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WIN 51711-Dependent Mutants of Poliovirus Type 3: Evidence that Virions Decay after Release from Cells Unless Drug Is Present

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Twenty-two spontaneous mutants of the Sabin strain of poliovirus type 3 were selected for drug resistance by plating on HeLa cell monolayers in the presence of WIN 51711, an uncoating inhibitor. When replated in the presence and absence of drug, two classes of mutants were observed; mutants displayed either a drug-dependent or a non-drug-dependent phenotype, in the proportion 14:8. Non-drug-dependent mutants plaqued with equal efficiency in the presence or absence of drug. By contrast, drug-dependent mutants made no plaques in the absence of drug, except for revertants. In single-step growth curve experiments, however, drug-dependent mutants grew as well in the absence of drug as in its presence. This paradoxical behavior of dependent mutants was traced to extreme thermolability at 37°C (12- to 30-s half-life) in the absence of drug. Thermolability was exhibited only after the virus was released from the cell, implying the presence of a cell-associated protective factor, possibly pocket factor. Thus, in the absence of a thermostabilizing drug, drug-dependent mutants decayed too rapidly after release to permit spread in the plaque assay. The thermodecay product was shown to consist of 135S particles lacking VP4.

Picornaviruses are small, icosahedral viruses with 60 copies each of four capsid proteins, VP1, VP2, VP3, and VP4, surrounding the genomic RNA. The structures of several picornaviruses have been determined crystallographically; these include Mahoney poliovirus type 1 (19), human rhinovirus 14 (36), and the Sabin strain of poliovirus type 3 (11). The major capsid proteins, VP1, VP2, and VP3, are each folded into an eight-stranded beta barrel with various decorations that determine the topography of the inner and outer surfaces (37).

One of the problems of virology at the molecular level is the assignment of functions to specific regions of the protein subunits. In nonenveloped viruses, the viral coat must package the genome in a form protected from nucleases in the environment, deliver the genome to susceptible host cells by attachment to specific cellular receptors, and mediate the delivery of the genome across the cell membrane into the cytoplasm.

We chose to use the uncoating inhibitor WIN 51711 to study the regions of the viral capsid instrumental in the uncoating process. WIN 51711, or disoxaril (5-[7-[4-(4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-3-methylisoxazole), synthesized by Sterling Winthrop Pharmaceuticals Research Division, Rensselaer, N.Y. (25, 30), is one of a wide variety of hydrophobic drugs that have been shown to be inserted into the beta barrel of capsid protein VP1 under the "canyon" that encircles each fivefold axis of symmetry (1, 38). Insertion of these drugs into picornavirus capsids stabilizes the capsids against denaturation caused by heat and extremes of pH (3, 12, 14, 32). WIN 51711 also interacts with empty capsids (32, 34) and with the 14S pentamers of capsid polypeptides (33), preserving the native antigenicity of these structures against heat denaturation. Stabilizing effects on virions apparently lead to the primary antiviral effect of the drugs: prevention of viral uncoating (12, 26, 43).

A study of drug-resistant mutants has been carried out with human rhinovirus 14 and related drugs (16, 17). However, for this virus, the drugs inhibit attachment (17, 31). The attachment inhibition is strong enough to obscure any effect that the drugs may have on uncoating. Correlated with attachment inhibition is a drug-induced swelling on the floor of the canyon (1, 38), the acceptor site for the cellular receptor (4, 36).

One of the important outcomes of the study of drug escape mutants was the finding that resistance to WIN 52084 and WIN 52035 could be acquired via single amino acid substitutions (16). More significantly, many of these resistance mutations occurred in the region of the canyon floor that can be deformed by the drugs, suggesting that resistance mutations may provide a useful way to map regions of the coat protein important for functions that are blocked by drug action (16, 17).

Since we wished to study a virus whose drug escape mutants would provide information on the structures important in uncoating, we continued our studies on drug resistance with a virus whose attachment is unaffected by drug binding. We chose the Sabin strain of poliovirus type 3 because it is sensitive to the drug WIN 51711 and because its atomic structure is known to a very high resolution. This paper describes the properties of a special class of mutants that require the drug for survival after the particles are released from the cell.

MATERIALS AND METHODS

Cells and virus. HeLa cells, originally obtained from V. Hamparian of Ohio State University, were cloned in our laboratory and maintained in shake cultures in medium B (27). The Sabin strain of poliovirus type 1 was obtained from E. Seligmann, Jr., Bureau of Biologics, U.S. Food and Drug

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Administration, Bethesda, Md., and the Sabin strain of poliovirus type 3 was obtained from Olen Kew, Centers for Disease Control, Atlanta, Ga.

Solutions. WIN 51711 was a gift from Guy Diana, Sterling Winthrop Pharmaceuticals Research Division. It was dissolved at 8 mg/ml in dimethyl sulfoxide (DMSO) and diluted at least 1,000-fold into medium or phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.47 KH_2HPO_4, 4.86 mM Na_2HPO_4, 0.68 mM $CaCl_2$, 0.49 mM $MgCl_2$) containing 0.4% bovine serum albumin (BSA). The concentration of DMSO used in controls, typically less than 0.1%, was adjusted to be the same as that in the presence of drug. At 0.1%, DMSO had no measurable effect on cell viability or virus replication.

Mutant selection. Each mutant was isolated from a separate plaque-purified stock of wild-type virus. Each wild-type plaque was amplified by infecting a 100-mm-diameter monolayer of HeLa cells with 0.4 ml of virus stock and harvesting the virus when cytopathic effects were observed, 20 to 40 h later. The drug was allowed to diffuse into the virus by incubation of the virus in PBS containing 0.4% BSA and 2 μg of WIN 51711 per ml at room temperature for 1 h and in the refrigerator overnight. The virus-drug suspensions were then diluted in the same buffer and plated onto HeLa cell monolayers. Plaques were developed at 37°C under agar and liquid overlays containing supplemented medium P5 (see below) and 2 μg of WIN 51711 per ml as for the plaque assay (see below). Supplemented medium P5 is medium P5 (27) containing 0.4% BSA, 2 mM L-glutamine, 2 mM oxalacetic acid, 1 mM pyruvic acid, and 0.2% D-glucose. After 40 to 46 h, the cell monolayers were stained by replacement of the liquid layer with 0.01% neutral red–0.1% glucose in Earle's saline. The plates were incubated for another 1 or 2 h, and then the liquid layer was removed and agar plugs overlying the plaques were picked into 0.5 ml of PBS containing 0.4% BSA and 2 μg of WIN 51711 per ml. Viruses were released from cells by freezing and thawing.

Under these conditions, mutants appeared as normally sized plaques on a dense background of extremely tiny plaques of wild-type virus. Mutant plaques were subjected immediately to a second round of selection, which eliminated the background of tiny plaques.

Twenty-two mutants were selected in this way. Each was amplified on HeLa cell monolayers under liquid medium containing 2 μg of WIN 51711 per ml. When most of the cells had become round, they were scraped off the plates, and cells plus overlying medium were collected. Viruses were released by three cycles of freezing and thawing, and cellular debris was removed by centrifugation.

Plaque assay. Virus dilutions (200 μl) in PBS containing 0.4% BSA were applied to washed HeLa cell monolayers, and the virus was allowed to attach for 30 min at room temperature. The cells were then overlaid with 2.5 ml of supplemented medium P5 containing 0.8% Noble agar and 2.5 ml of the same medium without agar. WIN 51711 (2 μg/ml) was either present or absent in all buffers and media as described in the figure legends. The monolayers were incubated at 37°C for 40 to 50 h unless stated otherwise in the figure legends. About 1 ml of 4% formaldehyde was added to the liquid overlays to fix the cells, and 1 h or more later, the overlays were removed and the cells were stained with 0.1% crystal violet in 20% ethanol.

Attachment assays. Radiolabeled virus was incubated in buffer with or without 2 μg of WIN 51711 per ml for 1 h at room temperature and overnight at 4°C to allow diffusion of the drug into the virions. These virus stocks were mixed with HeLa cells at 10 PFU per cell, and the mixture was incubated for 30 min at room temperature with gentle agitation. Cells were sedimented and washed once in PBS with or without 2 μg of WIN 51711 per ml to remove unattached virus. The two supernatants were sampled in triplicate to determine radioactivity, and the cell pellet was resuspended in medium A (27) containing 0.4% BSA and with or without 2 μg of WIN 51711 per ml. Triplicate samples were treated with a 1/10 volume of 0.1 N NaOH to lyse the cells, and radioactivity was determined. The total recovery of initial radioactivity from the supernatants and the pellet was 75 to 95%.

Growth curves. Virus was complexed with drug in PBS containing 0.4% BSA, 0.1% DMSO, and 2 μg of WIN 51711 per ml by incubation for 1 h at room temperature and overnight in the refrigerator. Control virus was incubated in the same manner, but without drug. These virus solutions were used to infect HeLa cells (4 ml at 4×10^7 cells per ml in cold supplemented medium P5 with or without 2 μg of WIN 51711 per ml) at a multiplicity of infection of 5 to 15 PFU per cell for 30 min at room temperature. At the end of the attachment period, cells were pelleted and washed in PBS containing 0.4% BSA and with or without drug to remove unattached virus. Cells were resuspended to 4×10^6/ml in warm supplemented medium P5 with or without 2 μg of WIN 51711 per ml, sampled for the determination of infectivity, and incubated at 37°C.

Samples were taken at intervals thereafter. For independent determinations of cell-associated virus and released virus, samples were centrifuged at 12,000 rpm for 1 min in an Eppendorf model 5415 centrifuge. The supernatant was removed to a separate tube, and the cell pellet was resuspended in 0.5 ml of cold supplemented medium P5 with or without drug. All samples were frozen and thawed three times to release virus and diluted into PBS containing 0.4% BSA. Virus infectivity was determined by a plaque assay in the absence of drug for wild-type virus and in the presence of 2 μg of WIN 51711 per ml for mutant viruses.

Determination of thermostability. Virus was diluted to 10^8 PFU/ml with PBS containing 0.4% BSA or supplemented medium P5 as stated in the figure legends. Samples (150 μl) were pipetted into warmed, capped glass tubes immersed in a water bath and allowed to incubate for various times. The tubes were then withdrawn from the water bath and plunged into an ice bath for immediate cooling. Virus was diluted into drug-free PBS containing 0.4% BSA for wild-type virus and PBS containing 0.4% BSA and 2 μg of WIN 51711 per ml for mutant viruses, and infectivity was determined by a plaque assay. Heated samples were compared with control samples pipetted directly into tubes in an ice bath.

Sucrose gradient centrifugation. [^{35}S]methionine-labeled virus (100 μl) heated as described above in plaque assay medium (P5) or left at room temperature for a similar length of time was mixed with 5 μl of [^{3}H]leucine-labeled wild-type poliovirus type 3 (Sabin strain), and the mixture was layered onto a 4.5-ml 5 to 20% sucrose gradient in PBS containing 0.01% BSA. Gradients were centrifuged at 40,000 rpm (149,000 × *g*) for 40 min at 20°C in a Beckman SW50.1 rotor, 10-drop fractions were collected from the bottom directly into scintillation vials, and the radioactivity was counted in 5 ml of Ecoscint counting cocktail (National Diagnostics, Manville, N.J.).

RESULTS

High sensitivity of poliovirus type 3 to WIN 51711. Initial efforts to study drug-resistant mutants were directed to poliovirus type 1, for which there is a rich background of

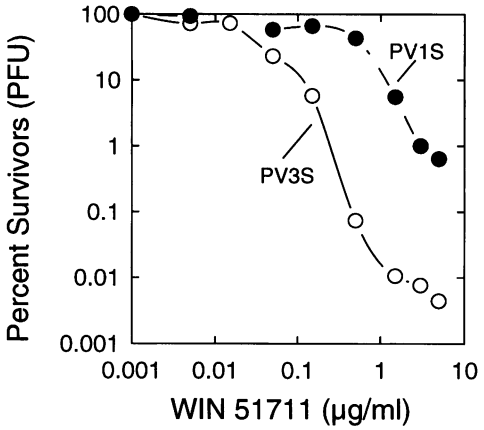

FIG. 1. Ability to form plaques in the presence of various concentrations of WIN 51711. Sabin polio vaccine virus types 1 (PV1S) and 3 (PV3S) were diluted to 10^8 PFU/ml and incubated with dilutions of the drug in PBS containing 0.4% BSA for 1 h at room temperature and then overnight in the refrigerator. Surviving infectivity was determined by a plaque assay on HeLa cells as described in Materials and Methods. Poliovirus types 1 and 3 were incubated for 45 and 50 h, respectively, before staining and counting of plaques were done. All dilutions and the overlay media were made by use of the indicated concentrations of drug.

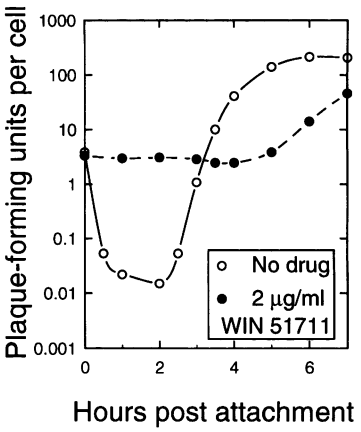

FIG. 2. Single-step growth curve for a HeLa cell suspension demonstrating that WIN 51711 inhibits the eclipse of Sabin poliovirus type 3. Virus pretreated at room temperature for 1 h and at 4°C overnight with or without WIN 51711 (2 μg/ml) was attached to HeLa cells as described in Materials and Methods. Virus and cells were mixed at 7 PFU per cell. Cells were washed free of unattached virus, resuspended in warm medium with or without drug, maintained at 37°C, and sampled periodically by quick-freezing of 0.5-ml samples in dry ice-ethanol. Infectivity was determined by a plaque assay in the absence of drug. The inhibitory effect of the drug was reversed by dilution of the virus 100-fold and incubation for at least 1 h at room temperature before plating.

information on neutralization by monoclonal antibodies (22, 29). However, poliovirus type 1 proved much less sensitive in plaque inhibition assays than type 2 or 3 (30). The different sensitivities of types 1 and 3 are illustrated in Fig. 1. At 1.5 μg/ml, the drug reduced the plaque titers of poliovirus type 3 to about 0.01% and of poliovirus type 1 to about 6% of the drug-free control value. All subsequent experiments were performed with poliovirus type 3.

It is worth noting that the shapes of the dose-response curves (Fig. 1) were affected by the time allowed for plaque development. When assay plates were incubated for 3 days instead of 2, the wild-type virus was able to produce tiny plaques even in the presence of the drug (data not shown). Thus, the drug delayed but did not completely prevent plaque development of the wild-type virus. This same delaying effect on virus replication emerged in other ways, as will be described below.

Evidence that WIN 51711 delays infectivity eclipse but does not affect attachment. Single-step growth experiments, such as that shown in Fig. 2, were useful in documenting the locus of drug action. In this experiment, approximately 50% of the inoculated virus was recovered after 30 min of attachment at room temperature and subsequent washing, and this recovery was not changed when attachment was accomplished in the presence of 2 μg of WIN 51711 per ml (Fig. 2, closed circles). In experiments with ^{35}S-labeled virus (see below), it was established that about 65% of purified radioactive particles attached in 30 min under standard conditions (4×10^7 HeLa cells per ml in growth medium); in these experiments, attachment was also not significantly affected by WIN 51711. Since the fraction of infectious particles that attached agreed within experimental error with the fraction of radiolabeled particles that attached, we concluded that eclipse during the standard 30-min attachment step was negligible.

However, the ability of the drug to block the rapid decline in infectivity (eclipse) that normally occurred when the temperature was raised to 37°C was evident. In the absence of drug, infectivity fell over 100-fold (Fig. 2, open circles); this decrease was followed by an increase beginning at about 2.5 h after infection and reaching a plateau at about 6 h. The eclipse period was almost completely obliterated by the drug, even as late as 4 h (Fig. 2, closed circles); nevertheless, the slow increase in infectivity beginning at about 5 h in the presence of drug implied that some uncoating must have taken place.

Progeny viruses grown in the presence of drug were still largely drug sensitive. When the titers of the material from the 7-h harvest of virus grown in the presence of drug were determined in the presence of 2 μg of WIN 51711 per ml, the result was 0.4% of the titer in the absence of drug. Therefore, we concluded that the eventual increase in infectivity in the presence of drug primarily represented the breakthrough of wild-type virus, although there was some enrichment for virus able to produce plaques in the presence of drug.

Selection and characterization of drug-resistant mutants. The progeny from single plaques of wild-type virus were incubated overnight in WIN 51711 and then plated on HeLa cell monolayers under an overlay containing drug. Agar plugs overlying 22 plaques representing drug-resistant mutants were picked, and the virus was released by freezing and thawing. This virus was passed through a second round of plating in the presence of drug to eliminate contaminating wild-type virus. Virus stocks were prepared, and titers were determined in the presence and absence of drug as described in Table 1.

As shown in Table 1, line 1, the drug reduced the plaque titer of wild-type virus over 1,000-fold, from 5.4×10^9 to 4.7×10^6 PFU/ml, yielding a drug plating index (see footnote *b*) of 0.00087. The drug-resistant mutants behaved quite differently and could be grouped into two distinct categories. Eight had plaque titers that were about the same in the presence or absence of drug, yielding a drug plating index of approximately 1 (mutants 1, 5, 6, 9, 11, 12, 14, and 21). This behavior was in sharp contrast to that of the other 14

TABLE 1. Titers of Sabin poliovirus type 3 mutant stocks[a] plated in the presence and absence of 2 μg of WIN 51711 per ml

Virus	Titer (10^7 PFU/ml)		Drug plating index[b]
	With drug	Without drug	
Wild type	0.47	540	0.00087
Mutant			
Non-drug dependent			
1	44	80	0.55
5	43	37	1.2
6	39	34	1.1
9	30	21	1.4
11	14	31	0.45
12[c]	39	56	0.7
14	70	38	1.8
21	22	30	0.73
Drug dependent			
2	66	0.17	390
3	43	0.093	460
4	35	0.07	500
7	38	0.065	580
8	20	0.019	1,050
10	57	0.07	810
13	23	0.011	2,090
15	26	0.037	700
16	27	0.041	660
17	21	0.026	810
18	14	0.025	560
19	49	0.10	490
20	25	0.10	250
22	26	0.065	400

[a] From amplified stocks. After the second round of plaque selection, virus was amplified in the presence of drug (except for the wild type) by plating of 0.4 ml of extract on HeLa cell monolayers (100-mm tissue culture dish) and harvesting of virus when cytopathic effects became evident as described in Materials and Methods.

[b] Defined as the plaque titer in the presence of drug divided by that in the absence of drug.

[c] Derived from a parent with a small-plaque phenotype.

mutants, with drug plating indices of 250 to 2,100; these latter mutants are hereafter called drug dependent.

Revertants of drug-dependent mutants. When HeLa cells were infected with drug-dependent mutants under plaque assay conditions in the absence of drug, viruses from the resulting plaques had drug plating indices of 0.09 to 0.00003 (Table 2). Since these progeny viruses were no longer drug dependent, we concluded that only revertants were able to form plaques in the absence of drug. Such revertants could theoretically be either resistant or sensitive to the drug. All of these revertants appeared to be drug sensitive, but their drug plating indices varied between that of wild-type virus (approximately 0.001 or less) and 0.01 to 0.09. These latter isolates were probably second-site revertants which, when sequenced, may provide further insights into the mechanisms of capsid stabilization.

TABLE 2. Generation of revertants by picking of plaques of drug-dependent mutants in the absence of drug

Parental drug-dependent mutant	Drug plating index[a] of progeny isolate selected without drug		
	1	2	3
4	0.0001	0.05	0.0002
8	0.0001	0.0003	0.04
13	0.09	0.0001	0.00003
16	0.004	0.0002	0.01
17	0.05	0.003	0.02
22	0.0001	0.00003	0.002

[a] See Table 1, footnote *b*.

TABLE 3. Attachment of radiolabeled virus to HeLa cells in the presence and absence of drug[a]

Expt	Virus	% Attachment	
		Without drug	With drug[b]
1	Mutant 13	36	64
	Mutant 22	40	73
2	Mutant 13	35	74
	Mutant 22	27	77
3	Wild type	66	62
	Mutant 22	58	57

[a] [^{35}S]methionine-labeled virus prepared and purified by pelleting through a sucrose cushion (see Materials and Methods) was mixed with cells (4×10^7/ml), and the mixture was incubated for 30 min at room temperature with gentle agitation. Radioactivity associated with washed cells was determined as described in Materials and Methods.

[b] Virus was loaded with drug by incubation with 2 μg of WIN 51711 per ml for 1 h at room temperature and then overnight at 4°C.

Dependent mutants 13 and 22 do not require drug for attachment. Our next experiments were directed towards determining which step in virus multiplication was drug dependent. The drug-binding pocket of wild-type poliovirus type 3 is normally occupied by an uncharacterized sphingosine-like molecule (pocket factor) of unknown function (11). Anticipating that this function might be related to an early step in infection and that the drug might replace the function of pocket factor in the dependent mutants, we first explored the requirement for drug at the attachment step.

However, examination of two arbitrarily selected dependent mutants, 13 and 22, revealed little if any effect of drug on attachment (Table 3). Experiments 1 and 2 suggested a 1.8- to 2.8-fold enhancement of attachment by the drug, and experiment 3 showed no effect of the drug and also confirmed the absence of an inhibitory effect of the drug on the attachment of wild-type virus. One possible explanation for the variability of these results is that several processes are going on at independent rates that are affected by small changes in temperature and perhaps other variables. These include virus denaturation, attachment to cells, elution from cells after interaction with the cellular receptor, and penetration into cells. Previous experiments with the wild-type virus had led us to conclude that at room temperature, all of these processes except for cellular attachment were quite slow, but this may not be true for the drug-dependent mutants in the presence of the cellular receptor. Regardless of the reasons for this variability, even a 3-fold enhancement of attachment in the presence of drug would be far too small to account for the 400- to 2,000-fold enhancement of plating efficiency for these two mutants.

Dependent mutants do not require drug when propagated under single-step growth conditions. Having established that drug was not required for attachment, we turned again to single-step growth curve experiments in the hope of identifying which subsequent step in the life cycle of dependent mutants was blocked in the absence of drug (Fig. 3). As shown in the lower panels, drug-dependent mutant 22 mul-

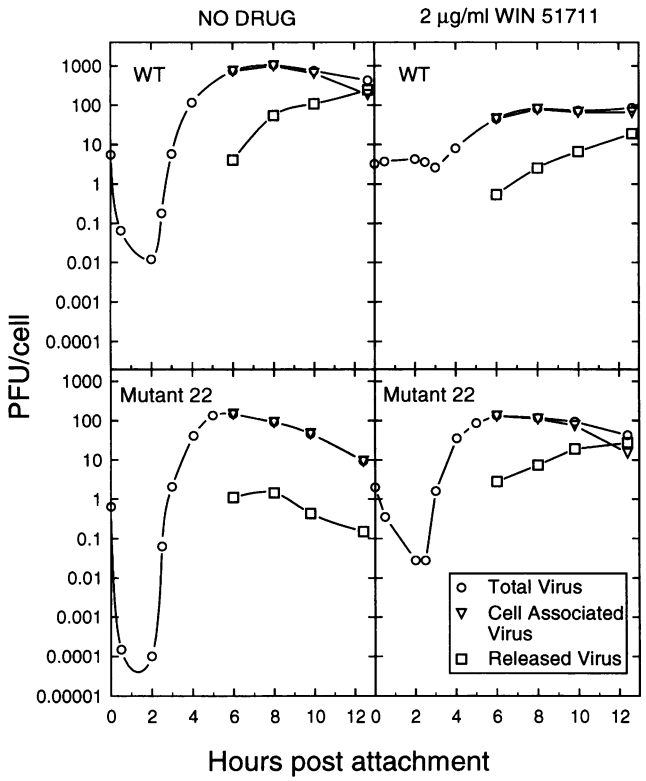

FIG. 3. Single-step growth curves of wild-type virus (WT) and drug-dependent mutant 22 in the presence and absence of 2 μg of WIN 51711 per ml. This experiment was carried out as described in the legend to Fig. 2 and Materials and Methods. Input multiplicities were 5 PFU per cell for wild-type virus and 6 PFU per cell for mutant 22. The wild-type virus titer was determined in the absence of WIN 51711; mutant virus titers were determined in the presence of 2 μg of WIN 51711 per ml.

tiplied as well in the absence of drug (left panel) as in its presence (right panel); under both conditions, this mutant reached maximum infectivity titers that were roughly 10% of the wild-type control titer. The lack of dependence on the drug for virus production under single-step growth conditions was shown to be general for all of the dependent mutants (Table 4). All 14 of these mutants were grown in the presence and absence of drug, harvested at 6.5 h after attachment, and released from cells by three cycles of freezing-thawing. When the titers of these stocks were determined in the presence of drug, the ratios of the yield of a mutant grown in the presence of drug to that of the same mutant grown in its absence ranged from 0.45 (mutant 3) to 3.0 (mutant 18); the average ratio for all of the dependent mutants was 1.2.

The ability of dependent mutant 22 to grow well in the absence of drug was unexpected in view of its inability to form plaques in the absence of drug. The first clue leading to an explanation of this result was provided from growth curves modified by measurement of the infectivities of cell-free and cell-associated viruses separately (Fig. 3). In the presence of drug (lower right panel, open squares), there was a steady increase in extracellular infectivity until it represented about 64% of total infectivity at 12 h. This result resembled the release of wild-type virus in the absence of drug (upper left panel). However, in the absence of drug, the

TABLE 4. Relative yields of dependent mutants grown in the presence and absence of drug under single-step growth conditions

Dependent mutant	Titer[a] (PFU/cell)		Relative yield[b]
	Without drug	With drug (2 μg/ml)	
2	113	115	1.0
3	75	34	0.45
4	140	100	0.71
7	73	130	1.8
8	80	70	0.88
10	160	160	1.0
13	148	145	0.98
15	220	125	0.57
16	90	88	0.98
17	73	130	1.8
18	28	85	3.0
19	138	173	1.3
20	70	105	1.5
22	145	83	0.57

[a] Mutant viruses were mixed with cells at 3 to 11 PFU per cell, allowed to attach for 30 min at room temperature at 4×10^7 cells per ml, diluted 10-fold with warm medium, and incubated in a shaking water bath for 6.5 h. Cells were sedimented and resuspended in a 1/10 volume of medium, and viruses were released by three cycles of freezing-thawing. All mutant virus titers were determined by a plaque assay in the presence of 2 μg of WIN 51711 per ml regardless of the presence of drug during growth.

[b] Defined as the titer for a mutant grown with drug divided by that for the same mutant grown without drug.

growth curve for mutant 22 showed one crucial difference. The infectivity of released virus did not increase with time; rather it remained at about 1 to 2% of total virus infectivity for all times sampled between 6 and 12 h (lower left panel, open squares).

Poliovirus type 3 is very thermolabile but can be stabilized by WIN 51711. One possible explanation for the failure of infectious particles to accumulate in the medium was that drug was required for the release of dependent virus; another was that dependent mutants were unstable in the absence of drug. The latter hypothesis seemed more plausible because WIN compounds and related molecules have been reported to stabilize picornaviruses against thermal inactivation (3, 17, 32). Moreover, experiments showed that poliovirus type 3 was rather thermolabile and that exposure to drug was strongly thermoprotective (Fig. 4). This result indicated that the virus was somewhat more thermolabile than reported by Macadam et al. (23), who used slightly different heating conditions and a different stock of virus. Such thermolability studies are highly dependent on assay conditions, especially the composition of the solution in which the virus is heated.

Sabin poliovirus type 3 was more sensitive to thermal inactivation than other picornaviruses that have been studied in our laboratory. In the absence of drug, after heating of the virus to 42°C for 4.5 min, about 0.1% of the infectivity of the virus remained (Fig. 4). For comparison, when we used the same procedure except for temperature, equivalent thermal inactivation was achieved after heating of Sabin poliovirus type 1 to 50°C for 4 min, Mahoney poliovirus type 1 to 49°C for 2 min (15), and human rhinovirus 14 to 52°C for 3 min (17).

Extreme thermolability is a common property of all drug-dependent mutants. The instability of the parental virus at 42°C suggested that even a small increase in thermolability in the mutants might render them labile at 37°C. As shown in Fig. 5, both wild-type Sabin poliovirus type 3 and drug-resistant mutant 5 were relatively stable at 37°C (closed

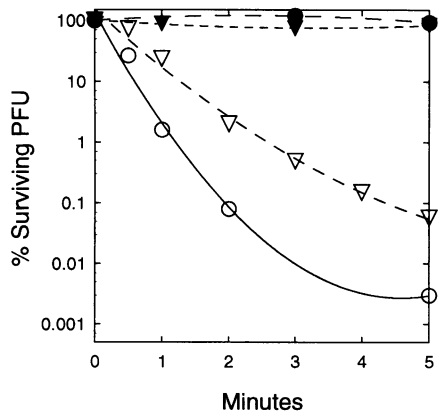

FIG. 4. Thermal inactivation of wild-type poliovirus type 3. Viral samples were diluted into PBS containing 0.4% BSA and 0.1% DMSO and with or without 2 μg of WIN 51711 per ml, allowed to set at room temperature for 1 h, and pipetted into glass tubes immersed in a water bath at 42 or 44°C. After the times shown, the tubes were removed to an ice bath and virus titers were subsequently determined. Symbols: ○ and ▽, no added drug; ● and ▼, virus preincubated with 2 μg of WIN 51711 per ml; ○ and ●, 44°C; ▽ and ▼, 42°C.

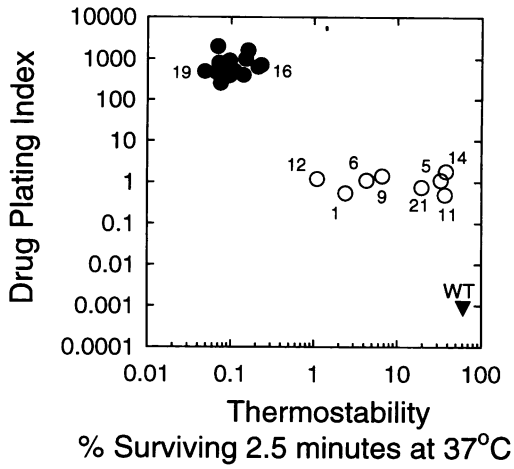

FIG. 6. Thermostability plot for Sabin poliovirus type 3 showing segregation into three separate clusters. Symbols: ▲, wild-type virus (WT); ●, drug-dependent mutants; ○, non-drug-dependent mutants. Values for the drug plating index are from Table 1. Those on the horizontal axis represent infectivity surviving at 37°C for 2.5 min under the conditions described in the legend to Fig. 5.

symbols); however, all four drug-dependent mutants examined (open symbols) were very thermolabile, losing about 90% of their infectivity per minute at 37°C.

Thermolability proved to be a general characteristic of the drug-dependent mutants. As shown in Fig. 6, all 14 of the drug-dependent mutants (closed circles) showed similarly low levels of survival; only 2 (16 and 19) are identified because differences in the thermosensitivities of the drug-dependent mutants were experimentally indistinguishable. The survival of non-drug-dependent mutants (open circles) spanned a wider range, with a few mutants (5, 11 and 14) showing thermostabilities near that of the wild-type virus.

Evidence that the thermosensitivity of drug-dependent mutants is expressed only after release from cells. It was difficult to understand how a drug-dependent mutant, such as mutant 22, could multiply at 37°C if, in the absence of drug, it lost

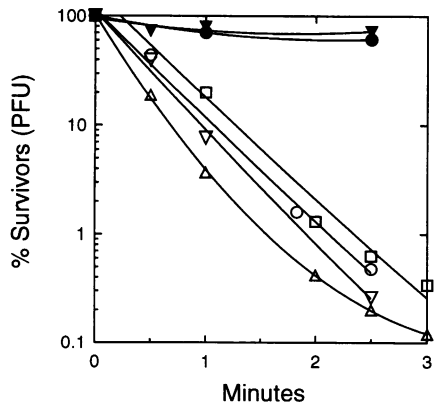

FIG. 5. Kinetics of thermal inactivation at 37°C. Viruses were grown in the absence of drug, diluted into plaque assay medium (P5), and pipetted into warmed glass tubes in a 37°C water bath. At various times thereafter, the tubes were withdrawn and plunged into an ice bath. Symbols: ●, wild-type Sabin poliovirus type 3; ▼, drug-resistant mutant 5; △, ▽, □, and ○, drug-dependent mutants 2, 13, 17, and 22, respectively.

90% of its infectivity per minute (Fig. 5), unless the mutant became thermolabile only after its release from the cell. This hypothesis would account for the growth behavior of mutant 22 (Fig. 3, lower panels), which showed a normal period of increase in cell-associated infectivity in the absence of drug but negligible accumulation of infectivity in the medium (left panel) unless drug was present (right panel).

For examination of this idea, cells infected in the absence of drug were harvested at 6 h, when 99% of the infectivity was still cell associated. The thermostability of virus heated in suspensions of intact or broken cells was then measured as infectivity relative to that of control virus that had been treated identically except for heating. Cells were broken by a single freeze-thaw cycle in either 100 cell volumes of medium (6×10^5 cells per 0.15 ml) or 5 cell volumes of medium (1.2×10^7 cells per 0.15 ml).

As shown in Fig. 7, when released by freezing-thawing into 100 cell volumes of medium, drug-dependent mutants 8 and 22 became markedly more thermolabile, both showing less than 10% survival (open bars). However, drug-dependent mutants 8 and 22, when present in intact cells, were as stable as wild-type virus or non-drug-dependent mutant 5 (closed bars); in this assay, differences of 25% in titer are in the same range as the statistical plaque counting error. The observation that drug-dependent viruses became thermolabile only after release from cells implies the presence in infected cells of a thermostabilizing agent that loses its protective activity in the medium, perhaps by dilution. Concentration of the infected-cell suspension 20-fold before freezing-thawing (hatched bars) substantially reduced the thermosensitivity of the drug-dependent mutants, supporting the notion that the protective factor from the cells could be diluted.

The thermodecay product is 135S particles lacking VP4. As shown in Fig. 8A, dependent mutant 13 cosedimented with native wild-type virus. After 2.5 min of heating at 37°C in plaque assay medium (P5) lacking drug (Fig. 8B), no intact virus survived and 95% of the recovered radioactivity sedimented as 135S particles. The recovery of radioactivity from the sucrose gradient was about 40%, perhaps reflecting the

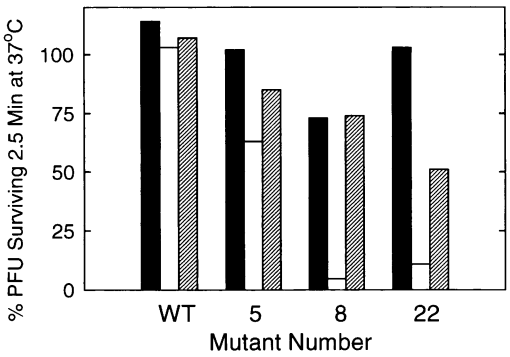

FIG. 7. Thermostability of wild-type virus (WT) and mutant viruses inside and outside cells. Infected-cell suspensions in 0.15 ml of medium were heated for 2.5 min in prewarmed glass tubes in a 37°C water bath after being treated as described in the text. Infectivities were normalized to that of unheated virus released from infected cells by one cycle of freezing-thawing. Symbols: ■, intact cells heated before breakage; □, cells broken in 100 cell volumes; ▨, cells broken in 5 cell volumes.

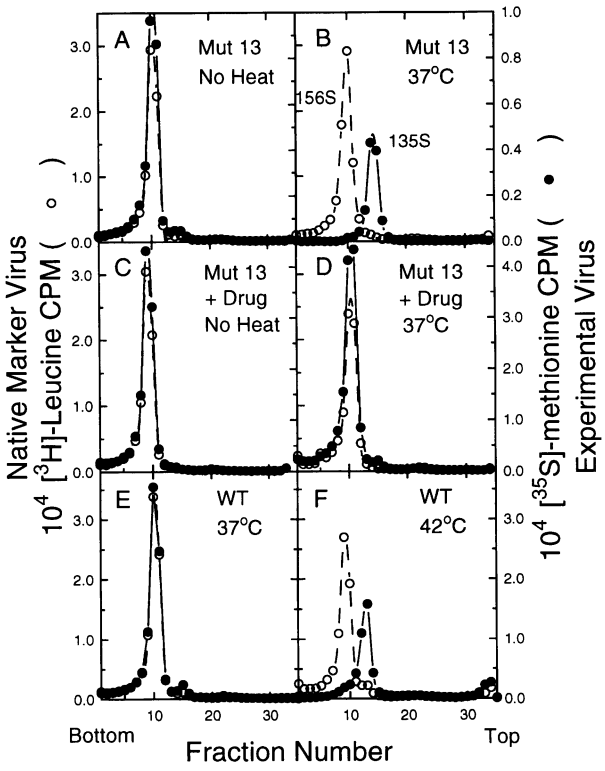

FIG. 8. Sedimentation of heated virus on sucrose gradients. Virus samples were prepared and centrifuged as described in Materials and Methods. Symbols: ○, [^{3}H]leucine-labeled native poliovirus type 3; ●, [^{35}S]methionine-labeled virus (A, unheated mutant [Mut] 13; B, mutant 13 heated to 37°C for 2.5 min; C, unheated mutant 13 in the presence of 2 μg of WIN 51711 per ml; D, mutant 13 heated to 37°C for 2.5 min in the presence of 2 μg of WIN 51711 per ml; E, wild-type virus (WT) heated to 37°C for 2.5 min; F, wild-type virus heated to 42°C for 2.5 min.

stickiness often shown by such particles (13). The incorporation of 0.1% Tween 20 into the sucrose gradients improved the recovery of radioactivity to about 75%, still as a single 135S peak (data not shown). The drug prevented the formation of 135S particles (Fig. 8D). Wild-type virus was stable when heated to 37°C (Fig. 8E) but was similarly converted to 135S particles when inactivated by being heated to 42°C (Fig. 8F). The presence of 135S particles and the absence of 80S particles were reproducible in eight experiments.

Electrophoretic analysis showed that capsid protein VP4 was missing from each of the 135S products shown in Fig. 8 (data not shown). Capsid protein VP1 was intact in unheated virions. However, after heating, the recovery of VP1 varied with the degree of virus purification; this was true for both wild-type and mutant viruses, suggesting that VP1 became highly labile after thermodecay of the particles, perhaps because of proteolytic contaminants.

DISCUSSION

Our results extend to poliovirus type 3 reports that WIN 51711 specifically targets the uncoating step; at 2 μg/ml, a concentration that inhibits eclipse at least 100-fold (Fig. 2), the drug had little or no effect on attachment of wild-type virus (Table 3). Earlier reports had shown that the compound also blocks the uncoating of poliovirus type 2 and human rhinovirus 2 (12, 43). The parent compound, arildone, has also been reported to block the uncoating of poliovirus types 1 and 2 (10, 26). Everaert et al. (10) reported that arildone specifically blocks the formation of 135S particles but not subsequent steps in uncoating.

Of 22 mutants selected for the ability to form plaques in the presence of WIN 51711, 14 required the drug for plaque formation. All 14 of these drug-dependent mutants were extremely thermolabile. We speculate that to escape the drug-imposed block to uncoating, the drug-dependent mutants have developed a capsid that allows the transition to A particles to be triggered spontaneously at 37°C in the absence of drug, without the usual participation of the viral receptor. During the development of a plaque on a cell monolayer, several cycles of cell-to-cell spread are required. We envision that when drug-dependent mutants are released from an infected cell in the absence of drug, they are quickly converted to noninfectious A particles in the extracellular medium before they can successfully infect another cell, thus preventing the formation of plaques. Ongoing sequencing studies on the locations of mutations in the drug-dependent mutants may provide insights into the capsid structures involved in triggering the conversion to 135S particles.

The ratios of drug-dependent to non-drug-dependent drug escape mutants vary widely with different picornaviruses. Almost two-thirds of the drug-resistant mutants of poliovirus type 3 were drug dependent. By contrast, no such drug-dependent mutants were observed in a collection of 69 drug-resistant mutants of human rhinovirus 14 (16) or in a collection of 15 drug-resistant mutants of the Sabin strain of poliovirus type 1 (28). Neither of these viruses was as thermolabile in our assays as the Sabin strain of poliovirus type 3. The natural thermolability of parental Sabin poliovirus type 3 may have made it possible to isolate drug-dependent mutants so easily.

Potential for crystallographic studies of A particles. Even brief exposure to 37°C converted dependent mutants to noninfectious particles with a sedimentation coefficient of approximately 135S. These heat-induced particles lacked VP4 and appeared to be somewhat more hydrophobic than native virus, since the addition of 0.1% Tween 20 to the sucrose gradients improved the recovery of the 135S parti-

cles; VP1 of these particles was apparently susceptible to proteolytic degradation by protease impurities. Thus, these particles resembled the A particles produced after the interaction of viruses with cellular receptors (5, 13, 21).

These mutants, and indeed the wild-type virus, are potential candidates for crystallographic studies of A particles. First, A particles can be produced in high yields simply by brief warming of the dependent mutants to 37°C. Second, because of the low temperature used, there is little evidence of smaller products, such as the 80S empty capsid reported by others after heating of polioviruses (2, 9, 18). However, the variable degradation of VP1 and the tendency of the particles to stick to hydrophobic surfaces are problems that need to be controlled before such studies are feasible.

Wetz and Kucinski (41) produced 135S particles from Mahoney poliovirus type 1 by heating the virus to 37°C for 1 h in 20 mM Tris-HCl (pH 7.5) with 2 mM $CaCl_2$. The advantage of the dependent mutants described here is that the conversion to A particles is complete in just a few minutes. McGregor and Mayer (24) heated poliovirus type 1 (LsC) to 45°C for 30 and 60 s and then observed particles in the electron microscope that appeared to be either intact virus or virions with "holes." Some of these particles may have been equivalent to 135S particles, but they were not sedimented on sucrose gradients.

What is the nature of the cell-associated factor that stabilizes drug-dependent mutants? The observation that drug-dependent mutants are stable at 37°C while inside the cell but become labile when released from the cell (Fig. 7, closed and open bars) implies the existence of a cell-associated stabilizing factor. The ability of cell-associated factors to stabilize polioviruses against changes induced by heating has been reported by others (6, 35), but the mechanism of this stabilization is not known. One possible candidate for the stabilizing factor is the pocket factor, a hydrophobic molecule observed in X-ray crystallographic studies of purified Sabin poliovirus type 3 (11). An extended hydrocarbon chain, possibly consisting of sphingosine or a mixture of lipids (11, 42), occupies the interior of the VP1 beta barrel. If pocket factor is the stabilizing factor inside the cell, the molecule must diffuse out of the mutants rapidly when the virus is released from the cell. These mutants might therefore have much-reduced affinities for pocket factor and perhaps for drug compared with those of the wild-type virus. Rombaut et al. (35) showed that a cell-associated factor responsible for the preservation of native antigens on heated virions was lost when poliovirus type 1 was extensively purified in a procedure that included extraction with butanol or Genetron (1,1,2-trichlorotrifluoroethane; Allied Chemical Corp., New York, N.Y.) or treatment with 0.5% sodium deoxycholate or 1% sodium dodecyl sulfate, all treatments that might extract natural pocket factor. Interestingly, Ward and Ashley (40) found that a variety of ionic detergents at 0.1% stabilized poliovirus type 2 against thermal degradation; it is tempting to speculate that some detergents can replace pocket factor.

Protective factors need not necessarily act at the drug-binding pocket, however. Various salts (7, 39), lysine and other diamines (8), and short-chain fatty acids (20) have all been shown to stabilize picornaviruses against conformational changes in the viral capsid.

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