have the potential to restrict the wider application of reverse genetics to the preparation of vaccines. For example, with influenza viruses, the overall patent rights on reverse genetics have been assigned to MedImmune, Inc. (Gaithersburg, MA, USA), although other individuals and companies hold subsidiary rights to individual patents. All patent holders have allowed ready access to this technology for research, but have indicated that they intend to claim future royalties for any commercial use by vaccine manufacturers.

In addition to concerns relating to intellectual property, a further major obstacle to the use of such vaccines could be their classification in certain jurisdictions as genetically modified organisms (GMOs) [79], which does not apply to vaccines prepared by traditional methods. Classification of a vaccine candidate as a GMO could mean the imposition of additional safety tests before release [79]. Despite these difficulties, it is widely believed that because of the continuing threats from pandemic influenza and the expanded need to prepare influenza vaccines, reverse genetics will be used for the preparation of influenza vaccine seeds in the near future [79]. Vaccine candidates for other respiratory viruses prepared by reverse genetics will probably be tested in humans within the next 5 – 10 years.

6. Expert opinion/possibilities for the future

Reverse genetics, although still a relatively new tool for the manipulation of negative-strand RNA viruses, is widely regarded as having great potential for the preparation of vaccines against many of the common respiratory viruses. Reverse genetics systems allow the direct modification of the genome of negative-stranded RNA viruses. They provide a powerful means for studying virus replication and assembly by allowing the function(s) of viral proteins to be studied after modification or deletion of single or multiple genes. Future applications should provide much more information than is available at present on the role of individual genes in virulence and on the mechanisms of viral pathogenicity.

This will allow a more rational basis for the selection of live vaccine strains than has been used in the past, where selection has been often based on purely empirical criteria. For inactivated vaccines against influenza, reverse genetics could be used to more precisely identify specific sequences concerned with enhanced growth of a vaccine reassortant in eggs or cell culture, which could contribute significantly to the cost and availability of pandemic vaccines. However, because vaccines produced by reverse genetics are classified as GMOs in some countries, extensive safety testing will be required and much debate will ensue before they will be licensed for human use.

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