JOURNAL OF VIROLOGY, Nov. 1996, p. 7984–7991 Vol. 70, No. 11
0022-538X/96/$04.00+0

Linkage between Reovirus-Induced Apoptosis and Inhibition of Cellular DNA Synthesis: Role of the S1 and M2 Genes

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Received 22 May 1996/Accepted 6 August 1996

The mammalian reoviruses are capable of inhibiting cellular DNA synthesis and inducing apoptosis. Reovirus strains type 3 Abney (T3A) and type 3 Dearing (T3D) inhibit cellular DNA synthesis and induce apoptosis to a substantially greater extent than strain type 1 Lang (T1L). We used T1L × T3A and T1L × T3D reassortant viruses to identify viral genes associated with differences in the capacities of reovirus strains to elicit these cellular responses to viral infection. We found that the S1 and M2 genome segments determine differences in the capacities of both T1L × T3A and T1L × T3D reassortant viruses to inhibit cellular DNA synthesis and to induce apoptosis. These genes encode viral outer-capsid proteins that play important roles in viral attachment and disassembly. To extend these findings, we used field isolate strains of reovirus to determine whether the strain-specific differences in inhibition of cellular DNA synthesis and induction of apoptosis are also associated with viral serotype, a property determined by the S1 gene. In these experiments, type 3 field isolate strains were found to inhibit cellular DNA synthesis and to induce apoptosis to a greater extent than type 1 field isolate strains. Statistical analysis of these data indicate a significant correlation between the capacity of T1L × T3A and T1L × T3D reassortant viruses and field isolate strains to inhibit cellular DNA synthesis and to induce apoptosis. These findings suggest that reovirus-induced inhibition of cellular DNA synthesis and induction of apoptosis are linked and that both phenomena are induced by early steps in the viral replication cycle.

Reovirus infections evoke a variety of cellular responses, including virus-induced cytopathic effects. For example, reoviruses induce apoptosis of infected cells, and prototype reovirus strains type 1 Lang (T1L) and type 3 Dearing (T3D) differ in this capacity (55). Reovirus infection is also associated with inhibition of cellular DNA (13, 17, 39, 47, 49), RNA (27, 48), and protein (8, 13, 33, 48) synthesis, alterations in cytoskeletal architecture (46), and changes in the number of cell surface signaling molecules (32, 52, 58). Signal transduction pathways play a role in efficiency of reovirus infection and the nature of virus-associated cytopathic effects. A receptor for strain T3D on R1.1 thymocytes exhibits tyrosine kinase activity, and engagement of this receptor is associated with inhibition of DNA synthesis and cell cycle arrest in the G_1-S phase (44). Transfected NIH 3T3 or 3T3-derived NR6 cells, which express functional epidermal growth factor receptor (52) or v-erbB protein (51), also exhibit enhanced reovirus infection. Both cases suggest that a binding event at the cell surface activates signal transduction pathways via protein tyrosine kinases to mediate a variety of virus-associated alterations in cellular metabolism.

Morphologic changes in nuclear structure, reported as a consequence of reovirus-induced inhibition of cellular DNA synthesis (4), closely resemble those associated with reovirus-induced apoptosis (55). These findings suggest an association between inhibition of host cell proliferation and DNA synthesis and the induction of apoptosis in reovirus-infected cells. Genetic studies using T1L × T3D reassortant viruses also support this association. Our previous work identified the viral S1 and M2 genome segments as the major determinants of differences in the capacities of T1L and T3D to induce apoptosis of L cells (55). An earlier study suggested that the S1 gene also determines differences in the capacities of T1L and T3D to inhibit cellular DNA synthesis (47). However, this study, which was limited by use of a small number of reassortant viruses, could not identify genes other than the S1 gene associated with strain-specific differences in DNA synthesis inhibition. An additional limitation of this study involved use of reassortant viruses derived from temperature-sensitive parental viruses generated by chemical mutagenesis (15). Results obtained in studies using reassortant viruses derived from mutagenized parental stocks have not always been in agreement with studies using reassortant viruses derived from nonmutagenized stocks (e.g., compare reference 43 with references 2 and 26 and reference 63 with reference 1).

We conducted experiments to investigate whether an association exists between the capacities of reoviruses to inhibit cellular DNA synthesis and to induce apoptosis. Because of the limitations of the studies described above, our genetic analyses of strain-specific differences in cellular DNA synthesis inhibition were performed with two large panels of reassortant viruses derived from crosses T1L × type 3 Abney (T3A) and T1L × T3D. We also examined the capacity of type 1 (T1) and type 3 (T3) field isolate strains to inhibit cellular DNA synthesis and to induce apoptosis. Our results indicate that there is a significant association between the capacities of both reovirus reassortants and reovirus field isolates to inhibit host cell DNA synthesis and to induce apoptosis. Furthermore, our genetic analyses indicate that these two properties are determined by the same viral genes, suggesting that inhibition of cellular

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DNA synthesis and induction of apoptosis are linked via the same or coordinated mechanisms.

MATERIALS AND METHODS

Cells and viruses. Spinner-adapted mouse L929 (L) cells were grown in Joklik's modified Eagle's minimal essential medium (Gibco BRL, Grand Island, N.Y.) supplemented to contain 5% heat-inactivated fetal bovine serum (Gemini Bioproducts, Calabasas, Calif.) and 2 mM L-glutamine (Gibco). Reovirus strains T1L, T3A, and T3D are laboratory stocks. T1L × T3A reassortant viruses were grown from stocks originally isolated by Tricia Jandris, Lynda Morrison, and Greame Wilson in the laboratory of Bernard Fields (63). T1L × T3D reassortant viruses were grown from stocks originally obtained from Kevin Coombs, Bernard Fields, and Max Nibert (3, 6). Reovirus T1 field isolate strains T1C11, T1C49, T1C62, T1C11936, and T1C12757 and T3 field isolate strains, T3C9, T3C18, T3C31, T3C43, T3C44, T3C45, T3C84, and T3C93 were originally isolated by Rosen and colleagues (9, 24, 40–42). Viral strains were plaque purified and passaged in L cells to generate working stocks as previously described (54).

Measurement of DNA synthesis. L cells were placed into 96-well plates (Costar, Cambridge, Mass.) at 6×10^3 cells per well in a volume of 100 μl in Joklik's modified Eagle's minimal essential medium supplemented to contain nonessential amino acids, 5% fetal bovine serum, 2 mM L-glutamine, 1 U of penicillin per ml, and 1 μg of streptomycin per ml and then incubated at 37°C overnight. The medium was removed, and cells were infected with viral strains at a multiplicity of infection (MOI) of 10 to 25 PFU per cell in a volume of 25 μl at 37°C for 1 h. Following viral adsorption, 75 μl of fresh medium and 1 μCi of [^{3}H]thymidine (25 Ci/mmol; Amersham, Arlington Heights, Ill.) were added to each well. Either cells were pulsed with [^{3}H]thymidine for the last 6 h of the indicated times postinfection (Fig. 1) or [^{3}H]thymidine was present for the final 24 h postinfection (Fig. 3 to 5; Tables 1 and 2). At various times, the medium was removed, 50 μl of 0.05% trypsin–0.02% EDTA in Hanks balanced salt solution (Biofluids, Rockville, Md.) was added to each well, and the cells were frozen at −70°C. DNA synthesis was measured by freeze-thawing cells and collecting DNA onto glass fiber filter strips (no. 240-1; Cambridge Technology, Watertown, Mass.) with a cell harvester (Cambridge Technology). Filters were dried, and incorporation of [^{3}H]thymidine-labeled DNA was measured by scintillation counting (Beckman LS3801; Palo Alto, Calif.). Results are shown either as total counts per minute per well or as percent DNA synthesis inhibition calculated as [1 − (mean cpm in virus-infected cells/mean cpm in mock-infected cells)] × 100. At least six wells were assayed for each viral strain.

Quantitation of apoptosis by fluorescent dye staining. Subconfluent monolayers (2×10^5 cells per well in 6-well plates, 9.4×10^4 cells per well in 12-well plates, and 3.7×10^4 cells per well in 24-well plates) of L cells were either mock infected or infected with viral strains at an MOI of 10 PFU per cell. The percentage of apoptotic cells was determined 48 h postinfection as previously described (12, 55). Briefly, cells were removed from tissue culture plates by trypsinization and resuspended at a concentration of 2×10^5 cells per ml. A 25-μl aliquot of cells was mixed with 1 μl of a dye solution containing 100 μg of acridine orange (AO; Sigma Chemical Co., St. Louis, Mo.) and 100 μg of ethidium bromide (Sigma) per ml in phosphate-buffered saline. Cells were examined by epifluorescence microscopy (Nikon Labophot-2; B-2A filter; excitation, 450 to 490 nm; barrier, 520 nm; dichroic mirror, 505 nm). Cells were scored as apoptotic when their nuclei contained uniformly stained condensed or fragmented chromatin (12, 55). At least 200 cells were counted for each viral strain. All experiments were performed in triplicate.

Statistical analysis. DNA synthesis inhibition and AO staining data were analyzed by using both nonparametric (Mann-Whitney [MW] test) and parametric (two-sample *t* test) statistical techniques. The contributions of individual reovirus genome segments were analyzed further by parametric stepwise linear regression analysis (18, 55). R^2 (percent variance) values were calculated for regression equations for all 10 reovirus genes and by using each gene individually as a predictor. Additional linear regression analysis was performed for all combinations of genes found to be statistically significant ($P < 0.05$) by MW test or *t* test, or as predictors in either 10-gene or single-gene linear regression analysis. Statistical tests were performed by using Minitab release 8 statistical software (Addison-Wesley, Reading, Mass.).

RESULTS

Reovirus strains T1L and T3A differ in the capacity to inhibit cellular DNA synthesis. It has previously been shown that reovirus strains T1L and T3D differ in the capacity to inhibit host cell DNA synthesis (17, 47). To determine whether strains T1L and T3A also differ in the capacity to inhibit cellular DNA synthesis, L cells were infected with each viral strain at an MOI of 25 PFU per cell, and DNA synthesis was measured by determining the amount of [^{3}H]thymidine incorporation at various intervals following adsorption (Fig. 1). Although infection with both viral strains resulted in cellular DNA synthesis inhibition, T3A was associated with substantially greater inhibition of cellular DNA synthesis than T1L. For cells infected with T3A, maximum DNA synthesis inhibition was detected 48 h after adsorption.

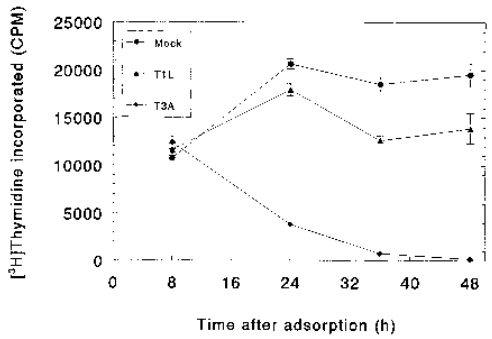

FIG. 1. Reovirus-induced inhibition of cellular DNA synthesis. L cells were either mock infected or infected with T1L or T3A at an MOI of 25 PFU per cell. [^{3}H]thymidine was added to the culture medium for the last 6 h of the infection. Cells were harvested at the indicated times, and incorporation of [^{3}H]thymidine incorporation was measured. Results are presented as total counts per minute per well. Error bars indicate standard errors of the means.

DNA synthesis inhibition by T1L × T3A reassortant viruses. To identify viral genes associated with differences in the capacities of T1L and T3A to inhibit cellular DNA synthesis, we tested 20 T1L × T3A reassortant viruses for the capacity to inhibit DNA synthesis by using the [^{3}H]thymidine incorporation assay (Table 1). The results demonstrate a significant association between the capacities of reassortant viruses to inhibit cellular DNA synthesis and the derivation of the viral S1 gene (MW test, $P < 0.0001$; *t* test, $P < 0.001$) and a less significant association between inhibition of DNA synthesis and the M2 gene (MW test, $P = 0.05$; *t* test, $P = 0.10$). No other viral genes were significantly associated with differences in DNA synthesis inhibition in this analysis (MW test and *t* test, $P > 0.05$). To investigate whether the S1 and M2 genome segments contributed independently to the capacity of T1L × T3A reassortant viruses to inhibit DNA synthesis, a parametric stepwise linear regression analysis was performed. We obtained R^2 values of 74.7% for the equation using all 10 reovirus genes, 66.3% ($P < 0.001$) for the equation using the S1 and M2 genes, 58.1% ($P < 0.001$) for the equation using the S1 gene alone, and 22.3% ($P = 0.035$) for the equation using M2 alone. These results indicate that both the S1 and M2 genome segments contribute independently to differences in the capacities of T1L and T3A to inhibit cellular DNA synthesis but identify the S1 gene as the primary determinant of these differences.

DNA synthesis inhibition by T1L × T3D reassortant viruses. A previous study using a small panel of T1L × T3D reassortant viruses linked the viral S1 gene to differences in the capacities of T1L and T3D to inhibit host cell DNA synthesis (47). We tested a larger panel of T1L × T3D reassortants ($n = 37$) for the capacity to inhibit cellular DNA synthesis in infected L cells to determine whether the viral S1 and M2 genes are significantly associated with this property (Table 2). Similar to our findings for T1L × T3A reassortant viruses, we found a statistically significant association between the capacities of T1L × T3D reassortant viruses to inhibit cellular DNA synthesis and the derivation of the S1 gene (MW test, $P < 0.0001$; *t* test, $P < 0.0001$). The M2 gene also was significantly associated with DNA synthesis inhibition (MW test, $P = 0.0125$; *t* test, $P = 0.005$) as was the L1 gene (MW test, $P = 0.008$; *t* test, $P = 0.004$). The M1 gene showed a less significant association

TABLE 1. Capacities of T1L × T3A reassortant viruses to inhibit cellular DNA synthesis and to induce apoptosis

Virus strain	Genome segment[a]										% DNA synthesis inhibition[b]	Rank[c]	% of AO-stained cells[d]	Rank[c]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4				
T1L	1L	1L	1L	1L	1L	1L	1L	1L	1L	1L	10	P	4.5	P
T3A	3A	3A	3A	3A	3A	3A	3A	3A	3A	3A	99	P	50	P
GW49	1L	1L	1L	3A	3A	3A	3A	3A	3A	3A	97	1	43	3
GW7	1L	1L	1L	1L	3A	3A	3A	1L	3A	3A	80	2	41.5	4
GW28	1L	1L	1L	1L	3A	1L	3A	3A	1L	1L	78	3	36	6
GW12	3A	1L	3A	3A	1L	3A	3A	3A	3A	1L	58	4	45	2
GW13	3A	3A	3A	3A	1L	3A	3A	3A	3A	3A	55	5	39.5	5
GW31	3A	1L	1L	3A	3A	1L	1L	3A	3A	1L	54	6	—[e]	—
GW24	3A	1L	1L	1L	3A	3A	3A	3A	3A	3A	51	7.5	46	1
GW15	3A	3A	3A	3A	3A	1L	1L	1L	3A	1L	51	7.5	—	—
GW524	1L	3A	3A	1L	1L	1L	1L	3A	3A	3A	46	9	24.5	9
GW27	3A	1L	1L	3A	1L	3A	3A	3A	3A	3A	44	10	20.5	10
GW10	1L	1L	3A	1L	1L	3A	1L	3A	1L	1L	36	11	19.5	11
GW11	3A	1L	1L	1L	1L	3A	1L	3A	3A	1L	28	12	13	13
GW4	3A	1L	3A	3A	1L	3A	1L	3A	3A	3A	19	13	8.5	15
GW45	1L	1L	3A	1L	1L	1L	1L	1L	1L	1L	17	14	27.5	7.5
GW54	1L	1L	1L	1L	1L	1L	1L	1L	3A	1L	14	15	27.5	7.5
GW14	1L	1L	3A	1L	3A	3A	1L	3A	3A	1L	13	16.5	—	—
GW26	1L	1L	3A	3A	1L	1L	1L	1L	1L	1L	13	16.5	12	14
GW34	1L	1L	1L	1L	1L	1L	1L	1L	1L	3A	12	18	15.5	12
GW16	3A	3A	3A	3A	3A	3A	1L	3A	3A	1L	8	19	—	—
GW32	1L	3A	1L	1L	1L	1L	1L	1L	1L	1L	0	20	—	—

[a] The parental origin of each genome segment in the reassortant strains: 1L, genome segment derived from T1L; 3A genome segment derived from T3A (63).

[b] L cells were infected with viral strains at an MOI of 10 PFU per cell and assayed for incorporation of [^{3}H]thymidine 48 h after adsorption. The percent DNA synthesis inhibition was calculated as [1 − (mean cpm in virus-infected cells/mean cpm in mock-infected cells)] × 100. The results represent the means for at least six wells for each viral strain.

[c] Viruses are ranked from highest to lowest on the basis of their capacities to inhibit cellular DNA synthesis and induce apoptosis. P indicates parental strain (T1L or T3A). Parental strains were not included in statistical analysis.

[d] L cells were infected with viral strains at an MOI of 10 PFU per cell and stained with AO 48 h after adsorption. The percentage of AO-stained cells was determined by fluorescence microscopy. The results represent the means for at least two independent experiments for each viral strain.

[e] —, reassortant virus was not tested.

with DNA synthesis inhibition (MW test, $P = 0.049$; t test, $P = 0.045$). No other viral genes were significantly associated with differences in the capacities of T1L and T3D to inhibit cellular DNA synthesis (MW test and t test, $P > 0.05$). We used parametric stepwise linear regression analysis to determine whether the S1, M1, M2, and L1 genes contributed independently to the capacities of T1L × T3D reassortant viruses to inhibit cellular DNA synthesis. We obtained R^2 values of 82.2% ($P < 0.001$) for the regression equation using all 10 reovirus genes; 71.8% ($P < 0.001$) for the S1, M1, M2, and L1 genes, 71.6% ($P < 0.001$) for the S1, M2, and L1 genes; 71.3% ($P < 0.001$) for the S1 and M2 genes; and 64.7% ($P < 0.001$) for the S1 gene alone. These results indicate that the S1 gene is the major determinant of differences in the capacities of reovirus strains T1L and T3D to inhibit cellular DNA synthesis. The M2 gene makes a smaller but detectable contribution; the M1 and L1 genes do not contribute significantly to the capacities of the S1 and M2 genes to mediate strain-specific differences in cellular DNA synthesis inhibition.

Apoptosis induction by reovirus strains T1L and T3A. Differences in the capacities of reovirus strains T1L and T3D to inhibit cellular DNA synthesis and to induce apoptosis have led to the suggestion that these phenomena are different manifestations of the same cellular response to reovirus infection (55). To determine whether T1L and T3A also differ in the capacity to induce apoptosis, L cells were infected with either T1L or T3A at an MOI of 10 PFU per cell, and apoptosis was quantitated by AO staining (55). We have previously shown that quantitation of apoptosis by AO fluorescent dye staining following reovirus infection in L cells is associated with ultrastructural evidence of apoptosis, the presence of DNA fragmentation, and the appearance of oligonucleosomal laddering of host cell DNA following electrophoresis in agarose gels (55). A significantly greater percentage of cells infected with T3A than of those infected with T1L exhibited condensed chromatin indicative of apoptosis (Fig. 2). For both strains, maximum apoptosis was detected 48 h after adsorption.

Apoptosis induction by T1L × T3A reassortant viruses. To determine whether the S1 and M2 genes are associated with differences in apoptosis induction by T1L and T3A, 15 T1L × T3A reassortant viruses were tested by the AO staining assay for the capacity to induce apoptosis of L cells (Table 1). Both the S1 gene (MW test, $P = 0.004$; t test, $P = 0.0005$) and the M2 gene (MW test, $P = 0.019$; t test, $P = 0.0006$) were significantly associated with the capacities of T1L × T3A reassortant viruses to induce apoptosis of L cells. No other viral genes were significantly associated with differences in apoptosis induction in this analysis (MW test and t test, $P > 0.05$). Using linear regression analysis, we obtained R^2 values of 89.1% for the regression equation using all 10 reovirus genes, 67.8% for the equation using the S1 and M2 genes ($P = 0.001$), 64.7% for the equation using the S1 gene alone ($P < 0.001$), and 42.8% for the equation using M2 alone ($P = 0.008$). Similar to our findings in studies of strain-specific differences in cellular DNA synthesis inhibition, these results indicate that the S1 gene is the major determinant of differences in the capacities of T1L and T3A to induce apoptosis. The M2 gene makes a smaller contribution in determining these differences.

Inhibition of cellular DNA synthesis is correlated with induction of apoptosis. To determine whether the capacities of reassortant viruses to inhibit cellular DNA synthesis correlate with their capacities to induce apoptosis, we compared the

TABLE 2. Capacities of T1L × T3D reassortant viruses to inhibit cellular DNA synthesis

Virus strain	Genome segment[a]										% DNA synthesis inhibition[b]	Rank[c]	% AO-stained cells[d]	Rank[e]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4				
T1L	1L	1L	1L	1L	1L	1L	1L	1L	1L	1L	10	P	3	P
T3D	3D	3D	3D	3D	3D	3D	3D	3D	3D	3D	67	P	48	P
EB129	3D	3D	1L	3D	3D	1L	3D	1L	1L	3D	89	1	74	11
EB138	3D	1L	1L	3D	3D	1L	3D	3D	1L	1L	87	2	91	6
EB62	3D	3D	3D	3D	3D	3D	3D	1L	3D	1L	84	3	94	4.5
EB13	3D	3D	3D	3D	3D	3D	3D	3D	3D	1L	76	4	94	4.5
KC150	3D	1L	1L	1L	3D	1L	3D	3D	1L	3D	74	5	98	2
EB88	3D	3D	3D	3D	1L	3D	3D	3D	3D	3D	70	6.5	87	8
EB97	3D	3D	1L	3D	3D	3D	3D	3D	3D	1L	70	6.5	90	75
H15	1L	3D	3D	1L	3D	3D	3D	3D	3D	1L	69	8	95	3
EB28	3D	3D	1L	3D	3D	3D	3D	1L	3D	3D	62	9	81	9
EB86	1L	3D	3D	3D	3D	1L	3D	3D	3D	1L	58	10	96	1
EB120	3D	3D	3D	1L	1L	3D	3D	3D	1L	1L	53	11	73	12
E3	3D	3D	3D	3D	1L	3D	3D	3D	3D	3D	42	12	69	14
EB39	1L	3D	3D	1L	3D	3D	3D	3D	3D	3D	41	13	76	10
EB143	3D	1L	1L	1L	1L	1L	3D	1L	1L	1L	39	14	70	13
H9	3D	3D	1L	3D	1L	1L	3D	3D	3D	3D	37	15	62	15
KC9	3D	3D	3D	3D	3D	3D	1L	3D	3D	3D	35	16	45	19
EB68	1L	3D	1L	1L	3D	1L	1L	1L	3D	3D	34	17	25	22
EB18	3D	3D	1L	3D	3D	3D	1L	1L	3D	1L	31	18	42	20
H14	1L	1L	3D	1L	1L	1L	1L	1L	3D	1L	30	19.5	7	24
G2	1L	3D	1L	1L	1L	1L	3D	1L	1L	1L	30	19.5	50	17.5
EB144	1L	1L	1L	1L	3D	3D	1L	1L	3D	1L	29	21	51	16
EB132	3D	3D	1L	3D	3D	1L	3D	3D	3D	1L	26	22.5	30	21
EB109	3D	3D	1L	3D	3D	3D	1L	3D	3D	3D	26	22.5	50	17.5
EB67	1L	3D	1L	1L	1L	3D	1L	1L	1L	1L	13	24	8	23
EB145	3D	3D	3D	3D	3D	1L	1L	3D	3D	3D	10	25	6	25
H27	1L	3D	3D	1L	3D	1L	1L	1L	1L	1L	6	27	1	36.5
EB113	1L	1L	1L	3D	1L	1L	1L	1L	3D	1L	6	27	5	26
H24	1L	1L	1L	1L	1L	1L	1L	1L	1L	3D	6	27	2	33
G16	1L	1L	1L	3D	1L	1L	1L	1L	1L	1L	3	29	2	33
EB85	1L	1L	1L	1L	1L	3D	1L	3D	1L	1L	0	33.5	2	33
H17	3D	3D	3D	1L	3D	3D	1L	3D	3D	1L	0	33.5	3	30
H41	3D	3D	1L	1L	1L	3D	1L	3D	3D	1L	0	33.5	0	36.5
EB31	1L	1L	1L	3D	1L	1L	1L	3D	3D	1L	0	33.5	0	33
EB121	3D	3D	1L	3D	1L	3D	1L	3D	3D	3D	0	33.5	0	33
EB98	1L	3D	1L	1L	1L	1L	1L	3D	1L	3D	0	33.5	0	28
EB1	1L	3D	1L	1L	3D	1L	1L	1L	3D	1L	0	33.5	0	28
EB47	1L	3D	1L	1L	1L	1L	1L	1L	1L	1L	0	33.5	0	28

[a] The parental origin of each genome segment in the reassortant strains: 1L, genome segment derived from T1L; 3D, genome segment derived from T3D (3, 6, 61).

[b] L cells were infected with viral strains at an MOI of 10 PFU per cell and assayed for incorporation of [^{3}H]thymidine 48 h after adsorption. The percent DNA synthesis inhibition was calculated as [1 − (mean cpm in virus-infected cells/mean cpm in mock-infected cells)] × 100. The results represent the means for at least six wells for each viral strain.

[c] Viruses are ranked from highest to lowest on the basis of their capacities to inhibit cellular DNA synthesis. P indicates parental strain (T1L or T3D). Parental strains were not included in statistical analysis.

[d] L cells were infected with viral strains at an MOI of 10 PFU per cell. The percentage of apoptotic cells was determined by AO staining 48 h after infection. Results are from reference 55 and are presented to facilitate comparison with DNA synthesis inhibition.

[e] Viruses are ranked from highest to lowest on the basis of their capacities to induce apoptosis. P indicates parental strain (T1L or T3D).

extent of DNA synthesis inhibition with the extent of apoptosis for 15 T1L × T3A reassortant viruses (Fig. 3). Using stepwise parametric linear regression analysis, we obtained an R^2 value of 59.6% ($P = 0.001$) ($r = 0.772$) for the regression equation: % DNA synthesis inhibition = 0.1 + 1.52 (% apoptosis). We also compared the extent of DNA synthesis inhibition with our previously published data (55) on the extent of apoptosis for 37 T1L × T3D reassortant viruses (Fig. 4). Using this larger panel of reassortant viruses, we obtained an R^2 value of 87.8% ($P < 0.001$) ($r = 0.937$) for the regression equation: % DNA synthesis inhibition = 1.65 + 0.736 (% apoptosis). These results indicate that there is a strong linear relationship between the capacities of T1L × T3A and T1L × T3D reassortant viruses to inhibit cellular DNA synthesis and to induce apoptosis.

DNA synthesis inhibition and induction of apoptosis by T1 and T3 field isolate reovirus strains. Both T3A and T3D are associated with substantially greater inhibition of cellular DNA synthesis and induction of apoptosis than T1L. To determine whether inhibition of cellular DNA synthesis and apoptosis induction are serotype-dependent properties of reovirus, we tested five T1 field isolate strains (T1C11, T1C49, T1C62, T1C11936, and T1C12757) and eight T3 field isolate strains (T3C9, T3C18, T3C31, T3C43, T3C44, T3C45, T3C84, and T3C93) for their capacities to inhibit cellular DNA synthesis and to induce apoptosis (Table 3). Each of the T3 strains tested, except T3C9, inhibited DNA synthesis to a greater extent than any of the T1 strains. In addition, each T3 strain induced apoptosis to a greater extent than any of the T1 strains. The correlations between serotype and DNA synthesis inhibition (MW test, $P = 0.001$; $R^2 = 68.1\%$, $P < 0.001$, $r = 0.825$) and serotype and apoptosis induction (MW test, $P = 0.001$; $R^2 = 75.7\%$, $P < 0.001$, $r = 0.870$) were highly signifi-

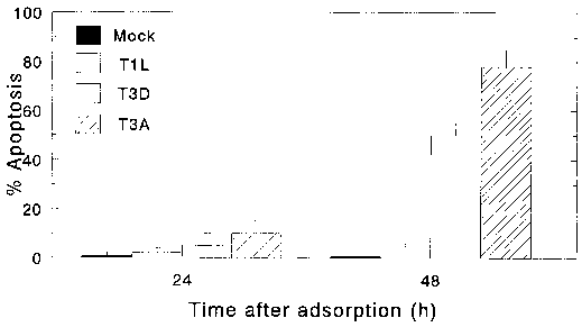

FIG. 2. Reovirus-induced apoptosis of L cells as detected by AO staining. L cells were either mock infected or infected with T1L, T3A, or T3D at an MOI of 25 PFU per cell. After incubation at 37°C for 24 or 48 h, cells were harvested and processed for AO staining. The results are expressed as the means for three independent experiments. Error bars indicate standard errors of the means.

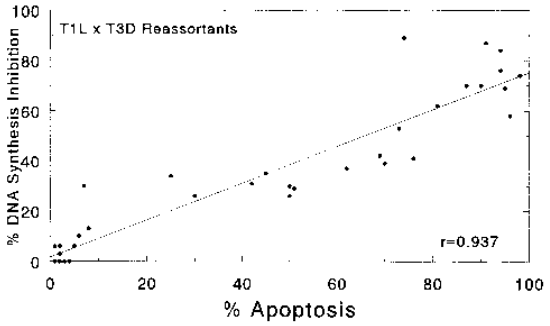

FIG. 4. Correlation between the capacities of T1L × T3D reassortant viruses to inhibit cellular DNA synthesis and to induce apoptosis. Each point represents a single reassortant virus. Data are derived from Table 2 and reference 55.

cant. We also examined the correlation between the capacities of individual field isolate strains to inhibit cellular DNA synthesis and to induce apoptosis (Fig. 5). DNA synthesis inhibition and apoptosis induction were highly correlated (R^2 = 72.5%, $P < 0.001$, $r = 0.851$) for the regression equation: % DNA synthesis inhibition = 16.6 + 0.913 (% apoptosis). These findings strongly suggest that reovirus-induced apoptosis and inhibition of cellular DNA synthesis are associated with viral serotype, a property determined by the S1 gene (60), and provide further support for a link between these reovirus-induced cellular responses.

DISCUSSION

In this report, we show that (i) the reovirus S1 and M2 genes determine differences in the capacities of both T1L × T3A and T1L × T3D reassortant viruses to inhibit cellular DNA synthesis and to induce apoptosis, (ii) inhibition of DNA synthesis and induction of apoptosis are serotype-specific properties of reovirus, and (iii) there is a significant correlation between the capacities of both reassortant viruses (T1L × T3A and T1L × T3D) and field isolate strains (T1 and T3) to inhibit cellular DNA synthesis and to induce apoptosis. These findings, taken in conjunction with earlier studies (see below), suggest that that inhibition of cellular DNA synthesis and induction of apoptosis are linked as part of a coordinated cellular response to reovirus infection. Since the S1 and M2 genes encode viral outer-capsid proteins that play important roles in viral entry into cells (21, 22, 35), these results also suggest that both DNA synthesis inhibition and apoptosis induction are mediated by early steps in the viral replication cycle.

Previous studies of reovirus-induced inhibition of cellular DNA synthesis indicated that the block to DNA synthesis occurs during initiation of DNA replication and that transcription is unaffected (7, 14, 20, 39). Reovirus-induced inhibition of host cell DNA synthesis is detectable as early as 8 h postadsorption and reaches 80% of maximal levels within 12 to 24 h postadsorption (7, 14, 17, 19, 39, 44) (Fig. 1). The morphological and biochemical changes characteristic of apoptosis are not detected in reovirus-infected cells until 24 to 48 h postadsorption (55). Thus, inhibition of cellular DNA synthesis occurs early in the course of reovirus infection and precedes detectable signs of apoptosis.

Apoptosis can be induced by dysregulated control of the cell cycle or by addition or removal of various growth-regulatory signals (5, 16, 28, 56). For many cell types, programmed cell death occurs after cells arrest in an early phase (e.g., G_0 or G_1) of the cell cycle (16, 30, 56). Similarly, reovirus infection in-

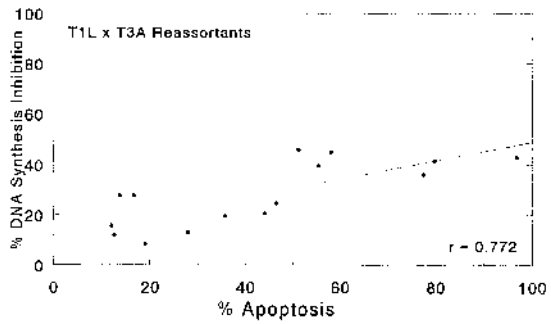

FIG. 3. Correlation between the capacities of T1L × T3A reassortant viruses to inhibit cellular DNA synthesis and to induce apoptosis. Each point represents a single reassortant virus. Data are derived from Table 1.

TABLE 3. Capacities of reovirus field isolate strains to inhibit cellular DNA synthesis and to induce apoptosis

Virus strain	% DNA synthesis inhibition[a]	% AO-stained cells[b]
T3A	100	99
T3C93	100	84
T3C43	100	43
T3C45	98	77
T3C18	90	83
T3C31	83	75
T3C44	70	54
T3C84	67	61
T3D	67	50
T1C11936	57	24.5
T3C9	47	67
T1C62	38	11.5
T1C12757	27	27
T1C49	24	27
T1C11	10	4.5
T1L	10	4.5

[a] L cells were infected with viral strains at an MOI of 10 PFU per cell and assayed for incorporation of [^{3}H]thymidine 48 h after adsorption. The percent DNA synthesis inhibition was calculated as [1 − (mean cpm in virus-infected cells/mean cpm in mock-infected cells)] × 100. The results represent the means for at least six wells for each viral strain.

[b] L cells were infected with viral strains at an MOI of 10 PFU per cell and stained with AO 48 h after adsorption. The percentage of apoptotic cells was determined by fluorescence microscopy. The results represent the means for at least two independent experiments for each viral strain.

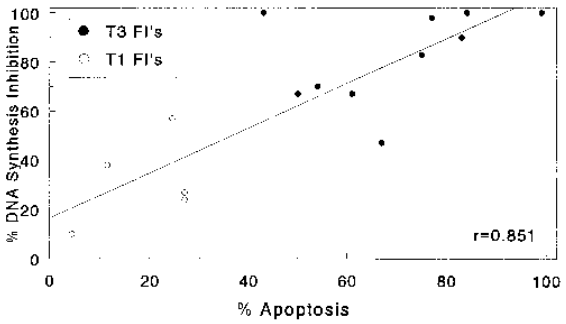

FIG. 5. Correlation between the capacities of T1 and T3 field isolate strains (FI's) to inhibit cellular DNA synthesis and to induce apoptosis. Data are derived from Table 3.

duces a time-dependent reversible arrest at the G_1-S phase of the cell cycle (44). It is possible that reovirus-mediated inhibition of host cell DNA synthesis disrupts the normal cell cycle and as a result induces apoptosis. Alternatively, DNA synthesis inhibition and apoptosis induction might be triggered by engagement of cellular receptors capable of signaling both events. In this latter scenario, the signaling pathway required for DNA synthesis inhibition mediates its effects with faster kinetics than the pathway required to induce apoptosis. Second messengers known to mediate growth suppression and induce apoptosis, which might participate in these pathways, include ceramide (36, 38) and members of the Jun kinase/stress-activated protein kinase family (25, 64).

It has been proposed that DNA synthesis inhibition in reovirus-infected cells requires cross-linking of cellular receptor molecules, which in turn activates as yet unidentified receptor-linked signaling pathways involving protein tyrosine kinases (17, 44). Reovirus-induced inhibition of cellular DNA synthesis and inhibition of cellular proliferation can be induced both by purified σ1 protein (44) and by a mouse monoclonal anti-reovirus T3 receptor antibody (17, 44). Viral replication is not required for either inhibition of cellular DNA synthesis (17, 47, 49) or induction of apoptosis (55), provided a sufficient quantity of replication-incompetent virus is adsorbed to cells. All reovirus strains that inhibit cellular DNA synthesis also induce apoptosis, and the extents to which both phenomena occur are determined primarily by the σ1 and μ1 proteins, which mediate binding and penetration of the virus, respectively. These findings suggest that reovirus inhibition of DNA synthesis and induction of apoptosis are linked by similar mechanisms, involving engagement of a cell surface receptor, which initiates both processes.

The potential role of tyrosine kinases in reovirus replication, and perhaps in apoptosis and inhibition of cellular DNA synthesis, is suggested by several recent studies. Strong et al. demonstrated that the efficiency of reovirus infection in NIH 3T3 cells is enhanced by the expression of functional epidermal growth factor receptors (52) or the v-erbB protein (51). This enhancement is also blocked by the tyrosine kinase inhibitor genistein (51). Saragovi et al. found that binding of reovirus to a receptor expressed on R1.1 thymocytes is associated with tyrosine kinase activity (44). These studies suggest that reovirus infection is associated with enhanced activity of tyrosine kinases. Since tyrosine kinases play a critical role in the regulation of cell growth and differentiation (57), perturbation by reovirus infection of a tyrosine kinase-dependent signaling pathway may result in impairment of cellular proliferation by inhibiting DNA synthesis. The resulting disruption in the cell growth cycle may serve to trigger apoptosis (5, 16, 28, 56). In addition, early events in reovirus infection that influence intracellular signaling pathways via alteration of protein tyrosine kinase activity might also serve to facilitate virus replication, either as a result of or despite inhibition of host cell DNA synthesis and induction of apoptosis.

Insight into the relationship between inhibition of DNA synthesis and induction of apoptosis during reovirus infection may also be provided by a nonviral model of apoptosis. Treatment of L cells with etoposide, a topoisomerase II inhibitor, results in rapid inhibition of DNA synthesis followed by apoptosis (31). The kinetics of these events are strikingly similar to those observed following reovirus infection. In both cases, DNA synthesis inhibition occurs within hours of initial exposure to ligand, while apoptosis is observed after several days. The data from both systems raise the possibility that apoptosis is induced in response to disruption of DNA synthesis. However, the phenotype of T3C43 would not be easily explicable by this model. TC43 produces nearly maximal DNA synthesis inhibition but only 43% apoptosis. This finding suggests that even maximal virus-induced inhibition of DNA synthesis may not be sufficient to induce maximal virus-induced apoptosis.

An alternative model, in which apoptosis and DNA synthesis inhibition are triggered by the same signal but are independent events, is provided by the recently described sphingomyelin cycle of signal transduction (36, 38, 45). In this system, binding of ligand to cell surface receptors results in activation of a sphingomyelinase which hydrolyzes membrane sphingomyelin and generates ceramide. It has been suggested that a pathway involving ceramide-activated protein phosphatase is central to ceramide's antiproliferative effects, whereas a distinct pathway involving ceramide-activated protein kinase is involved in apoptosis-induction (see references 36 and 38 for reviews). Such a model, in which the pathways involved in DNA synthesis inhibition and apoptosis induction are triggered by the same initial event but subsequently involve separate signaling pathways, might account for examples, such as that provided by T3C43, in which these two events are partially dissociated.

Our previous studies of reovirus-induced apoptosis of L cells indicate that both the viral S1 and M2 genes are determinants of strain-specific differences in apoptosis induction (55). In the current study, we found that these same viral genes determine strain-specific differences in reovirus-associated inhibition of cellular DNA synthesis. In assays using independent panels of reassortant viruses, the viral S1 gene was found to be the primary determinant of differences in inhibition of cellular DNA synthesis exhibited by different reovirus strains. On the basis of the R^2 values obtained by linear regression analysis, it can be estimated that the reovirus S1 and M2 genes together account for 79 to 89% of the genetically determined variance in reovirus-induced DNA synthesis inhibition and 76 to 91% of the genetically determined variance in apoptosis induction. Thus, although the S1 and M2 genes are the primary determinants, other reovirus gene segments play a contributory role in these events. In this regard, it should also be noted that the phenotypes of some reassortant viruses appeared to be exceptions to the general pattern. This type of phenotypic variation among reassortant viruses may reflect innate biologic and experimental variability, the presence of undetected mutations (50), or the effects of certain particular combinations of gene segments on phenotypic expression (reviewed in reference 59).

The S1 gene encodes the viral attachment protein, σ1, and a nonstructural protein, σ1s, in overlapping reading frames (35). The capacity of purified, UV-inactivated reovirus virions (which lack σ1s and the capacity to mediate its synthesis) to induce apoptosis (55) and to inhibit DNA synthesis (17, 47, 49)

indicates that σ1, not σ1s, is critical for these effects. Similarly, the capacities of purified σ1 and of anti-T3D receptor antibody to inhibit cellular DNA synthesis and cellular proliferation (17, 44) also indicates that σ1, not σ1s, is the key determinant of these processes. This contention is also supported by the link between viral serotype, which is determined by the σ1 product of the S1 gene (60), and the capacities of field isolate reovirus strains to inhibit cellular DNA synthesis and to induce apoptosis. These results suggest that receptor binding by σ1 triggers a signal that results in inhibition of cellular DNA synthesis and induction of apoptosis. Furthermore, the observation that strain-specific differences in DNA synthesis inhibition and apoptosis induction are strongly correlated suggests that the same cellular receptor is required for initiation of both processes.

Although the S1 gene is the primary determinant of the capacity of reoviruses to inhibit DNA synthesis and induce apoptosis, it is possible that this capacity is modulated by interactions, at either a structural or a biological level, between σ1 and the M2-encoded μl protein. The μ1 protein is a 76-kDa N-terminal myristoylated protein that is a major component of the virion outer capsid (35). The processed forms of this protein play important roles in reovirus penetration of cell membranes (21, 22, 29, 34, 53). The μ1 protein is also an important determinant of the stability of the reovirus outer capsid (10, 11, 62). The same two proteins are also responsible for determining differences in the neurovirulence of T3 reoviruses (23). These findings suggest that differences in neurovirulence exhibited by reovirus strains in vivo are related to differences in their capacities to inhibit cellular DNA synthesis and to induce apoptosis. Our preliminary studies (37) show that reovirus strain T3D induces apoptosis in the central nervous system of infected mice, which suggests that apoptosis is an important mechanism of reovirus-induced disease. Experiments are in progress to determine whether reovirus strains that vary in the capacity to inhibit cellular DNA synthesis and to induce apoptosis also vary in virulence. Improved understanding of the mechanisms used by these viruses to injure cells in vivo will contribute important information about how viruses produce neurologic disease.

ACKNOWLEDGMENTS

This work was supported by a Merit Review Award from the Department of Veterans Affairs (K.L.T.), the core tissue culture and media facilities of the University of Colorado Cancer Center (K.L.T.), a virology-molecular biology training grant (NIH T32 NS0731) from the National Institutes of Health (K.L.T. and S.M.O.), a fellowship grant from the American Cancer Society and University of Colorado Cancer Center (S.M.O.), Public Health Service award AI32539 from the National Institutes of Allergy and Infectious Diseases (T.S.D.), and the Elizabeth B. Lamb Center for Pediatric Research (T.S.D.).

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