Genomic analysis of chimeric human cytomegalovirus vaccine candidates derived from strains Towne and Toledo

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28   **Abstract**   Human cytomegalovirus (HCMV) is an important opportunistic pathogen in

29   immunocompromised patients and a major cause of congenital birth defects when acquired *in utero*. In

30   the 1990s, four chimeric viruses were constructed by replacing genome segments of the high passage

31   Towne strain with segments of the low passage Toledo strain, with the goal of obtaining live attenuated

32   vaccine candidates that remained safe but were more immunogenic than the overly attenuated Towne

33   vaccine. The chimeras were found to be safe when administered to HCMV-seronegative human

34   volunteers, but to differ significantly in their ability to induce seroconversion. This suggests that

35   chimera-specific genetic differences impacted the ability to replicate or persist *in vivo* and the

36   consequent ability to induce an antibody response. To identify specific genomic breakpoints between

37   Towne and Toledo sequences and establish whether spontaneous mutations or rearrangements had

38   occurred during construction of the chimeras, complete genome sequences were determined. No major

39   deletions or rearrangements were observed, although a number of unanticipated mutations were

40   identified. However, no clear association emerged between the genetic content of the chimeras and the

41   reported levels of vaccine-induced HCMV-specific humoral or cellular immune responses, suggesting

42   that multiple genetic determinants are likely to impact immunogenicity. In addition to revealing the

43   genome organization of the four vaccine candidates, this study provided an opportunity to probe the

44   genetics of HCMV attenuation in humans. The results may be valuable in the future design of safe live or

45   replication-defective vaccines that optimize immunogenicity and efficacy.

46

47   **Keywords**   cytomegalovirus, recombinant, vaccine, attenuation, virulence

48

49   Human cytomegalovirus (HCMV) infections are an important cause of birth defects among newborns

50   infected *in utero* and of morbidity and mortality in transplant and AIDS patients. Despite receiving the

51   US Institute of Medicine's highest priority designation in 2000 (1), and after half a century of research,

52   development of an HCMV vaccine remains an unmet medical need of considerable importance to public

53   health.

54   Among the first HCMV vaccine candidates was the live attenuated strain Towne vaccine produced by

55   >125 passages in cultured human fibroblasts (2). This vaccine has been administered safely to nearly

56   1,000 human subjects at doses as high as 3,000 plaque-forming units (pfu), and has never been

57   recovered from an immunized subject, even following immune suppression (3-5). In contrast, the Toledo

58   strain passaged only 4 or 5 times in cultured fibroblasts exhibited virulence characteristics in HCMV-

59   seronegative volunteers at a dose of only 10 pfu (6), and was capable of superinfection, replicating, and

60   persisting in the context of pre-existing natural immunity (6, 7). Although administration of Towne

61   vaccine prior to renal transplantation reduced post-transplant HCMV-associated disease, it did not

62   prevent HCMV infections (3), and it failed to protect immunocompetent mothers from acquiring HCMV

63   infections from their children (8). These results suggest that the immunogenicity of the Towne vaccine

64   may be overly attenuated due to mutations acquired during serial passage *in vitro* (9-11).

65   With the goal of increasing the immunogenicity of the Towne vaccine, four genetic chimeras were

66   constructed by systematically replacing Towne genome segments with segments from Toledo (12). Each

67   chimera was shown to be safe when administered at a dose of 1,000 pfu to healthy HCMV-seropositive

68   human volunteers. However, failure to recover any chimera from blood, urine, or saliva following

69   inoculation, combined with the inability of the chimeras to boost humoral or cellular immune responses,

70   suggested that none retained the superinfection properties of the Toledo strain (12).

71   A phase 1 trial of the four chimeras in healthy HCMV-seronegative subjects was recently completed

72   (13). Each vaccine was administered to a total of nine subjects, with groups of three subjects receiving

73   doses of 10, 100, or 1,000 pfu by the subcutaneous route. There were neither local nor systemic

74   reactions nor serious adverse events, and none of the subjects shed infectious virus in urine or saliva. In

75   general, cellular and humoral immune responses were comparable to those reported previously for the

76   Towne vaccine, and none of the chimeras appeared to be more virulent or immunogenic than the

77   Towne vaccine. However, with regard to seroconversion, chimeras 2 and 4 were clearly more

78   immunogenic than chimeras 1 or 3: seven of the nine subjects who received chimera 4 seroconverted,

79   as did three of the nine subjects who received chimera 2, while only one of the nine subjects who

80   received chimera 1 seroconverted, and none of the nine subjects who received chimera 3 seroconverted

81   (13).

82   These results suggest that genetic differences among the four chimeras significantly impacted their

83   ability to replicate or persist *in vivo* to an extent necessary to induce an antibody response. Although the

84   approximate locations of junctions between Towne and Toledo sequences in the chimeras have been

85   reported (12), the precise breakpoints and any spontaneous mutations that may have arisen during

86   recombinant virus construction were unknown. Therefore, we determined the complete sequences of

87   all four chimeras.

88   Table 1 summarizes genome information for the chimeras and complete (or substantially complete)

89   Towne and Toledo sequences that were derived previously or during the present study. The Towne

90   genomes represent two major variants, of which varS, in comparison with varL, has a large deletion at

91   the right end of the U_L region (commonly called U_L/b' (11)) associated with an inverted duplication of a

92   sequence from the left end of U_L (9). Passage of HCMV in cell culture is known invariably to result in

93   mutation of RL13 and also of UL128, UL130, or UL131A (14-16), the latter three genes encoding subunits

94   of a pentameric complex necessary for efficient entry of HCMV into cells of the epithelial, endothelial, or

95   myeloid lineages (17-22). Towne is mutated in RL13 and UL130, as well as in UL1, UL40, and US1 (9, 10),

96   and the form of varS from which the chimeras were derived is also mutated in UL36 (23). Toledo is

97   mutated in RL13 and UL128 (the latter by the inversion of a large region of the genome) (11, 24, 25), as

98   well as in UL9. Mapping the components of the chimeras was informed in particular by accessions

99   FJ616285 and GQ121041 for Towne (9, 10) and accessions GU937742 and KY002201 for Toledo.

100 GU937742 represents the standard form of Toledo from which the chimeras were derived (at passage

101 8), and KY002201 represents a variant (obtained via transfection of a Toledo DNA stock followed by

102 plaque purification) that has a different mutation in gene RL13. The fact that more than one RL13

103 mutant was selected during isolation of Toledo is consistent with similar observations made with other

104 strains, and indicates that adaptation of wild-type HCMV to cell culture involves a complex, gradual

105 process of genetic selection (14-16). Thus, both Towne and Toledo apparently carried mutations that

106 had accumulated due to passage in fibroblasts.

107   The genetic maps of the chimeras are shown in Figure 1A. The parental strains are both non-

108 epitheliotropic and non-endotheliotropic due to the mutations disrupting expression of UL130 (Towne)

109 or UL128 (Toledo) (10, 17, 26). The consequent failure to express a functional pentameric complex is

110 speculated to contribute to attenuation of the Towne vaccine by limiting the range of host cell types

111 available for replication *in vivo*, and to Towne's insufficient efficacy, as the pentameric complex is an

112 important immunogen for eliciting antibodies that neutralize the entry of HCMV into cells of the

113 epithelial, endothelial, and myeloid lineages (22, 27-29).

114   By design (12), all four chimeras contain Toledo U_L/b' and within this a disrupted copy of UL128.

115 However, prior to the present study it was unclear whether chimeras 1 and 2 might contain an intact

116 copy of UL128 within the upstream Towne sequences, potentially rendering them epitheliotropic and

117 endotheliotropic. However, as the sequence data indicate that Towne UL128 is absent from all four

118 chimeras, none of them is genetically capable of expressing a functional UL128 protein or pentameric

119 complex, even though the UL130 and UL131A proteins, which contain neutralizing epitopes (30), may be

120 expressed. Consistent with this, phenotypic analysis revealed that all four chimeras fail to enter ARPE-19

121 epithelial cells efficiently (Figure 1B and (31)). By extension, the inability to express the pentameric

122 complex is consistent with the phase 1 trial findings that the chimera vaccines induced neutralizing titers

123 to entry into epithelial cells similar to those of Towne and significantly lower than those induced by

124   natural infection (13). In addition to the previously recognized mutations in the parental strains, the

125   sequences revealed three novel mutations. The first disrupts UL147A in chimera 4, the second is a short

126   duplication within the Towne-derived noncoding RNA4.9 in chimeras 1, 3, and 4 (with two duplications

127   in chimera 4), and the third is an intragenic deletion between US34A and TRS1 in chimera 1. A few other

128   minor differences were also noted, as specified in the legend to Figure 1.

129   Examination of the mutations highlighted in Figure 1A revealed no obvious association between the

130   presence of particular mutations and the efficacy of the chimeras in inducing seroconversion. For

131   example, the fact that chimeras 2 and 3 contain the same mutations except for one impacting UL40

132   might suggest that an inability to express UL40 renders chimera 3 unable to induce seroconversion.

133   However, the same mutation is present in chimera 4, which is the most immunogenic of the vaccines.

134   Indeed, each of the mutations present in chimera 3 is also present in immunogenic chimeras 2 or 4.

135   Therefore, the ability to induce seroconversion is likely associated with the distribution of parental

136   sequences among the chimeras rather than with specific mutations. For example, sequences from US16

137   to the right genome terminus are derived from Toledo in chimera 4 and from Towne in the other

138   chimeras. This region contains immune evasion genes (32) and perhaps other elements that may

139   contribute to the relatively enhanced immunogenicity of chimera 4.

140   Although the phase 1 chimera trial did not include Towne vaccine, comparison to historical data

141   suggested that all four chimeras are attenuated to a level similar to that of the Towne vaccine (13). This

142   indicates that the virulence characteristics associated with Toledo are multifactorial, in that none of the

143   Toledo sequences appeared measurably to enhance virulence when inserted into the Towne genome.

144   Alternatively, it is possible that the RL13 or UL128 mutations present in Toledo passage 8 and the

145   chimeras did not fully pervade the viral population present in the Toledo passage 4 or 5 stocks that

146   proved virulent in humans; that is, that some unmutated virus may have remained at this stage and was

147   responsible for the biological effect. Unfortunately, Toledo passage 8 has not been tested in humans,

148   and samples of earlier passages are no longer available.

149   The construction and testing of the four chimeric vaccine candidates has provided a rare

150   opportunity to study the genetics of viral pathogenesis in humans. While no specific virulence gene

151   emerged from this limited study, the data suggest that relatively few genetic changes are capable of

152   producing a virus that is highly attenuated and yet capable of replicating *in vivo* to an extent required to

153   induce both humoral and cellular immune responses. These findings may be valuable for rationally

154   designing live attenuated or replication-defective vaccines that maximize safety while optimizing

155   immunogenicity and efficacy.

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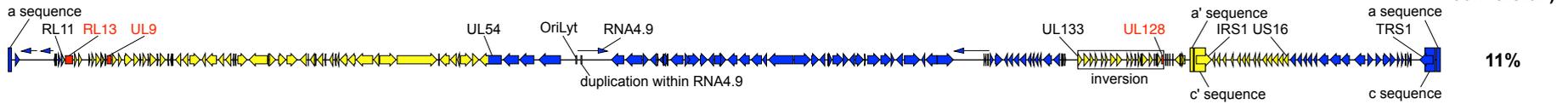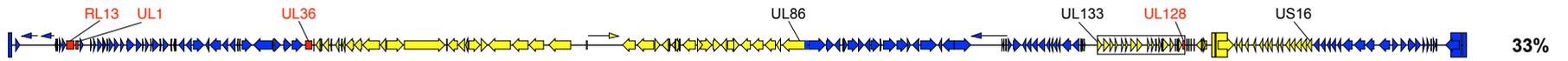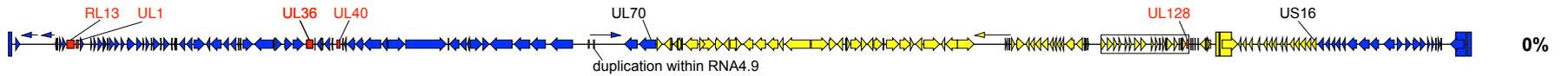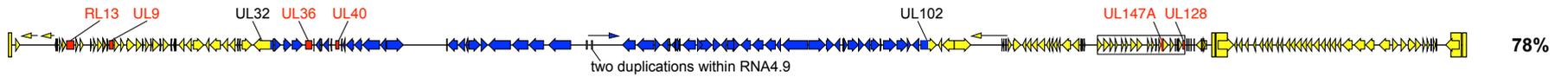247   **Table 1.** Partial and complete genome sequences of HCMV strains Towne and Toledo[a].

248

Strain	Genome	Accession	Size (bp)	Release date	Reference
Towne	BAC varS	AC146851	229,483	14-Oct-2003	(33)
Towne	BAC varS	AY315197	222,047	01-Dec-2003	(34)
Towne	Virus varL	FJ616285	235,147	07-Feb-2009	(9)
Towne	BAC varL	GQ121041	238,311	17-Jun-2009	(10)
Towne	BAC mutant (UL96) varS	KF493877	233,028	18-Aug-2013	(35)
Towne	Virus mutant (UL96) varS	KF493876	232,948	18-Aug-2013	(36)
Toledo	BAC	AC146905	226,889	21-Oct-2003	(33)
Toledo	Virus	AH013698	158,133	08-Mar-2004	(36)
Toledo	Virus	GU937742	235,404	10-Mar-2010	Present work
Toledo	Virus variant	KY002201	235,681	15-Nov-2016	Present work
Toledo	Virus mutant (RNA2.7)	KY002200	233,779	15-Nov-2016	Present work
Towne/Toledo	Virus chimera 1	KX101021	235,882	08-Jun-2016	Present work
Towne/Toledo	Virus chimera 2	KX101022	234,441	08-Jun-2016	Present work
Towne/Toledo	Virus chimera 3	KX101023	235,354	08-Jun-2016	Present work
Towne/Toledo	Virus chimera 4	KX101024	236,269	08-Jun-2016	Present work

249

250   [a]Genomes were sequenced as bacterial artificial chromosomes (BACs), viruses, virus variants, virus

251   mutants, or virus chimeras, and in varS or varL form for Towne. The two Towne BAC varS sequences

252   describe the same BAC but differ in size because they lack different parts of the vector. The chimeras

253   that had been used to inoculate seronegative human subjects (13) were amplified by passaging twice in

254   MRC-5 human fibroblast cells, and virion DNA was isolated from culture supernatants as described

255   previously (37). Sequence data were obtained for these and the other viruses examined in the present

256   work by using the Illumina MiSeq platform, and assembled and validated as described previously (38).

257   Additional information is available in the GenBank accessions.

258   **Figure 1.** (A) Sequence-based genetic maps of the four Towne/Toledo chimera vaccine strains. Open

259   arrows indicate open reading frames, and lines with arrowheads indicate noncoding RNAs. Tall

260   rectangles indicate inverted repeats (*a/a'* and *c/c'*), and these and other features (oriLyt, RNA4.9, IRS1,

261   and TRS1) are labeled on chimera 1. Genes containing disrupting mutations are labeled in red, and genes

262   located at breakpoints are labeled in black (these include UL36 in chimera 2). Additional differences

263   among regions derived from the same original strain are not marked. These include a large noncoding

264   deletion between US34A and TRS1 in chimera 2, a small noncoding deletion between UL150A and IRS1

265   in chimera 3, a short region of Towne sequence at the beginning of the Toledo *a'* sequence in chimera 2

266   (probably as a result of recombination), a few differences in the lengths of noncoding G:C tracts, three

267   substitutions in intergenic regions (UL102/UL103 and UL124/UL128 in chimera 1 and UL23/UL24 in

268   chimera 4), one substitution in RNA5.0 in chimera 2, two synonymous substitutions in coding regions

269   (UL10 and TRS1 in chimera 1), four nonsynonymous substitutions (UL11 and US10 in chimera 1, UL47 in

270   chimera 2 and UL93 in chimera 4), and a small number (2-6 per genome) of nucleotide polymorphisms.

271   The recombinational breakpoint in US16 in chimeras 1, 2, and 3 is located in the same 255 bp sequence.

272   The values on the right indicate the relative immunogenicity levels of each chimera reported previously

273   (13). (B) MRC-5 fibroblast or ARPE-19 epithelial cells were mock-infected or infected with equivalent

274   amounts of the indicated viruses and after 4 d stained for HCMV immediate early proteins as described

275   previously (27). BAD*r*UL131 and TS15-rN are epitheliotropic variants of HCMV strains AD169 and Towne

276   varS, respectively (26, 39), and TS15 is a non-epitheliotropic variant of Towne varS (10).

A

Chimera 1

Efficacy
(% sero-
conversion)

a sequence
RL11 RL13 UL9   UL54   OriLyt   RNA4.9   UL133 UL128   a' sequence   a sequence
                                                                                                            IRS1 US16   TRS1

                                                                                                                                          11%

duplication within RNA4.9   inversion   c' sequence   c sequence

Chimera 2

RL13 UL1   UL36   UL86   UL133 UL128   US16

                                                                                                                                          33%

Chimera 3

RL13 UL1   UL36 UL40   UL70   UL128   US16

                                                                                                                                          0%

duplication within RNA4.9

Chimera 4

RL13 UL9   UL32 UL36 UL40   UL102   UL147A UL128

                                                                                                                                          78%

two duplications within RNA4.9

Towne sequences   Toledo sequences   ORFs containing disruptive mutations

B

| | mock | BAD*r*UL131 | TS15-rN | TS15 | Chimera 1 | Chimera 2 | Chimera 3 | Chimera 4 |

MRC-5

ARPE-19