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Altered Susceptibility to Tumor Necrosis Factor Alpha-Induced Apoptosis of Mouse Cells Expressing Polyomavirus Middle and Small T Antigens

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Infection with some virus types induces susceptibility to the cytotoxic effect of tumor necrosis factor alpha (TNF-α). To investigate whether expression of polyomavirus proteins has this effect on cells, the TNF-α sensitivity of C127 and L929 mouse cells transfected with viral DNA was analyzed. Expression of all three polyomavirus early proteins, the tumor (T) antigens, had no apparent effect. In contrast, middle T antigen by itself induced hypersensitivity to TNF-α. This effect was reversed by retransfection of the cells with DNA encoding small T antigen. Expression of this polypeptide also decreased the sensitivity of bovine papillomavirus type 1-transformed cells to TNF-α, showing that the protective function of the polyomavirus small T antigen was not strictly linked to a middle-T-antigen-induced event. Mouse and human TNF-α had the same effect on normal and transformed mouse cells, suggesting that this effect was mediated by TNF receptor 1. Consistent with this conclusion, all cell clones used in the experiments expressed TNF receptor 1 at similar levels, while we failed to detect TNF receptor 2. The amount of receptor on the cells was not influenced by binding of the ligand. Addition of TNF-α at cytotoxic concentrations to cells expressing middle T antigen by itself resulted in significant fragmentation of chromosomal DNA after only a few hours, indicating induction of apoptosis. Addition of the cytokine to these cells also leads to release of arachidonic acid, showing that phospholipase A_2 was activated. However, production of arachidonic acid did not appear to significantly precede loss of cell viability.

Polyomavirus is a small DNA virus that is ubiquitous in mammals. Polyomaviruses isolated from different animal species are host specific but have similar properties. Infection normally occurs early in life. After an acute phase, the infection becomes persistent. During the latter period, virus replication takes place at some preferred sites, especially in the kidney. Impairment of the immune system leads to increased virus growth, in some cases resulting in clinical symptoms of the infection (61). In experimental infections, polyomavirus induces tumors in newborn rodents at a relatively high frequency (63). However, malignancies caused by polyomavirus are rare under natural conditions (61). Most studies of polyomavirus have been carried out with cells in culture. Under these conditions, expression of the early proteins, the tumor (T) antigens, results in virus production and cytopathic effect within 48 h. When viral DNA replication is restricted, permanent expression of the T antigens results in malignant transformation of cells. Different polyomaviruses express two or three types of T antigen. Since viral DNA synthesis takes place only in cells during S phase, cellular DNA replication must be initiated after infection. All of the T antigens participate in the activation of quiescent cells. A second, essential function in productive infection is the initiation of viral DNA synthesis. Only large T antigen is directly involved in this process. It is likely that the T antigens have additional, as yet unknown functions, particularly activities that are redundant in vitro.

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Viruses that establish persistent infections must have ways to evade the host defense. Polyomavirus-infected cells that express viral proteins are susceptible to cytotoxic T lymphocytes (1, 45). Viral gene expression might make the cells sensitive to other effectors of the immune system, such as interferons and tumor necrosis factor (TNF). DNA viruses of higher genetic complexity than polyomavirus, such as members of the adenovirus, herpesvirus, and poxvirus families, have been shown to encode products that counteract the effects of the host defense (26). Adenoviruses have several functions of this type: the viral protein gp19, encoded by the E3 region, prevents major histocompatibility complex-dependent presentation of antigens on the cell surface (54, 70); the virus-associated RNAs abrogate the effect on protein synthesis by interferon (49); the E1B 19,000-molecular-weight protein (27, 78) and three proteins encoded by the E3 region (28, 29) are involved in protection of infected cells from TNF-α.

TNF-α is a multifunctional cytokine secreted primarily by mitogen-activated macrophages (71). Besides an immunoregulatory effect, the compound influences the function of many types of cells. Some of these effects are induction of an inflammatory response, necrosis of solid tumors in vivo (11, 19), and growth inhibition of normal and tumor cells in vitro (71, 73). TNF-α inhibits the multiplication of several DNA and RNA viruses in cultured cells, and it even lyses cells infected by some viruses (34, 50, 82). TNF-α exerts its effects by binding to specific cell surface receptors. Two receptors of 55 kDa (TNFR1) and 70 to 80 kDa (TNFR2) have been identified (46, 60, 64). Binding of TNF-α to TNFR1 causes most of the known biological effects (42, 80). The intracellular signals that result from binding of TNF-α to one or both of its receptors are just being unravelled. Binding of TNF-α to the transmembrane TNFR1 leads to oligomerization and association to the intra-

cellular effector protein TRADD. This event triggers secondary interactions with the TRAF2 and FADD proteins (12, 33). Binding to TRAF2 initiates a signal cascade that results in activation of the transcription factor NF-κB, while interaction of FADD mediates binding of a cysteine protease, FLICE/MACH, thus initiating a signal that results in apoptosis (6, 51). The cytotoxic response is linked to activation of proteases (reviewed in reference 23). The activity of cytosolic phospholipase A_2 is also correlated to TNF-α-induced cell lysis (75).

Information about the two signalling cascades leading to growth stimulation and apoptosis, respectively, and their possible interactions is still sketchy. Ceramide formed from membrane-bound sphingomyelin by TNF-activated sphingomyelinases (31, 36, 53) appears to be a necessary cofactor for apoptosis. In an apparently cell-type-specific manner, ceramide activates protein phosphorylation cascades that regulate transcription factor activity (74, 77, 84).

Treatment of cells in culture with TNF-α rarely leads to death (11, 69). To achieve cell killing, metabolic inhibitors such as actinomycin D or cycloheximide have to be added to the cultures (43). Mouse C127 cells originate from a mammary tumor, and L929 cells are derived from normal subcutaneous tissue. Both cells are sensitive to the cytotoxic effect of TNF-α in the presence of actinomycin D (11, 29). Moreover, C127 cells are protected from TNF-α cytotoxicity by adenovirus E3 proteins (29). In this report, we show that polyomavirus middle T antigen induces sensitivity to TNF-α-induced apoptosis and that small T antigen abrogates this effect.

MATERIALS AND METHODS

Chemical and immunological reagents. All chemicals used were of analytical grade. Restriction endonucleases, T4 DNA polymerase, T4 DNA ligase, and dimethylthiazol-diphenyltetrazolium bromide (MTT) were obtained from Promega. G418, okadaic acid, and recombinant human TNF-α were purchased from Gibco-BRL. Actinomycin D, cycloheximide, puromycin, and recombinant mouse TNF-α were from Sigma. Monoclonal antibodies specific for polyomavirus T antigens were prepared from the F4 hybridoma (55). Polyclonal TNFR1 and TNFR2 antibodies were from Santa Cruz Biotechnology. Horseradish peroxidase-linked anti-immunoglobulins were from DAKO.

Cells, cell culture, and viral genomes. Mouse C127 and L929 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, England). The bovine papillomavirus type 1 (BPV-1)-transformed C127 cell clone wh.2 (35) was obtained from Stanley Burnett. Animal cells were cultured in Dulbecco modified minimal essential medium (HyClone) containing 10% fetal bovine serum (HyClone).

Polyomavirus genomes were propagated in bacteria as recombinant plasmids. Polyomavirus DNA was inserted in plasmid pBR322, or one of its derivatives, via the single *Bam*HI cleavage sites. Polyomavirus mutant *dl*1023 has a deletion (nucleotides 5283 to 5291) that inactivates the origin of replication (47). It expresses all the three T antigens. Mutant *bc*1051 has a base change at a splice site. The mutant expresses middle and small T antigens with the conservative mutation V80I (52). Mutants MT-1 and ST-1 have complete intron deletions that limit early gene expression to middle and small T antigens, respectively (85). For selection of transfected clones, pSV2neo (66) and pSV2pac (72), conferring resistance to neomycin and puromycin, respectively, were used.

Gene transfer. C127 and L929 cells were transfected by the calcium phosphate coprecipitation procedure (81). Cell cultures in 5-cm-diameter petri dishes were transfected with 4 μg of DNA containing the polyomavirus genome and 1 μg of DNA with an antibiotic resistance gene. Selection of resistant cells was done in medium containing 1 mg of G418 or 2 μg of puromycin per ml. After 9 to 12 days, individual clones were isolated by trypsinization in glass cylinders and then expanded. Screening for expression of polyomavirus T antigens was done by immunoblot analysis, using the monoclonal antibody F4.

Immunoblot analysis. Cells from 5-cm-diameter petri dishes were washed and suspended in 100 μl of lysis buffer (0.5% Nonidet P-40, 100 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1.0% aprotinin, 1.0 mM dithiothreitol). The cell suspensions were vortexed, incubated for 30 min on ice, and centrifuged for 15 min. Supernatants were collected, and protein concentrations were determined (8). Samples with equal amounts of protein were loaded on a sodium dodecyl sulfate (SDS)–8% polyacrylamide gel. After polyacrylamide gel electrophoresis (PAGE), protein was transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in phosphate-buffered saline (PBS) and washed with 0.5% Tween 20 in PBS (TPBS).

Immunoglobulin at a final concentration of 1 mg/ml or monoclonal antibody was then added. After incubation for 1 h at 20°C, the filter was washed with TPBS and incubated with horseradish peroxidase-linked immunoglobulin directed against the primary antibody. After additional washes, bound secondary antibody was detected by using an enhanced chemiluminescence kit (Amersham).

Cytotoxicity assay. Approximately 2×10^4 cells were seeded in 96-well culture plates. The following day, dilutions of recombinant human or mouse TNF-α were added to the culture medium together with 2 μg of actinomycin D per ml. After 20 h, cells were examined by phase-contrast microscopy and the viability of cells, as reflected by mitochondrial function, was determined by assay of MTT conversion to the formazon product (2). The metabolite concentration was analyzed by spectrophotometry at 540 nm.

Flow cytometric analysis of DNA fragmentation. Strand breaks in DNA were traced by incorporation of fluorescein-dUTP in a reaction catalyzed by terminal deoxynucleotidyltransferase. C127 cells were treated with recombinant mouse TNF-α. The cells were trypsinized, washed twice with PBS containing 1.0% bovine serum albumin, and diluted to 2×10^7 cells/ml. Each cell sample was fixed in 4% paraformaldehyde in PBS (pH 7.4), washed once with PBS, and resuspended in 100 μl of permeabilization solution (0.1% [vol/vol] Triton X-100 in 0.1% [wt/vol] sodium citrate). After 2 min on ice, the cells were washed twice with PBS, resuspended in 50 μl of reaction mixture of a Boehringer Mannheim in situ cell death detection kit, and incubated for 60 min at 37°C in a humidified atmosphere in the dark. Finally, the samples were washed twice with PBS and transferred to a tube in a final volume of 500 μl of PBS. Cell size and green fluorescence, from incorporated fluorescein-dUTP, were analyzed in a FACScan (Becton Dickinson). Each diagram represents the result obtained with 2×10^4 cells.

Release of arachidonic acid and its metabolites. Cells were seeded in 12-well plates at a density of 1.5×10^5 cells per well. After 8 h of incubation, the growth medium was replaced with 0.45 ml of medium containing 0.15 μCi of [^{3}H]arachidonic acid (200 Ci/mmol; New England Nuclear), and incubation was continued for 12 h. The cells were then washed twice with Tris-buffered saline and incubated for 3 to 18 h in growth medium with human TNF-α and 10 μg of cycloheximide per ml. To determine the amount of released arachidonic acid, the medium was collected and radioactivity was analyzed in a liquid scintillation spectrometer.

RESULTS

Effect of polyomavirus T-antigen expression on TNF-α sensitivity. To investigate whether expression of polyomavirus T antigens affects the sensitivity of cells to TNF-α, cell lines were established by transfection of mouse C127 and L929 cells with polyomavirus DNA. Both of the original cell lines are susceptible to the effect of human TNF-α in vitro, and at least C127 cells are permissive to polyomavirus infection. Cells expressing only one T antigen, or two in combination, were made by transfection with plasmids carrying various mutant polyomavirus genomes. To prevent virus replication, the genomes expressing T antigens also carried the *dl*1023 deletion that inactivates the viral DNA replication origin. The cellular origin and T-antigen expression of all cell lines used in the experiments are summarized in Table 1. Before use in the experiments described below, the predicted pattern and level of T-antigen expression was confirmed by immunoblot analysis.

Clones of C127 cells were treated with mouse TNF-α and actinomycin D for 20 h, and cell viability was then determined (Fig. 1). A striking observation was that CNm41 cells, expressing polyomavirus middle T antigen, were susceptible to very low concentrations of TNF-α. Another cell clone, CNm82, had an identical phenotype (see Fig. 3). Figure 1 also shows that expression of middle and small T antigens from one viral genome (CNms4) was the same as for the parental cells. Expression of all three T antigens (CNp4 and CNp7) had no additional effect (data not shown).

Corresponding L929-derived cell lines were also tested (Fig. 2). Again, expression of middle T antigen made the cells (Lm8) susceptible to TNF-α, although the effect was less pronounced than with C127 cells. The protective effect of small T-antigen expression (Lms3) was also equivalent to the earlier observations. With L929 cells, we also investigated the effect of large T antigen by itself. Two cell clones (L15 and L16) were tested. Both had the same TNF-α susceptibility as the parental cells (Fig. 3).

TABLE 1. Cell lines and T-antigen expression

Cell line	Parental cell line	Expressed viral protein(s)[a]
C127		None
CNm41	C127	MT
CNm82	C127	MT
CNp4	C127	LT, MT, ST
CNp7	C127	LT, MT, ST
CNms4	C127	MT (V80→I), ST (V80→I)
CNms6	C127	MT (V80→I), ST (V80→I)
CNmPs2	CNm41	MT, ST
CNmPs5	CNm41	MT, ST
wh.2	C127	BPV1 (wt)
wh.2Ns1	C127	BPV1 (wt), ST
wh.2Ns3	C127	BPV1 (wt), ST
L929		None
Lm7	L929	MT
Lm8	L929	MT
Lms3	L929	MT (V80→I), ST (V80→I)
Lms6	L929	MT (V80→I), ST (V80→I)
L15	L929	LT
L16	L929	LT

[a] Predicted amino acid residues are indicated by one-letter amino acid code. LT, MT, and ST designate polyomavirus large, middle, and small T antigens, respectively.

The data suggested that expression of small T antigen could rescue middle-T-antigen-expressing cells from TNF-α susceptibility. To clarify this point, CNm41 cells were transformed with the polyomavirus mutant genome ST-1, encoding small T antigen. Puromycin resistance, conferred by plasmid pSV2pac, was used as a selectable marker. Clones of puromycin resistant cells were analyzed for small-T-antigen expression. Immunoblot analysis of the selected clones (CNmPs2 and CNmPs5) demonstrated that they expressed middle and small T antigens (data not shown). Expression of small T antigen, in addition to middle T antigen, by CNmPs5 increased the tolerance to TNF-α (Fig. 1), albeit not to the level achieved by coordinate expression of the viral proteins from one viral genome (CNms4).

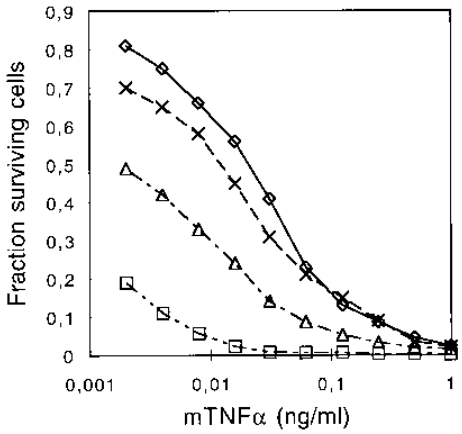

FIG. 1. Effects of T antigens on TNF-α sensitivity of C127 cells. Cell lines expressing polyomavirus T antigens in various combinations were established by transfection of C127 or CNm41 cells with viral DNA. Cells were treated with mouse TNF-α at a range of concentrations, and cell viability was determined 20 h later by analysis of MTT conversion. All values represent the means of triplicate determinations. The variance was less than 2%. The curves show results with parental C127 cells (no T antigen; ◇), CNm41 cells (middle T antigen; □), CNms4 cells (middle and small T antigens; ×), and CNmPs5 cells (CNm41 cells retransfected to express small T antigen in addition to middle T antigen; △).

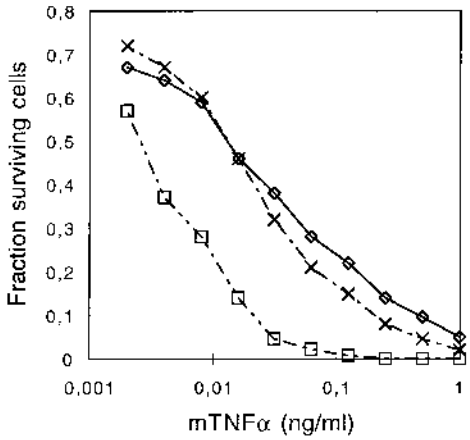

FIG. 2. Effects of T antigens on TNF-α sensitivity of mouse L929 cells. The experiment was performed as described in the legend to Fig. 1. The curves show results with parental L929 cells (no T antigen; ◇), Lm8 cells (middle T antigen; □), and Lms3 cells (middle and small T antigens; ×). The variance of triplicate values was less than 2%.

Small and middle T antigens associate with protein phosphatase 2A (PP2A) and modify its activity. However, it is possible that the two proteins have different effects on PP2A. Okadaic acid at low concentrations is a relatively specific inhibitor of PP2A. To test the hypothesis that the individual effects of middle and small T antigens on TNF-α susceptibility are mediated by PP2A, CNm41 cells were analyzed in the presence of 20 nM okadaic acid. The TNF-α dose response curves in the absence and presence of okadaic acid were superimposable (experiment not shown), suggesting that PP2A activity was not directly involved in transduction of the cytotoxic TNF-α signals.

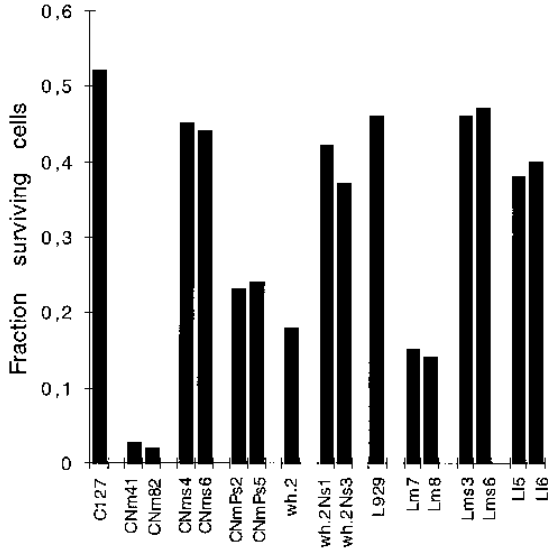

FIG. 3. Summary of TNF-α sensitivity data. A compilation of data from mouse TNF-α cytotoxicity tests displayed in Fig. 1 and 2 and analyses performed in parallel are shown. Due to the extreme sensitivity of cells expressing only middle T antigen, data on cell survival at 0.016 ng of TNF-α per ml are used instead of the toxic dose at which 50% of the cells survived. Each bar represents the average of three determinations. The variance of triplicate values was less than 2%. The origin and T-antigen expression of the cells are shown in Table 1.

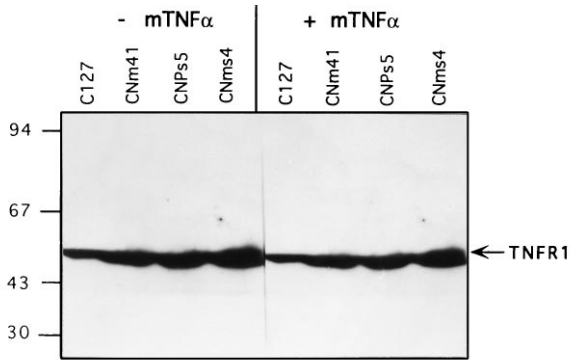

FIG. 4. Effects of T antigens and TNF-α on TNFR1 expression. Parental C127 cells and clones expressing middle T antigen by itself (CNm41) or middle and small T antigens together (CNmPs5 and CNms4) were analyzed for expression of TNFR1 before and 3 h after addition of 1 ng of mouse TNF-α per ml. Extracted protein was resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with anti-TNFR1 immunoglobulin. Bound antibodies were detected by chemiluminescence. Sources of protein extracts and TNF-α treatment are indicated at the top. Positions of protein size markers are indicated in kilodaltons at the left, and the expected position of TNFR1 (55 kDa) is indicated at the right.

BPV-1-transformed C127 cells have increased TNF-α sensitivity. For comparison to the effect of T antigens on TNF-α sensitivity, we used BPV-1-transformed C127 cells. Transformation with the complete BPV-1 genome (wh.2) induced increased sensitivity to TNF-α (Fig. 3). However, these cells were not as sensitive as those expressing only polyomavirus middle T antigen. To investigate the effect of polyomavirus small T antigen on the BPV-1-induced TNF-α susceptibility, wh.2 cells were transfected with pST-1 DNA. The resulting cell lines (wh.2Ns1 and wh.2Ns3) were tested and found to have the same TNF-α tolerance as the parental C127 cells (Fig. 3).

TNF-α receptors on T-antigen expressing cells. A possible mechanism for control of TNF-α sensitivity is by receptor expression. TNFR1 is the predominant receptor on cells not belonging to the immune system. We investigated whether polyomavirus T antigens influenced TNFR1 expression in C127 cells, either constitutively or after receptor occupation. Extracts were prepared from parental cells and from C127 clones expressing middle T antigen (CNm41), middle and small T antigens from separate genomes (CNmPs5), and middle and small T antigens from the same genome (CNms4). Proteins were resolved by SDS-PAGE, and TNFR1 was analyzed by immunoblotting. The results (Fig. 4) show that all cells expressed similar levels of TNFR1 and that treatment with 1 ng of TNF-α per ml for 3 h before harvest did not perturb the expression. Analysis of TNFR2 in the same protein extracts did not yield detectable signals (data not shown).

Induction of apoptosis by TNF-α treatment. Binding of TNF-α to TNFR1 activates intracellular signals that are transduced to the nucleus and induce apoptosis. In apoptosis, fragmentation of chromosomal DNA can be observed before cell death. The increased TNF-α susceptibility induced by middle T antigen suggests that the intracellular apoptotic signals are reinforced by the activity of the viral protein. To confirm that middle-T-antigen-expressing C127 cells indeed undergo apoptosis after TNF-α treatment, fragmentation of chromosomal DNA was tested. Cultures of CNm41 cells, expressing middle T antigen, and of CNms4 cells, expressing middle and small T antigens, were treated with 10 ng of mouse TNF-α per ml. At this dose, the cytokine is completely cytotoxic to both types of cells when incubation is extended to 20 h. However, in this experiment, the cells were collected and fixed after 3 h. Free 3′-OH ends in cellular DNA were extended with fluorescein-conjugated dUTP, using terminal nucleotidyltransferase. Cells were then analyzed by flow cytometry. Figure 5A shows the results with TNF-α treated CNm41 cells. Approximately 25% of the cells showed increased DNA fragmentation in comparison to the untreated cells (Fig. 5B). Expression of small T antigen in CNms4 cells appeared to delay DNA fragmentation. TNF-α treatment of these cells for 3 h (Fig. 5C) did not lead to a detectable increase in strand breaks compared to the control cells (Fig. 5D).

The flow cytometric analysis also showed the relative size distribution of the cells. TNF-α treatment did not affect the average cell size of either clone. However, CNm41 and CNms4 cells had different size profiles. In the latter population, there was a distinct class of cells with almost twice the size of the majority. We have not analyzed whether the large cells are in the G_2 phase of the cell cycle or represent a separate population.

Release of arachidonic acid in TNF-α induced apoptosis of C127 cells. Release of arachidonic acid is an important factor in the cellular inflammatory response. Experiments with TNF-α-treated HL-60 cells show that arachidonic acid activates sphingomyelinase, leading to accumulation of ceramide (37). This result suggests that release of arachidonic acid is a primary effector in TNF-α toxicity. The TNF-α-protecting early gene products from the adenovirus E3 region have been shown to inhibit the release of arachidonic acid that precedes cell lysis (41, 86). To investigate whether the polyomavirus middle T antigen stimulates release of arachidonic acid and whether small T antigen counteracts this effect, we used the clones derived from C127 cells.

In this experiment, we used control C127 cells and cells expressing middle T antigen (CNm41) and both middle and small

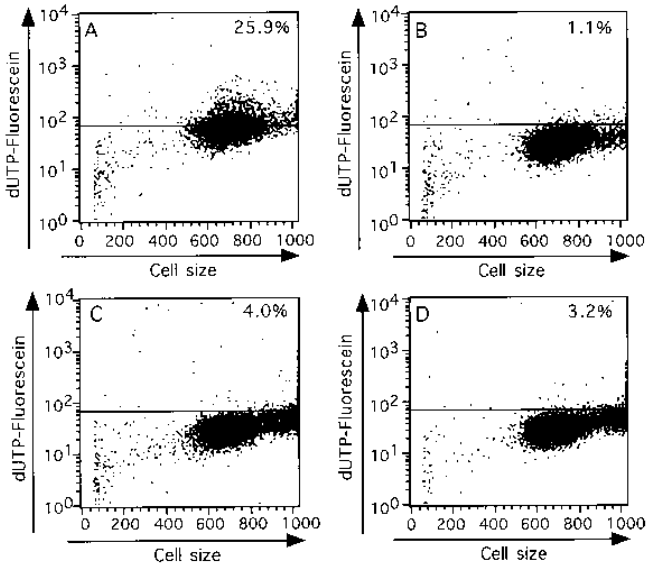

FIG. 5. Analysis of TNF-α-induced DNA fragmentation by flow cytometry. CNm41 cells (A and B) and CNms4 cells (C and D) were analyzed for breaks in chromosomal DNA before (B and D) and after (A and C) 3-h exposure to 10 ng of mouse TNF-α per ml. Actinomycin D at 1 μg/ml was added to all cultures. Cells were detached by trypsin treatment, fixed, permeabilized, and incubated with fluorescein-dUTP and terminal deoxynucleotidyltransferase. Cells were analyzed in a flow cytometer for light scatter and intensity of green fluorescence. The percentage of cells with green fluorescence intensity above the value indicated by the horizontal line is shown in each panel.

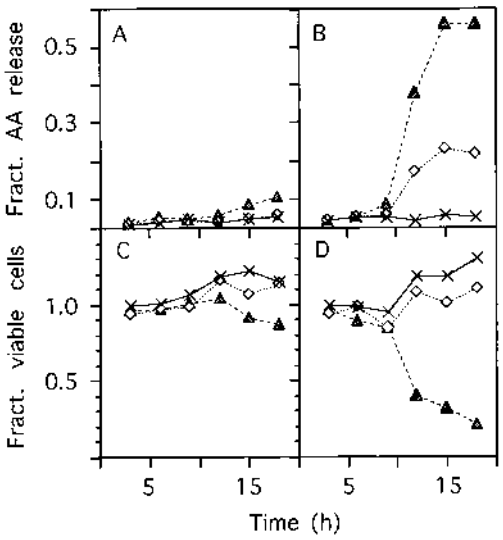

FIG. 6. Effects of T antigens on TNF-α induced release of arachidonic acid. C127 cells (A and C) and CNm41 cells (B and D), expressing middle T antigen, were labeled with [^{3}H]arachidonic acid and treated with human TNF-α at a concentration of 0.3 (◇) or 3 (▲) ng/ml. Control cells were not subjected to TNF-α (×). All cultures were also treated with 10 μg of cycloheximide per ml. Released arachidonic acid (AA) was analyzed at different time points after addition of TNF-α (A and B). Simultaneously, cell viability was determined by analyzing conversion of MTT to its formazon derivative (C and D). All values represent the mean of determinations in duplicate or triplicate. Variation of duplicate values is shown by error bars.

T-antigens (CNms4). The cells were labeled with [^{3}H]arachidonic acid and then treated with TNF-α at two concentrations. Release of arachidonic acid into the culture medium and cell viability were then analyzed. The results obtained with C127 and CNm41 cells are presented in Fig. 6. The CNms4 cell data are not shown, since these cells responded like the control C127 cells. At 9 h posttreatment, the cell line CNm41 started to release arachidonic acid, and at 12 h the effect became obvious (Fig. 6B). This release appeared to be parallel to loss of cell viability (Fig. 6D). Release of arachidonic acid from control cells and from CNms4 cells was much lower (Fig. 6A). Most of these cells also remained viable during the course of the experiment.

DISCUSSION

Polyomavirus establishes persistent infection and probably has mechanisms for evading the host defense against infection. Such functions are needed in latently infected cells and are probably expressed by viral early proteins. In polyomavirus, the three early proteins, the T antigens, are essential for efficient productive infection of most mouse cell types. For this reason, we did not use infection with mutant virus in these experiments but instead established cell lines that expressed T antigens. To investigate the effect of the inflammatory cytokine TNF-α on cells that express polyomavirus proteins, the two mouse cell lines C127 and L929 were used, since the cytokine is toxic to these cells in vitro.

Transfection of C127 or L929 cells with wild-type polyomavirus DNA resulted in loss of contact inhibition as a sign of malignant transformation. However, the susceptibility to TNF-α was not altered. Expression of middle T antigen alone from a mutant viral genome induced similar transformed cell growth properties. However, with these cells a strong cytotoxic effect of TNF-α was apparent at 10- to 100-fold-lower concentrations than with the progenitors (Fig. 1 and 2). Expression of large T antigen alone did not induce neoplastic growth properties or TNF-α susceptibility of the cells (Fig. 3). Expression of small T antigen alone was not tested due to its cytotoxic effect (48, 58). However, together with middle T antigen, small T antigen is tolerated, and in the experiment, it abrogated the hypersensitivity to TNF-α (Fig. 1). The relative synthesis of middle and small T antigens appeared to be important. Cell clones expressing the proteins from separate genomes (CNmPs2 and CNmPs5) were more susceptible to TNF-α than those transfected with viral DNA that directed the synthesis of middle and small T antigens coordinately (CNms4 and CNms6). Thus, the work reported here provides an additional example of cooperation between the T antigens.

The C127 and L929 cells responded to TNF-α by activation of TNFR1. All C127 cell lines were demonstrated to express this receptor (Fig. 4), while TNFR2 was undetectable. Moreover, human and mouse TNF-α had the same activity, and the human cytokine binds only to TNFR1 on murine cells (44). One effect of TNFR1 occupation is activation of phospholipase A_2, leading to release of arachidonic acid. This compound is a mediator of the cellular inflammatory response, and the phospholipase A_2 activation is correlated with TNF-α cytotoxicity (75). In our experiments, TNF-α treatment of C127 cells expressing middle T-antigen induced apoptosis (Fig. 5). After addition of the cytokine, the cells started to release arachidonic acid within 10 h. Coexpression of small T antigen counteracted this effect (Fig. 6). In the susceptible cells, release of arachidonic acid and death appeared to be roughly parallel events. In contrast to our observations, TNF-α treatment of HL-60 cells resulted in an immediate but transient release of arachidonic acid (37), consistent with a primary signalling function of the fatty acid. Although unlikely, our experiment does not rule out the possibility that arachidonic acid was transiently released and later taken up by the cells. A more probable interpretation of the result is that immediate phospholipase A_2 activation either was below the detection level or was not an initial event in TNF-α toxicity to C127 cells expressing middle T antigen. The E3 gene products of human adenovirus also protect cells against TNF-α and inhibit arachidonic acid release (41, 86). Also in these studies, the release of arachidonic acid started a few hours, rather than a few minutes, after addition of TNF-α to the cells. However, our data provide no information as to whether adenovirus E3 proteins and polyomavirus small T antigen protect the cells by the same mechanism.

In the initial study of TNF-α, the connection between its cytotoxic effect and the growth properties of cells was observed (11). Later, Shisler et al. (62) proposed that mechanisms which drive quiescent cells into S phase are the same as or similar to those which induce susceptibility to TNF cytolysis. These investigators showed that the adenovirus E1A protein induces TNF-α sensitivity and that the ability of the protein to form a complex with the cellular p300 or $p105^{Rb}$ is necessary for both the susceptibility to the cytokine and the induction of host cell DNA synthesis. The effect of E1A was the same whether cells were virus infected or cell lines that stably expressed the protein were used. The latter condition is comparable to our experiments.

Polyomavirus cannot replicate unless quiescent cells are induced to enter S phase. All three polyomavirus T antigens have various mitogenic activities. Large T antigen, like the adenovirus E1A protein, binds the cellular protein $p105^{Rb}$ or p300. Loss of binding activity results in partially defective function (21, 57, 65). However, expression of large T antigen did not alter the TNF-α susceptibility of mouse cells (Fig. 3), suggest-

ing that the effect of the cytokine on polyomavirus-transformed cells was not primarily dependent on $p105^{Rb}$ and p300 activity.

Middle T antigen, although devoid of known catalytic activities, has several mitogenic effects on cells. Association with the C-terminal part of $pp60^{c\text{-}src}$ (7, 15, 17, 18) and other members of the Src family (40) leads to increased protein tyrosine kinase activity and to phosphorylation of several tyrosine residues on middle T antigen itself. Cellular proteins with SH2 domains bind to these sites and become activated. Binding of the SHC polypeptide to middle T antigen results in Ras activation (9, 20). Binding of the phosphatidylinositol 3-kinase regulatory p85 subunit and of phospholipase C-γ1 leads to activation of these enzymes (16, 38, 68, 79). All three interactions are involved in the transforming activity of middle T antigen. In addition, middle and small T antigens bind to PP2A (56, 76) by displacing a regulatory subunit (59). This interaction leads to inhibition or altered substrate specificity of PP2A (83).

Middle T antigen might amplify TNF-α-induced signals or increase the sensitivity of the cells to such signals. In our experiments, the cytotoxic effect of TNF-α was tested in the presence of actinomycin D or cycloheximide. Therefore, the middle-T-antigen-induced property of the hypersensitive cells was not dependent on gene expression after addition of the cytokine. There are several possible connections between the intracellular effects of middle T antigen and TNF-α. Activation of the c-Src protein tyrosine kinase leads to an increased expression of the c-*myc* gene (3). Deregulation of c-*myc* expression is associated with an increased tendency of the cells to undergo apoptosis (22) and an increased sensitivity to TNF-α (39). On the other hand, expression of the constitutively active v-Src protein has been demonstrated to protect cells against TNF-α (10, 25). Thus, whether activation of c-Src, or related proteins, by middle T antigen increases the sensitivity to TNF-α-induced signals cannot be determined.

The SHC-mediated activation of the c-Ras protein by middle T antigen might lead to increased TNF-α susceptibility of the cells by intracellular signal amplification. A downstream target of c-Ras is the transcription factor c-Jun. In cells expressing middle T antigen, it is activated by phosphorylation of serine residues in the N-terminal part and dephosphorylation of serine and threonine residues in the DNA binding domain (67). The c-Jun protein is similarly affected by cellular stress or by TNF-α. In these situations, the protein kinase JNK/stress-activated protein kinase is activated and the N-terminal part of the c-Jun polypeptide becomes phosphorylated (74). Thus, middle T antigen and TNF-α might act in synergy to activate c-Jun. In cells expressing middle T antigen (Fig. 1 to 3), the substrate of JNK/stress-activated protein kinase might be activated before addition of TNF-α.

Polyomavirus small T antigen abolished the TNF-α susceptibility induced by both middle T antigen and the early proteins of BPV-1 (Fig. 3). Although the alterations in gene expression that lead to cellular transformation by polyomavirus and BPV-1 may show similarities, the viral transforming proteins act by different mechanisms (14, 32). Hence, small T antigen was not solely an antagonist of middle T antigen, something that might be expected from the colinear structures of the two proteins. Expression of small T antigen did not increase the TNF-α tolerance beyond the level of parental C127 or L929 cells. Therefore, it is unlikely that the protein interfered with the relay of signals from the TNF receptor.

We have not identified the activity of small T antigen that protects polyomavirus transformed cells against TNF-α. The polypeptide has at least two activities (48). One is binding to and inhibition of PP2A (56, 83). The biochemical mechanism of other small-T-antigen activities is still unknown. Unfortunately, no mutant forms of small T antigen that specifically fail to modify PP2A activity have been described. Instead, we tested okadaic acid at a concentration that selectively inhibits PP2A (5, 30). This compound by itself can induce apoptosis in some cell types (4). However, it had no effect on the TNF-α-induced apoptosis of CNm41 cells expressing middle T antigen. In this experiment, okadaic acid and TNF-α were added to the cells at the same time. Hence, PP2A was not involved in the TNF-α-induced cytotoxic signal. However, it is still possible that an altered activity or specificity of PP2A induces a state of decreased TNF-α susceptibility that would have been apparent only after pretreatment of the cells with okadaic acid.

Mutants of polyomavirus that do not express small T antigen form infectious virions in only a limited number of cell types (24, 48). This defect precludes a straightforward analysis of whether small T antigen affects the in vivo sensitivity of polyomavirus-infected cells. Analysis of polyomavirus-transformed cell lines can be extended to animal studies. An investigation of mesothelioma induction in hamsters by simian virus 40 showed that small-T-antigen expression was required (13). It is possible that small T antigen increased cell growth by making the cells more tolerant to effectors of the immune system.

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