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Analysis of varicella zoster virus attenuation by evaluation of chimeric parent Oka/vaccine Oka recombinant viruses in skin xenografts in the SCIDhu mouse model

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Abstract

Varicella-zoster virus (VZV) is the only human herpes virus for which a vaccine has been licensed. A clinical VZV isolate, designated the parent Oka (pOka) strain was passed in human and non-human fibroblasts to produce vaccine Oka (vOka). The pOka and vOka viruses exhibit similar infectivity in cultured cells but healthy susceptible individuals given vaccines derived from vOka rarely develop the cutaneous vesicular lesions characteristic of varicella. Inoculation of skin xenografts in the SCIDhu mouse model of VZV pathogenesis demonstrated that vOka had a reduced capacity to replicate in differentiated human epidermal cells in vivo (Moffat, J.F., Zerboni, L., Kinchington, P.R., Grose, C., Kaneshima, H., Arvin A.M., 1998a. Attenuation of the vaccine Oka strain of varicella-zoster virus and role of glycoprotein C in alphaherpesvirus virulence demonstrated in the SCID-hu mouse. J Virol. 72:965–74). In order to investigate the attenuation of vOka in skin, we made chimeric pOka and vOka recombinant viruses from VZV cosmids. Six chimeric pOka/vOka viruses were generated using cosmid sets that incorporate linear overlapping fragments of VZV DNA from cells infected with pOka or vOka. The cosmid sets consist of pOka and vOka DNA segments that have identical restriction sites. As expected, the growth kinetics and plaque morphologies of the six chimeric pOka/vOka viruses were indistinguishable in vitro. However, the chimeric viruses exhibited varying capacities to replicate when evaluated in skin xenografts in vivo. The presence of ORFs 30–55 from the pOka genome was sufficient to maintain wild-type infectivity in skin. Chimeric viruses containing different vOka components retained the attenuation phenotype, suggesting that vOka attenuation is multi-factorial and can be produced by genes from different regions of the vOka genome.

Keywords: Varicella-zoster virus; Oka strain; Varicella vaccine; IE62 localization; SCIDhu mice

Introduction

Varicella-zoster virus (VZV) belongs to the genus Varicellovirus of subfamily Alphaherpesvirinae in the family *Herpesviridae*. VZV has a large double-stranded DNA genome of approximately 125 kb that encodes at least 71 genes. Like other herpesviruses, the VZV genome has two unique regions, referred to as unique long (U_L) and unique short (U_S), which are flanked by repeated sequences (Davison and Scott, 1986). Three VZV open reading frames (ORFs) are located within the inverted repeat regions; the ORFs 62, 63, and 64 from the internal repeat region are

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duplicated at the end of the VZV genome as ORFs 71, 70, and 69. The immediate early 62 protein (IE62) encoded by the duplicate ORF62/71 is the major VZV transactivator; IE62 protein is required for transcription of all viral genes that have been evaluated (Kinchington et al., 1992).

VZV has a very limited host range, producing disease in humans only (Arvin, 2001a, 2001b). Initial replication is thought to occur in epithelial cells of the upper respiratory tract where VZV may gain access to tonsil T cells (Ku et al., 2002). Infected T cells appear to transport the virus to the skin where it causes the characteristic vesicular rash of varicella (chicken pox) (Koropchak et al., 1989; Ku et al., 2004; Ozaki et al., 1986; Soong et al., 2000). VZV exhibits the neurotropic characteristics of the other alphaherpesviruses, and establishes latency in sensory nerve ganglia (Kennedy et al., 1998; Cohen and Straus, 2001). The reactivation of latent VZV causes zoster. Zoster is usually associated with a vesicular rash and acute pain in one cutaneous dermatome, indicating that VZV migrates along axons from a single ganglion. Even though varicella is usually uncomplicated, prior to vaccine licensure, annual varicella epidemics resulted in hospitalization of 5 per 1000 children and an average of 100 deaths each year in the United States. Zoster is often associated with the morbidity of postherpetic neuralgia (PHN) and immunocompromised individuals may develop life-threatening dissemination of the virus to lung, liver, brain, and other organs during VZV reactivation.

After 15 years of clinical trials, a live attenuated VZV vaccine (Oka-Merck; VARIVAX) was licensed in the United States in 1995. The varicella vaccine was generated by serial passage of a wild-type VZV isolate, referred to as the parent Oka (pOka) virus, in human and guinea pig embryo fibroblasts. This procedure resulted in attenuation when susceptible children were inoculated with vOka (Takahashi et al., 1974; Johnson et al., 1997). In contrast, early reports showed that injection of VZV directly from varicella skin lesions caused typical varicella (von Bokay, 1909). Subcutaneous inoculation of the Oka-Merck preparation of varicella vaccine induced seroconversion in 95% of healthy children and varicella vaccine efficacy has been approximately 85% in pre- and post-licensure evaluations of protection against wild-type VZV. Vaccination was associated with a mild vesicular rash in fewer than 5% of vaccine recipients (White, 1996). The incidence of zoster may also be reduced among vaccine recipients (Broyer et al., 1997; Kamiya et al., 1984; Plotkin et al., 1989; Sauerbrei et al., 1997; White, 1996).

The clinical experience with all varicella vaccines, including the Merck, Biken, and SKB preparations, has established that vOka in the vaccine preparations is attenuated. However, the mechanisms that account for this clinical phenotype of reduced virulence are not understood. In general, while the sequence of the VZV genome (Dumas strain) was determined in 1986, investigating the molecular biology of VZV has been complicated by the extremely cell-associated nature of its replication in vitro (Arvin, 2001a, 2001b; Davison and Scott, 1986). This characteristic hindered the construction of VZV recombinants with mutations in the viral genome by standard methods of homologous recombination. This problem has been solved by using cosmid-based methods for VZV mutagenesis that allow efficient generation of viral mutants (Cohen and Seidel, 1993; Kemble et al., 2000; Mallory et al., 1997; Niizuma et al., 2003). In parallel, the development of the SCIDhu model for studies of VZV pathogenesis has made it possible to overcome the barriers posed by the restricted host range of VZV and to assess VZV virulence in human skin and T cells located within intact tissue microenvironments in vivo (Moffat et al., 1995). Using this SCIDhu model, we found that the varicella vaccine (Oka-Merck) was attenuated for replication in skin when compared to pOka, but retained infectivity for differentiated human T cells (Moffat et al., 1998a, 1998b). Since SCID mice lack the capacity to mount an adaptive immune response, these experiments indicated that the clinical attenuation of the varicella vaccine was intrinsic, reflecting genetic changes that altered virulence in skin, rather than being due to other factors, such as the infectious virus dose or route of inoculation.

The purpose of these experiments was to explore the attenuation of varicella vaccines derived from vOka. Recent sequencing information indicates that Biken and Oka-Merck vaccine preparations contain a mixture of VZV genomes that have heterogenous changes when compared to pOka (Gomi et al., 2002; Quinlivan et al., 2004). We used our VZV cosmids, which consists of overlapping fragments of VZV DNA from cells infected with pOka or vOka, to further investigate the altered virulence of vOka. Six chimeric pOka/vOka viruses were generated and evaluated for their relative infectivity in human skin xenografts in the SCIDhu model in vivo.

Results

Generation and sequencing of pOka/vOka chimeric viruses

The method for generating vOka recombinant viruses from four overlapping fragments of genomic DNA in SuperCos1 cosmid vectors (Stratagene) has been described (Kemble et al., 2000; Mallory et al., 1997); subsequently, we made a set of four pOka cosmids with the same restriction sites as used for vOka cosmids, as well as five cosmid systems from vOka and pOka (Besser et al., 2003; Niizuma et al., 2003; Sato et al., 2003). Chimeric viruses were generated by co-transfection of combinations of the pOka and vOka cosmid sets (Fig. 1). Six pOka/vOka recombinants were generated from these cosmids (Fig. 2A). The heterogenous junctions, designated junctions 1, 2, and 3, of the chimeric viruses were sequenced along with approximately 600 bp on each side of the overlapping regions and the results were compared to the Oka-Merck vaccine sequence. These comparisons showed complete matches, with only a few exceptions (Fig. 2B). Differences

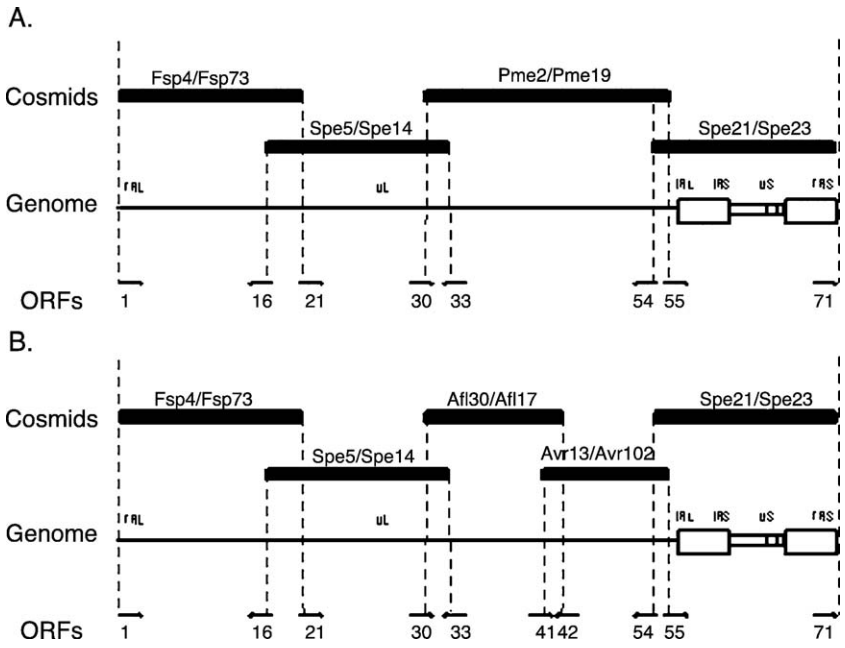

Fig. 1. Cosmids used to generate pOka/vOka recombinant viruses. Panel A depicts the four cosmid sets for vOka and pOka, with the designations used for the vOka cosmid indicated first, for example, Fsp4, followed by the pOka cosmid designation after the '/' mark, for example, Fsp73. Panel B shows the five cosmid sets generated from vOka and pOka DNA.

were identified with reference to the position number of the nucleotide in the vOka sequence. Of the chimeric viruses that had an overlap at junction 1, the G and H chimeras had an A (pOka) instead of G, found in vOka, at position 26075. The A to G change from vOka in these chimeras was in ORF18, which encodes VZV ribonucleotide reductase-2; the substitution did not alter the amino acid. As the vOka vaccine constitutes mixtures of VZV genomes, our sequence comparison identified some localized sequence heterogeneity in the Oka-Merck vaccine sequence. Among the chimeras with an overlap at junction 2, the D, G, K, and M chimeras had G instead of G/A in vOka at position 58474. Chimera D had G, chimeras K and M had A, and chimera G had G/A polymorphism at position 58793. All chimeras had A instead of G/A in vOka at position 59166. These changes were in ORF31, which encodes glycoprotein B; the changes at 58474 and 58793 affected the amino acid sequence of gB by substituting valine for isoleucine (I58474V) and glutamine for arginine (Q58793R), respectively; the change at 59166 did not alter the amino acid. The vOka sequence had six A's at positions 60147–152 in the presumed non-coding region between ORF32 and ORF33, while chimera M had 11 A's, and the other chimeras had 10 A's. Of the chimeras that had an overlap at junction 3, D, G, H, and M had T instead of the C found in vOka at position 94032, which was within ORF54. The change did not alter the leucine residue. Sequencing of ORF62, and its repeat, ORF71, in the pOka and vOka cosmids identified two nucleotide changes in vOka compared to pOka that resulted in altered amino acids, including A to G at position 2872 (A2872), changing arginine to glycine at amino acid 958, and T to C at position 3172 (T3172C) changing tyrosine to histidine at amino acid 1058 (Y1058H) (Sato et al., 2003).

Growth characteristics of pOka/vOka chimeric viruses in vitro

Analysis by infectious center assay demonstrated that the six pOka/vOka chimeric viruses had growth kinetics that were indistinguishable in melanoma cells (Fig. 3). These observations were expected because replication, as well as plaque morphologies, of pOka and vOka do not differ in vitro. All six chimeras were transferred to HELF cells for inoculation of SCIDhu skin xenografts. No differences in growth or plaque morphology were observed in fibroblasts.

Growth characteristics in SCIDhu skin implants

Whereas replication is not altered in cultured cells, attenuation of vOka can be demonstrated in SCIDhu skin xenografts in vivo (Moffat et al., 1998a, 1998b). The comparison of the pOka and vOka viruses generated from pOka and vOka cosmids, respectively, showed that the recombinant viruses retained these differences (Fig. 4). When infectious virus yields were assessed by infectious center assay over a 28-day time course, the mean titer for pOka replication in skin at day 28 was 2.8×10^4 pfu/implant compared to 0.9×10^4 pfu/implant for vOka ($P < 0.05$). The six chimeric pOka/vOka viruses were evaluated for replication in skin and compared to pOka and vOka (Fig. 4). By this analysis, chimeras D and G had mean day 28 titers of 3.0×10^4 and 2.9×10^4 pfu/implant, which were comparable to pOka. Chimera K had a more intermediate

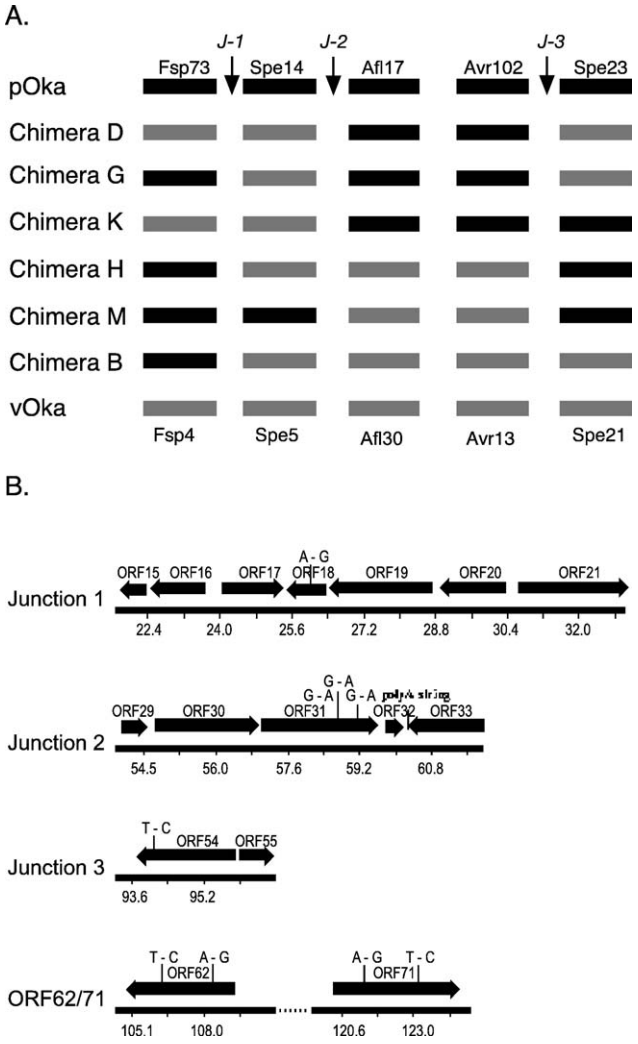

Fig. 2. A schematic representation of the pOka/vOka chimeric viruses and sequencing of junction regions. (A) Black rectangles indicate that the cosmid fragment was pOka in origin. Shaded rectangles indicate that the cosmid fragment was vOka in origin. J-1, J-2, and J-3 designate the junction regions between pOka and vOka cosmids for chimeric viruses in which pOka/vOka cosmids are overlapping. (B) Sequence differences that were detected between the chimeric viruses, based on comparison with the vOka sequence, are illustrated in regions where the recombinant viruses have an overlapping junction between a pOka and a vOka cosmid (Junctions 1, 2, and 3). Sequence differences that were detected in the vOka cosmid identified by Sato et al. (2003) in ORF62/71 based on comparison with the pOka cosmid (ORF62/71).

growth phenotype with a titer in skin of 2.3×10^4 pfu/implant at day 28. Chimeras H and M grew less well than Chimera K with day 28 mean titers of 1.7×10^4 and 1.5×10^4 pfu/implant, respectively. Chimera B resembled vOka, with a mean titer in skin of 1.1×10^4 pfu/implant at day 28 (Fig. 4). The mean peak titers of the three pOka-like viruses (pOka, Chimera D, and Chimera G) is significantly greater than the mean peak titers of the vOka-like viruses (vOka, Chimera H, Chimera M and Chimera B), with a p value of 0.018 (Student's *t* test).

These growth characteristics of chimeric pOka/vOka viruses in skin were analyzed in relation to the components of the recombinant viruses that were derived from the pOka and vOka cosmids (Table 1). All of the three chimeras, D, G and K, that incorporated the pOka cosmids, Afl117 and Avr102, had infectious virus yields of $\geq 2.3 \times 10^4$ pfu/implant. Afl17 and Avr102 included ORFs 30–55 of the VZV genome (Fig. 1). These segments were derived from vOka in the chimeras H, M, and B. In contrast to D, G and K, these chimeras had mean peak titers in skin that were $\leq 1.7 \times 10^4$ pfu/implant. Chimera D, which had a mean peak titer of 3.0×10^4 pfu/implant, contained only these two fragments from pOka, whereas chimeras G and K contained pOka Afl17 and Avr102 in addition to one other pOka fragment. This additional fragment was either Fsp73 (Chimera G) or Spe23 (Chimera K). Thus, ORFs 30–55 from pOka were sufficient to yield a pOka-like infection in skin. The more restricted growth phenotypes of chimeras H, M, and B in skin xenografts suggested that other regions did not compensate for the absence of pOka sequence in the ORF30–55 region.

Experiments with chimeras D, G, and K indicated that pOka-like infectivity was achieved without pOka Spe14 genes, which include ORFs 21–30. The results with chimera B indicate that the presence of the pOka genes contained in Fsp73 alone were not sufficient to overcome its vOka-like phenotype; these genes include ORFs 1–16.

The two chimeras, D and G, that were as infectious as pOka in skin, were constructed with the Spe21 cosmid from vOka. Since the Spe21 cosmid carried ORF62/71 and ORF63/70, as well as the unique short region of the VZV genome, which includes genes for gE and gI, the results suggested that these regions were not dominant over other regions of the VZV genome in creating the attenuation phenotype of vOka in skin. Conversely, growth of chimeras

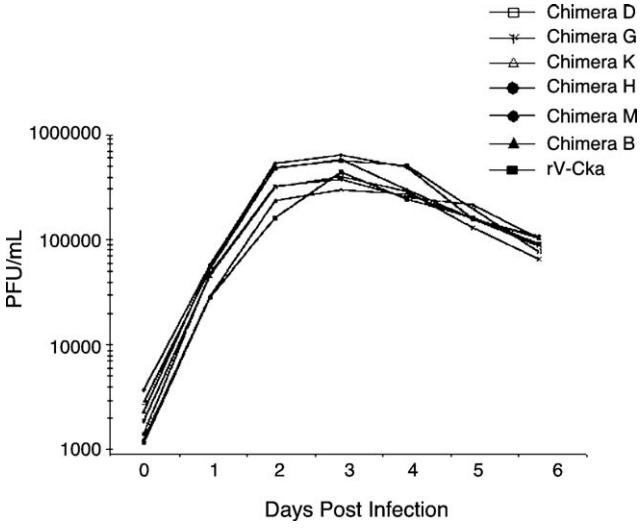

Fig. 3. Replication of pOka/vOka chimeric viruses in vitro. Yields of infectious virus were determined for 6 days after inoculation of melanoma cells with 10^3 pfu of the recombinant viruses; titers are given as the mean for quadruplicate wells at each time point. The *X* axis shows the days after inoculation when infected cell monolayers were harvested and the *Y* axis shows pfu/ml.

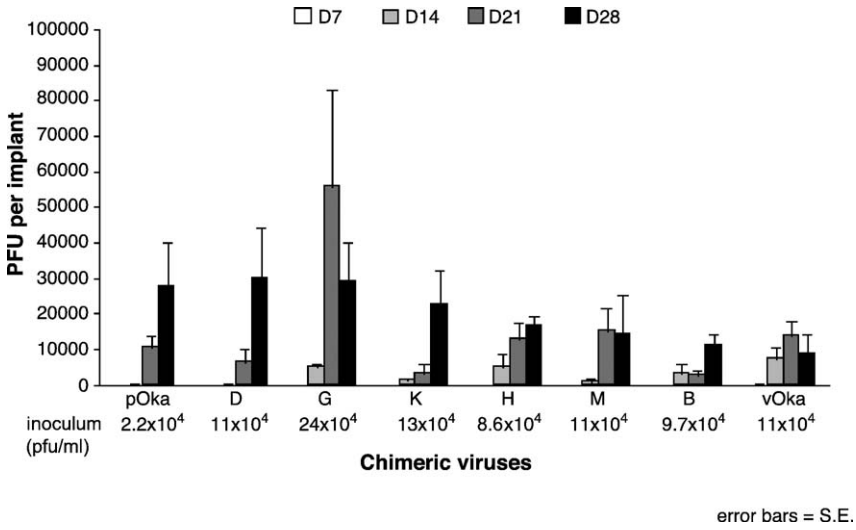

Fig. 4. Growth of pOka/vOka chimeric viruses in skin xenografts in SCIDhu mice. Skin xenografts were inoculated with pOka, the chimeric viruses, or vOka. Each bar represents the mean titer of infectious virus recovered from skin implants harvested at 7, 14, 21, or 28 days after inoculation; error bars indicate the standard error (SE). Inoculum titers (PFU/ml) are given for each virus tested.

H and M was reduced even though the Spe23 genome segment was from pOka.

Skin histology

Infection of skin xenografts with varicella vaccine was characterized by a relative restriction of replication to the epidermis, with limited penetration of the dermal layers, compared to much more extensive cytopathic changes associated with pOka infection (Moffat et al., 1998a, 1998b). In the current experiments, recombinant pOka and each of these chimeric viruses tested demonstrated extensive replication in epidermal tissues with limited penetration into dermal layers at 21 days after infection. By day 28, the lesions had expanded significantly with disruption of the basement membrane at the epidermal/dermal junction and replication in dermal cell layers and hair follicles. In comparison, replication of recombinant vOka at day 28 resembled pOka lesions at day 21 with extensive epidermal replication but limited progression into the dermis (Fig. 5). Our results indicate that, like the parent Oka strain, recombinant pOka retains the ability to spread deep into dermal tissues, whereas recombinant vOka, like varicella vaccine, exhibits a delayed penetration phenotype.

Intracellular localization of IE62 protein in vitro

The inoculation of melanoma cells with recombinant pOka and vOka showed a marked difference in the intracellular localization of IE62 protein, which was characterized by a prolonged nuclear retention of IE62 protein in vOka-infected cells. In our experiments, IE62 protein was predominantly cytoplasmic by 20 h in cells infected with recombinant pOka, while IE62 protein remained predominantly nuclear at 30 h in vOka-infected cells (Fig. 6) and had only a limited expression in cytoplasm at 48–72 h. Chimera B, which was constructed from vOka cosmids, with the exception of the pOka Fsp73 fragment, had the same IE62 localization phenotype as vOka. Chimera B was also the most attenuated of the chimeric viruses for infectivity in skin xenografts. Chimera G, which contained the vOka ORF62, was also predominantly nuclear. However, the IE62 nuclear localization phenotype could not be attributed solely to the presence of ORF62 from the vOka

Table 1
Relationships between patterns of growth in skin, the pOka composition of the chimeric viruses, and localization of immediate early 62 (IE62) protein in infected melanoma cells

	Peak titer in skin (cfu/implant)	pOka Fsp73	pOka Spe14	pOka Afl17	pOka Avr102	pOka Spe23	IE62 localization in melanoma cells (30 h)
pOka	2.8×10^4	+	+	+	+	+	C
D	3.0×10^4	–	–	+	+	–	C
G	2.9×10^4	+	–	+	+	–	N
K	2.3×10^4	–	–	+	+	+	C
H	1.7×10^4	+	–	–	–	+	C
M	1.5×10^4	+	+	–	–	+	C
B	1.1×10^4	+	–	–	–	–	N
vOka	0.9×10^4	–	–	–	–	–	N

N: nuclear localization, C: cytoplasmic localization.

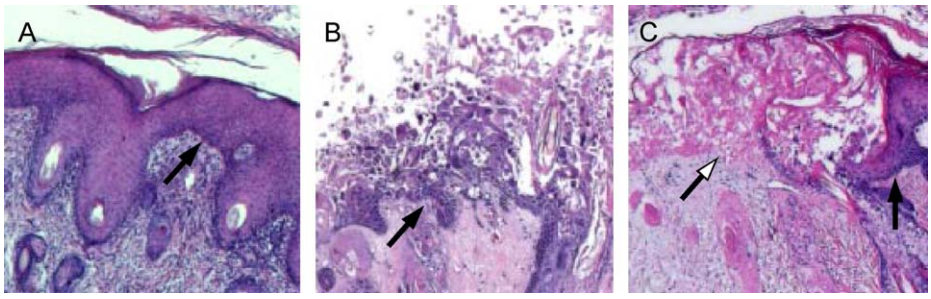

Fig. 5. Hematoxylin and eosin staining of skin xenografts. Hematoxylin and eosin staining of uninfected (A), recombinant vOka inoculated (B) or recombinant pOka inoculated (C) skin xenografts at 28 days post infection. Intact basement membrane representing the epidermal/dermal junction is indicated by a black arrowhead. Disruption of basement membrane and spread of VZV lesion into dermal layers is indicated by a white arrowhead.

cosmid because chimera D had typical cytoplasmic expression of IE62. Chimeras K, H, and M, all of which contain pOka ORF62, have cytoplasmic expression of IE62 protein as well. All of these observations were consistent for each chimeric virus tested in three separate assays.

Discussion

The live attenuated varicella vaccine is the first vaccine to be licensed in the United States for prevention of a human herpesvirus infection. Safety, as demonstrated by the significant reduction in the capacity of vOka to cause varicella, was documented in early clinical trials in Japan (Takahashi et al., 1974). The clinical evidence for vOka attenuation is that vaccine-associated rashes occur in only about 5% of healthy children and adults and that vOka replication does not occur or is relatively restricted in vaccinees who have leukemia or other immunosuppressive conditions that predispose to life-threatening wild-type VZV infection (Gershon, 2001; White, 1996). Infection of skin xenografts in SCIDhu mice with vOka was associated with lower yields of infectious virus, decreased viral protein synthesis and slower progression of cutaneous lesions compared to pOka and to VZV-S, another low passage clinical isolate. Therefore, we have proposed that the

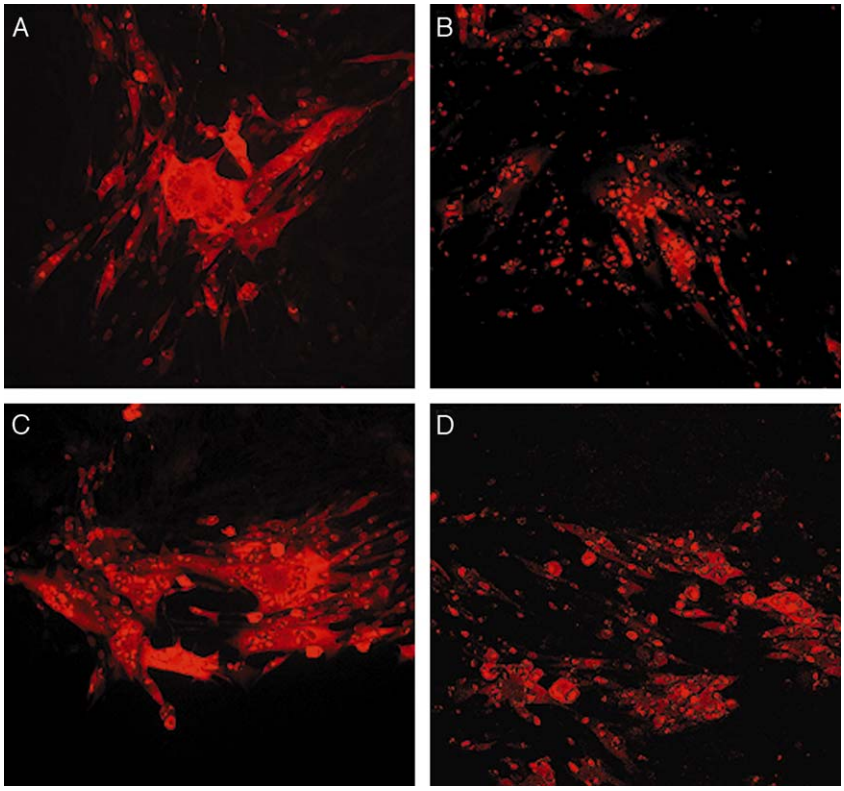

Fig. 6. Localization of IE62 in melanoma cells infected with pOka/vOka chimeric viruses. Melanoma cells were infected, incubated for 30 h. Monolayers were fixed and stained with anti-IE62 mAb and counterstained with Texas Red conjugated-anti-mouse IgG. The panels show IE62 localization for (A) pOka, (B) vOka, (C) chimera H, and (D) chimera B. Magnification 20×.

attenuation of vOka vaccines results from a reduced capacity to replicate in skin, which allows a longer interval for the adaptive immune response to develop after viral inoculation. According to this hypothesis, infectious vOka may be cleared before skin lesions are formed and before cell-associated viremia, with spread to other skin sites of replication, can occur (Arvin, 2001a, 2001b).

These experiments with chimeric pOka/vOka recombinant viruses suggest that vOka attenuation in human skin is multi-factorial. Diminished replication in skin was observed with chimeras M, B, and to a lesser degree with chimera H, even though different components of the genomes of these chimeric viruses were derived from vOka cosmids. Conversely, chimera D was as infectious as pOka despite containing only two pOka segments in the recombinant genome. These observations are consistent with consensus sequencing which suggests the presence of multiple nucleotide differences between pOka and vOka that are predicted to change amino acid residues (Argaw et al., 2000; Faga et al., 2001; Gomi et al., 2000). Our comparative analysis of the vOka and pOka consensus sequence data indicated that 32 viral proteins in vOka have predicted amino acid substitutions (cmgm.stanford.edu/~jjones/) (Table 2). Changes have been identified that could affect all three putative kinetic classes of VZV proteins, including immediate early regulatory proteins, early proteins and the late glycoproteins. Genetic heterogeneity has also been demonstrated when selected VZV ORFs were sequenced using viral DNA recovered from single plaques in tissue culture monolayers that were infected with vOka vaccine preparations (Gomi et al., 2000). Importantly, vOka viruses recovered from different individuals who had vaccine-related skin lesions have also demonstrated sequence heterogeneity when several ORFs were analyzed (Quinlivan et al., 2004). These observations suggest that vOka vaccines constitute mixtures of VZV genomes with varying genetic alterations that have accumulated during serial passage in cultured cells. As a result, the VZV genes and non-coding regions present in each of the five vOka cosmids, which were made from cells infected with the varicella vaccine, are expected to have changes that represent a subset of this mixed population of vOka genomes. The recombinant vOka virus was attenuated in skin. Thus, one combination of changes in the VZV genome that were induced by passage in human and non-human fibroblasts appears to be sufficient to confer the attenuation that is observed with the mixed population in varicella vaccine preparations. The fact that recombinant pOka remained virulent in skin xenografts suggests that the steps required to make VZV cosmids from pOka-infected cells did not introduce attenuating mutations into the pOka genome.

Nucleotide changes appear to be relatively common in ORF62/71 in vOka subpopulations. Sequencing of ORF62 in plasmid clones made from cells infected with the varicella vaccine Oka virus revealed considerable heterogeneity (Gomi et al., 2000). The A2872G substitution found in ORF62 in our vOka cosmid was identified in some ORF62 plasmids derived from vaccine Oka virus stocks, but the T3172C mutation had not been reported previously (Sato et al., 2003). Nevertheless, our experiments suggest that ORF62 changes were not sufficient or required to diminish vOka virulence in skin. Chimera M had this attenuation phenotype even though ORF62 was derived from pOka. IE62 protein has been observed within distinct small, dense nuclear bodies in cultured cells infected with vOka and occurred with our recombinant vOka virus (Kinchington et al., 2000). Our experiments suggest that this IE62 localization phenotype in vitro is not related to vOka attenuation.

Table 2
Analysis of consensus sequence data for V-Oka and P-Oka

ORF	Protein	aa	ORF	Protein	aa	ORF	Protein	aa
			24	(UL34)	1	51	ori bp frame shift[a]	
6	DNA rep	2	28	DNA pol	3	52	helic/primase	3
8	dUTPase	1	29	ssDNA bp	3	54	(UL6)	2
9	(UL49/VP22)	1	31	gB	1	56	(UL4)	1
10	transactivator	3	33	protease	3			
11	(aTIF aux)	insert/delete[b]	35	(UL24)	1	58	(UL3)	3
14	gC	insert/delete[b]	36	thy kinase	1	60	gL	2
16	DNA bp	1	37	gH	1	62/71	IE62	3
17	(UL41/vhs)	3[c]	38	(UL21)	1	64/69	(US10)	1
18	ribo reductase	1	39	(UL20)	frame shift[a]	68	gE	1
21	(UL37)	1	44	(UL16)	1			
22	(UL36)	11[d]	48	alk exonucl	2			

Parentheses indicate HSV homologue if VZV protein has not been characterized. aa = number of amino acid residues in the protein that are changed by the altered vOka sequence.

Sequence comparisons are based on the Merck consensus sequence for vaccine Oka and the Yaminishi consensus sequence for parent Oka.

Adapted from Cohen and Straus, 2001.

[a] Frame shift changes C-terminus.

[b] Insertions/deletions in R-repeats.

[c] With a 3-nt deletion.

[d] Two R-repeat insertion/deletion regions with 11 aa differences.

Chimera G had nuclear retention of IE62 but was as infectious as pOka in skin xenografts.

Quinlivan et al. (2004) demonstrated that rashes following vOka vaccination were derived from single clones of the vaccine virus mixture. Our experiments with chimeric pOka/vOka viruses suggest that VZV genomes with varying combinations of pOka-derived and vOka-derived DNA exhibit the attenuation phenotype in skin whereas other chimeras replicate as well as pOka. These observations are consistent with the evidence that the vaccine-related rashes are due to single viral clones and suggest that the limited number of cutaneous lesions that occur in some vaccine recipients represent an in vivo selection of the vOka genomes that retain infectivity for skin. The importance of the overall attenuation of the vOka mixture in skin is suggested by our evidence that VZV passage in fibroblasts attenuated VZV virulence in skin but not T cells (Moffat et al., 1998a, 1998b). VZV-Ellen, which has been passaged more than 100 times in cultured cells was even more impaired for replication in skin xenografts than vOka. However, vOka and pOka did not differ in their infectivity for human T cells in thymus/liver xenografts in vivo. Combining our findings with those of Quinlivan et al. may provide some further insights about the etiology of varicella vaccine related rashes. That is, vOka clones that can replicate more efficiently in skin would be expected to have retained the capacity to infect T cells that traffic through the vaccination site; if these T cells become infected, the selected virus can be transported to new skin sites of replication, creating additional cutaneous lesions that would represent the same vOka clone.

Our studies using recombinants made from cosmids in the SCIDhu model of VZV pathogenesis demonstrate that VZV infectivity in skin can be diminished even by a single amino acid substitution, such as disrupting ORF47 kinase function in the mutant, rOka47DN, or altering C-terminal residues in gE (Besser et al., 2003, Moffat et al., 2004). Changes in non-coding regions can also decrease skin tropism, as shown with gI promoter mutants (Moffat et al., 2002; Ito et al., 2003). These findings indicate that VZV attenuation in skin can be achieved by very restricted alterations in the genome sequence. The traditional approach to making a live attenuated viral vaccine by tissue culture passage has resulted in the production of a varicella vaccine containing mixtures of viral genomes. As shown with our chimeric pOka/vOka viruses, the presence of some vOka genome segments may not confer attenuation, whereas others do. These observations suggest that a "next generation" varicella vaccine could be made as a recombinant derived as a pOka/vOka chimera and representing a single VZV genome. As long as the vaccine contains a predominance of VZV genomes with mutations that reduce skin virulence, the result is the clinical experience of vaccine safety that has been observed with Oka-derived varicella vaccines.

Materials and methods

VZV Cosmids

The method for generating vOka recombinant viruses from four overlapping fragments of genomic DNA in SuperCos1 cosmid vectors (Stratagene) has been described (Kemble et al., 2000; Mallory et al., 1997) (Fig. 1A); subsequently, we made a set of four pOka cosmids with the same restriction sites as used for vOka cosmids, as well as five cosmid systems from vOka and pOka (Besser et al., 2003; Niizuma et al., 2003; Sato et al., 2003). To generate the five cosmid system, the largest of the four cosmids (Pme19) was first digested with AflII and AscI (Fig. 1B). A 26.0-kb AscI/AflII fragment, representing the left half of the Pme19 cosmid was isolated. An AflII/AscI linker was annealed to the isolated DNA and this fragment was ligated to the 6.8-kb AscI-digested SuperCos vector to generate the cosmid pAfl30. After digestion with AscI and AvrII, a 24-kb fragment representing the right half of the Pme19 cosmid was isolated, as well as the 4.6-kb AvrII/AscI fragment of the SuperCos vector. Following isolation, the two DNA fragments were ligated together to generate the cosmid pAvr13. The two cosmids, pAfl30 and pAvr13, generated from Pme19 contain a 7.5-kb overlapping region. An identical protocol was used to generate two new cosmids, pAfl17 and pAvr102, from the largest pOka cosmid (Pme2).

Generation of recombinant viruses

Cosmids were electroporated into Top 10F' E. Coli cells (Invitrogen) and DNA was purified using a Qiagen Maxiprep kit. The method for transfection of VZV cosmid DNA has been previously described (Mallory et al., 1998). In brief, cosmid DNA was digested with Asc1, with the exception of Avr102 and Avr13 which were digested with Asc1 and AvrII to release the viral DNA. In order to generate recombinant pOka virus, the pOka cosmids were mixed to a final concentration of 100 ng/μl of Fsp73, Spe14, Afl17 and Avr102, 50 ng/μl of Spe23 and 252 mM $CaCl_2$ in a total of 250 μl. Human melanoma cells were cotransfected with the intact pOka cosmids (Niizuma et al., 2003). The cells were grown at 37 °C for up to 28 days, passing at a 1:3 ratio every 3 to 4 days, by which time viral plaques were evident. The same protocol was used to generate recombinant vOka virus using the vOka cosmids Fsp4, Spe5, Afl30, Avr13, and Spe21. In order to produce chimeric viruses, cosmids from pOka and vOka were mixed prior to transfection. Chimeric viruses were analyzed by PCR and sequencing of the junctions between pOka and vOka cosmids.

Replication and IE62 localization in cultured cells in vitro

VZV recombinants were assessed for growth kinetics by infectious center assay (Mallory et al., 1998; Moffat et al., 1995). Briefly, VZV-infected melanoma cells were seeded

onto fresh monolayers of melanoma cells in a 6-well plate. At days 1 through 6 after infection, the infected cell monolayer was trypsinized, serially diluted, and added to uninfected Vero cell monolayers in a 24-well plate. After 5 or 6 days, wells were stained with crystal violet, plaques were counted and the number of infectious center-forming units (pfu) per ml of inoculum was calculated.

To examine localization of the IE62 protein, melanoma cells were seeded in 1 ml chamber slides, allowed to grow to near confluency, and inoculated with the test viruses. After 20, 30, and 48 h, cells were fixed in 2% paraformaldehyde with 0.1% Triton in phosphate-buffered saline (PBS) for 1 h, washed five times with PBS for 5 min each, blocked with 5% donkey serum in PBS for 30 min, and incubated overnight with mouse anti-IE62 antibody (1:750) with 1% donkey serum in PBS. Chamber slides were washed again five times in PBS and incubated with a secondary anti-mouse Texas red conjugated antibody (Jackson Immuno-Research) for 1 h. After washing, coverslips were mounted and slides were viewed and photographed at 200×. This assay was done three times for each test virus.

Infection of skin xenografts in vivo

Construction of human skin xenografts in SCID mice and subsequent inoculation with VZV or mock-infected cells was done as described previously (Moffat et al., 1998a, 1998b). Protocols for animal studies were approved by the Stanford University Administrative Panel on Laboratory Animal Care; human fetal tissues were obtained with informed consent according to federal and state regulations. Briefly, 3–5 weeks after implantation, mice were anesthetized and bilateral skin xenografts were inoculated with VZV-infected human lung fibroblasts (HELF) cells by scarification and direct injection using a 27-gauge needle. Control xenografts were inoculated with an equal number of uninfected HELF cells. At days 7, 14, 21, and 28 after inoculation, xenografts were removed and prepared for infectious center assay and immunohistochemistry (Moffat et al., 1998a, 1998b). Five implants were evaluated for each virus per time point. For viral titrations, half of the xenograft was mechanically homogenized in 1 ml of phosphate-buffered saline (PBS, pH 7.4). An equal volume of the homogeneous lysate (100 μl) was serially diluted for plaque assay. Plaque assays were performed in triplicate for each implant. For immunohistochemistry, the remainder of the xenograft was formalin-fixed and paraffin-embedded. VZV proteins were detected with a high-titer polyclonal human anti-VZV IgG; human IgG from a VZV non-immune donor was used as a negative control.

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