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Large-Population Passages of Vesicular Stomatitis Virus in Interferon-Treated Cells Select Variants of Only Limited Resistance

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Vesicular stomatitis virus (VSV) populations were repeatedly passaged in L-929 cells treated with alpha interferon (IFN-α) at levels of 25 U/ml. This IFN-α concentration induced a 99.9% inhibition of viral yield in standard infections. Analysis of viral fitness (overall replicative ability measured in direct competition with a reference wild-type VSV) after 21 passages in IFN-treated cells showed only a limited increase or no increase in fitness, compared with the greater increase upon parallel passage in cells not treated with IFN-α. However, this limited increase in fitness was more pronounced when competition assays were carried out with IFN-α-treated cells, suggesting the selection of VSV populations with a low level of resistance to IFN-α. Thus, despite the extensively documented capacity of VSV to adapt to changing environments, the antiviral state induced by IFN-α imposes adaptive constraints on VSV which are not readily overcome.

Interferons (IFNs) are a group of cytokine proteins secreted by cells. They constitute one of the earliest responses after viral invasion, and they play a critical role in the clearance of some viral infections (31, 49). Gamma IFN (IFN-γ) and alpha and beta IFN (IFN-α and -β) act through different pathways to establish an antiviral state in cells stimulated by IFN binding to specific receptors. Many different antiviral proteins are induced by IFN, including the Mx proteins, the 2′-5′-oligoadenylate synthetase complex, and P1/eF-2 protein kinase (33, 52). IFN also upregulates the expression of major histocompatibility complex antigens (33, 52) and activates a double-stranded RNA adenosine deaminase (48). The editing function of the latter enzyme might hypermutate (and destabilize) RNA virus genomes (6). Thus, the antiviral actions of INF are multiple, although the sensitivity of any particular virus to each of those mechanisms varies. Some DNA viruses carry accessory genes with anti-IFN activity (1, 2, 7, 12, 22, 34, 44, 58). RNA viruses have long been known for their potential to adapt to different environments (10, 11, 16, 26–29, 57). This adaptability is due mainly to their high mutation rates (16, 17, 29, 54), and their small genomic size does not usually allow the acquisition of genes mediating specific resistance to antiviral host functions. Despite this limitation, several RNA viruses have developed mechanisms for partial resistance to IFN. There are several reports of RNA virus proteins with anti-IFN activity (3, 24, 30, 32, 38, 50). Natural isolates of a particular virus often show variable sensitivity to IFN (4, 5, 21, 23, 36, 45, 46, 51), and patients undergoing IFN therapy often show different degrees of responsiveness to the treatment (23, 46). Understanding whether and how IFN influences RNA virus evolution is relevant to its use in antiviral therapy, especially in the treatment of hepatitis B and C virus and human immunodeficiency virus infections (13, 25, 35, 37). In the present report, we describe the selection of vesicular stomatitis virus (VSV) populations displaying slight resistance to IFN. However, adaptation to growth in the presence of IFN also limits viral adaptation to the host cell type in which the viruses replicate repeatedly.

The Mudd-Summers (M-S) strain (Indiana serotype) of VSV was used in this study. The wild-type virus was a population derived from a clone, and it was partially adapted to mouse L-929 cells by four diluted passages before use. We have assigned a fitness value (W) of 1 (the neutral internal control value) to this wild-type VSV population. A relative fitness value is experimentally measured by coinfection passages of the test virus in direct competition with wild-type VSV. The test virus was a genetically marked (I1 monoclonal antibody resistant) subclone of wild-type VSV. The parental wild-type clone of VSV was employed as the competing reference virus. All virus assays were done in triplicate, both in the absence and in the presence of I1 monoclonal antibody in plaque overlay medium (27). The proportion of test and reference virus was measured initially (before passages) and after one or more competition passages. Changes in ratios of test to reference virus allowed the calculation of the relative fitness value (for further details, see references 8, 9, 18–20, and 27). It should be noted that in population biology, a fitness gain (or loss) of 10% is considered to be biologically highly meaningful (39). Two monoclonal antibody-resistant mutant (MARM) viral populations, MARM X and MARM E, were used in this study. We used a previously described clone, MARM X, which is an I1-antibody-resistant derivative of wild-type VSV. MARM X initially exhibited a low-fitness phenotype in L-929 cells ($W = 0.035 \pm 0.004$). The MARM E clone was obtained after 21 diluted passages of MARM X on L-929 monolayers and initially exhibited a high-fitness phenotype in that cell line ($W = 3.7 \pm 0.4$). Preparation of I1 monoclonal antibody stocks has been described elsewhere (20). Mouse IFN-α was obtained

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TABLE 1. Fitness changes of MARM X virus populations during large population passages in 25-U/ml IFN-treated and untreated L-929 cells[a]

Series	Result with IFN in competition	Fitness (W)	t^b	df	P
Control (untreated)					
A	−	3.1 ± 0.3			
	+	3.4 ± 0.1	0.6165	5	0.2823
B	−	3.4 ± 0.2			
	+	3.3 ± 0.3	0.2233	7	0.4148
C	−	0.89 ± 0.02			
	+	ND[c]			
D	−	2.6 ± 0.3			
	+	2.2 ± 0.1	1.1461	7	0.1447
E	−	4.6 ± 0.8			
	+	4.3 ± 0.7	0.3255	7	0.3772
F	−	2.5 ± 0.1			
	+	2.44 ± 0.08	0.5005	7	0.3160
IFN-treated					
B	−	0.31 ± 0.07			
	+	1.02 ± 0.03	9.0879	7	<0.0001
C	−	0.47 ± 0.08			
	+	1.1 ± 0.6	0.9762	8	0.1788
D	−	0.5 ± 0.1			
	+	0.9 ± 0.3	1.3700	8	0.1040
E	−	0.49 ± 0.09			
	+	5 ± 1	3.7908	7	0.0034
F	−	1.0 ± 0.2			
	+	2.9	2.1347	8	0.0327

[a] The results were analyzed by ANCOVA (55). Parental MARM X has a fitness of 0.035 ± 0.0.004 in untreated L-929 cells, and fitness values in the presence of IFN are not statistically different ($P = 0.414$).
[b] Student's t test employed for comparison of each population's competition in the presence and absence of IFN.
[c] ND, not determined.

from Lee Biomolecular Research, Inc. Twenty-one consecutive large virus population (control) transfers in the absence of IFN were done as described previously (8, 9, 18–20, 27). Also, 21 passages in IFN-pretreated cells were carried out in the same manner. Virus yields were diluted after each passage so that approximately 10^5 to 10^6 infectious particles were present in each transferred population. Cells were pretreated with 1.5 ml of a 25-U/ml IFN solution for 24 h. After pretreatment, IFN was removed and the monolayers were immediately infected with the large virus populations. IFN inhibition of virus yield was about 1,000-fold (99.9% yield reduction). Viral titers were determined after each passage, so that appropriate dilutions were used in the next passage to keep the population transfer size approximately constant (10^5 to 10^6 infectious particles) at a multiplicity of infection of about 0.1.

MARM X populations passaged 21 times in IFN-treated cells showed significant increases in mean fitness in untreated L-929 cells ($W = 0.51 \pm 0.06$; $P < 0.0001$) compared with the level in the control parental population ($W = 0.035 \pm 0.04$), as shown by an analysis of covariance (ANCOVA) test (55). However, such increases were significantly lower than those observed in populations passaged in the absence of IFN ($W = 2.3 \pm 0.3$; $P < 0.0001$). Fitness gains of populations passaged in IFN-treated cells were higher when competitions were carried out in the IFN-treated cells than in the untreated cells (1.7 ± 0.4 versus 0.51 ± 0.06; $P = 0.001$) (Table 1). This difference indicated the selection of populations with slightly increased resistance to IFN. Results with MARM E populations were less significant (Table 2). First, MARM E populations passaged in IFN-treated cells did not show significant overall increases in fitness in L-929 cells ($W = 6.0 \pm 1$) compared with unpassaged MARM E populations ($W = 3.7 \pm 0.3$). The results of each series were very heterogeneous, with values higher than, lower than, or about equal to those for parental MARM E (Table 2). The possible generation of an IFN-resistant population was statistically analyzed by ANCOVA ($P = 0.0525$) and ANOVA ($P = 0.0233$). Both results are close to the 0.05 level of significance, indicating that only very limited resistance was developed in MARM E populations.

Next, we carried out experiments with cells pretreated with low concentrations of IFN (1.5 ml of a 1-U/ml solution for 6 h, causing about 60% inhibition of viral yield). Neither MARM X nor MARM E virus showed any evidence of resistance after 21 passages at low levels of IFN, and their adaptation to L-929 cells was comparable to that observed in untreated controls in every case (data not shown).

The present study shows that, at best, only partially IFN-resistant variants of VSV can be selected even when a strong selective pressure is applied. This very limited acquisition of IFN-α resistance is probably due to the pleiotropic effects of IFN: inhibition of protein synthesis, RNA degradation by endonucleases, RNA editing, and inhibition of viral entrance in the case of VSV (56, 59). The multiple effects of IFN apparently severely constrain the possibility of generating highly resistant VSV genomes. Presumably, multiple mutations at several genomic loci would be needed to overcome the multiple biochemical blocks in IFN-treated cells. This would be a low-probability occurrence in our viral quasispecies populations (15). VSV is known to be very sensitive to IFN, and it is

TABLE 2. Changes in fitness of MARM E virus populations during large population passages in 25-U/ml IFN-treated and untreated L-929 cells[a]

Series	Result with IFN in competition	Fitness (W)	t^b	df	P
Control (untreated)					
A	−	9 ± 2			
	+	11 ± 3	0.3996	8	0.35
B	−	13 ± 2			
	+	11.2 ± 0.3	0.5646	8	0.2939
C	−	19 ± 3			
	+	8.7 ± 0.8	3.9085	8	0.0022
D	−	11 ± 2			
	+	15 ± 3	0.9631	8	0.1819
E	−	16 ± 3			
	+	15 ± 2	0.1488	6	0.4433
F	−	28 ± 2			
	+	21 ± 3	1.7442	8	0.0596
IFN treated					
A	−	17 ± 2			
	+	18 ± 8	0.0811	8	0.4687
B	−	8.5 ± 0.2			
	+	31 ± 15	1.527	8	0.0826
C	−	24 ± 8			
	+	28 ± 15	0.2068	7	0.4210
D	−	4 ± 1			
	+	21 ± 5	3.3826	8	0.0048
E	−	3.0 ± 0.9			
	+	14 ± 5	2.2025	7	0.0317
F	−	0.7 ± 0.2			
	+	3.7 ± 0.4	6.1426	6	0.0004

[a] Results were analyzed by ANCOVA (55). Parental MARM E has a fitness of 3.7 ± 0.3 in untreated L-929 cells, and fitness values in the presence of IFN are not statistically different ($P = 0.483$).
[b] Student's t test employed for comparison of each population's competition in the presence and absence of IFN.

routinely used to test IFN activity, indicating a lack of genetic functions conferring IFN resistance. In some cases, as described for mengo virus, resistant phenotypes are simply explained by the inability of a viral strain to induce IFN in infected cells (40). VSV Indiana can induce IFN production in vivo (60), but not in cell culture (43). In fact, VSV Indiana strains are often supressors of IFN production in cell culture (41–43, 53). However, in our case, IFN is supplied exogenously; thus, our resistant populations are not due to variants that have lost the capacity to induce IFN.

In summary, despite their great adaptability and remarkable ability to gain fitness exponentially (47), RNA viruses have only a feeble capacity to increase fitness in the presence of IFN. Furthermore, this slight gain in fitness in the presence of IFN is achieved only at the cost of an overall decrease in gains of fitness in the host cell. Our results demonstrate that the adaptability of RNA viruses encounters insurmountable limitations when antiviral activities are directed to multiple viral functions and/or to independent targets on the viral genome. They provide further support for the notion that whole-virus vaccines able to trigger a multivalent immune response or combination therapy with multiple antiviral inhibitors represents the most logical approach for the control of highly variable RNA viruses (14, 15).

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