Different Levels of Immunogenicity of Two Strains of Fowlpox Virus as Recombinant Vaccine Vectors Eliciting T-Cell Responses in Heterologous Prime-Boost Vaccination Strategies

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The FP9 strain of Fowlpox virus has been described as a more immunogenic recombinant vaccine vector than the Webster FPV-M (FPW) strain (R. J. Anderson et al., J. Immunol. 172:3094–3100, 2004). This study expands the comparison to include two separate recombinant antigens and multiple, rather than single, independent viral clones derived from the two strains. Dual-poxvirus heterologous prime-boost vaccination regimens using individual clones of recombinant FP9 or FPW in combination with recombinant modified Vaccinia virus Ankara expressing the same antigen were evaluated for their ability to elicit T-cell responses against recombinant antigens from Plasmodium berghei (circumsporozoite protein) or human immunodeficiency virus type 1 (a Gag-Pol-Nef fusion protein). Gamma interferon enzyme-linked immunospot assay and fluorescence-activated cell sorting assays of the responses to specific epitopes confirmed the approximately twofold-greater cellular immunogenicity of FP9 compared to FPW, when given as the priming or boosting immunization. Equality of transgene expression in mouse cells infected with the two strains in vitro was verified by Western blotting. Directed partial sequence analysis and PCR analysis of FPW and comparison to available whole-genome sequences revealed that many loci that are mutated in the highly attenuated and culture-adapted FP9 strain are wild type in FPW, including the seven multikilobase deletions. These “passage-specific” alterations are hypothesized to be involved in determining the immunogenicity of Fowlpox virus as a recombinant vaccine vector.

Fowlpox virus (herein abbreviated as Fowlpox) is the type species of the Avipoxvirus genus of the Poxviridae. Infected birds develop lesions on unfeathered areas of the skin and on internal mucous membranes, in the case of the more severe and sometimes fatal diphtheritic form. While vaccine strains have been available for several decades as prophylactics for use in domestic poultry, the virus is undergoing a resurgence of interest as a recombinant vaccine vector for use in mammals, including humans. The virus has the ability to accept multiple large recombinant inserts (9) and has an excellent inherent safety profile. Avian poxviruses such as Fowlpox (and Canarypox virus) are apathogenic and replication deficient in mammals due to their natural host range restriction, but they still have the ability to enter mammalian cells (including dendritic cells [11]), reach an early stage of morphogenesis, and express exogenous genes (44). The efficacy of recombinant Fowlpox subunit vaccines was originally demonstrated in veterinary settings using Fowlpox alone (43, 49), but current clinical interest principally centers upon their use in combination with other vectors.

The heterologous prime-boost approach relies upon immunization with two or more different viral or nonviral antigen delivery systems to enhance synergistically the production of T-cell responses against recombinant antigens (31, 53). This approach is anticipated to permit the development of novel vaccines against cancer (20) and pathogens, such as human immunodeficiency virus (HIV) (29), Mycobacterium tuberculosis (30), and the malaria parasite (34), that are able to resist the humoral immunity typically evoked by traditional vaccines. The evaluation of Fowlpox as a component of a prime-boost strategy typically involves its administration in conjunction with DNA plasmid vaccination and/or recombinant modified Vaccinia virus Ankara (MVA), an antigenically dissimilar poxvirus vector whose replication is also blocked in mammalian cells (28, 46). A DNA-prime, Fowlpox-boost HIV vaccination strategy has been demonstrated to protect macaques against HIV type 1 (HIV-1) challenge (23) and is currently undergoing clinical trials in humans (12). Phase I trials of prime-boost vaccine strategies for liver-stage malaria (35) have recently demonstrated that priming with recombinant Fowlpox, but not with DNA, prior to a recombinant MVA boost can elicit complete sterile protection against Plasmodium falciparum (51, 52).

A number of different strains of Fowlpox are being evaluated for use as recombinant vaccine vectors. The proprietary platform TRICOM, used by Therion Biologics Inc. for vaccines against HIV and cancer (17), is based upon a commercial
vaccine strain called POXVAC-TC, produced by Schering-Plough. In Australia and Thailand, a large HIV vaccine initiative (23) is using a strain called FPV-M3, which was derived by plaque purification of the FPV-M (“mild”) vaccine strain produced by Cyanamid Webster Ltd. (13). The well-characterized FP9 strain is in use primarily by academic and commercial research groups in the United Kingdom (15, 50, 52). This paper presents a comprehensive comparison of the capacity of the FP9 strain to elicit T-cell responses against recombinant antigens with that of the Cyanamid Webster FPV-M strain (hereafter abbreviated FPW). The different levels of performance of these two strains present an opportunity for the identification of viral factors influencing immunogenicity, thereby offering potential future routes for vector optimization.

The FP9 strain was derived by plaque purification (33) of a highly tissue culture-adapted strain produced by Anton Mayr and K. Malicki, who subjected a field isolate known as HP-1 Munich to 438 blind passages in chicken embryo fibroblasts (CEFs), after which the virus retained no pathogenicity (27). The genome of FP9 has been sequenced (24) and compared to the genome of a North American strain (FPV-US) described as pathogenic (2), revealing that it has accumulated numerous mutations, including deletions totalling 25 kb (8.5% of the genome) during its extensive adaptation to growth in culture. Mayr et al. also produced MVA via similar treatment of Vaccinia virus (28), which, like FP9, is highly attenuated and has lost a comparable proportion (31 kb) of its genome to large-scale deletions (4, 32).

The genome sequence of strain FPW is unknown, and its passage history is unclear compared to strain FP9. It has probably undergone some adaptation to growth in CEFs and/or embryonated eggs, but it has not been subjected to a plaque purification step. Unlike FP9, it retains pathogenicity in chicks, but it has undergone some degree of attenuation, since it is less virulent than the Webster FPV-S (“standard”) strain or wild-type isolates (10). Here we show that, in concordance with this phenotype, FPW does not possess the extensive genomic deletions and many of the less drastic mutations that are characteristic of the highly attenuated FP9 strain.

A previous publication (3) reported that the magnitude of murine CD8+ T-cell responses against recombinant Plasmodium berghei circumsporozoite protein (PbCSP) was dependent upon the strain of Fowlpox used as a vector. When given as a prime-boost in conjunction with MVA carrying this malarial antigen, an FP9.PbCSP recombinant elicited approximately two-fold-greater frequencies of specific gamma interferon (IFN-γ)-secreting CD8+ T cells compared to FPW.PbCSP, with some dependence upon the order of administration of the two heterologous vectors.

We have expanded this comparison of strains FP9 and FPW in two important ways, using a similar dual-poxvirus prime-boost vaccination regimen in mice. First, using PbCSP recombinants, we compared the cellular levels of immunogenicity of multiple independent clones of each strain in order to dismiss the possibility that the characteristics of the particular viral clones, rather than a true strain-specific difference, were responsible for the initial observation that FP9 is more immunogenic that FPW. Second, using a recombinant HIV-1 Gag-Pol-Nef polyprotein, we verify that the superior immunogenicity of FP9 is not confined to the PbCSP antigen and demonstrate that this increased immunogenicity is reflected in the magnitude of responses to CD8+ and CD4+ T-cell epitopes. By Western blotting, we show that the difference in performance of FP9- and FPW-based recombinant vaccines is not attributable to an inequality of antigen expression in infected murine cells. We conclude that the superior immunogenicity of FP9 is determined by viral genetic factors that differ as a result of the distinct laboratory histories of the two strains.

MATERIALS AND METHODS

Antigens and MVA recombinants. The PbCSP antigen from Plasmodium berghei and an MVA.PbCSP recombinant have been described previously (42). The GPN construct consists of a scrambled and inactivated fusion of the gag, pol, and nef genes of HIV-1 (clade B, HXB2 strain [40]). In order to abolish protein function without destroying T-cell epitopes, the genes were fused and inactivated in a redundant fashion. The p17- and p24-coding regions of gag were fused in the reverse of their natural order, and the myristoylation site of p17 was removed by a single-aminooxy-acid substitution. The transcriptase activity of p51, encoded by pol, was ablated by splitting the sequence near the active site and swapping the order of the two resulting fragments, with duplication of a central 12-residue sequence at the novel termini to prevent loss of T-cell epitopes in this region. Finally, in the case of nef, the gene was divided into eight segments, with duplication of eight-codon sequences at the junctional regions, again to retain T-cell epitopes present at the breakpoints. In addition to these three inactivated but fully immunogenic constructs, the ~120-kDa GPN fusion protein carries at the C terminus an eight-residue sequence from HIV gp160 (encoded by env) that is a known CD8+ T-cell epitope in BALB/c mice (see Table 1).

An MVA recombinant carrying this construct at the thymidine kinase insertion locus under control of the Vaccinia virus p7.5 early/late promoter with lacZ as a selectable marker was prepared using the pOPK6s shuttle vector (A. van Maurik et al., unpublished data).

Recombinant Fowlpox production. The Cyanamid Webster FPV-M strain of Fowlpox (FPW) was obtained from Solvay Animal Health and was not subjected to plaque purification prior to isolation of recombinant clones. FP9 was derived by plaque purification of the attenuated strain HP-438-Munich (27) at the Institute for Animal Health in the late 1980s (33). For introduction into Fowlpox, the antigen constructs were ligated into the Smal site of the pEFL29 shuttle vector (38), which carries lacZ as a marker and places the antigen gene under the control of the p7.5 promoter at the p002 locus. Independent clones of recombinant viruses were produced by replicate transfection of primary chicken embryo fibroblasts with the shuttle vector and subsequent infection with FP9 or FPW, followed by isolation by repeated plaque purification on CEFs using 5-bromo-4-chloro-3-indolyl-p-tolylgalactopyranoside (X-Gal) selection as previously described (38). The viruses were amplified by three passages in CEFs and purified by centrifugation through sucrose cushions. The concentration and homogeneity of the resulting stocks were determined by titration using duplicate dilution series on two separate preparations of CEFs followed by X-Gal staining.

Antigen expression was verified by Western blotting of infected CEFs.

Immunizations. Female BALB/c mice (H-2b; 6 to 8 weeks) were used in all experiments and kept in individually ventilated cages in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Doses of 1 × 106 PFU of recombinant virus were administered intravenously in 100 μl injection-grade phosphate-buffered saline (PBS). Control animals received PBS alone. Boost immunizations were performed 14 days after priming immunizations. On day 28, the animals were sacrificed by cervical dislocation, and the spleens were removed for processing.

Ex vivo IFN-γ enzyme-linked immunospot assay (ELISPOT). Splenocytes from individual mice were prepared as previously described (3). The cells were enumerated using a CASY automated cell counter (Scharf Systems GmbH) and diluted to equalize concentrations. Appropriate numbers of splenocytes from each mouse were pipetted in duplicate into the wells of Millipore Multiscreen-IP plates previously coated with the anti-mouse IFN-γ antibody AN-18 (Mabtech). A twofold dilution series of the splenocytes was performed in the wells using a multichannel pipette. Target cells (5 × 104 per well) from naive mice were treated (1 μg/ml) with T-cell epitope peptides (obtained from NMI IT GmbH and listed in Table 1) and added to the plates, which were then agitated and placed in a CO2 incubator at 37°C. The following day, the assay was developed by labeling with biotinylated anti-mouse IFN-γ antibody R4-6A2 (Mabtech) and alkaline phosphatase-conjugated streptavidin. The spots were counted using...
TABLE 1. Synthetic peptides used to stimulate T cells

| Peptide | Sequence | Antigen | MHC restriction |
|---------|----------|---------|-----------------|
| Pb9     | SYIPSAEKI | PbCSP | H2-K\(^{d}\)     |
| AMQ    | AMQMLKETI | HIV-1 GPN (Gag) | H2-K\(^{d}\)     |
| RGP    | RGPGRAFVTI | HIV-1 GPN (Env) | H2-D\(^{d}\)H2-IA\(^{d}\) |
| NPP    | NPPPVPHEL- | HIV-1 GPN (Gag) | Unknown class II |

\(^{a}\) Synthetic peptides used to stimulate T cells in ex vivo ELISPOTs of splenocytes from mice immunized with recombinant Fowlpox and MVA expressing \(\text{PbCSP} (\text{Pb9})\) or GPN (AMQ, RGP, and NPP). Note that unlike the MHC class I-restricted peptides \(\text{Pb9} (41, 42)\) and AMQ (26), RGP has a dual MHC restriction in \(H-2^{d}\) mice (48). Therefore, NPP (26) is the only exclusively \(H-2^{d}\) MHC class II-restricted peptide used.

RESULTS

Comparison of \(\text{FP9, PbCSP}\) and \(\text{FPW, PbCSP}\) recombinants in prime-boost immunization regimens. The superior immunogenicity of \(\text{FP9, PbCSP}\) compared to \(\text{FPW, PbCSP}\) as a component of dual-poxvirus prime-boost regimens was described by Anderson et al. (3), but the possibility remained that a clone-specific effect, rather than a strain-specific effect, was responsible for the difference. In order to investigate this question, we prepared three new independent clones each of \(\text{FP9, PbCSP}\) and \(\text{FPW, PbCSP}\) and performed a head-to-head comparison of the abilities of these six recombinants to elicit murine T-cell responses when given in combination with \(\text{MVA, PbCSP}\) as a heterologous prime-boost immunization. We employed a prime-boost regimen, rather than single immunizations, in order to improve the signal-to-noise ratio in immune readouts and because Fowlpox is used as a combination vaccine in clinical settings. Both orders of administration, Fowlpox-MVA (FM) and MVA-Fowlpox (MF), were tested, using an intravenous dose of \(10^6\) PFU and a 14-day interval between the prime and boost. The cellular immune responses to the \(\text{PbCSP}\) antigen were determined at day 28 by ex vivo IFN-\(\gamma\) ELISPOT of splenocytes stimulated with the known immunodominant \(H-2^{d}\)-restricted peptide Pb9 (Table 1) (41, 42).

Figure 1 shows the results, expressed as spot-forming cells (SFC) per million splenocytes, of the Fowlpox-MVA (FM) prime-boost vaccinations, in comparison to a control treatment consisting only of MVA,\(\text{PbCSP}\). The data were analyzed using a one-way analysis of variance (ANOVA) followed by a Newman-Keuls post hoc test for each pairwise comparison. While the responses produced by \(\text{FP9}\) priming were similar between independent clones (\(P > 0.05\) for all \(\text{FP9,FP9 pairwise comparisons}\)), there was considerable variability in those produced by \(\text{FPW}\). In particular, clone 2 of \(\text{FPW, PbCSP}\) was as immunogenic as any clone of \(\text{FP9, PbCSP}\) (\(P > 0.05\)); however, in contrast, the response to clone 3 was as low as that elicited by \(\text{MVA, PbCSP}\) alone (\(P > 0.05\)) and was significantly lower than the responses elicited by clone 2 of \(\text{FPW, PbCSP}\) (\(P < 0.001\)) or any clone of \(\text{FP9, PbCSP}\) (0.001 < \(P < 0.01\)). Presenting an intermediate immunogenicity, clone 1 of \(\text{FPW, PbCSP}\) is more effective than clone 3 (\(P < 0.05\)), but not as effective as clone 2 of \(\text{FPW, PbCSP}\) (\(P < 0.05\)).

When Fowlpox was used as a boost, rather than a prime, in combination with \(\text{MVA (MF)}\), a somewhat different picture emerged, as shown in Fig. 1 (six mice per group, instead of four, were used in this experiment). In this case, no clone performs significantly differently from any other clone of the
same strain ($P > 0.05$, Newman-Keuls), and every recombinant FP9, PbCSP clone performs significantly better than every FPW, PbCSP clone ($0.001 < P < 0.01$). To clarify, using MF compared to FM prime-boost, the intrastain (clone-specific) differences are less pronounced, and the interstrain differences are more pronounced. There appear to be two reasons for this: (i) the variablepriming performances of FPW, PbCSP clones and (ii) the superior boosting performance of FP9, PbCSP, which produces markedly higher responses (average about 6,500 SFC/million compared to 2,500) when given MF compared to FM, unlike FPW, PbCSP (for which the responses are only slightly increased using MF).

To aid interpretation of the results, Fig. 1 also presents the same data pooled by strain. From these graphs, it is clear that, overall, the FP9 strain elicits higher Pb9-specific T-cell responses than FPW, whether used as a prime or boost in combination with MVA, PbCSP. As revealed by Table 2, which shows the difference in immunogenicity as a ratio (FP9 response/FPW response) together with the 95% confidence intervals for the difference, both of these comparisons are significant ($P = 0.04$ for FM; $P < 0.0001$ for MF).

The magnitude of the difference between FPW and FP9 immunogenicity is comparable to that previously reported using single PbCSP clones in the two backgrounds (3). The FPW, PbCSP recombinant described by Anderson et al. (3) behaved similarly to clone 1 of FPW, PbCSP (which showed intermediate immunogenicity) when used as a prime, and for FM administration, the overall difference in efficacy presented here is very similar to that previously reported. Using the reverse order of administration (MF), however, the magnitude of the strain efficacy difference observed in the current study is somewhat greater than that previously reported. Nevertheless, the available data taken together clearly demonstrate that the virus strain is a key determinant of the immunogenicity of Fowlpox PbCSP recombinant vaccines, though there is also evidence for clonal variation, especially in the FPW strain.

**Comparison of multiple clones of FP9, GPN and FPW, GPN recombinants.** The next objective was to ascertain whether the superior immunogenicity of strain FP9 is observed when the virus carries a different recombinant antigen. In order to increase the number of T-cell epitopes available for analysis and to include a CD4+ epitope, we selected an artificial polyprotein derived from HIV as the alternative antigen. Briefly, this construct, abbreviated GPN, consists of an inactivated fusion of the gag, pol, and nef genes from HIV-1 (van Maurik et al., unpublished). Further information can be found in Materials and Methods.

In a series of experiments analogous to those carried out using PbCSP recombinants, we analyzed the abilities of FP9, GPN and FPW, GPN recombinants to elicit T-cell responses in heterologous prime-boost regimens with MVA, GPN. In this case, four independent clones of FPW, GPN and FP9, GPN were tested. These were given either as prime (FM) or boost (MF) immunizations using an intravenous dose of 106 PFU and a 14-day interval between the prime and boost as before. Splenocytes from immunized mice were analyzed at day 28 for T-cell responses by ex vivo IFN-γ ELISPOT and additionally by FACS using a fluorescent MHC pentamer.

The immune assays for T-cell responses to GPN were conducted using peptides corresponding to three T-cell epitopes present in the construct. The amino acid sequences and MHC restriction of these peptides, abbreviated AMQ, RGP, and NPP, are given in Table 1. These sequences have been previously identified as immunodominant HIV-1 T-cell epitopes in BALB/c mice (26, 48) and are present in the scrambled and inactivated GPN construct. Experiments using overlapping peptides have confirmed the immunodominance of the CD8+ epitopes in the BALB/c T-cell response to the GPN construct (van Maurik et al., unpublished). Responses to all three peptides were determined in IFN-γ ELISPOTs, and RGP was additionally used as an MHC pentamer for FACS assays. RGP is somewhat unusual: in H-2d mice, it is a dual CD8+ and CD4+ epitope, and the peptide is presented by MHC class I (H2-D9) and class II (H2-IA5) (48). As mentioned above, RGP is encoded by env and was included in the GPN (gag-pol-nef) construct as a control marker epitope for BALB/c mice. In contrast, AMQ and NPP (both encoded by gag) are conventional CD8+ and CD4+ T-cell epitopes, respectively (26).

Figure 2 shows IFN-γ ELISPOT analysis of the frequencies of antigen-specific T-cell responses to these epitopes elicited by immunization with the four independent clones of FP9, GPN and FPW, GPN as a prime-boost with MVA, GPN, using both orders of administration. It is immediately apparent from the data pooled by strain (see also Table 2) that FP9 elicits a greater response against all three epitopes than FPW, establishing that the superior immunogenicity of FP9 is not solely restricted to PbCSP. For both orders of administration, a two-way ANOVA (strain versus peptide) indicates that this difference is highly significant ($P < 0.0001$, FM; $P < 0.003$, MF). Bonferroni post-tests on these comparisons reveal that there is a strain-specific difference in the responses against RGP for both FM ($P < 0.001$) and MF administration ($P < 0.01$) but that the responses against AMQ are significantly different only using FM ($P < 0.01$) and not MF ($P > 0.05$), though a consistent trend is observed. In the case of NPP, it is justifiable to consider the responses against this class II-restricted peptide as an independent measure of immunogenicity and to conduct separate statistical analysis (such that the variance in the other two peptide groups is not taken into account).

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**Table 2. Differences in the immunogenicity of strain FP9 compared to FPW as components of a prime-boost with MVA**

| Antigen | Peptide | Mean ratio of immunogenicity (FP9/FPW) in a prime-boost with MVA given in the following order: |
|---------|---------|--------------------------------------------------------------------------------------------------|
|         |         | Fowlpox-MVA                                                                                      |
|         |         | MVA-Fowlpox                                                                                        |
| PbCSP   | Pb9     | 1.59 ± 0.58                                                                                     |
| GPN     | AMQ     | 1.68 ± 0.47                                                                                        |
|         | RGP     | 2.11 ± 0.51                                                                                        |
|         | NPP     | 1.45 ± 0.58                                                                                        |

$^a$ Mean ratios (FP9 response/FPW response) ± 95% confidence intervals of immunogenicity of strain FP9 to FPW as components of a prime-boost with MVA. The frequencies of specific IFN-γ-secreting T cells produced by strain FP9 versus FPW prime-boost in combination with MVA (depicted in Fig. 1 and 2) were used to calculate these values, after subtraction of the responses observed using MVA vaccination alone (indicated by dashed lines in Fig. 1 and 2). The values therefore reflect differences in immune responses elicited by the Fowlpox component of prime-boost vaccinations.

$^b$ These results were not significant statistically (see the text).
tests reveal a significant difference in the NPP responses using MF administration ($P < 0.03$) but not FM (though the pooled data reflect a trend consistent with the CD8$^+$ responses). The increased immunogenicity of FP9 compared to FPW therefore extends across both types of T-cell response.

In comparison with PbCSP, the effect of the order of administration upon the strain-specific difference in immunogenicity of Fowlpox GPN recombinants is not as pronounced. For PbCSP, FP9 was about 1.6 times more immunogenic than FPW when given as a prime, but it was about 2.7 times more immunogenic when given as a boost (Table 2) as discussed above; however, for GPN, the difference in immunogenicity of FP9 relative to FPW is similar (about 1.6-fold on average, with some dependence upon peptide; Table 2) regardless of the order of administration. In other words, the relative priming efficacy of the strains is independent of antigen, but the difference in the relative boosting efficacy of FP9 and FPW is greater when the antigen is PbCSP than when it is GPN. The conclusion is therefore that differential strain efficacy is a general phenomenon but that its magnitude under specific circumstances depends upon the antigen.

Statistical analyses of pairwise comparisons of the eight GPN recombinants are complex due to the multiple peptide-specific responses measured. An exhaustive approach is not necessary to perceive that it is not the case that all clones of FP9.GPN recombinant are more immunogenic than all clones of FPW.GPN and that this is the result of variation among clones of the same background. For example, clone B of FPW.GPN is as immunogenic as any clone of FP9.GPN when used as a prime (except for the RGP-specific response elicited by clone C of FP9.GPN), and when used as a boost, it is as immunogenic as three of the four FP9.GPN clones (A, B, and

\[\text{FIG. 2. Frequencies of AMQ-, RGP-, and NPP-specific spot-forming cells per million splenocytes determined by IFN-\(\gamma\) ELISPOT following prime-boost immunization with four independent clones of FP9.GPN or FPW.GPN. Fowlpox was given either as a prime (FM) or boost (MF) immunization in combination with MVA.GPN. Left-hand panels show individual data points (one circle per mouse), with horizontal bars indicating the mean values. Right-hand panels show pooled data for all four clones of each strain, with error bars indicating standard errors of the means. Dashed lines indicate the mean response observed after immunization with MVA.GPN alone.}\]
Indeed, the highest CD8⁺ responses were observed in mice boosted with clone C of FPW.GPN, though the scatter in this group is exceptionally high (it also contains two of the lowest responders). On the other hand, clone A of FPW.GPN is inferior to all clones of FP9.GPN both as a priming and boosting vector. This contrasts with the situation observed using PbCSP, where equal strain efficacy (for one FPW clone) was observed only using FM prime-boost. Furthermore, the within-strain variation observed using GPN is not confined to the FPW strain (as it was using PbCSP) and is apparent both in MF and FM prime-boosts (also unlike PbCSP). To exemplify this, clone D of FP9.GPN is a relatively rather poor priming agent, but it elicits the highest CD8⁺ responses when used as a boost: it is the only GPN clone of any strain for which there is a higher response using MF versus FM administration (as was the case for FP9.PbCSP). In addition, clone A of FP9.GPN produces outstandingly high responses against NPP when used as a prime, even though it elicits average CD8⁺ responses. To summarize, in addition to the overall difference in strain efficacy, there are (in both strains) clone-specific differences, which may be apparent only using a particular antigen or order of administration. However, the variation inherent in the experimental approach makes it harder to characterize these “secondary” viral phenomena than the clearer overarching effects of the strain background.

An additional correlate of immunogenicity was obtained by using an RGP H2-Dβ1† pentamer in FACS analysis of the CD8⁺ T-cell responses in the mice given FPW.GPN and FP9.GPN prime-boost immunizations. It is simplest to consider only the pooled data (shown in Fig. 3) due to the lower signal-to-noise ratio compared to that of ELISPOTs. The mean differences in frequencies of RGP-binding CD8⁺ cells elicited using FP9.GPN compared to FPW.GPN are 2.5-fold (FM) or 1.7-fold (MF), and these differences are highly significant (P < 0.0001, ANOVA). These values are comparable to the mean ratios of frequencies of RGP-specific IFN-γ-secreting T cells shown in Table 2 (2.1 for FM; 1.6 for MF), despite the lack of discrimination between CD4⁺ and CD8⁺ responses in the ELISPOT (RGP is an epitope of both populations of T cells [26]). The pentamer data provide a useful corroboration of the ELISPOT responses by a different readout parameter.

**Antigen expression levels in FP9- and FPW-infected cells.** In order to verify that the increased immunogenicity of FP9 compared to FPW is not attributable to an inequality of expression of antigen in murine cells by the two strains, we conducted Western blotting of infected mouse fibroblast (3T3) and chicken embryo fibroblast cells. The blots were probed using antibodies against the recombinant antigen and the viral protein Fpv168, an essential virion core protein (the orthologue of Vaccinia virus A4L) that is produced abundantly during late gene expression (7). As shown previously, this protein has a different molecular weight in FP9 and FPW due to diversity in the copy number of a nonapeptide repeat sequence (8).

Figure 4 shows the Western blots obtained when an equal multiplicity of infection with the eight Fowlpox GPN recombinants, plus MVA.GPN for comparison, was used; similar results were obtained using the PbCSP recombinants (not shown). In CEFs (in which Fowlpox can replicate), the four FP9 clones produce large and equal amounts of Fpv168, but FPW generally produces less protein and exhibits greater variability in the amount expressed by the individual clones. Since fpv168 is a late gene, its expression levels at the time of lysis (24 h postinfection) are indicative of the replication rates of the viruses, and indeed the relative abundance of Fpv168 shows some correlation with the virus yield obtained during productive infections (data not shown). The clonal levels of GPN polyprotein correlate well with those of the viral protein, but the recombinant antigen is expressed more evenly by the two strains, since its expression is driven by an early/late promoter. These observations have no bearing on immunogenicity in mammals but rather reflect (i) the very different behaviors of the two strains in cell cultures derived from the natural host (a result of the extensive tissue culture adaptation of FP9) and (ii) variation in growth rates of individual clones derived from the non-plaque-purified FPW strain.

When infected 3T3 cells (in which viral replication is blocked) are analyzed, a rather different picture emerges. Here, despite their very different behaviors in permissive CEFs, FPW and FP9 produce nearly equal levels of Fpv168, and there is less variability among the FPW clones. Most importantly, the amount of GPN produced is similar across all eight clones; while small variations are evident in both strains,
it is not the case that FP9 produces larger amounts of recombinant antigen than FPW does in mouse cells in vitro. Quantitation of these GPN bands using ImageJ (1) confirms that FP9 produces larger amounts of recombinant antigen than FPW does in mouse cells in vitro. Quantitation of bands in GPN-labeled immunoblots of infected 3T3 cells. The graph shows the mean area of the GPN band in the blot shown in panel A and in an additional blot (not shown). Error bars show standard errors of the means. The table to the right shows the individual values obtained for each GPN band in these two immunoblots.

FIG. 4. (A) Western blots of chicken (CEF) and murine (3T3) fibroblast cells infected at an equal multiplicity of infection (1.0) with clones A to D of FP9.GPN or FPW.GPN, with MVA.GPN or mock infected (–), immunolabeled for the GPN fusion protein (≈120 kDa) and the endogenous viral protein Fpv168 (168). The molecular mass of Fpv168 is ≈39 kDa in strain FP9 and it is ≈36 kDa in strain FPW due to differences in the copy number of a repeat sequence (S). Cells were lysed for analysis 24 h postinfection or after 48 h for detection of Fpv168 in 3T3 cells. Note that GPN expression is controlled by a synthetic early/late promoter but that fpv168 is under control of its endogenous late promoter. (B) ImageJ (1) quantitation of bands in GPN-labeled immunoblots of infected 3T3 cells. The graph shows the mean area of the GPN band in the blot shown in panel A and in an additional blot (not shown). Error bars show standard errors of the means. The table to the right shows the individual values obtained for each GPN band in these two immunoblots.

The restriction map data also suggest that strain FPW is similar, though not identical to strain FPV-US. There is an extra PstI site in fragment A, and there are possibly significant differences in the sizes of fragments B and F (the size difference of fragment G is due to the shorter repeat region in FPV-US than in FP9). To verify this analysis, we generated PCR products spanning the junctional regions to confirm an FPV-US-type, nondeleted genotype at all six loci. The most striking FP9 passage-specific mutations are seven multikilobase deletions, cumulatively responsible for the removal of more than 25 kb of coding sequence and the elimination or severe disruption of 17 open reading frames (out of 260 in FPV-US). These dramatic mutations are amenable to analysis by restriction mapping. Table 3 presents a comparison of PstI-digested Fowlpox genome fragment sizes, using both actual agarose gel migration data for strains FPW and FP9 (13, 33) or data generated in silico from the genomic sequences for FP9 and FPV-US (2, 24). The discrepancies between the two FP9 maps are indicative of the inaccuracy inherent in the traditional method, but it is clear that FPW does not possess any of the large-scale deletions. There is no reduction in the sizes of fragments D, L, and C with deletions 9, 11, and 13, respectively. Fragment A, which has deletions 22 and 24, is accounted for in full by the two FPW fragments A and D’ (FPW having an extra PstI site here). Fragment G, which contains the inverted terminal repeats (ITRs) and diploid deletions 2 and 25, is not reduced in size compared to the corresponding sequence in FP9, though the situation is complicated by the presence of a shorter noncoding repeat region in the ITR of FPV-US than in FP9 (2, 24). To verify this analysis, we generated PCR products spanning the junctional regions to confirm an FPV-US-type, nondeleted genotype at all six loci and in three cases (deletions 9, 11, and 24) obtained sequence demonstrating identity with FPV-US at these regions.

The genome sequences of the FP9 and FPV-US strains of Fowlpox have been determined, but that of FPW remains unknown. In a comparative analysis, Laidlaw and Skinner (24) categorized the differences in FP9 relative to the FPV-US strain into lineage- and passage-specific mutations by sequencing the HP-1 strain (the FP9 progenitor) at those loci. The passage-specific mutations arose during the attenuation of HP-1 by extensive passage in CEFs (27), whereas the lineage-specific mutations are a reflection of the different origins of the strains. We have conducted a partial screen of FPW for these passage-specific mutations using restriction mapping and sequencing of selected PCR-amplified regions.

Limited screen for FP9-specific mutations present in FPW.
TABLE 4. Limited sequencing of the FPW strain at loci corresponding to FP9 passage-specific mutations identified by comparison with HP-1 and FPV-US strains (24)*

| Mutation type | Total no. of mutations inFP9 | No. of passage-specific mutations | No. of loci sequenced inFPW |
|---------------|-----------------------------|----------------------------------|----------------------------|
| Small-scale deletion* | 18                          | 12                               | 6                          |
| Insertion     | 15                          | 5                                | 1                          |
| Substitution  | 77                          | 25                               | 11                         |

*Selected FP9 mutation loci were analyzed in FPW by sequencing of PCR products, and in all cases investigated, the FPW sequence was identical to that of FPV-US.

**Only small-scale (1- to 27-bp) deletions are listed (not the seven deletions greater than 1 kb).

expression levels in infected mouse cells (at least as determined by Western blotting).

What viral genetic factors could be responsible? Our results show that, at all loci inspected, FPW does not possess the mutations acquired by FP9 during its extensive passage through tissue culture, including the dramatic multikilobase deletions. The 46 open reading frames affected by these passage-specific mutations (24) therefore represent strong candidates for genes whose loss or mutation is implicated in the superior vector phenotype of FP9. Like FP9, MVA acquired multikilobase deletions during its extensive passage in CEFs (28, 32) and has been reported to be a more immunogenic recombinant vector than the less attenuated Wyeth (19) and Western Reserve (16, 39) strains of *Vaccinia virus*. This may be related to MVA’s ability, unlike standard strains, to activate mammalian dendritic cells (14) (a behavior shared by FP9 [11, 36]). This behavior has been attributed to the absence in MVA of several important immunomodulators, including soluble receptors for tumor necrosis factor, IFN-γ, IFN-α/β, and CC chemokines (6), a hypothesis that is further supported by the recent discovery that deletion of the retained soluble interleukin 1β receptor from MVA results in increased murine CD8+ T-cell responses to the virus (45). In contrast, such a hypothesis is not immediately suggested by the identities of the genes lost from FP9 during passage. These include, for example, ankyrin repeat proteins, C-type lectins, immunoglobulin domain genes, and members of the N1R/p28 family (24). Furthermore, FP9 still possesses IFN-γ-binding (37) and putative interleukin 18-binding proteins, as well as chemokine-like proteins (21). The interpretation is hindered by incomplete understanding of the avian immune system, quite apart from the more complex question of the function of avian viral immunomodulators in a xenologous host.

In addition to confirming the generality of the difference in the vector efficacy of the FP9 and FPW strains, the results presented here also indicate interactions with other factors. For example, the superior immunogenicity of FP9 compared to FPW is especially marked when using *PhCSP* antigen in an MVA-Fowlpox prime-boost (a difference of nearly threefold compared to less than twofold for all the other combinations [Table 2]). This superenhanced relative performance was not observed using FP9/PhCSP priming or with GPN as the recombinant antigen (except for clone D of FP9/GPN), suggesting a complex antigen-specific effect that is confined to one strain and one order of administration. A variation in antigen pre-
sentation or immunodominance hierarchy between the two strains might present itself in a “unidirectional” (i.e., boost-only) fashion by virtue of differential synergy with an MVA-prime-primed immune response, possibly additionally involving anti-vector responses (3, 51).

There is also considerable variability in the responses elicited by individual recombinant clones of both strains. The general overlap between the two strains is likely to be attributable to experimental factors (e.g., CEF batch, titration, immunization, and ELISPOT) and other nonviral factors (e.g., mice and antigen processing), as is the often quite large unavoidable experimental error in the immune responses of individual mice in the same experimental group (e.g., clone WC given as a boost). Despite this occasionally very pronounced scatter, there are striking examples of clone-specific effects, such as the widely divergent levels of immunogenicity of the three recombinant FPW.PbCSP clones as primes (but not boosts) and the more subtly distinctive behaviors of clones A and D of FP9.GPN. These results cannot be attributed to stochastic factors, since there is a dependence upon antigen, epitope, or order of administration that provides an “internal control” for comparison. They are therefore indicative of polymorphism among individual viral clones of the same strain. Since the poxviral spontaneous mutation rate is rather low (47), it is likely that these genetic differences reflect heterogeneity in the populations from which the recombinant clones were derived, rather than mutation during their isolation and passage in vitro. Such heterogeneity has been observed in the DryVax strain of vaccinia virus (R. Regnery, personal communication), as well as the FPW (10) and HP-1 strains of Fowlpox (S. Jarmin et al., unpublished data). The large disparity in rates of production of viral protein in CEFs by the four clones of FPW.GPN (a correlate of their growth rates in avian cells) is strong evidence for polyphyletism in the FPW strain. Though more pronounced in FPW, there is also evidence for heterogeneity in the vaccinological behavior of FP9, despite the expectation of uniformity in the plaque-purified, tissue culture-adapted strain. In this case, it is not clear when putative polymorphisms may have arisen, but the implication is that adaptive mutations may continue to accumulate even after several hundred tissue culture passages. Further investigation is needed to determine how extensively these factors impinge upon the production of useful recombinant vaccines using the two strains.

A prime-boost vaccination strategy for HIV developed in Australia (9) and currently undergoing clinical trials in Thailand (23) uses DNA in combination with a Fowlpox vector known as FPV-M3. This strain was derived from FPW by isolation of a single plaque (13). No conclusions about the relative efficacy of this strain can be made on the basis of the results described here, because of the heterogeneity in the FPW strain described above, quite apart from any considerations relating to the different antigen, insertion site, promoter, culture conditions, and vaccination strategy.

An important limitation of this study is the unaddressed question of whether the increased frequency of IFN-γ-secreting and specific pentamer-binding T cells produced by immunization with strain FP9 correlates with augmented protection against pathogenic challenge. Notably, it has been reported that FM vaccination of mice with FP9.PbCSP recombinant resulted in greater protection against stringent P. berghei challenge than MF, even though the frequencies of Pb99-specific T cells observed in ELISPOT and tetramer assays were equal, or slightly higher for MF, using the two orders of administration (3). A related question is whether the frequency of circulating effector cells 14 days after the boost would correlate with future levels of central or effector memory cells (25), though a recent clinical study has demonstrated that there is a correlation between the responses to dual-poxvirus prime-boost vaccination determined by ex vivo and cultured ELISPOTs (though the kinetics of the responses are very different) (51). A comparison of FP9 and FPW taking these considerations into account remains to be performed; indeed, the validation of the laboratory measures of vaccine efficacy that can conveniently be deployed during a program of vector development is an important wider consideration.

What are the possibilities for further improvement of strain FP9 as a recombinant prime-boost vector? As mentioned above, a rational approach, such as has been taken for MVA, is hampered by the difficulty of predicting a priori the effect of avian immunomodulators in mammals (even if their presumed functions in the natural host were fully understood). An investigation of the role of the FP9 passage-specific mutations, here identified as prime candidates for genes involved in determining vector efficacy, may provide a hypothetical basis for further rational development of FP9, especially when combined with the introduction of known immunomodulators (54). In addition, although their causes are less clear, the rather subtle clonal effects described here (dependent upon antigen or order of administration) also indicate that further gains may be possible, especially in terms of optimizing the synergistic interaction of the immune responses elicited by MVA and FP9.

In conclusion, we have demonstrated that, compared to strain FPW, strain FP9 elicits enhanced CD4+ and CD8+ T-cell responses to transgenic antigen when given either as a priming or boosting dose in combination with recombinant MVA. We suggest that the passage-specific mutations (including large-scale deletions) acquired by FP9 during extensive attenuation by cell culture passage and absent from FPW are responsible for this difference. Underlying this overall difference, there are variations in the behavior of individual clones of both strains that are dependent upon antigen and order of administration. These demonstrate the advisability of screening several candidate clones and are hypothesized to be the result of heterogeneity in strain populations. In addition to highlighting the current requirement for an empirical approach to vaccine design, these findings also represent possible future avenues for the rational enhancement of Fowlpox as a vector for clinical use.

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