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Identification of Envelope Protein Epitopes That Are Important in the Attenuation Process of Wild-Type Yellow Fever Virus

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Monoclonal antibodies (MAbs) have been prepared against vaccine and wild-type strains of yellow fever (YF) virus, and envelope protein epitopes specific for vaccine (MAbs H5 and H6) and wild-type (MAbs S17, S18, S24, and S56) strains of YF virus have been identified. Wild-type YF virus FVV, Dakar 1279, and B4.1 were each given six passages in HeLa cells. FVV and B4.1 were attenuated for newborn mice following passage in HeLa cells, whereas Dakar 1279 was not. Examination of the envelope proteins of the viruses with 87 MAbs showed that attenuated viruses gained only the vaccine epitope recognized by MAb H5 and lost wild-type epitopes recognized by MAbs S17, S18, and S24 whereas the nonattenuated Dakar 1279 HeLa p6 virus did not gain the vaccine epitope, retained the wild-type epitopes, and showed no other physical epitope alterations. MAb neutralization-resistant (MAb^r) escape variants generated by using wild-type-specific MAbs S18 and S24 were found to lose the epitopes recognized by MAbs S18 and S24 and to acquire the epitope recognized by vaccine-specific MAb H5. In addition, the MAb^r variants became attenuated for mice. Thus, the data presented in this paper indicate that acquisition of vaccine epitopes and loss of wild-type epitopes on the envelope protein are directly involved in the attenuation process of YF virus and suggest that the envelope protein is one of the genes encoding determinants of YF virus pathogenicity.

Yellow fever (YF) virus is the prototype of the family *Flaviviridae* and is responsible for the disease YF. The development of safe live attenuated vaccines has resulted in YF diminishing as a major public health problem. The 17D vaccine has proved very effective and safe (reviewed in reference 1) and was developed by passage of the wild-type YF virus Asibi through chicken embryo tissue (18). Two distinct substrains of 17D are recognized; they are known as 17D-204 and 17DD and were derived by differing passage series of 17D. The 17D-204 vaccines are obtained between passages 233 and 240 from parent wild-type strain Asibi, whereas 17DD vaccines are obtained between passages 286 and 288. In addition to the 17D vaccine, French workers have derived a live attenuated YF vaccine virus, known as the French neurotropic vaccine (FNV) virus, by passage of the wild-type French viscerotropic virus (FVV) through mouse brain (reviewed in reference 1).

Hahn et al. (10) have compared the nucleotide and deduced amino acid sequences of wild-type strain Asibi and the 17D-204 vaccine substrain and identified 68 nucleotide changes coding for 32 amino acid substitutions (including 12 in the envelope [E] protein gene). Which of these nucleotide and/or amino acid changes are responsible for the attenuated phenotype of the 17D vaccine has not been elucidated.

The YF virus E protein, which contains antigenic determinants eliciting the biological activities of neutralization, hemagglutination, and passive protection, has been investigated in detail by using monoclonal antibodies (MAbs) (2, 4, 8, 9, 15) and the spatial location of epitopes on the E protein reported previously (5, 16). Also, Lobigs et al. (12) have used MAb neutralization-resistant escape variants to demonstrate that amino acids 70 and 71 constitute part of a YF virus type-specific epitope on the E protein.

Previously, Gould et al. (8) described epitopes that were 17D-204 substrain (MAb 864) and vaccine (17D-204, 17DD, and FNV) (MAb 411) specific, and Schlesinger et al. (15) also described a 17D-204 substrain (MAb 8A3)-specific epitope. More recently, Gould et al. (9) have described a YF virus wild-type (MAb 117)-specific epitope on the envelope protein of YF virus. We (3) used these MAbs to investigate the attenuation of wild-type YF virus Asibi after six passages in HeLa cells (Asibi HeLa p6 virus) and found that the latter virus had gained the vaccine epitope recognized by MAb 411 and lost the wild-type epitope recognized by MAb 117. Whether such envelope protein epitopes were involved in the attenuated phenotype or were coincidental was unclear from these studies.

To investigate further the involvement of the E protein in the molecular basis of attenuation of YF virus, we have generated panels of MAbs against both wild-type and vaccine strains of YF virus and identified MAbs that specifically recognize vaccine and wild-type epitopes on the envelope protein of the YF viruses. In this paper we report the involvement of vaccine-specific epitopes in the attenuation of wild-type YF virus.

MATERIALS AND METHODS

Viruses. The viruses used in this study and their titer determination in infectivity assays have been described previously by Barrett et al. (2, 4). Wild-type YF virus Dakar 1279 and the French viscerotropic virus are termed D1279 and FVV, respectively. B4.1 is a plaque-purified preparation

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TABLE 1. Reactivity of HeLa-passaged YF viruses with anti-YF virus MAbs

MAb	Specificity	Result of IIF last measuring reactivity[a] of:										
		Wild-type YF virus				HeLa-passaged YF virus				YF vaccine virus		
		Asibi	FVV	B4.1	D1279	Asibi	FVV	B4.1	D1279	17D-204/W	17DD/B	FNV-NT
H5	YF vaccine	−	−	−	−	+	+	+	−	+	+	+
H6	17D vaccine	−	−	−	−	+	−	−	−	+	+	−
H10	17DD vaccine	−	−	−	−	−	−	−	−	−	+	−
S17	Wild type	+	+	+	+	−	−	−	+	−	−	−
S18	Wild type	+	+	+	+	−	−	−	+	−	−	−
S24	Wild type	+	+	+	+	−	−	−	+	−	−	−
S56	Wild type	+	+	+	+	−	+	−	+	−	−	−

[a] +, positive reaction; −, negative reaction.

(13) of the wild-type strain 1899/81, a human isolate from Peru (13).

MAbs. MAbs were produced against YF vaccine virus strain 17DD manufactured in Brazil (17DD-Brazil) and wild-type virus B4.1 by using the methods described by Barrett et al. (4), except that NSO myeloma cells were used for the B4.1 fusion.

Characterization of MAbs. MAbs were characterized by indirect immunofluorescence (IIF) assays, hemagglutination inhibition (HAI) assays, passive protection tests, and plaque reduction neutralization tests. IIF assays were performed as described by Barrett et al. (4) with acetone-fixed, virus-infected Vero cells. HAI assays were performed by using ascitic fluids as described by Clarke and Casals (7) and as modified for use in microtiter plates by Sever et al. (17). Plaque reduction neutralization tests were done as described by Barrett et al. (4) on the monkey kidney cell line LLC-MK2. Plaques were visualized by addition of neutral red to the agar overlay, and titers were expressed as the $\log_{10}$ reduction in virus infectivity. Passive protection tests were performed as described by Barrett et al. (4).

Passaging of viruses in HeLa cells. Wild-type YF virus D1279, FVV, and B4.1 were passaged six times in HeLa cells as described by Barrett et al. (3).

Virulence of HeLa-passaged and non-HeLa-passaged YF viruses for newborn mice. Newborn (outbred TO) mice, 24 to 48 h old, were inoculated intraperitoneally with 50 μl containing 100 PFU of virus diluted in phosphate-buffered saline (PBS) and observed for up to 21 days postinoculation.

Cluster analysis. The viruses used in the studies were examined with the panels of MAbs generated in HAI assays. HAI endpoint titers were normalized by using $\log_{10}$ transformation, and the data were used to compute similarity ratios between all the viruses. This similarity matrix was transformed by an unweighted pair-group method, and results were visualized as a dendrogram. All computational operations were performed by using Clustan version 3.2 running on a prime mainframe computer (19).

MAb neutralization-resistant escape variants. Antigenic variants were selected after mixing virus with the appropriate MAb in plaque reduction neutralization tests as described by Barrett et al. (4). The MAb neutralization-resistant escape variants were cloned by plaque purification as described by Wiktor and Koprowski (20) and amplified in SW13 cells. MAb neutralization-resistant escape variants were then reidentified by repeating the antibody treatment procedure, as described above, to confirm that the variants were no longer neutralized by the selecting MAb.

RESULTS

Preparation of MAbs. Panels of hybridoma cell lines were generated against two strains of YF virus. A total of 24 MAbs were raised against 17DD-Brazil (series H) and 34 were raised against the B4.1 plaque pick of wild-type strain 1899/81 (series S). Most of the MAbs were flavivirus intermediate (i.e., recognized some but not all flaviviruses examined) or flavivirus group common (i.e., recognized all flaviviruses examined) in reaction. Three of the series H (H5, H6, and H10) and four of the series S (S17, S18, S24, and S56) MAb-secreting hybridoma cell lines were examined in detail.

Antigenic specificities of MAbs. Both panels of MAbs were characterized by IIF tests by using representatives of four antigenic complexes of the family *Flaviviridae*. Table 1 shows the results of IIF tests with the selected H and S MAbs. MAb H10 was found to be specific for the 17DD vaccine substrain, whereas H6 recognized the 17D (17D-204 and 17DD) YF vaccine strain. MAb H5 reacted with all YF vaccine viruses (17D-204, 17DD, and FNV) but no wild-type YF virus. MAbs S17, S18, S24, and S56 were specific for both African and South American wild-type strains of YF virus (Asibi, FVV, B4.1, and D1279).

Passage of wild-type strains of YF virus in HeLa cells. We have previously reported that wild-type YF virus Asibi becomes attenuated for monkeys and mice and loses its

TABLE 2. Virulence of HeLa- and non-HeLa-passaged YF virus strains for newborn mice

Virus	Virulence	
	No. dead/total no. inoculated[a]	% Mortality
Asibi		
p0	13/13	100
p6	1/10	10
FVV		
p0	14/14	100
p6	0/10	<10
B4.1		
p0	9/9	100
p6	0/13	<8
D1279		
p0	10/10	100
p6	10/10	100

[a] Number of newborn mice inoculated intraperitoneally with 100 PFU of virus diluted in PBS.

TABLE 3. HAI titers of MAbs against flaviviruses

MAb	HAI titer[a] against:											
	Wild-type YF virus					YF vaccine virus			HeLa-passaged virus				JE virus[b]
	Asibi	FVV	B4.1	D1279	Y5	17D	17DD	FNV	Asibi	FVV	B4.1	D1279	
H3	2.2	2.2	1.9	2.2	2.2	2.2	2.2	2.2	1.9	1.9	1.9	2.2	–
H5	–[c]	–	–	–	1.9	1.9	1.9	1.9	1.9	1.9	1.9	–	–
H6	–	–	–	–	–	1.9	1.6	–	1.9	–	–	–	–
H10	–	–	–	–	–	–	1.6	–	–	–	–	–	–
H25	–	–	–	–	–	–	–	–	–	–	–	–	–
H33	1.9	2.2	1.9	1.9	2.2	1.9	2.2	2.2	1.9	2.2	1.9	2.2	–
S1	3.1	3.1	3.1	3.1	1.3	1.3	2.2	1.9	1.3	1.3	1.3	1.3	2.8
S2	1.6	1.9	2.2	1.6	1.3	1.6	1.6	1.6	1.6	1.6	1.9	1.6	–
S3	1.9	1.9	2.2	1.6	1.3	–	1.6	1.6	2.2	1.9	2.2	1.3	1.6
S5	2.2	2.2	2.5	1.9	2.8	1.3	1.6	2.5	2.5	2.8	2.8	2.2	–
S6	2.8	1.9	2.5	2.2	–	1.3	1.6	1.6	1.6	1.6	1.6	1.3	–
S11	1.3	1.3	1.9	1.3	–	1.6	2.2	1.3	1.3	1.3	1.9	2.2	1.3
S12	2.2	2.2	2.2	1.6	1.6	1.3	1.3	1.3	1.6	1.6	1.9	1.6	–
S15	2.2	2.2	2.5	2.2	1.9	1.9	1.9	2.2	2.2	1.9	2.2	2.5	–
S17	1.9	1.9	1.9	1.9	1.6	–	–	–	–	–	–	1.9	–
S18	2.2	2.2	2.2	1.9	1.6	–	–	–	–	–	–	2.5	–
S19	2.8	2.5	3.1	2.5	2.5	2.2	1.9	2.2	2.2	2.2	2.5	3.1	–
S20	–	1.6	1.9	1.3	2.8	1.3	1.3	1.3	1.6	1.6	1.6	1.3	–
S24	1.9	2.2	3.1	2.2	1.3	–	1.3	–	–	–	–	2.2	–
S29	2.5	2.2	3.1	1.9	1.9	2.2	1.9	1.6	2.8	2.5	2.5	2.8	–
S30	1.9	1.9	3.1	1.3	2.2	1.6	–	1.6	1.6	1.6	1.9	1.3	–
S32	1.9	1.6	1.6	1.6	1.3	1.3	1.6	1.9	2.2	2.2	2.5	–	–
S34	–	1.6	1.3	–	1.9	1.6	1.6	1.9	1.3	1.3	1.3	–	–
S37	1.9	2.2	1.6	1.6	1.6	1.6	1.6	1.6	1.3	1.9	1.3	1.6	–
S38	1.6	1.9	2.2	1.6	1.6	1.3	1.6	1.6	1.6	1.3	1.9	1.9	1.6
S39	1.6	1.9	1.6	1.6	1.3	1.3	1.3	–	1.6	1.9	1.3	1.3	–
S40	1.3	1.9	1.6	1.6	–	1.3	1.3	–	1.3	1.3	1.6	1.6	–
S41	1.3	1.3	1.9	1.6	2.2	–	–	1.3	1.3	1.3	1.3	1.6	–
S42	–	–	–	–	–	–	–	–	–	–	–	–	–
S43	2.2	2.2	1.6	1.9	–	1.6	1.9	1.6	2.2	1.9	2.5	2.2	–
S44	2.2	2.2	3.1	1.9	2.5	1.9	1.9	1.9	1.9	1.6	1.9	2.5	–
S46	1.3	1.6	2.2	1.6	1.6	1.3	1.6	1.3	1.3	1.3	1.3	1.3	–
S47	1.3	1.6	1.9	1.6	1.3	1.3	1.3	1.3	1.9	2.2	1.9	–	–
S48	–	1.3	2.5	–	1.3	1.3	1.6	1.6	1.6	1.6	1.6	–	–
S49	1.6	1.6	1.6	1.6	1.9	1.3	1.6	1.6	1.6	1.3	1.6	–	–
S50	1.3	1.9	2.2	1.6	1.6	1.9	1.9	1.6	1.6	–	1.9	1.3	–
S53	1.6	1.3	2.2	1.3	1.6	1.9	1.6	1.9	1.3	1.3	1.6	1.3	1.9
S56	1.9	1.9	1.9	2.5	1.9	1.3	1.3	1.3	–	1.6	–	3.1	–
S72	1.9	1.9	1.6	1.6	1.9	1.3	1.3	1.6	1.3	1.3	1.6	1.6	–
S80	1.9	1.9	1.9	1.9	1.9	1.6	1.6	1.9	1.6	2.2	1.9	1.9	1.9

[a] Titers are expressed as $\log_{10}$ HAI titers.
[b] JE virus, Japanese encephalitis virus.
[c] –, <1.3.

ability to infect mosquitoes after six passages in HeLa cells (3, 14). Three other wild-type strains of YF virus (FVV, D1279, and B4.1) were each given six passages in HeLa cells (HeLa p6 virus), and their virulence was examined by using newborn mice. Non-HeLa-passaged (HeLa p0) or HeLa p6 virus (100 PFU for each virus strain) was inoculated intraperitoneally into 1- to 2-day-old mice (Table 2). All three HeLa p0 viruses were virulent, with no mice surviving the challenge, whereas FVV HeLa p6 and B4.1 HeLa p6 viruses were avirulent, with no mice dying after the challenge. In comparison, D1279 HeLa p6 virus was still virulent and killed all mice after the challenge.

The reactivities of the HeLa p0 and HeLa p6 viruses (including Asibi HeLa p6 reported previously [3]) were examined with the MAbs described above (Table 1). Asibi, FVV, and B4.1 HeLa p6 viruses all lost the wild-type-specific epitopes recognized by MAbs S17, S18, and S24; Asibi and B4.1 HeLa p6 viruses also lost the wild-type epitope recognized by MAb S56. All three HeLa p6 viruses gained the vaccine epitope recognized by MAb H5, and Asibi HeLa p6 also gained the 17D epitope recognized by MAb H6. In comparison, D1279 HeLa p6 virus retained all four wild-type epitopes and did not acquire the vaccine epitopes recognized by MAbs H5 and H6. None of the HeLa-passaged viruses gained the 17DD substrain-specific vaccine epitope recognized by MAb H10. These viruses were also examined with a large panel of E protein-reactive MAbs (those described in this paper and in references 4 and 11, totaling 87 MAbs), and all but those described above were found to react with every YF virus used in these studies (data not shown).

Reactivity of the MAbs in HAI tests. Six H MAbs and 34 S MAbs were examined for their ability to exhibit HAI activity with the viruses used in these studies (Table 3). All but H25

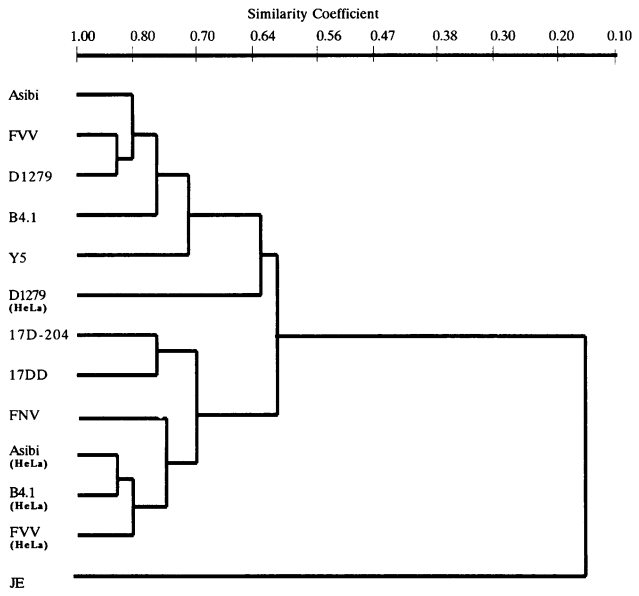

FIG. 1. Dendrogram showing the relationship between HeLa-passaged YF viruses and other strains of YF virus as determined by HAI assays with two panels of monoclonal antibodies.

and S42 exhibited HAI activity with one or more of the viruses in the study, depending on the antigenic specificity of the MAb. MAb H5 exhibited HAI activity with Asibi, FVV, and B4.1 but not D1279 HeLa p6 viruses, whereas the wild-type MAbs S17, S18, S24, and S56 lost HAI activity with the HeLa p6 viruses. Thus, the HAI activity results (Table 3) support the results obtained in the IIF tests (Table 1).

The HAI data in Table 3 show that some virus strains appear to be similar whereas others are more distinct. However, the data are complex, and to give a quantitative view of the differences or similarities between individual strains, the data were subjected to cluster analysis to determine similarity coefficients between the viruses (Fig. 1). This technique has been used to great effect in the analysis of sequence data from wild-type isolates of Japanese encephalitis virus (6). The results show that the different strains of YF virus examined shared a minimum of 60% similarity in their reactions with this particular panel of MAbs. However, the wild-type strains of YF virus (Asibi, FVV, B4.1, Y5, and D1279) were more similar in their reactions and were distinct from both the vaccine derivatives (17D-204, 17DD, and FNV) and the attenuated HeLa p6 viruses. The attenuated strains were more related to each other than to the wild-type strains of YF virus. The D1279 HeLa p6 virus was similar to non-HeLa-passaged D1279 virus.

Neutralization of YF viruses by MAbs. The neutralizing activity of the MAbs was examined by screening viruses against 100 PFU of selected YF viruses (Table 4). Most MAbs, including H5, S17, and S56, did not neutralize any of the viruses examined. MAbs that did exhibit neutralization are shown in Table 4; they included wild-type-specific MAbs S18 and S24 and MAb H6. Since wild-type-specific MAbs S17 and S56 do not exhibit neuralizing activity, it is unlikely that they recognize the same epitope as that recognized by MAbs S18 and S24. Three of the MAbs (H3, H10, and H25) neutralized one or more of the vaccine strains. MAb H3 neutralized vaccines derived from the YF 17DD substrain, whereas MAb H10 neutralized 17DD-Brazil but not 17DD-Colombia or 17DD-Senegal vaccine substrains. MAb H25 neutralized 17D-204-WHO. Nine of the S series of MAbs (S6, S18, S19, S20, S24, S29, S32, S44, and S46) possessed neutralizing activity against one or more of the YF viruses examined. Flavivirus intermediate MAbs (S6, S19, and S46) and two YF type-specific MAbs (S32 and S44) neutralized the wild-type strains of YF virus but not the vaccine strains.

TABLE 4. Reactivity of HeLa- and non-HeLa-passaged YF viruses with MAbs in neutralization assays

MAb	Specificity	Reactivity (neutralization titer)[a] of:												
		YF wild-type virus				HeLa-passaged virus				YF vaccine virus				
		Asibi	FVV	B4.1	D1279	Asibi	FVV	B4.1	D1279	17D-204/W	17DD/B	17DD/C	17DD/S	FNV-NT
H3	YF type	–[b]	–	–	–	–	–	–	–	–	3.0	2.8	3.0	–
H5	YF vaccines	–	–	–	–	–	–	–	–	–	–	–	–	–
H6	17D vaccine	–	–	–	–	–	–	–	–	1.8	0.8	nt[c]	nt	–
H10	17DD vaccine	–	–	–	–	–	–	–	–	–	1.0	–	–	–
H25	Flavivirus group common	–	–	–	–	–	–	–	–	2.2	–	–	–	1.8
S6	Flavivirus intermediate	1.3	1.2	1.6	1.2	1.3	0.8	1.4	1.2	1.0	1.0	nt	nt	1.0
S17	YF wild type	–	–	–	–	–	–	–	–	–	–	nt	nt	–
S18	YF wild type	1.2	1.1	1.3	1.0	–	–	–	1.1	–	–	nt	nt	–
S19	Flavivirus intermediate	0.9	0.9	1.0	0.7	1.0	0.9	0.8	0.9	1.0	0.8	nt	nt	1.0
S20		–	–	1.0	–	–	–	–	–	–	–	nt	nt	–
S24	YF wild type	1.5	1.5	1.0	1.1	–	–	–	1.3	–	–	nt	nt	–
S29	Flavivirus group common	–	–	0.8	–	–	–	0.4	–	–	–	nt	nt	–
S32	YF type	0.9	0.8	1.0	0.7	1.0	0.8	0.8	0.7	0.5	0.7	nt	nt	0.6
S44	YF type	0.8	1.1	1.0	1.2	1.1	1.0	0.7	1.0	0.7	0.6	nt	nt	0.9
S46	Flavivirus intermediate	0.6	–	–	–	–	–	–	–	–	–	nt	nt	–
S56	YF wild type	–	–	–	–	–	–	–	–	–	–	nt	nt	–

[a] Neutralization titers are expressed as $\log_{10}$ reductions in virus infectivity titers.
[b] –, neutralization <0.3.
[c] nt, not tested.

TABLE 5. Reactivity of MAb^r variants with YF E protein-reactive MAbs in IIF assays

MAb	Specificity	Result of IIF test measuring reactivity[a] of:									
		Wild-type parent virus			MAb^r variant				YF vaccine virus		
		B4.1	FVV	D 1279	B4.1-S18^r	FVV-S24^r-A	FVV-S24^r-B	D1279-S18^r	17D-204/W	17DD/B	FNV-NT
S17	YF wild type	+	+	+	+	+	+	+	−	−	−
S18	YF wild type	+	+	+	−	−	−	−	−	−	−
S24	YF wild type	+	+	+	−	−	−	−	−	−	−
S56	YF wild type	+	+	+	+	+	+	+	−	−	−
S44	YF type	+	+	+	+	+	+	+	+	+	+
H3	YF type	+	+	+	+	+	+	+	+	+	+
H5	YF vaccines	−	−	−	+	+	+	+	+	+	+
H6	17D vaccine	−	−	−	−	−	−	−	+	+	−
H10	17DD vaccine	−	−	−	−	−	−	−	−	+	−
H25	Flavivirus group common	+	+	+	+	+	+	+	+	+	+

[a] +, positive reaction; −, negative reaction.

Passive protection of mice against virus challenge. To investigate the role of MAbs H5, H6, and H10 in protective immunity, we analyzed the MAbs in passive protection tests against 17DD-Brazil. Although all the mice died after challenge with 17DD-Brazil virus, the average survival time of mice that had received MAb H5, H6, or H10 was significantly longer (9.0 ± 0.28, 10.0 ± 0.2, and 11.0 ± 0.2 days, respectively) than that of the control nonreactive MAb K23-treated mice (6.9 ± 0.3 days) ($P < 0.01$).

MAb neutralization-resistant escape variants. MAbs S18 and S24 were used to neutralize wild-type strains FVV, D1279, and B4.1 to generate MAb neutralization-resistant (MAb^r) escape variants. Three MAb^r variants were selected for FVV with MAb S24, three for B4.1 with MAb S18, and three for D1279 with MAb S18. Each variant was examined for the loss of neutralization activity with the selecting MAb. Of the nine plaque-purified viruses selected, four, selected as described in Materials and Methods, were found to be resistant to neutralization to the selecting MAb after plaque purification. Two were against FVV (FVV-S24^r-A and FVV-S24^r-B), one was against B4.1 (B4.1-S18^r), and one was against D1279 (D1279-S18^r). All four variants had lost the wild-type epitopes recognized by MAbs S18 and S24 and gained the vaccine epitope recognized by MAb H5 (Table 5). This indicates that the MAbs S18 and S24 recognize the same epitope or that the epitopes recognized by the two MAbs are functionally or spatially linked. It follows that the wild-type-specific epitopes recognized by MAbs S17 and S56 are distinct from those recognized by the other wild-type-specific MAbs. The virulence of the variants was examined by intraperitoneal inoculation of 100 PFU into newborn mice. All the parent viruses were lethal, whereas all four variants were attenuated and killed none of the mice (Table 6).

DISCUSSION

In the work described in this paper, panels of MAbs have been prepared against vaccine and wild-type strains of YF virus and used to examine attenuation of wild-type strains of YF virus. From our panel of E protein-reactive MAbs raised against 17DD-Brazil, three important epitopes were identified. MAb H10 recognized a 17DD substrain-specific epitope and differentiated among 17DD manufactured in Brazil, Colombia, and Senegal in neutralization tests. Thus, we have identified the existence of a substrain-specific epitope similar to that described for 17D-204 (MAbs 864 [8] and 8A3 [15]). As with the 17D-204 epitopes, the 17DD epitope exhibited HAI, passive protection, and neutralization. The 17DD epitope was not found on the Asibi HeLa p6 virus and presumably is not associated with attenuation. MAb H6 recognized an epitope that is 17D (i.e., recognizes 17D-204 and 17DD substrains) YF vaccine specific. This is the first time such an epitope has been reported. This epitope was also present on the attenuated Asibi HeLa p6 virus, which does not cause viscerotropic disease (3). Thus two separate attenuation processes of the Asibi strain (chicken tissue and human HeLa cells) give rise to the same epitope, suggesting that this epitope may be involved in the attenuation process of wild-type YF virus Asibi.

MAb H5 also showed a unique pattern of reactivity with the attenuated strains of YF virus. This MAb is specific for an epitope that appears conserved among all YF vaccine viruses (i.e., 17D-204, 17DD, and FNV); the Asibi HeLa p6, FVV HeLa p6, and B4.1 HeLa p6 viruses; and the wild-type YF virus Y5. Previously, Gould et al. (8) identified MAb 411, which was prepared against FNV-NT and reacted like MAb H5, being YF vaccine and Asibi HeLa p6 specific. However, MAb 411 did not exhibit HAI or neutralization activity. In contrast, MAb H5 is HAI reactive, suggesting that the two MAbs do not recognize the same epitope, although it is likely that the epitopes are part of the same antigenic site on the E protein. Three different attenuation processes of wild-type YF virus (passage of FVV in mouse brain to produce FNV; passage of Asibi in chicken tissue to produce 17D; and

TABLE 6. Virulence of MAb^r variants and their parent viruses for newborn mice

Virus	Virulence	
	No. dead/total no. inoculated[a]	% Mortality
B4.1	9/9	100
B4.1-S18^r	0/11	<9
FVV	14/14	100
FVV-S24^r-A	0/16	<6
FVV-S24^r-B	0/14	<7
D1279	16/16	100
D1279-S18^r	0/9	<11

[a] Newborn mice inoculated intraperitoneally with 100 PFU of virus diluted in PBS.

passage of Asibi, FVV, and B4.1 in human HeLa cells) all result in acquisition of the same E protein epitope recognized by MAb H5. Therefore, in addition to the epitope recognized by MAb H6, it is likely that the epitope recognized by MAb H5 plays a role in the attenuation process of YF virus. In support of this proposal, D1279 HeLa p6 virus did not acquire the epitope recognized by MAb H5 and remained virulent for newborn mice. This also shows that the appearance of the epitope recognized by MAb H5 is not an artifact produced by passage of YF virus in HeLa cells. Why D1279 virus was not attenuated after passage in HeLa cells is not clear; it will be analyzed further in a separate study (7a).

Four wild-type-specific MAbs were identified (S17, S18, S24, and S56) since they were present on all four wild-type strains of YF virus examined, including South American and African strains, but not the vaccine strains 17D-204, 17DD, and FNV. Of the four HeLa cell-passaged wild-type strains of YF virus, three (Asibi, FVV, and B4.1) were attenuated and all lost the epitopes recognized by MAbs S17, S18, and S24, while Asibi and B4.1 lost the epitope recognized by MAb S56. In comparison, the HeLa nonattenuated virus, D1279, retained the epitopes recognized by all four of the MAbs. Thus, these results support the proposal that loss of wild-type epitopes is associated with attenuation of YF virus. Also, the results suggest that the four wild-type-specific MAbs do not recognize the same epitope. The epitope recognized by MAb S56 was lost only by passage of Asibi and B4.1 viruses in HeLa cells whereas MAbs S18 and S24 appear to recognize the same or strongly associated epitopes. Therefore at least three wild-type epitopes are identified on the E protein of wild-type YF virus. The cluster analysis of the HAI results suggests that attenuation of YF virus by any of the three routes (chicken embryo, mouse brain, or HeLa) results in an alteration in the antigenicity of the E protein of the viruses (Fig. 1). Possibly this results in a change in conformation in the E protein such that wild-type epitopes are lost and vaccine epitopes appear. This possibility is supported by the results obtained with the MAb^r variants. Loss of the wild-type epitope(s) recognized by MAbs S18 and S24 was found to coincide with acquisition of the vaccine epitope recognized by MAb H5 and attenuation of virulence for newborn mice. How attenuation takes place is unclear. The alteration in antigenicity and the loss or gain of particular epitopes may affect the interaction of the virus attachment site on the E protein with the cell receptor of YF virus. Studies are under way to investigate this possibility.

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