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Rejection of reovirus-treated L1210 leukemia cells by mice*

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Summary. L1210 leukemia cells were treated in vitro with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and reovirus to determine their interactive effects on rejection of these tumor cells by mice. The cells were treated with BCNU at concentrations of 0, 3, or 10 μM, incubated for 48 h, then treated with reovirus at a multiplicity of infection of 0, 10, 30, or 100 for 2, 6, or 12 h. The survival of mice injected with cells treated with any amount of reovirus, regardless of BCNU treatment, was greater than that of mice injected with untreated cells. Exposure of the cells to reovirus for 6 or 12 h increased the survival of mice injected with these cells as compared with that of mice injected with cells exposed to reovirus for 2 h. Of the survivors, 76% were resistant to subsequent challenge with untreated L1210 cells. These results suggest that activities associated with reovirus replication may cause modifications of L1210 cells that enable them to induce an immune response, thus facilitating their rejection. A lack of correlation between differences in DNA synthesis (measured by ^{3}H-thymidine uptake) by treated cells and the ability of those cells to kill recipient mice indicates that rejection of cells treated with reovirus or BCNU is not due to a decrease in their ability to proliferate or, presumably, to generate lethal tumors. The survival of mice injected with treated L1210 cell preparations containing as few as 2.9% reovirus-infected cells was enhanced to the same degree as that of mice injected with those containing as many as 14.6% infected cells, indicating that modification of only a minor component of the tumor cell population is sufficient to alter the ability of the cells to generate a lethal tumor.

Introduction

Reovirus has been demonstrated to act synergistically with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) to provide therapy effective against the murine lymphoma, EL4, and the murine leukemia, L1210 [11, 12]. In vivo treatment of immunocompetent mice with BCNU and reovirus caused regression of the tumor in 40% to 75% of the mice. BCNU/reovirus-treated mice survived 80 days after tumor injection and developed tumor-specific immunity, as shown by their resistance to challenge with a lethal dose of the tumor cells originally injected. BCNU alone provided only limited survival; reovirus alone was ineffective in increasing survival of the mice. The therapy observed with the BCNU/reovirus combination could be due to modification of the tumor cells which makes them recognizable to the host, to modification of the host immune system which enables it to recognize the tumor cells and eliminate them, or to some combination of these mechanisms.

This study was designed to gain insight into the mechanisms of this therapy system by determining the effect of in vitro treatment of L1210 cells with BCNU and reovirus on their rejection by mice. The treated cells were injected into mice, whose survival was monitored. To demonstrate that increased survival of mice receiving treated cells was not due to damage sustained by L1210 cells during in vitro treatment, the ability of the cells to proliferate was determined by assessing their ^{3}H-thymidine uptake. In addition, the number of plaque-forming units of reovirus associated with reovirus- and BCNU/reovirus-treated cells was determined using an infectious center assay.

Materials and methods

Animals. Adult male BDF1 mice (Jackson Laboratories, Bar Harbor, Me.) were used in all experiments. Animals were fed water and Purina Rodent Laboratory Chow 5001 ad libitum.

Tumor cells. Mouse leukemia L1210 cells were obtained from the cell culture collection at Purdue University. Ascites tumors were induced by i.p. injection of 10^5 tumor cells in 0.2 ml phosphate buffered saline (PBS; 0.075 M sodium phosphate, 0.075 M NaCl, pH 7.2)/mouse. L1210 cells were passaged in mice and harvested as needed for each experiment. After development of tumors, between 9 and 14 days after injection, the ascitic fluid was collected by aspiration from the peritoneal cavity and placed in Hanks' balanced salt solution. After sedimenting the cells by centrifugation at 300 × *g* for 10 min, erythrocytes were lysed by treatment with 0.144 M NH_4Cl in 0.017 M Tris (pH 7.2) for 2 min at room temperature [14]. Heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Logan, Utah) was layered under the cell suspension and the cells sedimented by centrifugation at 300 × *g* for 10 min. These cells were washed twice in PBS, then resuspended in

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tissue culture medium (TCM) consisting of RPMI 1640 (K. C. Biological, Inc., Lenexa, Kan.) containing 100 units/ml penicillin and 100 µg/ml streptomycin and 5% or 10% (v/v) FBS. The cells were incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2 in air before use in any experiment.

BCNU. BCNU lot 11-107-003, was kindly donated by Bristol-Myers Company (Syracuse, NY). It was dissolved in absolute ethanol to a concentration of 33 mg/ml and brought to 3.3 mg/ml in sterile water. BCNU was then diluted to appropriate concentrations in PBS for use in experiments. The final concentration of BCNU used was 3 or 10 µM.

Preparation of reovirus. The Dearing Strain of reovirus type 3 was propagated and purified from suspension cultures of L-929 cells. The cells, grown in 3-l flasks to a density of 10^6 cells/ml, were infected with third passage lysate at a multiplicity of infection (MOI) of 10 to 20 plaque-forming units/cell. Reovirus was purified essentially as described by Kollmorgen et al. [11]. Frozen pellets of infected cells were resuspended in homogenizing solution (0.25 M NaCl, 0.01 M 2-mercaptoethanol, 0.01 M Tris, pH 8) and Freon (kindly provided by DuPont; Wilmington, Del.). The homogenate was centrifuged at 2500 × *g* for 10 min. Following centrifugation, the organic phase was reextracted twice with homogenizing solution. The aqueous phases were pooled and diluted with Freon. Following homogenization and centrifugation, the aqueous phase was decanted and saved. The organic phase was reextracted with homogenizing solution, after which the organic phase was discarded. The aqueous phases were pooled and reextracted 3 times with Freon. The final aqueous phase was layered on a 14-ml preformed CsCl gradient (1.2–1.4 g/ml) and centrifuged for 2 h at 21,000 rpm in an SW 27 rotor. The virus band was collected and further purified using a 20%–40% glycerol gradient centrifuged for 45 min at 21,000 rpm using an SW 27.1 rotor. The virus bands were collected and dialyzed against saline sodium citrate buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.4) at 4 °C for 48 h. Purified reovirus was titrated by the procedure of Duncan et al. [2].

In vitro treatment of L1210 cells. L1210 cells at a concentration of 5×10^5 cells/ml in TCM containing 5% FBS, were treated with PBS or BCNU at concentrations of 3 or 10 µM for 1 h. The cells were then washed 3 times with PBS. The cells were counted and brought to 5×10^5 cells/ml in fresh TCM containing 10% FBS. After 48 h the cells were counted and adjusted to 5×10^6 cells/ml in TCM without FBS. PBS or sonicated reovirus, at an MOI of 10 or 30, was added to appropriate tubes. After a 1-h adsorption period at 37 °C in 5% CO_2, the cells were adjusted to 5×10^5/ml in TCM containing 5% FBS and incubated for 1, 5, or 11 h at 33 °C. The cells were washed 3 times in PBS, then viable cells enumerated using trypan blue exclusion. The cells were then resuspended at 5×10^5 viable cells/ml.

Tumor generation in mice. Mice were divided into groups of 10. Each mouse was injected i.p. with 0.2 ml of a suspension containing 5×10^5 viable cells/ml, treated as described above. Mice were monitored daily for mortality.

^{3}H-thymidine uptake. At 2, 6, and 12 h following infection, treated L1210 cells were counted and 5×10^5 cells were added to each of three tubes for each cell treatment. The cells were sedimented by centrifugation at 300 × *g* and washed once in PBS. The cells were resuspended to 1 ml in TCM containing 10% FBS and 5 µCi/ml ^{3}H-thymidine (69 Ci/mmole; ICN; Irvine, Calif.). The cultures were incubated at 37 °C for 1 h and then placed on ice for several minutes. The cellular DNA was then precipitated onto glass filters with cold 10% trichloroacetic acid. The filters were rinsed with cold 95% ethanol, placed in vials, and dried. Econofluor (New England Nuclear; Boston, Mass.) was added to each vial. Vials were then counted to 2% sigma error in a Beckman model LS 7500 liquid scintillation counter.

Infectious center assay. At 12 h after infection with reovirus, L1210 cells were sedimented by centrifugation at 300 × *g*. The cells were washed three times with PBS and resuspended in Earles minimum essential medium containing 5% FBS and kanamycin (100 mg/l). The cell suspensions were serially diluted and 2 ml of medium containing 10^4, 10^3, or 10^2 treated cells was added to confluent monolayers of L-929 cells in 6-well plates and allowed to incubate overnight at 37 °C to facilitate cell attachment. The medium was then removed carefully from the wells and the monolyers were overlayed as described for the plaque assay [2]. Plaques were counted and the percent of infected cells determined.

Analysis of results. The 60-day survival of mice injected with treated tumor cells was compared using the SPSS statistical system and the Lee-Desu χ^2 analysis program [7]. The effect of BCNU on percent viability and number of viable cells, the ^{3}H-thymidine uptake of treated cells, and the percent infection of treated cells was analyzed by one-way analysis of variance [16]. All statements relative to significance are based on accepting $P < 0.05$.

Results

BCNU treatment of L1210 cells

L1210 cells were treated with BCNU at selected concentrations to determine the lowest concentration that inhibited their growth and viability. The cells were monitored over a 72 h period, the length of time they were to be maintained in vitro during subsequent experiments. Treatment with 30 µM BCNU caused a significant decrease in both the number of viable cells and in the percent viability at 24, 48, and 72 h post-treatment (Table 1). Treatment with 10 µM BCNU caused a significant, but lesser, decrease in both the number of viable cells and in the percent viability at 24, 48, and 72 h post-treatment. Treatment with 3 µM BCNU caused a slight, but insignificant, decrease in both the number of viable cells and in the percent viability at 24, 48, and 72 h post-treatment. Based on these results, the concentrations of BCNU used in subsequent experiments were 3 µM and 10 µM, the concentrations at which BCNU just began to be demonstrably toxic to the L1210 cells.

Survival of mice injected with treated L1210 cells

Mice injected with cells treated with reovirus exhibited greater survival than mice injected with cells not treated

Table 1. Effect of treatment of L1210 cells with BCNU at selected concentrations on number of viable cells and percent viability[a, b]

BCNU (μM)	24 h Number of viable cells ± SD (X 10^{-6})	48 h Number of viable cells ± SD (X 10^{-6})	72 h Number of viable cells ± SD (X 10^{-6})	*n*
0.00	1.45 ± 0.32 AB	2.65 ± 0.32 A	3.65 ± 0.41 AB	4
0.03	1.86 ± 0.26 A	3.12 ± 0.11 A	4.91 ± 1.34 A	2
0.10	1.37 ± 0.41 AB	2.25 ± 0.86 AB	3.20 ± 1.06 AB	4
0.30	1.64 ± 0.56 AB	2.74 ± 0.90 A	3.93 ± 1.50 A	4
1.00	1.35 ± 0.73 AB	2.34 ± 0.14 AB	3.41 ± 1.15 AB	4
3.00	1.08 ± 0.57 BC	1.83 ± 0.36 BC	2.41 ± 1.55 BC	4
10.00	0.56 ± 0.53 CD	0.69 ± 0.72 D	0.99 ± 1.12 CD	4
30.00	0.06 ± 0.08 D	0.01 ± 0.003 D	0.01 ± 0.01 D	2

BCNU (μM)	24 h %V ± SD	48 h %V ± SD	72 h %V ± SD	*n*
0.00	95.3 ± 0.5 A	94.3 ± 1.3 A	94.3 ± 1.0 A	4
0.03	94.5 ± 0.7 A	93.5 ± 0.7 A	93.5 ± 2.1 A	2
0.10	93.8 ± 1.0 A	94.0 ± 1.4 A	93.8 ± 1.0 A	4
0.30	93.8 ± 1.0 A	95.0 ± 1.4 A	92.5 ± 0.6 A	4
1.00	92.3 ± 1.7 A	92.8 ± 1.3 A	92.3 ± 1.3 A	4
3.00	86.5 ± 10.9 A	84.3 ± 13.0 A	86.3 ± 9.2 A	4
10.00	62.3 ± 31.9 B	56.5 ± 35.9 B	56.5 ± 37.6 B	4
30.00	4.0 ± 0.0 C	2.0 ± 1.4 C	2.5 ± 0.7 C	2

[a] For each experiment, the mean values within a time group which share a common letter are not significantly different

[b] This experiment was repeated with similar results

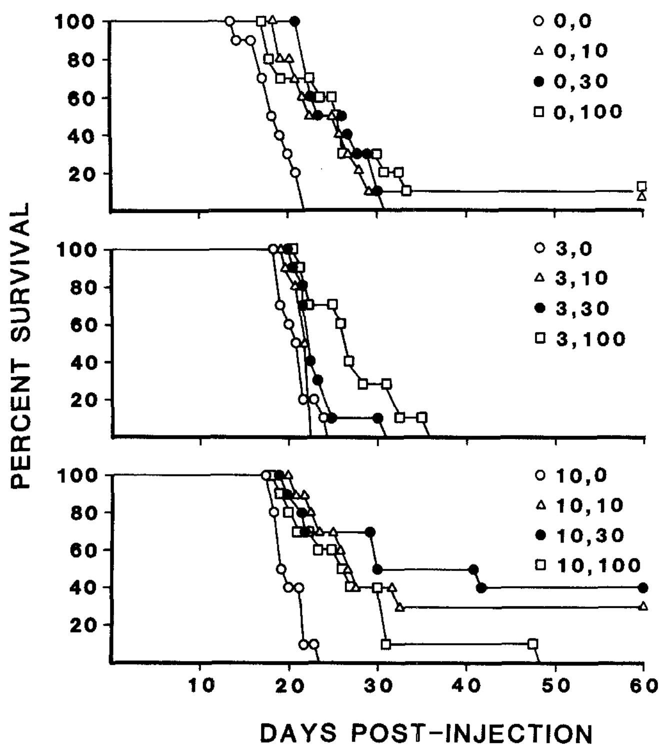

Fig. 1. Survival curves for mice injected with in vitro treated L1210 cells. Length of reovirus exposure was 2 h. For each treatment, the first number represents the BCNU concentration (μM) and the second number represents the amount of reovirus (MOI) used to treat cells. There were 10 mice in each group. Where no reovirus was added, values from the 6 h data are presented to facilitate comparison

with reovirus (Figs. 1, 2, and 3; Table 2). All the doses of reovirus used seemed to enhance the survival of the tumor cell recipients to the same degree. None of the mice which received cells not exposed to reovirus survived. Thus, BCNU treatment alone had no effect on survival. Further, no synergism between the BCNU and reovirus treatments was apparent. When comparisons were made within each treatment group, it was seen that mice injected with cells exposed to reovirus for 6 h (Fig. 2) or 12 h (Fig. 3) had significantly greater survival than mice injected with cells exposed to reovirus for 2 h (Fig. 1). This was true for all treatment groups, except those in which the mice received cells treated with 10 μM BCNU and MOI 10 or 30 reovirus; for these two treatment groups, there was no significant increase in survival with increased length of exposure to reovirus. Within each treatment group the survival of mice that received cells exposed to reovirus for 6 h was not significantly greater than that of mice which received cells exposed to reovirus for 12 h (Table 2).

Challenge of survivors

When the mice which survived the initial experiments were challenged with 10^5 untreated L1210 cells, approximately 76% survived (data not shown). There was no apparent difference in the survival of any of the groups of challenged mice. Thus, having been injected with cells treated with BCNU, in addition to reovirus, during the initial experiments did not influence the ability of the mice to survive this subsequent challenge with a lethal dose of L1210 cells.

Proliferation capability of treated cells

The ability of the treated cells to proliferate in vitro was determined by assessing their ^{3}H-thymidine uptake at 2, 6,

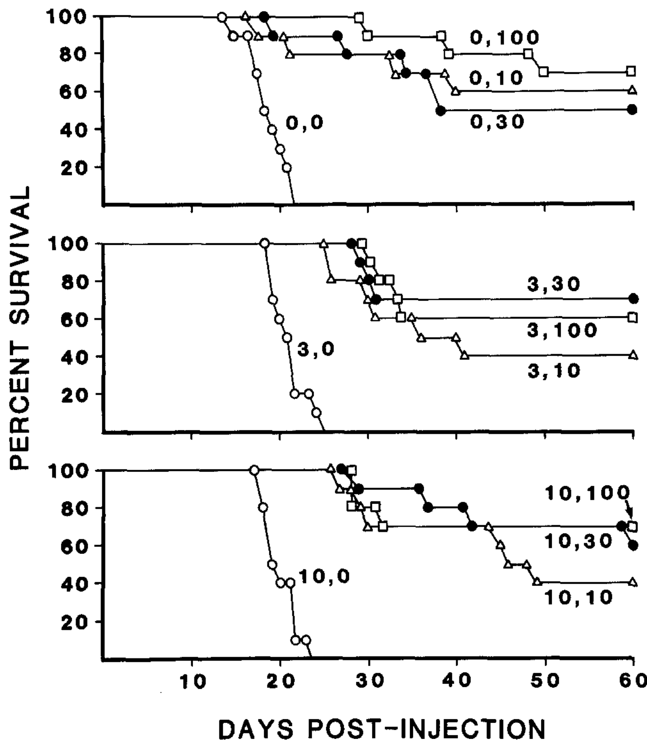

Fig. 2. Survival curves for mice injected with in vitro treated L1210 cells. Length of reovirus exposure was 6 h. For each treatment, the first number represents BCNU concentration (μM) and the second number represents the amount of reovirus (MOI) used to treat cells. The number of mice in each group was 10

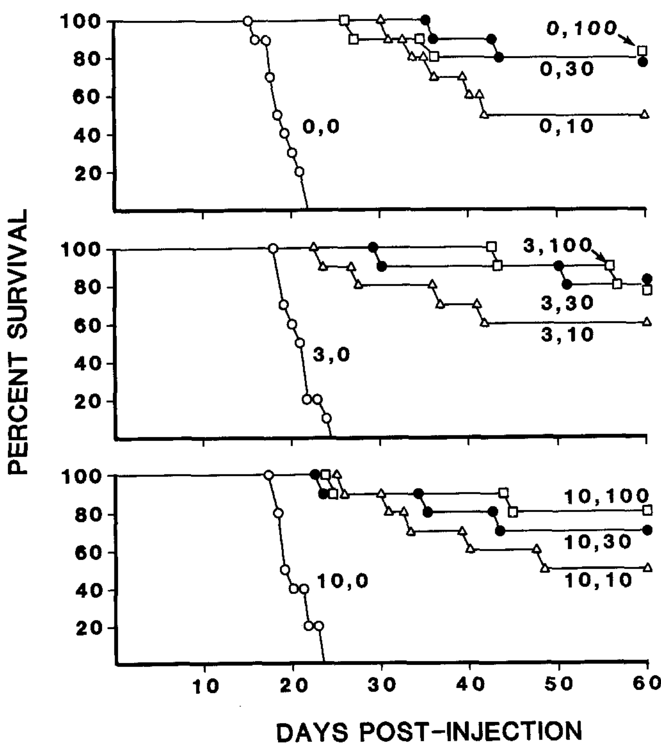

Fig. 3. Survival curves for mice injected with in vitro treated L1210 cells. Length of reovirus exposure was 12 h. For each treatment, the first number represents the BCNU concentration (μM) and the second number represents the amount of reovirus (MOI) used to treat cells. There were 10 mice in each group. Where no reovirus was added, values from the 6 h data are presented to facilitate comparison

Table 2. Median survival times in days for mice injected with L1210 cells treated in vitro with BCNU and reovirus

Treatment[a]		Time of exposure to reovirus[b]		
BCNU (μM)	Reovirus (MOI)[c]	2 h	6 h	12 h
0	0	19.0 A	19.0 A	19.0 A
0	10	23.0 DEH	*> 60.0 C*	*43.0 B*
0	30	28.0 H	*39.0 C*	*> 60.0 B*
0	100	27.0 DEH	*> 60.0 C*	*> 60.0 B*
3	0	22.0 BCD	22.0 B	22.0 A
3	10	22.0 BCDF	*37.0 C*	*> 60.0 B*
3	30	22.7 DFG	*> 60.0 C*	*> 60.0 B*
3	100	27.5 H	*44.0 C*	*> 60.0 B*
10	0	20.0 AB	20.0 AB	20.0 A
10	10	*28.0 H*	*47.0 C*	*49.0 B*
10	30	*31.0 EGH*	*> 60.0 C*	*> 60.0 B*
10	100	27.0 EFH	*> 60.0 C*	*> 60.0 B*

[a] Median survival times sharing a common underline between cell exposure times are not significantly different
[b] Median survival times sharing a common letter for a cell exposure time are not significantly different
[c] In systems to which reovirus was not added, data for the 2 h and the 12 h time points were taken from 6 h data

and 12 h after infection with reovirus. There were no significant differences in ^{3}H-thymidine uptake among the groups of cells at 2 h post-infection (Table 3). At 6 and 12 h post-infection, there were some significant differences among the groups of treated cells, but they did not correlate with the other effects of either BCNU or reovirus treatment. When these data were compared within each treatment group over time, the ^{3}H-thymidine uptake of the 10 μM BCNU/MOI 10 reovirus-treated cells was significantly lower at 6 and 12 h than it was at 2 h of exposure to reovirus.

Reovirus infection of L1210 cells

The percent infection of L1210 cells increased significantly as the reovirus MOI was increased from 10 to 30 to 100, regardless of BCNU treatment (Table 4). In contrast, cells treated with 3 or 10 μM BCNU appeared to have a lower percent infection than the control cells (although the differences were not always statistically significant). The minimum percentage of L1210 cells infected with reovirus observed during these experiments was 2.9%; the maximum percentage of cells infected with reovirus was 14.6%.

Discussion

The purpose of this study was to further elucidate the mechanisms involved in the therapy of L1210 leukemia observed when mice are injected with BCNU and reovirus [11, 12]. To accomplish this, L1210 cells were treated in vitro with BCNU and/or reovirus and their ability to generate lethal tumors in mice was determined. The survival of mice injected with L1210 cells treated with reovirus, regardless of BCNU treatment, was significantly greater than that of mice injected with untreated L1210 cells. Within the limits used in these experiments, enhanced survival appeared to be independent of the dosage of reovirus. Exposure of the cells to reovirus for 6 or 12 h signifi-

Table 3. Proliferation of L1210 cells treated in vitro with BCNU and reovirus[a]

BCNU (μM)	Revirus (MOI)	Duration of exposure of L1210 cells to reovirus[b]		
		2 h	6 h	12 h
		CPM ± SE (X 10^{-3})	CPM ± SE (X 10^{-3})	CPM ± SE (X 10^{-3})
0	0	71.0 ± 11.7 A	71.2 ± 14.4 ADEFGHIKL	70.3 ± 1.4 ACD
0	10	80.0 ± 3.0 A	84.0 ± 8.6 BCDEFK	80.7 ± 3.7 AB
0	30	76.5 ± 4.8 A	90.1 ± 5.4 BCE	67.3 ± 6.2 ACDF
0	100	70.9 ± 5.5 A	75.1 ± 5.7 ABDEFGKL	70.5 ± 1.5 CD
3	0	62.7 ± 5.8 A	78.8 ± 2.8 ABCDEFK	54.8 ± 7.8 EFGHIL
3	10	67.6 ± 5.0 A	73.3 ± 2.5 ABDEFGIKL	60.6 ± 5.0 CEFIL
3	30	71.9 ± 7.8 A	65.3 ± 5.4 ADFGHIKL	52.7 ± 2.0 EGHIJKL
3	100	68.2 ± 4.1 A	60.3 ± 4.3 AGHIJL	52.2 ± 3.7 EGHIJKL
10	0	57.8 ± 4.4 A	62.2 ± 4.5 AFGHIJL	55.7 ± 3.4 EFGHIL
10	10	64.5 ± 7.6 A	53.2 ± 4.9 HIJL	46.8 ± 1.8 GHJK
10	30	64.5 ± 4.4 A	73.6 ± 6.2 ABDEFGKL	46.4 ± 2.7 GHIJK
10	100	75.4 ± 6.7 A	65.2 ± 1.0 ADFGHIKL	54.9 ± 2.4 EFGHL

[a] Data presented are means ± SE of the radioactivity (CPM) incorporated by L1210 cells exposed to selected combinations of BCNU/reovirus prior to incubation for 1 h with 5 μCi/ml ^{3}H-thymidine in 2 independent experiments; each experiment had 3 determinations for each point

[b] Means which share a common letter for a reovirus exposure time are not significantly different. There were no significant differences among reovirus exposure period group values within any BCNU/reovirus treatment group

Table 4. Percent infection of L1210 cells following treatment with selected combinations of BCNU and reovirus[a]

BCNU (μM)	Reovirus (MOI)[b]		
	10	30	100
0	5.3 ± 0.3	7.8 ± 0.4 B	14.6 ± 0.8
3	3.3 ± 0.2 A	5.0 ± 0.4	11.6 ± 0.4 C
10	2.9 ± 0.4 A	6.6 ± 0.7 B	10.9 ± 0.5 C

[a] Data presented are means ± SE of 2 independent experiments; each experiment had 3 determinations for each point

[b] Mean percentages, within either a BCNU concentration group or a reovirus MOI group, which share a common letter are not significantly different

cantly increased survival of mice injected with these cells compared with that of mice injected with cells exposed to reovirus for 2 h. Of the mice which survived injection with reovirus-treated cells, 76% were resistant to subsequent challenge with a lethal dose of untreated L1210 cells.

Both reovirus [17] and BCNU [4, 8, 18] have been shown to inhibit cellular DNA synthesis under appropriate conditions. For assurance that the increased survival of mice which received reovirus-treated cells was not due to the inability of these cells to generate a lethal tumor per se, it was important to demonstrate that the treated cells were able to proliferate. As demonstrated by ^{3}H-thymidine uptake studies, treatment with BCNU and/or reovirus (at the concentrations used) had no significant effect on L1210 cell proliferation. This suggests that all the groups of treated cells were equally capable of proliferating, generating a tumor, and ultimately causing the death of the animals. These data thus support the idea that the reovirus-treated cells were not simply acting as a dead-cell vaccine to immunize the mice. Rather, it appears that the treated L1210 cells were able to proliferate, but that they induced an immune response that led to L1210 cell rejection prior to lethal tumor formation.

The fact that mouse survival increased significantly if the L1210 cells were in contact with reovirus for at least 6 h before injection of the tumor cells into mice suggested that the cells infected by reovirus were actively modified during the process of viral replication. Perhaps this was due to the generation of viral or viral-induced neoantigens similar to those found in reovirus-infected L-929 cells. Membranes of L-929 cells infected with reovirus contained four polypeptides not observed in uninfected cells; in addition, modification or deletion of two polypeptides was detected (Coleman et al. unpublished data). Changes such as these may be sufficient to allow host recognition and destruction of tumor cells in the L1210 cell system. Certainly, those mice that survived a subsequent challenge with a lethal dose of L1210 cells must be immune to the tumor cells. Presumably the immune cells active here would be the cytotoxic lymphocytes (CTL) detected in the in vivo therapy system of Kollmorgen et al. [11]. It seems likely that any neoantigens formed are closely associated with (or partially composed of) L1210 cell antigens, because the treated mice were immune to challenge with untreated L1210 cells. Thus, it would appear that the reovirus-treated cells induce CTL which recognize the antigens of untreated L1210 cells, as well as CTL which recognize reovirus antigens and CTL which recognize reovirus-induced neoantigens. If the mice were recognizing only the reovirus adsorbed to L1210 cells, they would probably not have been immune to challenge with untreated L1210 cells. The survival of mice injected with treated L1210 cell preparations containing as few as 2.9% reovirus-infected cells was enhanced to the same degree as that of mice injected with those containing as many as 14.6% infected cells. Therefore, it appears that modification of only a minor component of the tumor cell population is sufficient to alter the ability of the cells to generate a lethal tumor. Although relatively few of the treated cells were infected with reovirus at the time they were injected into the mice, it is possible that many more cells incorporated neoantigens into their membranes, but these cells were not detected in

the plaque assay because they were incapable of producing infectious virus.

Support for the concept that viral infection of tumor cells leads to decreased ability to generate tumors comes from another system in which rat tumor cells were infected with Friend virus in vivo [10]. In this system, decreased ability to generate tumors was parallelled by increased susceptibility to CTL [9], and by increased immunogenicity [19]. These changes were coupled with increased amounts of virus-associated antigen; however, the specificity of the lymphocytes was for tumor-associated antigen, the cell density of which remained unchanged. It was surmised that the virus-associated antigen enhanced the immunogenicity of the tumor-associated antigen [19].

Another possible explanation for the role of reovirus in this system is that it may modify the host immune system instead of, or in addition to, modifying the tumor cells. This modification may include induction of natural, nonspecific host defense mechanisms involving interferon, cytolytic macrophages, and/or natural killer cells. Reovirus is well-known as an interferon inducer [13], and interferon has been shown to potentiate the antitumor activities of both cytolytic macrophages and natural killer cells [1, 5, 6]. This nonspecific resistance might function to limit tumor cell proliferation until tumor-specific immunity, such as the tumor-specific CTL, can be developed. It is also possible that reovirus inhibits the activities of a population of host cells that suppress the immune response to tumor cells. This idea is supported by recent evidence indicating that reovirus selectively binds to the suppressor/cytotoxic subset of murine and human T cells [3].

In earlier in vivo therapy experiments [11, 12], injecting mice with reovirus alone had no effect on their survival with lethal tumors, despite the fact that it now appears that 50% of the L1210 cells in peritoneal washes from these tumor-bearing mice are infected with reovirus at the time of reovirus injection (Bryson and Cox unpublished data). Thus, there is a fundamental difference between the in vivo and in vitro systems. Perhaps the key to the difference(s) between these two systems lies in the function of BCNU. In the present study, treatment of L1210 cells with BCNU alone did not increase survival of the mice, and BCNU did not appear to act synergistically with reovirus. In addition, BCNU treatment did not increase the percent infection of L1210 cells; in fact, it appeared to decrease it. It is possible that the major role of BCNU involves interaction with host cells and not with the tumor cells themselves. Nagarkatti and Kaplan [15] recently demonstrated that BCNU treatment of B57BL/6 mice bearing LSA ascites tumors significantly increased CTL activity compared with that found in non-BCNU treated animals. This increase in CTL activity correlated with a lack of tumor-specific suppressor T cell activity and a significant decrease in nonspecific suppressor T cell activity. BCNU-treated mice were resistant to challenge with homologous tumor cells [15]. In the present system, suppressor T cells could not have been induced before the therapy began, because therapy and tumor induction began at the same time, when the mice were injected with treated L1210 cells. Perhaps reovirus-treated cells failed to produce lethal tumors in the present system because they were recognized as foreign, due to modification caused by reovirus infection, and were rejected before suppressor T cells had a chance to develop. This would explain both the lack of effect of BCNU in this in vitro therapy system and the synergistic effect of BCNU and reovirus in the in vivo systems [11, 12].

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