Fowlpox virus as a recombinant vaccine vector for use in mammals and poultry

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Live vaccines against fowlpox virus, which causes moderate pathology in poultry and is the type species of the *Avipoxvirus* genus, were developed in the 1920s. Development of recombinant fowlpox virus vector vaccines began in the 1980s, for use not only in poultry, but also in mammals including humans. In common with other avipoxviruses, such as canarypox virus, fowlpox virus enters mammalian cells and expresses proteins, but replicates abortively. The use of fowlpox virus as a safe vehicle for expression of foreign antigens and host immunomodulators, is being evaluated in numerous clinical trials of vaccines against cancer, malaria, tuberculosis and AIDS, notably in heterologous prime–boost regimens. In this article, technical approaches to, and issues surrounding, the use of fowlpox virus as a recombinant vaccine vector in poultry and mammals are reviewed.

Expert Rev. Vaccines 4(1), 63–76 (2005)

CONTENTS

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KEYWORDS:
avian influenza virus, avipoxviruses, DNA prime–boost vaccination, infectious bursal disease virus, interleukin-18, live attenuated vaccine viruses, Newcastle disease virus, nonreplicating poxvirus vectors

Fowlpox virus (FWPV) is the best-studied member and type species of the avipoxvirus genus of the *Poxviridae*. This avian virus was seized upon rapidly when techniques became available to construct recombinant poxviruses, with the hope of generating novel poultry vaccines. However, this virus now occupies an important niche in the development of novel vaccines against important human diseases such as cancer, malaria, tuberculosis and AIDS, as well as significant livestock diseases, such as bovine tuberculosis.

This review is addressed to all who are interested in using FWPV as a recombinant vector, particularly for vaccination and especially for those intending to use it in mammals, including humans. This review presents an introduction to specific aspects of FWPV and the generation of recombinant (r)FWPV, general aspects of the use of poxvirus vectors as vaccines having been reviewed previously [1]. Some examples of the use of rFWPV vaccines in poultry are provided, along with discussion of relevant issues and problems, although this review is neither intended to promote the agricultural use of such recombinant vaccines or provide a detailed and exhaustive critique of their use. Attempts to translate the technology for possible use in mammals, including humans, are described, highlighting the issues relevant to that sector. Numerous clinical trials are currently underway, or planned and thus references are provided to sources of up-to-date information and to published results of completed trials.

Although avipoxvirus infections have been reported to affect approximately 230 of the 9000 species of birds [3], little is known regarding the nature and phylogenetic relationships of the viruses causing those infections. Members of the avipoxvirus genus (canarypox, fowlpox, juncopox, mynahpox, pigeonpox, psittacinepox, quailpox, sparrowpox, starlingpox and turkeypox viruses [2]) do not cause disease in mammals, although they do cause mild-to-severe disease in birds. Fowlpox in chickens, spread mechanically by biting insects, is relatively mild in its cutaneous form but causes higher mortality in its diphtheritic form, due to occlusion of the oropharynx. In contrast, canarypox is highly virulent in canaries. These differences may represent a longer coexistence of FWPV with its chicken-like host and more

10.1586/14760584.4.1.63

ISSN 1476-0584

recent spread of canarypox virus to canaries from its normal avian host. Alternatively, it may be that the differences in virulence represent adaptations to hosts with very different lifestyles but these issues cannot be resolved without more knowledge of the molecular phylogeny and epidemiology of these viruses.

Vaccination against fowlpox was reported as early as 1928, using live FWPV or Pigeonpox virus [4], which are now known to be closely antigenically-related to FWPV. Monoclonal antibodies against the three major immunodominant structural antigens of FWPV virus have been isolated and characterized [5]. The genome sequences of the standard US Department of Agriculture (USDA) challenge strain of FWPV [6] and of an extensively culture passaged, attenuated, plaque-purified virus, FP9 [7], have been determined. The sequences have confirmed the extensive divergence and considerable differences between the avipoxviruses and mammalian poxviruses, typified by vaccinia virus. In fact, phylogenetic analysis demonstrates that avipoxviruses are most closely related to the molluscum contagiosum virus of humans. Avipoxviruses share several features with this virus, including the location of an ortholog of the large variola virus Bangladesh gene *B22R* between orthologs of vaccinia virus genes *E4L* and *E6R*, rather than at the near terminal location at which it is found in the other mammalian poxviruses (it is absent from vaccinia virus).

FWPV lacks orthologs of several vaccinia virus genes encoding envelope proteins, namely A33R, A36R, A56R and B5R, retaining only orthologs of F13L and A34R (as well as F12L). FWPV (i.e., canarypox virus and molluscum contagiosum virus) also lacks an ortholog of vaccinia virus A27L, which is required for envelopment and egress. Together, this probably explains the observation that the extracellular enveloped virus (EEV) particles of FWPV appear to be formed only by budding [8], whereas vaccinia virus EEV can be formed by exocytosis and budding [9,10]. It is apparent that there may be other major differences between vaccinia virus and FWPV in the function of some of their structural proteins [5,11]. There are also significant differences between vaccinia virus and FWPV in their complement of known or putative immunomodulators. FWPV (in common with canarypox virus and molluscum contagiosum virus) lacks orthologs of E3L and K3L interferon (IFN) resistance genes. The avipoxviruses encode proteins with strong similarity to transforming growth factor-β, as well as several proteins with similarity to chemokines and chemokine receptors. No genes encoding type I IFN-binding proteins have yet been identified in the avipoxviruses.

Host-range restriction of avipoxviruses

It is generally accepted that the avipoxviruses cause disease only in avian species, having been isolated only once from a mammal, from the skin of an already sick rhinoceros in a zoo [12]. The absence of FWPV replication and pathogenesis in mice, with only some mild pathology following intranasal inoculation, was reported as early as 1941 [13]. It is also generally accepted that avipoxviruses are unrestricted for entry to most mammalian cell types, but that they are restricted for productive replication in mammalian cells [14,15].

Early history of FWPV recombinant vaccines

Systems for isolating recombinant vaccinia virus were developed in the early 1980s [16], bringing the prospect of developing whole new generations of vaccines based on recombinant viruses. Shortly after, considerable interest emerged in developing FWPV as an equivalent recombinant vector for use in poultry [17–21]. This interest was broadened to use in mammals following the seminal and surprising observation by Taylor and colleagues [22], that rFWPV could enter mammalian cells, express foreign antigens and induce protective immunity in mammals. A subsequent study with canarypox virus indicated that this might be a common attribute of the avipoxviruses [23]. The stage at which replication is blocked in mammalian cells appears to differ depending on the cell type. For instance, FWPV DNA replication and late gene expression occurs in African green monkey Vero and CV-1 fibroblast cells, with replication blocked during virion morphogenesis. In contrast, only early gene expression occurs in human epithelial HeLa cells but some late expression was observed in human diploid MRC-5 fibroblast cells [15]. Since then, numerous recombinants of FWPV, and more frequently canarypox virus, have been designed to express antigens in mammalian cells and mammalian hosts. Some of these have progressed through veterinary and human clinical trials, and have thus undergone the required toxicity testing in animals with no reports of significant adverse effects [24].

Construction of recombinant FWPVs

FWPV strains

During the first few decades of the 20th century, considerable work on FWPV and the other avipoxviruses was conducted. By the late 1920s, vaccination against fowlpox was not uncommon. A fascinating insight into this early activity is provided by Beaudette [25]. However, a consequence of this activity is that our knowledge of the origins, histories and inter-relationships of viruses that subsequently found their way into academic laboratories and commercial vaccine producers is somewhat murky, particularly as the early vaccine producers were, as today, frequently spin-offs of academic activities. Much of the knowledge of the commercial strains is, even today, relatively inaccessible, complicated by the frequent acquisitions and mergers in this sector. However, vaccine strains of FWPV have been available from CEVA Laboratories, Cyanamid Webster (formerly Arthur Webster), Fort Dodge, Intercontinental Laboratories, Intervet, Merial, Schering-Plough, Select Laboratories, Solvay, Syntro-Zeon and Vineland Laboratories, among others. Some of these vaccines may have originated from isolates collected early in the 20th century, however, it has been reported that the Cyanamid Webster vaccines were derived from more recent field isolates collected in the mid 1960s [26]. Several of these commercial vaccine strains were the source material for the development of rFWPVs.

Arguably, FP9 is the best characterized of the FWPV strains used for recombinant vaccine purposes, with a well-documented history in academic publications. In addition, its

genome has been completely sequenced allowing identification of all of the differences (including deletions totaling 22 Kbp) between this strain and the USDA standard challenge virus [7]. Despite being described in the original sequence publication as pathogenic, the USDA describes its challenge virus as being derived from a fowlpox vaccine manufactured by a commercial firm in the early 1960s [6]. FP9 was obtained in the late 1980s by Tomley, Binns, Boursnell and Brown at the IAH Houghton Laboratory (St Ives, UK). It was derived by plaque purification of a virus that had been passaged some 438 times in chick embryo fibroblast (CEF) culture by Anton Mayr, the source isolate being HP-1 Munich [27]. Mayr demonstrated that any residual virulence (even for day-old chicks) had been lost by passage 350 [28]. Comparison of the genome of FP9 with partial sequence from its pathogenic progenitor, HP-1, allowed discrimination between the relatively mild mutations that marked differences between the US and European viruses and the more severe mutations that had occurred during tissue culture passage in the generation of FP9 from HP-1 [7].

Promoters

Vaccinia virus promoters appear to function well as FWPV promoters, although the levels of expression for the same gene from the same promoter are lower for rFWPVs than for recombinant vaccinia viruses, including modified vaccinia virus Ankara (MVA), even in CEFs [UNPUBLISHED DATA]. The vaccinia virus p7.5 early/late promoter is frequently used in rFWPVs, as are vaccinia virus-optimized synthetic early or early/late promoters. A very useful promoter element was first identified in FWPV by Kumar and Boyle [29]. This is a 38bp bidirectional early/late and late promoter, which drives the expression of the FWPV orthologs (FPV169 and FPV168) of vaccinia virus A5R and A4L. One would predict that an equivalent element should be present in other poxviruses. Other similar elements, which may prove useful in vector design, have also been identified in FWPV [30]. The promoter from FPV142, the ortholog of vaccinia virus H5R encoding VLTF-4, was demonstrated to have six-times the early promoter activity of the vaccinia virus p7.5 early/late promoter in FWPV-infected cells [31]. The late promoter from the FWPV homolog (FPV167) of vaccinia virus p4b has been used to direct expression of the *lac*Z gene used as a marker [32].

Sites for the insertion of foreign sequences

A major strength of the poxviruses as vectors is their large capacity for foreign gene insertion. The insertion sites (frequently identified as nonessential regions) that have been used to date are described, however, the authors believe that there are many more potential sites. As an illustration of this, six deletions (from 1.5 to 9 Kbp) resulted in the loss of 22 Kbp of genomic sequence from FP9 during its passage history in culture, removing or severely disrupting coding sequences for some 25 genes. From this alone, it may be reasonably inferred that 22 Kbp could be inserted at the 6 loci, in the same way that the major sites of deletion have been used as insertion sites for the construction of MVA recombinants. The insertion loci described below clearly extend both of these figures.

Although the thymidine kinase (TK) locus, a common insertion site for vaccinia virus, was used by several groups for construction of FWPV and other avipoxvirus recombinants, others reported problems in either obtaining recombinants or maintaining them at this insertion site [33,34]. Moreover, the lack of TK–CEFs removes the advantage of this site, since bromodeoxyuridine (BudR) selection cannot be used. It was later demonstrated that the FWPV TK gene (FPV086) is required for efficient FWPV replication [35], which is likely to be a contraindication for the use of this locus. However, a site immediately downstream of FPV086 has been used successfully [36].

A frequently used insertion site in FWPV FP9 has been the unique *Bgl*I site in open reading frame (ORF)1 of pB3ME [37], which carries the terminal 6 Kbp *Bam*HI restriction fragment of the FP9 genome [38]. This ORF is now known as FPV002. The FPV002 insertion site is used in the recombination plasmid pEFL29, which has had a good track record for successful recombinants since being used for expression of an avian pneumovirus fusion protein [32]. Expression of the foreign gene, inserted at a *Sma*I site, is driven from the vaccinia virus p7.5 early/late promoter and that of the *lac*Z marker from the FPV167 late promoter. There have been concerns regarding the stability of inserts within the terminal regions of poxviruses as these regions are highly variable and rearrangements have been observed in parental viruses. However, inserts in FPV002 in FP9 appear stable in laboratory conditions and during commercial propagation of recombinant vaccine for medium-scale clinical trials. This may be a consequence of the extensive passage history of FP9 in culture. As FWPV does not replicate in mammalian cells, the stability of the insert in vaccinated mammals should not be an issue. The long-term stability of FPV002 inserts in vaccinated poultry has not yet been addressed.

The FPV110 gene (ortholog of vaccinia virus F11L) has been used as an insertion site for a melanoma antigen [5] and insertion into the adjacent FPV109 gene (ortholog of vaccinia virus F12L) results in smaller plaques [39], offering the possibility of a selection system based on plaque size. Several other FWPV genes have directly demonstrated to be nonessential and therefore represent candidate insertion sites including; FPV030, FPV032 and FPV033 [40], FPV054 and FPV055 [41] and FPV158 [42]. Genes have also been inserted between FPV202 and FPV203, at the site of the reticuloendotheliosis virus (REV) long-terminal repeat (LTR) [43].

Insertion of foreign gene sequences

The large size of the genome of avipoxvirus genomes generally precludes direct DNA manipulation. Bacterial artificial chromosome vectors have not yet been constructed for avipoxviruses, although one has been made for vaccinia virus [44]. FWPV is becoming commonly used as a helper virus to rescue directly manipulated vaccinia virus, as the helper can be easily removed by passage through mammalian cells [45]. The authors are not

aware of any other helper virus that could similarly be purified away from rescued recombinant avipoxviruses by passage through the avian cells to which avipoxviruses are restricted. Recombinants are therefore isolated by DNA recombination between the virus genome and circular or linear DNA transfected into the avipoxvirus infected cell, relying on effective methods for selection or screening.

Recombinants from circular DNA can be obtained by single crossovers, integrating whole plasmids to form unstable viruses that subsequently resolve to stable viruses by a second crossover event, or by rarer double crossovers. Linear DNA, which can be obtained either by linearizing plasmids or by polymerase chain reaction amplification of appropriate templates, can only be integrated by a double crossover event.

Insertion of foreign gene sequences

The *Escherichia coli gpt* gene, conferring resistance to mycophenolic acid, is a useful selective marker, although its transcription needs to be driven by an early promoter. Immune responses may therefore be induced against it in vaccinees. However, *gpt* has been used as a marker in human Phase I clinical trials.

The *lac*Z gene (encoding β-galactosidase) has proved to be a convenient marker at any insertion locus, allowing identification of recombinant plaques which can be stained blue. Late expression of the *lac*Z marker (from the FPV167 promoter) is useful as there is consequently little immune response to the β-galactosidase in vaccinated animals [UNPUBLISHED DATA]. Moreover, β-galactosidase has been acceptable as a marker for vectors used in human Phase I clinical vaccine trials.

Fluorescent proteins (such as green fluorescent protein [GFP]) are becoming more popular as markers, allowing rapid isolation of cells carrying recombinant viruses by fluorescent flow cytometry [46]. This marker can be expressed from early or late promoters.

Careful consideration needs to be given to the choice of marker for isolation of recombinants. The requirement for the marker is likely to be evaluated as part of the review by national clinical trial authorities. The outcome may depend on national policy and precedent. It is possible that a broader range of markers may be acceptable for Phase I clinical trials than for Phase III trials. Finally, however, the marker will have to be approved by appropriate authorities responsible for licensing products in the selected markets.

It is possible to isolate recombinant viruses that do not carry marker genes. Such markerless viruses can be isolated using the transdominant selection method in which the *gpt* marker is used only to select an unstable intermediate virus, before the marker is lost by spontaneous recombination upon removal of the selection [47]. A similar approach can be used with linear DNA recombination constructs if the selection cassette is flanked by repeats. After the selectable marker has been used to isolate unstable intermediates, the selection can be removed allowing spontaneous elimination of the selection cassette [5].

For preclinical studies and Phase I clinical trials, it is advisable to monitor the recombinant virus at all stages, for the presence of the antigen gene (and/or expression of the antigen). Failure to perform such checks has resulted in the failure of some preclinical studies. The *lac*Z marker offers convenient monitoring, via blue/white plaque screening, for the presence of the linked antigen gene. The monitoring of viruses carrying markerless, *gpt* or GFP constructs is not as convenient,

Recombinants may have been isolated (and possibly subjected to Phase I clinical trials), using markers that might subsequently be deemed unnecessary or undesirable for late-stage trials or for clinical products. In this case there are methods for removal of the marker, such as the transdominant selection method described above [47].

Propagation of rFWPV

FWPV replicates only in avian cells, indeed, some vaccine strains, such as the Cyanamid Webster FPV-M vaccine, even display a preference for chick embryo skin (CES) cells over CEFs. FWPV FP9 has been effectively adapted to CEFs but has a distinct preference for primary as opposed to secondary CEFs [UNPUBLISHED DATA]. It fails to plaque and replicates poorly in the recently derived chicken fibroblast cell line, DF-1 [UNPUBLISHED DATA]. Replication is similarly poor in the chemically transformed cell line OU-2. It can be plaqued and replicated quite efficiently in quail cell lines, such as QT-35, but the presence in these cells of viable endogenous Marek's disease virus (MDV) means that their use for preparation of vaccines is not advisable [48]. Isolation of an MDV-free quail cell line would be a useful solution to this problem, and one such line has recently been isolated [49]. The potential of avian stem cell lines (termed EBx cells) for the propagation of avipoxviruses is currently being actively explored, particularly in the commercial sector. Of these possible cell substrates for avipoxvirus propagation, currently only CEF and CES cells are licensed for use in the production of human vaccines.

A recent publication describes the replication of avipoxviruses, the sources of which were not described, in embryonic bovine tracheal cells. Until corroborated, this report should be treated with some caution as replication was defined only by the presence of cytopathic effects, the cause of which was not confirmed by serologic or sequence analysis [116].

Recombinant FWPV vaccines for poultry

A number of important poultry viral pathogens became early targets for rFWPV vaccines, notably; avian influenza virus, Newcastle disease virus, infectious bronchitis virus, MDV and infectious bursal disease virus. In general, progress was swifter and more successful against the enveloped virus pathogens (such as avian influenza virus, Newcastle disease virus, infectious bronchitis virus and MDV) than against nonenveloped viruses (such as infectious bursal disease virus). Many factors might influence such success, in particular, the choice of antigen and pathogenesis of the disease are overriding considerations. It is likely, however, that this was due to the fact that protection against many of the viruses normally relies on humoral immunity, which generally requires that the proteins be expressed in native conformations.

Experience with poxvirus vectors demonstrates that viral envelope glycoproteins (gps) of foreign pathogens can frequently be expressed from vectors in isolation from other proteins of the pathogen and presented on the surface of the cell in a native conformation (this is not always the case and expression of accompanying pathogen proteins may also be required). Stimulation of humoral immunity against nonenveloped viruses using rFWPVs is clearly more problematic [50,51]. Coexpression of their capsid proteins is often required such that the proteins can fold and assemble into a capsid to adopt their native conformation. Even then, expression of empty capsids may be ineffective at stimulating humoral immunity, since the capsid proteins may only adopt their native conformation (capable of inducing protective antibodies) when the capsids mature on entry of the viral genomic RNA into the capsid or on export of virus from the cell.

It is not just viral pathogens of birds that have been the target for FWPV recombinants. Following the identification of a protein, mg*c*3, from *Mycoplasma gallisepticum* that induced protective antibodies, a FWPV recombinant has been constructed to express the protein [52]. However, most efforts have focused on viral diseases of poultry.

Recombinant vaccines against enveloped viruses of poultry

In the USA, rFWPV for Newcastle disease virus [37,53–56] and avian influenza virus [57,58] were produced in the early 1990s and have been licensed for commercial use by two companies (Merial and Syntro-Zeon). In addition, rFWPV against avian influenza has also been licensed for use by Merial in Mexico; indeed, 'since 1997, Mexico has used approximately 708 million doses of a killed H5N2 vaccine and an additional 459 million doses of a recombinant fowlpox-H5 vaccine in their H5N2 control program' [59]. rFWPV effective against MDV, turkey rhinotracheitis virus and reticuloendotheliosis virus have also been developed [60]. This is not to say that efforts to develop rFWPV vaccines against all enveloped viruses have been successful; attempts to derive effective vaccines against infectious bronchitis virus have met with only limited success [SKINNER, UNPUBLISHED DATA, [61]. It appears that expressing just the single coronavirus spike gp in a heterologous context was problematic, although it was possible to express the infectious bronchitis virus spike protein and stimulate the production of neutralizing antibodies in mice using vaccinia virus [62].

Recombinant vaccines against nonenveloped viruses of poultry

A large fragment of the VP2 capsid protein of infectious bursal disease virus was expressed as a β-galactosidase fusion protein in the FWPV recombinant fpIBD1 [51]. Vaccination with fpIBD1 did not induce the production of detectable antibody to VP2 but did result in protection of chickens against mortality subsequent to infectious bursal disease virus infection, although it did not protect from infection *per se*. It is likely that the protection against mortality is due to the induction of cell-mediated immunity rather than the humoral immunity normally induced after infection with infectious bursal disease virus (or with inactivated vaccine).

Humoral immunity has been induced against avian hemorrhagic enteritis virus (an adenovirus), but protection studies have not been reported, nor has there been any assessment of cell-mediated immunity [50].

Problems & limitations with the use of rFWPV in poultry

Pre-existing immunity to FWPV

One problem with using live vaccines, be they attenuated versions of the pathogenic agent or live recombinant vectors, is that pre-existing immunity may prevent the establishment of successful immunity, perhaps by preventing the establishment of infection by the live vaccine. The pre-existing immunity may be active in the vaccinee due to prior infection (humoral and/or cell-mediated immunity) or may be maternally derived immunity (humoral only). Previous vaccination of flocks with FWPV can apparently cause problems, at least using influenza rFWPVs; the influenza H5 rFWPV, vFP89, constructed by Taylor and colleagues [20], gave inconsistent protection against influenza after prior FWPV vaccination [63]. It is not known whether this was due to humoral immunity, cellular immunity or both. If humoral immunity was a factor then it could have implications for the vaccination of chicks with FWPV recombinants if the hen had been vaccinated against FWPV (due to the presence of maternal antibody). Whatever the mechanism of immunity, previous field exposure to circulating FWPV might also reduce the efficacy of the rFWPV vaccines. However, maternal immunity to FWPV or Newcastle disease virus did not prevent birds subsequently vaccinated with the recombinant from acquiring lifelong protective immunity to Newcastle disease virus, even if this was only 8 weeks for commercial broilers, concomitant with a slight fall in antibody titers [64].

Variant FWPVs

There are reports that outbreaks of fowlpox have arisen within flocks previously vaccinated with FWPV or pigeonpox virus vaccines [65]. This may be indicative of antigenic variation within the isolates, assuming of course that the vaccines had been stored and administered in an appropriate and effective manner. Virtually nothing is known about protective epitopes for humoral or cellular immune responses in FWPV and there has only been preliminary characterization of the variant isolates [65]. Monoclonal antibodies are now available against three major immunodominant FWPV antigens, allowing some discrimination between different FWPV (based on apparent molecular masses of the antigens), however, the monoclonal antibodies do not appear to be neutralizing [5,66].

Host genetics

A role for host genetics in the response against infectious bursal disease virus has been demonstrated [67]. Vaccination with fpIBD1 did not protect the inbred white leghorn chicken strain, line 15I, from infectious bursal disease virus-induced bursal damage (even at the lowest titer of challenge virus used) but did protect all other inbred white leghorn chickens examined (line 6[1], C.B4 and C.B12), as well as outbred Rhode Island Red

chickens. It remains to be seen whether the enhanced immunity induced by coexpression of chicken interleukin (IL)-18 with infectious bursal disease virus VP2.

Little is known regarding the influence of host genetics on the pathogenicity of FWPV, or of its function as a recombinant vaccine vector. It has recently been demonstrated that host genetics may play an important role in the efficacy of the vaccine, albeit possibly dependent on the antigen [68]. The study compared the efficacy of three rFWPV, each expressing the MDV gB antigen from MDV serotype 1, 2 or 3. The B*21/*21 chickens were protected more effectively by each of the three rFWPV vaccines than were the B*5/*5 or B*13/*13 chickens. The gB2 vaccine protected B*13/*13 chickens more effectively than B*5/*5 chickens, whereas the gB1 and gB3 vaccines provided similar protection to B*5/*5 and B*13/*13 chickens.

Both of the above studies were performed using inbred flocks. It is unclear how important such effects might be in commercial flocks. Moreover, such effects are unlikely to be restricted to poxvirus vectors and are likely to be observed with other types of vaccination, using vectors or otherwise.

Vaccine delivery in poultry

Although vaccination against a wide range of agents is routinely used in all sectors of the poultry industry, the difficulties of applying vaccines to extremely large numbers of birds poses major problems, unless the vaccine can be distributed in drinking water or as an aerosol. Application of FWPV vaccines to broiler breeders and commercial layers has traditionally involved wing-web scarification at 10–12 weeks of age. High titer vaccine (in this case, HP1 at passage level 200) can be introduced by the aerosol route [69], although this probably does not apply to more highly passaged and attenuated viruses such as FP9 [UNPUBLISHED DATA]. Application of the virus via drinking water appears to be even less reliable [70,71]. Thus, it is likely that effective vaccination with rFWPV will require introduction via scarification [72]. A recent innovation in poultry vaccination has been the development of methods for mass *in ovo* vaccination. A field trial of a multivalent *in ovo* vaccine, which includes a FWPV expressing Newcastle disease virus F and HN genes, has recently been reported [73], with promising protection achieved against both FWPV and Newcastle disease virus. However, it is by no means clear whether *in ovo* vaccination will be appropriate for all recombinant vaccines.

Coexpression by rFWPVs of host immunomodulators in poultry

Initial studies in mammals demonstrated that coexpression of host-derived cytokines, notably IFN-γ and other T-helper (Th)1-associated cytokines, by recombinant poxviruses was advantageous in the induction of immunity. Compared with mammalian cytokines, chicken cytokines have only been identified relatively recently, mainly due to high sequence divergence from their mammalian counterparts. Experiments exploring the potential of coexpressed host immunomodulators to improve the immunogenicity of rFWPVs in poultry are now, however, becoming more common. Coexpression of type I IFN reduced postvaccination loss of body weight but also reduced antibody induction against Newcastle disease virus [74]. A study into the affect of type I or II IFN coexpression by FWPV recombinants expressing Newcastle disease virus gps *in ovo* demonstrated earlier induction of Newcastle disease virus antibody responses by IFN-γ [75]. Recent studies demonstrated that expression of chicken IL-18 by rFWPV could strongly potentiate protective cellular immunity against infectious bursal disease virus [ELDAGHAYES I *ET AL.*, UNPUBLISHED DATA]

However, studies in mammals have raised concerns regarding the coexpression of some host-derived immunomodulators in replication-competent vector vaccines in replication-permissive hosts. It therefore remains unclear whether incorporation of all host immunomodulator genes into recombinant vaccine vectors will prove advisable or acceptable for licensed commercial vaccines. The results of Eldaghayes and colleagues, however, illustrate an alternative approach that may have similar, desirable consequences, without introducing possibly undesirable features. Potentiation of cell-mediated immunity that was weaker than that achieved by IL-18 coexpression, but still significant, was also achieved by deleting one of two genes encoding candidate IL-18-binding proteins [6,7].

Use of rFWPV in heterologous prime–boost regimens in poultry

Prime–boost regimens have received relatively little attention in poultry vaccination, probably due to the overheads and costs of delivering multiple vaccinations. However, use of rFWPV to boost recombinant MDV was seen to be effective in protecting against infectious bursal disease virus [76]. Both of these have potential as multivalent recombinant vectors and are pathogens in their own right, helping overcome the disadvantages of having to use two vectors.

Recombinant FWPV vaccines for mammals

History of FWPV vaccination of mammals: rabies & measles

Vaccination and protection of mammals by rFWPV was originally demonstrated using a recombinant that expressed the rabies virus G protein [22]. This protein is a potent inducer of the humoral response and an antibody response was detected in animals vaccinated with the rFWPV. However, the observation that recombinant canarypox virus expressing rabies virus G protein also stimulated the production of similar levels of antibody but with approximately 100-fold less virus, meant that work on canarypox virus received considerably more attention and commercial backing than that on FWPV [23].

Subsequently, it was demonstrated that a rFWPV expressing measles virus F protein could protect mice against a challenge infection [77]. However, in contrast to the rabies situation, no humoral response to the F protein could be detected. It could therefore be concluded that cell-mediated immunity was responsible, an observation that arguably prompted more studies on rFWPV as inducers of cell-mediated immunity.

Development of rFWPVs as mammalian vaccines

Studies regarding the use of rFWPV as vaccines for use in mammals have involved the application of innovations and developments that are considered in more detail in subsequent sections.

It needs to be remembered that the data available for rFWPV vaccines lags behind that available for recombinant vaccinia virus and even recombinant canarypox virus vaccines. This makes it difficult to draw sound general conclusions, particularly given the range of targets and approaches.

HIV, simian (S)HIV [36,78–84] and cancer antigens [85–87], have been the targets that have driven continuing work on rFWPV vaccines since the mid 1990s. DNA priming followed by rFWPV boosting has proved safe and immunogenic against immunodeficiency virus antigens, inducing cell-mediated immunity in particular, with some protection demonstrated in macaques [36,78,81,84].

Preclinical studies have demonstrated the ability of combinations of rFWPV and recombinant vaccinia virus expressing human cancer antigens and costimulatory molecules to stimulate antitumor immunity without inducing autoimmunity [85]. Clinical studies in melanoma patients demonstrated that immunity could be stimulated against a melanoma antigen, gp100, if the expressed epitope was modified, with complete tumor regression observed in three out of 12 patients when the rFWPV was followed by IL-2 treatment [86].

Subsequently, work has commenced with antigens from malaria parasites [24,88,89] and *Mycobacterium tuberculosis* [46,90]. Immunogenicity and significant protection against malaria was demonstrated in a murine model with a heterologous prime–boost regimen in which the circumsporozoite antigen was expressed by an rFWPV boost followed by a MVA boost [88]. There has also been work on bovine viral disease virus [91] and bovine respiratory syncytial virus [92]. The rFWPV are normally delivered intradermally, although intratumoral delivery has been explored for the anticancer recombinants [87].

Coexpression of host immunomodulators

Coexpression of host-derived cytokines, notably IFN-γ and other Th1-associated cytokines, by recombinant poxviruses has been demonstrated to be advantageous in the induction of immunity, as reviewed by Ramsay and colleagues [93]. There are, however, clear concerns regarding the expression of some host-derived immunomodulators in replication-competent vectors, especially the Th2-associated cytokines, IL-5, IL-6, IL-10 and particularly IL-4 [94–97]. Coexpression of IL-4 by poxviruses has been demonstrated to exacerbate the effect of the poxvirus infection severely, even breaking through pre-existing immunity [97]. However, the effects of this exacerbation were limited if the vectors had been attenuated, for instance by deleting the thymidine kinase gene [96]. If such immunomodulators are to be used in live vaccine vectors, the nonreplicating poxvirus vectors clearly provide a much safer and more acceptable vehicle for at least the initial clinical assessments. The inability of these vectors to replicate in mammals will ensure safety in the target mammalian recipient and contacts. There is always a remote possibility that the recombinant virus might be inadvertently transferred back to a permissive avian host, but the extensive sequence divergence of mammalian immunomodulators from their avian equivalents means that they are unlikely to have a significant effect on what is a highly attenuated avian virus.

The expression of IFN-γ by a FWPV recombinant expressing HIV *gag-pol* was shown to enhance T-cell proliferation to *gag* in HIV-infected macaques. Moreover, the recombinant was demonstrated to be safe in the macaques [36]. However, a more recent study demonstrated that coexpression of IFN-γ diminished the cell-mediated immunity and protection from a pathogenic SHIV challenge, obtained in macaques following a double DNA prime followed by an rFWPV boost [84].

Coexpression of IL-6 enhanced the humoral response to influenza hemagglutinin in mice, while coexpression of IFN-γ had an adverse effect [98]. Multiple costimulatory molecules (B7–1, interstitial cell-adhesion molecule-1 and leukocyte function-associated antigen-3, known collectively as the triad of costimulatory molecules [TRICOM]) appeared to enhance cell-mediated immunity against carcinoembryonic antigen (CEA) when expressed by rFWPV and recombinant vaccinia viruses in a prime–boost regimen [99]. Those rFWPV expressing TRICOM and CEA or Muc-1 are now being evaluated in many clinical trials against a wide range of cancers.

Prime–boost regimens involving FWPV

DNA & protein as prime–boosting agents with FWPV

Even in the case of rabies and measles vaccination with rFWPV, it was considered surprising that nonreplicating poxvirus vectors, used alone, could achieve the observed levels of immunogenicity and protection. However, against other disease agents, responses were generally relatively weak. The response to this was the gradual introduction of the use of heterologous prime–boost regimens, whereby different agents were used to deliver the same antigen, focusing the immune response on the common (hopefully protective) antigen of the pathogen rather than on scaffold antigens (which was normally the case with traditional homologous boosting regimens). Initially, regimens often involved combinations of recombinant vectors with recombinant proteins or with inactivated pathogens. However, as results with DNA vaccination were also proving less successful than had been hoped given initial successes in mice, it was perhaps not surprising that some of the early prime–boost regimens used DNA (or protein) vaccination in combination with nonreplicating poxvirus vectors [100]. This approach continues in the development of DNA/rFWPV prime–boost vaccines against SHIV/HIV [84]. Ramsay and colleagues reviewed the use of FWPV in prime–boost regimens with DNA as well as with coexpression of immunomodulators and the use of polyepitope strings (or minigenes) [93]. Such approaches appear particularly suited for the induction of cell-mediated rather than humoral responses.

Other viruses as prime–boost agents with FWPV

The use of vaccinia virus and FWPV recombinants as priming and boosting agents (or *vice versa*) developed further with the replacement of conventional strains of vaccinia virus by MVA, which is also a nonreplicating poxvirus in mammals. As with FWPV [101,102], MVA appears to be able to stimulate the maturation of human dendritic cells, since it appears to lack an inhibitor of dendritic cell maturation expressed by parental

strains of vaccinia virus [103]. The combination of MVA and FWPV vectors in prime–boost regimens appeared to be more potent than a combination of parental vaccinia virus and FWPV in the TRICOM/CEA model system [85]. The order of MVA and FWPV as priming and boosting agents appears to be important, although it may depend on the antigen and host combination. MVA priming and FWPV boosting appeared to deliver stronger $CD8^+$ enzyme-linked immunosorbent spot responses against a multiepitope string from the V3 loop of HIV *Env* [83]. There was some indication that MVA priming and rFWPV boosting gave stronger $CD8^+$ responses against the *Plasmodium berghei* circumsporozoite protein (PbCSP) in mice, but reversing the order (i.e., rFWPV-prime, MVA-boost) resulted in stronger protection against challenge by the parasite [88].

Differences between FWPV strains affect vaccine efficacy

It would appear that not all FWPV strains are equal when it comes to vaccination of mammals in prime–boost regimens. A rFWPV of strain FP9, expressing PbCSP, elicited stronger $CD8^+$ responses in mice than a recombinant expressing the same gene in the background of a commercial vaccine strain [88]. It is not clear why the FP9 PbCSP recombinant should outperform the recombinant in a commercial vaccine strain. Clearly, it might be due to loss of one or more of the genes deleted during the derivation of FP9, however, this must remain mere speculation until it is known whether the observation holds true for other FWPV strains and for other antigens, and until sequence data becomes available for other strains.

Improving the vaccine efficacy of existing FWPV strains

It is likely that there will be attempts to derive better FWPV-vaccines by direct, artificial methods. Work towards improving FWPV as a recombinant vector is likely to focus on the following areas; improvement in antigen presentation and immunogenicity, improvements in virus yield in culture and changes to environmental persistence. One area that is receiving attention, but which may not result in actual improvement, is the issue of integrated retrovirus sequences.

Role of integrated reticuloendotheliosis virus sequences

A near full-length, infectious progenome of the REV has been found in most, if not all, pathogenic isolates but in only one vaccine strain, FPV-S, that was already known to be contaminated with reticuloendotheliosis virus [26,43,65,104]. The majority of vaccine strains of FWPV (including 12 out of 12 unspecified commercial vaccines examined in one study [104] and the two completely sequenced viruses [6,7]) carry only non-infectious, LTR sequences of REV, apparently in two different size forms as a complete or a partial LTR [43,104]. The authors understand that work is already underway to delete the LTR found in the vaccine strains. The justification for this is not absolutely clear. It appears to be based on an implicit (and controversial) assumption that, because pathogenic isolates containing REV provirus have been found on farms vaccinated with LTR-containing vaccines, the pathogenic isolates are formed *de novo* by integration of REV proviral DNA into the vaccines, by recombination at the LTR. Removal of the LTR might thus prevent just such an occurrence. However, there is no direct evidence for *de novo* generation of pathogenic FWPV strains. Unfortunately, there are insufficient sequence data available to allow for the detailed comparison of cocirculating vaccine and pathogenic strains that might refute this proposition. It appears clear that pathogenic field strains carrying the full-length provirus are widespread and that the full-length provirus rapidly becomes deleted upon passage in cell culture (possibly even during embryo culture, as in the case of HP1 [7]), yielding one of two single LTR forms [26,43,65]. It is not known whether the loss of provirus sequences also occurs in the field – it is likely that the resulting viruses would be of low pathogenicity. However, the provirus and the LTR-only sequences have only ever been found at the same single locus (between FPV201 and FPV203, FPV202 being mainly encompassed by the LTR sequences). It therefore appears that a single, ancestral event inserted the REV provirus into the FWPV genome between FPV201 and FPV203, in contrast to the multiple REV insertions that have been observed in MDV [105–107]. The situation concerning the presence of REV LTR sequences in other avipoxviruses is unclear in the literature, possibly due to strain differences. There are no LTR sequences in the completely sequenced genome of canarypox virus, in which orthologs of FPV201 and FPV203 are separated by only 64 bp [108]. Kim and colleagues were unable to detect any REV sequences in one 1968 strain each of pigeonpox virus and canarypox virus [109]. They did, however, comment on unpublished results demonstrating the presence of REV LTR sequences in unspecified pigeonpox virus vaccine strains. Similarly, Moore and colleagues identified REV LTR sequences in all unspecified commercial pigeonpox virus vaccines and in one unspecified commercial canarypox virus vaccine but not in an unspecified commercial quailpox virus vaccine or in an unspecified canarypox virus isolate [104].

It is unclear whether there are any advantages to the removal of the REV LTR from vaccine strains of FWPV, it is by no means clear that the deliberate deletion of the LTR will be a neutral, let alone advantageous, modification in terms of vaccine efficacy. It remains to be seen whether loss of FPV202 (essentially encompassed by the REV LTR), or any regulatory changes to the nearby genes FPV201 and FPV203, will affect vaccine function.

Environmental stability of rFWPV

The attenuated FWPV vaccines that are being used as recombinant vectors in poultry and mammals, are unlikely to spread in the environment to susceptible hosts. However, such spread might be rendered even less likely if the environmental stability of the vector could be compromised without affecting its replicative ability. A (CPD)-photolyase (FPV158) encoded by FWPV has been identified. Its removal generated a virus that was less resistant to the effects of ultra violet. Such a phenotype

should reduce the likelihood of such a virus persisting in the environment [42]. Deletion of the A-type inclusion body protein (FPV190) might also be anticipated to reduce the resistance of virus shed in desquamated epithelium, to environmental pressures such as dessication, pH changes and chemical insults though, to the authors' knowledge, this has not been investigated experimentally.

Improvements in growth yield & expression

Any attempt to improve the yield of FWPV in conventional or novel cell substrates must consider that the immediate changes, or any changes inadvertently selected under the new conditions, may have an adverse effect on the genetic stability of the recombinant vaccine or on the efficacy of vaccination. Similarly any attempt to broaden the host range may affect vaccine efficacy or even compromise the fundamental safety of the FWPV vector.

Attempts have been made to relieve the early block to canarypox virus expression in HeLa cells, in an attempt to enhance levels of gene expression, by incorporating vaccinia virus IFN resistance genes *E3L* and *K3L* into the recombinant [110]. Attempts to improve the expression of genes carried by rFWPV in mammalian cells, or to develop new cell substrates for propagation of rFWPV, will benefit immensely from studies into the interaction of FWPV with the type I IFN and apoptotic systems of permissive avian and nonpermissive mammalian cells.

Improvements in antigen presentation & immunogenicity

Numerous attempts have been made to improve or modify the presentation of foreign antigens to the immune system by recombinant vectors. Initial attempts concentrated on the coexpression by the vectors of host-derived immunomodulators [74,75,78,111], as they were accessible and well characterized. However, another approach would be to delete viral immunomodulators already present in the vector. The inadvertent use of such an approach probably explains the success of MVA compared with parental vaccinia virus; MVA has lost most of the known viral immunomodulators possessed by traditional strains of vaccinia virus, including a factor that appears to inhibit maturation of human dendritic cells [103]. Deliberate application of this approach to the avipoxvirus vectors will be a slower process, as very few of their immunomodulators have been characterized, partly because they share no obvious sequence similarity with those found in mammalian poxviruses and because there are currently few host (i.e., chicken)-derived reagents to facilitate their characterization (although the recent determination of a first draft of the chicken genome sequence will help in this regard). The recent identification of an IFN-γ-binding protein depended on biochemical purification of the immunomodulator using capture with the chicken IFN-γ [112]. The identified gene (*FPV016*) had provided no previous indication, on the basis of its sequence, that it might encode a protein with IFN-γ-binding activity [6]. Furthermore, although the authors have demonstrated that FWPV is resistant to avian type I IFN [113], there are no clues from the virus sequence or from studies with other poxviruses how this is achieved; there are no homologues of vaccinia virus genes *E3L, K3L* or of the type I IFN-binding proteins. In general, the utility of deleting virus immunomodulators is difficult to predict due to species-specific differences between the host ligands for these viral immunomodulators. Thus, cross reactivity between avipoxvirus immunomodulators and potential mammalian ligands will have to be evaluated on a case-by-case basis. It is interesting to note that the FWPV IFN-γ-binding protein demonstrated crossreactivity against human but not murine IFN-γ [112]. However, the failure to identify immunomodulators that are expected to be encoded by FWPV does provide hope that when they are eventually identified, they may represent novel immunomodulatory mechanisms that may provide new targets for manipulation.

Clinical trials

A few clinical trials using rFWPV have been reported. Those against HIV were well covered in a recent review article in this journal [114]. However, many more are now underway and, until publication, their progress is best monitored at the various clinical trials websites.

The US Federal Clinical Trials website currently lists 26 clinical trials (completed, underway or recruiting) mainly involving FWPV-TRICOM recombinants, mainly targeted against a wide range of neoplasms [201]. The University of Oxford Malaria Vaccines Trials Group lists some six clinical trials conducted in the UK and the Gambia [202].

Expert opinion

Considerable progress has already been achieved in inducing cell-mediated immunity to foreign antigens using rFWPV in poultry and mammals but the potential of these vectors is such that they warrant much more investigation. Apart from its high safety profile, the major strength of FWPV, as with any poxvirus, is its high capacity for foreign gene inserts. To take advantage of this we need a clear understanding of the biology and immunology of the pathogens, to identify where we need to express multiple antigens from any one pathogen. It may be possible to optimize foreign antigen expression by using native FWPV promoters. We also need to learn more about competition between the antigens expressed by the rFWPV, be they foreign antigens or FWPV antigens, to help construct multivalent rFWPV effective against multiple pathogens. Further development of prime–boosting should help us identify and then predict optimal combinations of delivery vehicles (including DNA, protein and conventional vaccines). New combinations should also be explored; fowlpox and canarypox viruses are highly diverged and thus it would be interesting to see how well they would interact. Among the many other avipoxviruses there may be other useful candidate vectors [3], although they would require attenuation by traditional or molecular methods. Finally, we have to stress that little is known regarding the biology of the avipoxviruses. A recent paper may herald further studies to elucidate the phylogeny of the avipoxviruses [115].

They have many similarities to the better-studied mammalian poxviruses but demonstrate clear differences and thus there is a need to study them more closely and more extensively in cells permissive and nonpermissive for replication, if we are to fully exploit their potential as recombinant vaccine vectors.

Five-year view

For mammalian vaccination, we anticipate increased use, both of rFWPV that coexpress host immunomodulators and of rFWPV from which FWPV immunomodulators (such as binding proteins for IFN-γ and IL-18) have been deleted. We expect to see more evaluation of FWPV heterologous prime–boost regimens in livestock. Studies involving FWPV itself are likely to concentrate on improving virus yield and elucidating how the virus evades the avian type I IFN response. It is also possible that the mechanisms for the superior induction of humoral immunity by canarypox virus will be investigated.

Acknowledgements

The authors would like to acknowledge the support of the Biotechnology and Biological Sciences Research Council. The authors also wish to acknowledge the support of the Libyan Government to Ibrahim Eldaghayes in the form of a postgraduate fellowship. Oxxon Therapeutics sponsor the work of Matthew Cottingham. We are grateful to Sarah Gilbert and Keith Howard for helpful discussions.

Declaration of competing interests

Oxxon Therapeutics sponsor Matthew Cottingham's research project in the Skinner laboratory. Michael Skinner and Stephen Laidlaw hold inventors' rights on FWPV intellectual property, for which they are rewarded via the UK Government-approved Biotechnology and Biological Sciences Research Council's Rewards for Inventors scheme. None of the authors are a director or consultant or knowingly a shareholder of any company involved in exploitation of recombinant FWPV technology.

Information resources

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- HIV Vaccine Trials Network: fowlpox vector vaccines http://chi.ucsf.edu/vaccines/vaccines?page = vc-01–03 (Accessed January, 2005)
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Key issues

- Recombinant fowlpox viruses are effective means of delivering protective immunity in poultry – with licensed commercial products against two major pathogens.
- Fowlpox virus is nonreplicating in mammalian cells; it is safe for use even in immunocompromized individuals.
- The high safety profile of fowlpox virus means that it is a safe vehicle for coexpression of host immunomodulators that might not be acceptable in a replicating, or potentially replicating, vector.
- Deletion of endogenous fowlpox virus genes encoding virus immunomodulators offers an alternative means of improving immunogenicity, especially in poultry (in which fowlpox virus replicates) where coexpression of host immunomodulators may be contraindicated.
- Fowlpox virus has, as with other poxviruses, potential for the incorporation of many foreign genes, allowing production of multivalent recombinant vaccines.
- Heterologous prime–boost regimens, especially with other viruses, offer considerable potential for tailoring responses to individual antigens.

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