Intracellular Reovirus Survives Cytotoxic T Lymphocyte-mediated Lysis of its Host Cell

By DONNA M. HOWELL AND ERIC MARTZ*

Department of Microbiology and Program in Molecular and Cell Biology, University of Massachusetts, Amherst, Massachusetts 01003, U.S.A.

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SUMMARY

Cytotoxic T lymphocytes (CTLs) induce rapid, extensive internal disintegration in target cells and this is unique among immune lytic mechanisms studied. This raises the question of whether CTLs are uniquely capable of halting virus infections by inducing damage within the target cell causing inactivation of intracellular virus. Reovirus infection of mouse P815 cells provided a suitable system for evaluating this question. An increase in cell-associated infectious virions began 8 h after infection and increased until 20 h post-infection, at which time the titre levelled off at about 100- to 1000-fold higher than the initial value. The infectious activity was compared between host cells killed by CTLs and those killed by sonication at various points in the infection cycle. The presence of reovirus within the target cell did not inhibit the usual internal disintegration events associated with the death of a target killed by CTLs. Nevertheless, the results indicated that CTLs were incapable of inactivating intracellular reovirus at any point in the life cycle of the virus: CTL-induced cytolysis simply released the infectious virions into the medium. Thus, at least in the case of reovirus, the utility of direct killing by CTLs would appear to be limited to reduction of the virus yield by lysis of the host cell before virus replication and assembly is completed.

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) are believed to play an important role in combating viral infections (Zinkernagel et al., 1978; Finberg et al., 1979; Blanden, 1974; Bukowski & Welsh, 1985). CTLs can specifically recognize and destroy allogeneic or virus-infected syngeneic cells in conjunction with the cognate class I major histocompatibility complex gene products (Zinkernagel & Doherty, 1975). Although a considerable number of studies have addressed the mechanism by which CTLs recognize and kill target cells, much less attention has been directed to the mechanism by which CTLs may clear a viral infection.

The conventional view holds that lysis of infected cells by CTLs reduces the yield of virus, thereby slowing the progress of the infection (Zinkernagel & Althage, 1977; Byrne & Oldstone, 1984). This would be most effective if the CTLs can recognize virus-specific antigens on the cell surface early in the infection, before large numbers of progeny can be produced. If the attack occurs during the eclipse period, it might entirely prevent the release of any infectious particles. More probably, some virus progeny will have been assembled by the time of CTL attack, and may be released as infectious particles during target cell lysis. Since there is no evidence that CTLs can inactivate free virions, it would apparently be left to non-CTL mechanisms, such as antibodies, complement and phagocytes (Clouston & Kerr, 1985) to inactivate the extracellular virus and bring the infection to a halt.

Clearly, CTLs would be substantially more effective if they were able to inactivate the already completed virus particles which typically would have accumulated inside the target cell prior to the time of attack (Russell, 1983; Clouston & Kerr, 1985). Such inactivation might involve the induction, by the CTLs, of indiscriminately destructive processes within the target cell prior to
Evidence that CTLs may indeed be capable of inducing such processes came first from Russell's work demonstrating rapid CTL-induced disintegration of target cell nuclear DNA (Russell et al., 1980, 1982; Russell & Dobos, 1980). The DNA is solubilized and ultimately fragmented to the nucleosomal level. This nuclear disintegration occurs to a large extent within the target cytoplasm prior to plasma membrane breakdown and lysis, at least for mouse haematopoietic target cells. This phenomenon is unique among immune and non-immune lytic processes studied to date. In particular, antibody plus complement-mediated lysis does not induce nuclear disintegration or DNA breakdown, nor does any chemically or physically induced cytolytic process examined to date. Cohen and colleagues (Cohen & Duke, 1984; Cohen et al., 1985; and reviewed by Duke & Cohen, 1987) have demonstrated DNA degradation when thymocytes are subjected to low dose irradiation or cortisone but the DNA degradation requires hours whereas CTL-mediated killing degrades DNA within minutes. DNA degradation occurring when certain target cells are attacked by CTLs is less dramatic than for others. Using methods similar to those employed by Russell, Christiaansen & Sears (1985) were unable to detect DNA degradation in human targets killed by CTL attack. However, recent work by Gromkowski et al. (1986) demonstrates that DNA damage does occur in the form of DNA strand nicking and this again is unique to CTL-mediated death. We find that the degree of nuclear disintegration depends on the target cell type, but does not correlate with the origin (i.e. human or mouse) of target cells (Howell & Martz, 1987).

The present study is, to the best of our knowledge, the first to evaluate directly the possibility that CTL-mediated lysis might inactivate fully assembled virions present within the target cell at the time of attack. Uncoated, replicating or recently replicated viral nucleic acids seem likely to be susceptible to the same CTL-induced catabolic processes which degrade cellular DNA (the fate of RNA, proteins and lipids has not been evaluated). However, naked nucleic acids are generally much less infectious to healthy cells than are fully assembled virions. Since loss of infectivity is the most meaningful criterion for the success of immunological control, we utilized a p.f.u. assay in the present study, rather than evaluating the fate of uncoated viral nucleic acids.

The virus to be used needed to meet several technical requirements. (i) Large numbers of infectious particles must accumulate within the target cytoplasm in order to guarantee that productive infection is occurring. This criterion eliminates most enveloped viruses, which become infectious only after acquiring membrane during exit from the cell. (ii) The particles cannot be rapidly released from the living cell. Rapid release during CTL attack would confound quantification of the fate of the intracellular particles, since extracellular virus would not be susceptible to intracellular destructive processes. (iii) We wanted the virus to infect haematopoietic tumour cells which have been best characterized as targets in the CTL assay, and which display the most dramatic and rapid nuclear disintegration. Fibroblasts, for example, display relatively little nuclear disintegration (Howell & Martz, 1987).

We first attempted to use polyoma virus, a DNA virus which meets criteria (i) and (ii). However, it did not appear to infect P815 cells productively. More seriously, we were unable to generate plaques sufficiently clearcut for precise quantification. Thus, criterion (iv) is that the virus should produce unambiguous plaques. Considerable work was also invested in herpes simplex virus type 1. It failed criteria (ii) and (iii), and interpretation of a slight reduction in p.f.u. was further confounded by a profound eclipse phase. Reovirus, a cytoplasmically replicating RNA virus (Zarbl & Millward, 1983), was the only one of seven viruses considered that met all four criteria quite well and was otherwise technically feasible.

We thought it important to be able to produce rapid CTL-mediated lysis early, as well as later in the infection cycle. This argued for the use of allospecific CTLs, rather than typically much weaker virus-specific CTL populations. Moreover, initially we wished to evaluate the effects of the purely CTL-induced destructive processes (e.g. nuclear disintegration) independently of inactivation which might depend on the presence of virus-specific T cell receptors. Finally, we wished to use CTLs which had not been 'hyperactivated' by prolonged culture in exogenous interleukin 2, which may have acquired properties not found among CTLs in vivo (Havele et al., 1986; Berke & Rosen, 1987).
We find that cell-associated reovirus is released unharmed during allospecific CTL-mediated killing of the host cell at any time during the infection cycle. This result has been presented elsewhere in preliminary form [Peters (Howell) & Martz, 1986].

METHODS

Virus. Working stocks of reovirus type 3 (Dearking strain) were produced from virus obtained from Dr B. N. Fields (Harvard Medical School, Boston, Mass., U.S.A.) following methods used in his laboratory. Briefly, virus was plaque-purified on a mouse L cell monolayer by removing a plug from an isolated plaque, resuspending the plug, and inoculating the suspension onto a confluent L cell monolayer. When 80 to 90% c.p.e. was reached (3 to 4 days), the culture was frozen and thawed three times (passage 1). The virus was passed again in a similar manner, and the resulting passage 2 preparation was utilized as the working stock for the experiments reported.

Cells and media. P815, a mastocytoma cell line derived from DBA/2 mice (Dunn & Potter, 1957), was used for host and target cells. P815 cells were produced intraperitoneally in syngeneic mice. Mouse L cells (Sanford et al., 1948) were utilized as control cells in place of CTLs in some experiments as well as for the indicator cells in the plaque assays. L cells were grown in suspension in Eagle’s MEM (Joklik’s modification; Irvine Scientific, Santa Ana, Ca., U.S.A.) supplemented with 2 mM-L-glutamine, 5% foetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin. All experiments were performed in RPMI 1640 with 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin (RB; Gibco). CTLs were generated in CTLM medium consisting of RPMI 1640 with 2 mM-L-glutamine, 5 x 10^-4 M-2-mercaptoethanol, 2 g/l glucose, 5% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Generation of allogeneic CTL. P815 cells (1 x 10^7) (H-2^d) were injected intraperitoneally into C57BL/6 (H-2^b) mice. Three to 6 months later the splenocytes were harvested (responders). Stimulator cells were splenocytes from DBA/2 mice. The isolated cells were irradiated with 1400 rad. Responders (8 x 10^7) and 3 x 10^7 stimulators were cultured in 25 cm^2 polystyrene tissue culture flasks (Corning) in 25 ml CTLM medium, and incubated upright in 10% CO_2 at 37 °C for 4 to 6 days. Living cells were separated from dead cells prior to use by flotation on lymphocyte separation medium (1000 g, 7 min; Litton Bionetics, Kensington, Md., U.S.A.).

Preparation of target cells. [51]Cr-labelled P815 cells, designated P815*, were prepared by incubating 1 x 10^7 cells in 1 ml RB with 150 μCi of Na_2S_35O_4 (New England Nuclear) for 45 min in a 37 °C waterbath and then washing twice. These cells were then infected with reovirus, the infected cells being designated Reo-P815*. Virus at a multiplicity of infection of 5 p.f.u./cell were allowed to adsorb to P815 (7.5 x 10^6 cells/ml) for 60 rain at 4 °C. At this temperature virus adheres to the plasma membrane but does not enter the cell (Silverstein & Dales, 1968). (When the cells were later warmed to 37 °C the virus entered in a synchronized fashion.) Cells were then washed three times with cold RB, resuspended at 1 x 10^5 cells/ml and divided into 2 ml aliquots into 17 x 100 mm polystyrene tubes. The tubes were capped tightly and maintained at 4 °C.

Experimental procedure. Tubes containing Reo-P815* cells were moved from 4 °C to a 37 °C, 10% CO_2 incubator at designated intervals and the caps loosened. After 0 to 24 h incubation post-infection, either a cell-associated p.f.u, assay or a CTL-mediated killing assay was conducted.

Cell-associated p.f.u. assay. Following incubation at 37 °C, 10% CO_2, all tubes were centrifuged at 500 g for 5 min and 1 ml of supernatant was drawn off. The supernatant and pellet were sonicated and a 6 day plaque assay was performed on dilutions of the pellet and supernatant (Ramig & Fields, 1979). Sonication was for 1 min in a Bioso nik IV bath (VWR Scientific, Boston, Mass., U.S.A.) at a power setting of 70.

CTL-mediated killing plus total residual p.f.u. assay. Following the incubation at 37 °C, 10% CO_2, all tubes were centrifuged once and the cells resuspended in 2 ml of fresh RB. One-hundred μl of effectors [effector to target cell ratio (E/T) 7] or L cells (control) were added to the tubes. The tubes were vortexed, centrifuged at 500 g for 3 min, capped, incubated in a 37 °C waterbath for 90 min, centrifuged at 1000 g for 5 min and 1 ml of supernatant was removed into new tubes. Samples in the pellet and supernatant tubes were sonicated and the latter were counted in a gamma scintillation spectrometer (Beckman Gamma 4000) to determine [51]Cr release. Percentage corrected [51]Cr release was calculated as [100 (e - c)/(t - c)] where 'e' is experimental release (CTL), 'c' is control spontaneous release (L cells) and 't' is total initial cell-associated [51]Cr per tube. After counting, the supernatants were pooled with the corresponding pellet tubes and a 6 day p.f.u. assay was used to determine total residual p.f.u.

Susceptibility of infected cells to CTL-mediated lysis. To determine whether the subpopulation of P815 cells productively infected with reovirus was lysed by CTLs, the procedure outlined for the CTL-mediated killing assay was modified. Reo-P815* cells were incubated for 16 h post-infection at 37 °C, 10% CO_2. The cells were washed once and resuspended in 2 ml fresh RB prior to the CTL-mediated killing assay. Following the 90 min assay the tubes were vortexed and centrifuged at 1000 g for 5 min. The supernatant was decanted into a new tube and 2 ml fresh RB was added to the pellet. The radioactivity in the supernatant tubes was measured to determine the level of [51]Cr release. Samples in the supernatant and pellet tubes were sonicated and a 6 day plaque assay was performed.

Extended CTL-mediated killing assay. To determine whether slow killing of infected cells by CTL would increase the susceptibility for inactivation of intracellular reovirus, the procedure outlined for the CTL-mediated killing
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assay was modified. Reo-P815* cells were incubated for 16 h post-infection at 37 °C, 10% CO₂. The cells were then washed once and resuspended in 2 ml fresh RB. One tube was sonicated immediately to determine the p.f.u. present before the killing assay. One-hundred µl of effectors (E/T 1) or L cells (control) was added to the remaining tubes. The tubes were vortexed, centrifuged at 500 g for 3 min, and incubated in a 37 °C waterbath for 6 h. Following the 6 h incubation, centrifugation, supernatant removal and gamma counting were as above.

DNA solubilization. The fate of the virus-infected host cell's DNA when killed by CTLs was investigated using the procedure of Russell et al. (1980) with minor modifications. Briefly, P815 cells were infected with reovirus or mock-infected in parallel. Half of the cells were labelled with [3H]thymidine, and the other half with 51Cr. Twenty h post-infection, 2 × 10⁴ target cells (100 µl) were mixed with 6 × 10⁵ effector cells (100 µl), centrifuged for 3 min at 500 g and incubated in a 37 °C waterbath for 0, 20, 40 or 60 min. One ml of RPMI 1640 was added to 51Cr-labelled targets and 1 ml of RPMI 1640 containing 0.2% Triton X-100 (T 6878, Sigma) was added to [3H]thymidine-labelled cells. Total uptakes were determined by adding 1 ml of 0.1% SDS to 100 µl of 51Cr-labelled targets. For [3H]thymidine total uptake, targets were incubated in 1 ml RB containing 0.2% Triton X-100 and 100 µl of 30 µg/ml bovine pancreatic DNase I (D 5025, Sigma) in a 37 °C waterbath for 1 h.

All experimental tubes were centrifuged at 1000 g for 10 min, 0.5 ml of the supernatant was removed and counted for gamma emissions (51Cr) or, after the addition of 1.5 ml of Scinti-Verse (Fisher Scientific, Boston, Mass., U.S.A.) a Beckman LS 1801 liquid scintillation counter for [3H]thymidine.

RESULTS

Kinetics of reovirus infection

P815 cells have been shown to be susceptible to infection by reovirus (Noseworthy et al., 1983; Kauffman et al., 1983); our results confirm this and extend previous reports by showing that P815 cells were capable of supporting viral replication as measured by increases in p.f.u./cell over time. Fig. 1 illustrates the kinetics of a reovirus infection of P815 cells, which is similar to that reported for L cells (Gomatos et al., 1962). At the end of the 1 h adsorption period, approximately 10% of the p.f.u. supplied had become cell-associated. Reduction of p.f.u. due to eclipse, if any, was never more than a few-fold. After 8 h cell-associated p.f.u. began increasing, reaching a plateau of approximately a 10²- to 10³-fold increase by 20 h.

During the eclipse period (up to 6 to 8 h in Fig. 1), 80% of the virus was cell-associated, and this increased to more than 99% near the end of the first day. Thus, virus was not released into the medium during the first day. After substantial new p.f.u. had been produced (15 h) it is assumed that the majority of cell-associated virus was intracytoplasmic.

After 24 h, more than 70% of the infected cells remained viable as estimated by 51Cr release. At 2 and 3 days, the percentages of cells stained by trypan blue were 13% and 24% for uninfected cells, and 29% and 57%, respectively for reovirus-infected cells. Thus, reovirus-induced death did not begin until the second day.

Effects of CTLs on reovirus infectivity

Fig. 2 illustrates the effect of CTL attack on reovirus within P815 cells. At no point during the course of the infection did the killing of the infected P815 cells by CTLs inactivate intracellular infectious virions. Killing of infected P815 cells by CTLs (followed by sonication) left the same number of p.f.u. as did killing by sonication.

The inability of the CTLs to inactivate the virus was not due to their inability to kill reovirus-infected P815 cells. Table 1 shows that CTLs could kill these cells at any time during the course of the infection. Table 2 demonstrates that the reovirus-infected subpopulation of P815 cells (which might be a minority of the total 51Cr-labelled cells) was susceptible to CTL-mediated lysis as indicated by the release of virus from lysed cells.

Reovirus infection of the target cells did not interfere with induction of pre-lytic nuclear disintegration by the CTLs (Fig. 3). As reported for uninfected P815 cells (Russell & Dobos, 1980), nuclear disintegration occurred within minutes after CTL attack and preceded 51Cr release. In contrast, DNA was not solubilized during lysis induced by freezing and thawing (confirming Russell & Dobos, 1980) or by sonication (data not shown).

One could argue that the rapidity with which the CTLs destroyed the infected targets in experiments exemplified by Fig. 2 (probably less than 1 h) did not allow enough time for
Reovirus survives CTL-induced host cell lysis

Fig. 1. Kinetics of reovirus p.f.u. production within P815 cells. Reovirus at an m.o.i. of 5 p.f.u./cell was adsorbed to P815 cells (7.5 × 10⁶ cells/ml) for 1 h at 4 °C. The cells were washed with ice-cold medium and resuspended at 1 × 10⁶ cells/ml. Aliquots of 2 ml were distributed to 14 × 100 mm polystyrene tubes and placed loosely capped at 37 °C, 10% CO₂. After the indicated times at 37 °C, cell-associated p.f.u. (▲) and supernatant-associated p.f.u. (■) were determined by assay on L fibroblasts. For the first 6 to 8 h post-infection 80% of the total virus was cell-associated. This increased to > 99% by 24 h. Similar results were obtained in two other comparable experiments.

Fig. 2. CTL-mediated killing of reovirus-infected P815 cells does not inactivate infectious intracellular virions. Following the 1 h, 4 °C adsorption and washes, the reovirus infection in P815 cells was allowed to proceed for the indicated intervals of time at 37 °C, 10% CO₂. The cells were then washed and resuspended in fresh medium (thereby discarding any spontaneously released ⁵¹Cr or reovirus). CTLs (■; E/T 7) or L cell controls (▲) were added; a CTL-mediated killing assay (90 min, data in Table 1) and a total residual p.f.u. assay were performed. See Table 1 for ⁵¹Cr release values for each time point. P.f.u. were divided by the initial number of P815 cells/tube (2 × 10⁵) to obtain p.f.u./cell. Killing reovirus-infected P815 cells by CTL plus sonication or sonication alone resulted in equal numbers of infectious reovirus. Similar results were obtained in three other comparable experiments.

Table 1. Killing of a reovirus-infected P815 cell population by allogeneic CTLs*

<table>
<thead>
<tr>
<th>Time post-infection (h)</th>
<th>Corrected ⁵¹Cr release (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>84.6</td>
</tr>
<tr>
<td>2</td>
<td>78.8</td>
</tr>
<tr>
<td>4</td>
<td>83.7</td>
</tr>
<tr>
<td>6</td>
<td>74.6</td>
</tr>
<tr>
<td>8</td>
<td>82.7</td>
</tr>
<tr>
<td>12</td>
<td>75.0</td>
</tr>
<tr>
<td>16</td>
<td>76.9</td>
</tr>
<tr>
<td>20</td>
<td>70.9</td>
</tr>
</tbody>
</table>

* These data were obtained in the experiment described in Fig. 2. Spontaneous ⁵¹Cr release was <7% in all cases.

Table 2. Effect of CTLs on the subpopulation of P815 cells productively infected with reovirus*

<table>
<thead>
<tr>
<th></th>
<th>P.f.u./cell</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>- CTL</td>
<td>Supernatant</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Pellet</td>
<td>25.0</td>
</tr>
<tr>
<td>+ CTL</td>
<td>Supernatant</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>Pellet</td>
<td>14.0</td>
</tr>
</tbody>
</table>

* See Methods. The m.o.i. was 5, the corrected ⁵¹Cr release was 48% and the spontaneous release was 5%. 
Fig. 3. Reovirus infection does not alter pre-lytic nuclear disintegration induced by CTL-mediated killing. Comparison of $^{51}$Cr release (Δ, ▲) and Triton-soluble $[^3]$H]thymidine release (□, ■) for reovirus-infected (20 h post-infection, Δ, □) and uninfected P815 cells (▲, ■). CTL-mediated killing (E/T 3) was for the indicated times. Freezing and thawing released $^{51}$Cr immediately (●), but did not render the DNA Triton-soluble even when the cells were incubated for 60 min at 37 °C after the freeze-thaw (○).

Table 3. Effect of slow killing on inactivation of reovirus*

<table>
<thead>
<tr>
<th>Target treatment</th>
<th>P.f.u./cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 h post-infection</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>16 h post-infection plus 6 h with L cells</td>
<td>15.0 ± 3</td>
</tr>
<tr>
<td>16 h post-infection plus 6 h with CTL</td>
<td>11.0 ± 2</td>
</tr>
</tbody>
</table>

* E/T cell ratio was 1. Corrected $^{51}$Cr release was 56%. Spontaneous $^{51}$Cr release was 16%. Values are given ± standard error of the mean, which is calculated from the square root of the total number of plaques counted.

inactivation of the virus prior to lysis of the target cell. Since damage may be delivered to target cells in sublethal stages which must accumulate to reach a lethal level, slower killing may prolong the intracellular disintegration events induced by CTLs over a longer time within each target cell. Consistent with this notion, it has been shown that lysis of individual target cells is accelerated when they are conjugated with more than one CTL simultaneously (Zagury et al., 1979). Table 3 demonstrates that slowing the killing process by decreasing the E/T ratio from 7 (as in Fig. 2) to 1 (the length of the killing assay was extended from 1.5 to 6 h to permit lysis of a large percentage of the target cells) also failed to inactivate reovirus.

DISCUSSION

The major result in this paper is the failure of CTL-mediated lysis of reovirus-infected cells to reduce the p.f.u. present at the time of CTL attack. This was found for all points in the reovirus infection cycle. Our interpretation is that, despite the induction of nuclear disintegration, CTLs are unable to induce any intracellular damage capable of inactivating virions which have progressed far enough in their assembly to have become infectious particles. For this interpretation to be valid, three additional points must be established. First, the virions must have been within the target cell during CTL attack; if they had already exited from the cell, they would not be susceptible to the intracellular processes, notably nuclear disintegration, which provide the motivation for this investigation. Second, we must be certain that the virus-infected
Reovirus survives CTL-induced host cell lysis

Cells were lysed. Third, we must show that virus infection does not somehow prevent the induction of nuclear disintegration by the CTLs.

With regard to the first point, we focused our experiments on the period beginning immediately after infection and continuing until the increase in p.f.u./cell reached a plateau at 20 h post-infection. Reovirus-induced cytolysis and release of viruses from the cells did not begin until the second day post-infection. During the first day post-infection, 80 to 99% of the virus remains cell-associated (Fig. 1). Furthermore, we washed the infected cells prior to applying the CTLs, hence ensuring the reduction of any free virus to insignificant levels. Therefore, reovirus did not avoid CTL-induced inactivation by exiting from the target cells prior to CTL attack. Our evidence suggests that the reovirus was intracytoplasmic during CTL-mediated lysis of the host cell, and hence subject to whatever destructive processes the CTLs induced within target cells, including those responsible for nuclear disintegration.

Secondly, it was necessary to be certain that virus-infected P815 cells are actually killed by the CTLs. The 51Cr release assay indicated that at least 75 to 85% of the cells were killed by the CTLs. [51Cr release assays probably underestimate plateau levels of cytolysis (Martz, 1977).] CTL-induced DNA solubilization (Fig. 3) confirms that at least 90% of the cells were killed. If 10% or fewer of the cells resisted CTL-mediated killing, it is possible that these were the only cells productively infected with reovirus. Hence, the CTLs might have failed to kill any of the reovirus-infected cells. We attempted to establish that over 10% of the P815 cells were infected, using an infectious centre assay. However, this assay is likely to underestimate the percentage for non-adherent cells such as P815; in any case, we were unable to establish a value above 10% (data not shown). We were able to resolve this dilemma by demonstrating that 30% of the p.f.u. associated with the P815 cells were released from the cells during CTL-mediated killing (48% corrected 51Cr release, Table 2). Thus we can be certain that the productively infected cells were killed.

Finally, it was necessary to verify that virus infection did not prevent the nuclear disintegration process previously reported for uninfected cells (Russell et al., 1980, 1982; Russell & Dobos, 1980). We showed that at least 90% of the P815 nuclei disintegrated. We cannot exclude the possibility that only 10% of the P815 were infected, and that despite the cytolysis of this subpopulation as certified above, the nuclei of these cells did not disintegrate. However, we have no reason to believe that this might be the case. On the population level, at least, nuclear disintegration is unaltered by reovirus infection (Fig. 3). Therefore, our results suggest that the reovirus is fully exposed to the nuclease and possibly protease activities stimulated by CTL attack.

Survival of the reovirus may have been due in part to its rather unusual structure. The viral genome is never completely exposed to nucleases or proteases present within the host cell; rather, it apparently remains within an inner protein coat (Skup et al., 1981) which has been shown to be tenaciously resistant to destruction by a number of treatments including chymotrypsin, trypsin and excessive heat (Spendlove et al., 1970; Wallis et al., 1964). Early during the infection, this inner protein coat may have been responsible for protecting the replicating parental virus from events within the target cell initiated by the CTLs. Alternatively, most of the p.f.u. observed before the increase in p.f.u./cell may represent virions that attached to the cells but failed to initiate replication, and hence remained infectious.

If CTLs cannot inactivate intracellular virions, then their roles would appear to be limited to (i) reducing the yield of virus by killing infected cells before the maximum yield has been produced, and (ii) exposing intracellular virus to render it susceptible to extracellular immune controls (Clouston & Kerr, 1985). Neutralizing and haemagglutination-inhibiting antibodies can be detected in serum approximately 1 week after the introduction of reovirus into mice (Fields, 1982). Natural killer cells and activated macrophages may also contribute to control of virus infections, as has been shown for other viruses using athymic mice (Zinkernagel & Blanden, 1975).

Role (i) above requires that CTLs be able to recognize low levels of virus antigen expressed on the host cell surface early in the infection. CTLs may be unique among immune effector mechanisms in their ability to recognize minute densities of antigen. Most of the target cell H-2
can be removed without any reduction in allospecific CTL-mediated killing (Gromkowski et al., 1983; Stulting et al., 1978). The mere binding of inactivated Sendai virus to the cell surface renders the cell susceptible to virus-specific CTL-mediated killing (Palmer et al., 1977).

Zinkernagel & Althage (1977) studied the effects of virus-specific CTL on vaccinia virus-infected cells. They found that CTLs can recognize and kill infected cells before the assembly of infectious progeny, thereby reducing the yield. Vaccinia virus is a DNA virus which acquires a host-derived envelope and becomes infectious while still within the cytoplasm (Prescott et al., 1971). At the time of their study, the unique nuclear disintegration induced by CTLs had not been discovered. Therefore, they did not address whether the virus p.f.u. are reduced, or the viral DNA is degraded when CTLs are applied during viral replication, nor is any comparison made with other forms of immune lysis such as that caused by antibody and complement. However, the data show that cytolysis mediated by CTLs was not accompanied by a reduction in p.f.u. below the starting level either early or late in the infection. No determinations were made of whether or not the p.f.u. were intracellular at the time of the CTL-mediated attack. The reduction in subsequent virus yield suggests that the virus-infected subpopulation was actually killed, although interferon released by the CTLs could also have been involved. Overall, while not definitive, the results do suggest that vaccinia virus p.f.u., like reovirus p.f.u., are unscathed when their host cell succumbs to attack by CTLs.

There is another possible mechanism by which nuclear disintegration may limit virus replication. CTLs are highly motile cells and in vivo, where the E/T ratio may be quite low, the CTLs may not remain in contact with any particular target cell long enough to effect rapid lysis. If brief contact were enough to initiate internal/nuclear disintegration in the absence of rapid cytolysis, however, the replication of virus would be stopped despite the fact that the target cell would not lyse for many hours. Thus, internal/nuclear disintegration might be a more important effect of CTLs than the rapid cytolysis typically assayed. This hypothesis is currently being investigated in our laboratory.

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