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TNF-Related Apoptosis-Inducing Ligand (TRAIL) Induces Apoptosis in Fas Ligand-Resistant Melanoma Cells and Mediates CD4 T Cell Killing of Target Cells[1]

Wayne D. Thomas and Peter Hersey[2]

We have previously shown that melanoma cells were resistant to apoptosis induced by TNF family members Fas ligand (FasL), TNF-α, and CD40L. FasL also was not involved in CD4 T cell-mediated killing of melanoma cells. In the present study, we have tested melanoma cells for their susceptibility to apoptosis induced by human TNF-related apoptosis-inducing ligand (TRAIL) and the ability of a mAb against TRAIL to inhibit apoptosis and CD4 CTL-mediated killing of melanoma and Jurkat target cells. The results show that TRAIL-induced apoptosis in cells from 7 of 10 melanoma cell lines tested as well as in Jurkat T cells. Susceptibility to apoptosis was increased in some of the cell lines by treatment with cyclohexamide or actinomycin D. The melanoma cells were resistant to apoptosis induced by FasL, TNF-α, and CD40L. mAb M180 against TRAIL inhibited apoptosis induced by TRAIL. It was also found to inhibit CD4 CTL-mediated killing of Jurkat T cells as well as autologous and allogeneic melanoma cells. The degree of inhibition produced by the mAb varied between different clones of CTL and according to the susceptibility of the target cells to TRAIL-induced apoptosis. These results suggest that TRAIL is an important mediator of cell death induced by CTL and may have an important therapeutic role against human melanoma. *The Journal of Immunology,* **1998, 161: 2195–2200.**

The TNF receptor ligand family consists of at least 10 different transmembrane glycoproteins (1, 2). Several members of the TNF receptor family are capable of inducing programmed cell death or apoptosis because of intracytoplasmic (death) domains that bind proteins in the cell death pathway (3). These include receptors for TNF-α (CD120a,b) and Fas ligand (FasL)[3] (CD95), as well as a more recently described protein referred to as TNF-related apoptosis-inducing ligand or TRAIL (4, 5). The latter was found to be more widely distributed in tissues than FasL but was similar to FasL in its ability to induce apoptosis in a number of cell lines, particularly of hemopoietic origin (4, 6, 7).

At least three receptors have been described for TRAIL. R1 or death receptor 4 (DR4) was found to be expressed in most tissues, including activated T cells. It did not interact with the common adaptor molecule Fas-associated death domain (FADD), which binds to TNFR-1 and Fas (8). The second receptor, R2 (DR5), was also found to be widely distributed on tissues and, in contrast to R1, induced apoptosis by mechanisms that involved interaction with FADD (9). The third receptor, R3, differed mainly from R1 and R2 in not having a cytoplasmic domain and being linked to glycosyl phosphatidylinositol in the cell membrane. Interaction of TRAIL with R3 did not induce apoptosis (10). Expression of the latter was therefore postulated to protect cells against TRAIL-induced apoptosis (11, 12). This decoy role for R3 (or DcR1) was supported by its high expression on a variety of normal tissues but not on tumor cell lines (11). Similar findings were independently reported by a second group who referred to the receptors as TRID (13) (TRAIL receptor without an intracellular domain).

We have previously examined the role of the FasL/Fas pathway in the killing of melanoma target cells by CD4 CTL. This followed reports that the latter was the principal cytotoxic mechanism used for killing by CD4 T cells (14). Our results indicated, instead, that although the melanoma cells expressed Fas, they were resistant to killing by FasL and that mAb against FasL did not inhibit killing by the CD4 T cell clones (15). The CD4 T cells appeared to use different mechanisms against autologous and allogeneic melanoma cells. In the present study, we have investigated the susceptibility of melanoma cells to TRAIL-induced apoptosis and the role of TRAIL in the killing of melanoma and other target cells by CD4 T cells. We report that a high proportion of melanoma cells were susceptible to apoptosis induced by TRAIL and that the latter appears to mediate cytotoxic activity by CD4 T cells against Jurkat and melanoma target cells.

Materials and Methods

Cell lines

The series of melanoma cells with the prefix Mel were isolated from patients attending the Newcastle and Sydney Melanoma Units. Mel-FH, Mel-RM, Mel-JS, Mel-FC, Mel-CV, and Mel-WB were isolated from lymph nodes. These cell lines had been in culture from 2 to 6 mo at the time of these studies. The MM200 cell line was kindly supplied by Drs. Pope and Parsons (Queensland Institute for Medical Research, Queensland, Australia) and were isolated from primary melanoma. ME1007, ME10538, and ME4405 were kind gifts from Dr. Parmiani (National Cancer Institute, Milan, Italy) and were established from primary melanoma. IgR3 was provided by Dr. Hope (Genetics Department, University of Adelaide, Adelaide, Australia); it was established from a primary melanoma and is described elsewhere (16). The Jurkat T cell leukemia line was obtained from

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[3] Abbreviations used in this paper: FasL, Fas ligand; CD40L, CD40 ligand; FADD, Fas-associated death domain; LCL, lymphoblastoid cell lines; FLICE, Fas-associated death domain-like ICE (caspase 8); FLIP, FLICE-inhibitory protein.

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Dr. Robert Gallo (National Cancer Institute, Bethesda, MD). Lymphoblastoid cell lines (LCL) were obtained by EBV transformation of blood lymphocytes by culture in supernatants from the B95-8 marmoset leukocyte line (ATCC CRL 1612). Melanoma cells and LCL were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia).

mAbs and recombinant proteins

Recombinant human TRAIL (lot 6321-19) prepared as described elsewhere (4) and human CD40L (lot 5753-56) were supplied by Immunex (Seattle, WA). Each preparation was supplied as a leucine zipper fusion protein, which required no further cross-linking for maximal activity. The mAb M3 (IgG1) (lot 5323-12.COA) against Fas (4), which blocks Fas-mediated lysis, and mAb M180 (IgG1) against TRAIL (9) were supplied by Immunex as purified mAb preparations. Recombinant human FasL (huFasL), produced from isolated cDNA (GenBank accession No. U08137) in vector pDC409 and transfected into COS cells, was kindly supplied as sterile supernatants by Immunex. It produced 50% lysis of Jurkat T cells at dilutions of 2 to 4 (17). rTNF-α cytokines and control mAb anti-trinitrophenyl (anti-TNP; IgG1) were purchased from PharMingen (Bioclone, Marrickville, Australia).

Cytotoxic T cells

The generation and cytotoxic activity of the CD4 T cell clones used in the study have been fully described elsewhere (15). In brief, they were generated from the blood of patient FH (60-yr-old male, HLA-2, B44, 62 DR-15, DR-51) ~2 yr after surgical removal of metastatic melanoma in the right axillary lymph nodes. The cloned T cells were maintained in DMEM + 10% AB serum + 25 IU IL-2 and were restimulated every 14 days by adding mitomycin C-treated autologous melanoma and autologous LCL (1:2), which had been previously incubated together for 24 h and then irradiated (10,000 R). The generation and specificity of the CD8 CTL clone CO is described elsewhere (16). It is restricted by HLA-A1 and appears specific for melanoma.

Cytotoxic assays

The cytotoxic activity of cultured T cells was tested in triplicate or quadruplicate in 4- or 18-h ^{51}Cr release assays. Target cells were labeled with 100 uCi of Na$_2$ ^{51}Cr0$_4$ (NEN, Boston, MA) for 1.5 h at 37°C, washed three times, and resuspended in 10% FCS + 25 IU IL-2 (complete medium) at a concentration of 3×10^4/ml. A volume of 0.1 ml of target cells was added to 0.1 ml of T cells (6×10^5/ml) in a 96-well V-bottom plate (Medos, Lidcombe, Australia). The plates were centrifuged at $100 \times g$ for 2 min and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 18 h. For each target system, spontaneous release as well as maximal ^{51}Cr activity release was determined. After incubation, 100 μl of supernatant was harvested and counted in an automated gamma counter. Percent lysis was calculated as follows: % specific cytotoxicity = [experimental release (cpm) − spontaneous release (cpm)]/[maximal release (cpm) − spontaneous release (cpm)].

Inhibition of CTL activity with mAbs was performed likewise in a total volume of 200 μl. CTLs or target cells were preincubated with mAb for 30 min at 37°C at 20 μg/ml. Cultures were maintained for 18 h at 37°C in the continued presence of mAbs diluted four times. Inhibition induced by the mAb was calculated as the reduction in percent specific cytotoxicity/specific cytotoxicity × 100.

Apoptosis

Apoptotic cells were determined by the propidium iodide method (18). In brief, melanoma cells were adhered overnight in a 24-well plate (Falcon 3047; Becton Dickinson, Lane Cove, Australia) at a concentration of 1×10^5/well in 10% FCS. Cells in suspension were added on the day of the assay. Medium was removed, and 500 μl of fresh medium + 10% FCS containing the appropriate mAb was added for 30 min at 37° before the addition of TRAIL, FasL, CD40L, or TNF-α. Cells were incubated for a further 24 h at 37°C, and the medium removed and adherent and suspended cells washed 1 × with PBS. The medium and PBS were placed in 12 × 75 mm Falcon polystyrene tube and centrifuged at $200 \times g$. A hypotonic buffer 1 ml (propidium iodide, 50 μg/ml, in 0.1% sodium citrate plus 0.1% Triton X-100; Sigma, St. Louis, MO) was added directly to the cell pellet of cells grown in suspension or to adhered cells in the 24-well plate and gently pipetted off, then added to the appropriate cell pellet in the Falcon tube. The tubes were placed at 4°C in the dark overnight before flow cytometric analyses. The propidium iodide fluorescence of individual nucleic was measured in the red fluorescene using a Facscan flow cytometer (Becton Dickinson, Mountain View, CA) and the data registered in a logarithmic scale. At least 10^4 cells of each sample were analyzed. Apoptotic nuclei appeared as a broad hypodiploid DNA peak, which was easily distinguished from the narrow hyperdiploid peak of nuclei in the melanoma cells.

Cyclohexamide (10 μg/ml) and actinomycin D (3 μg/ml) were added to cells 2 h before adding apoptotic mediating reagents.

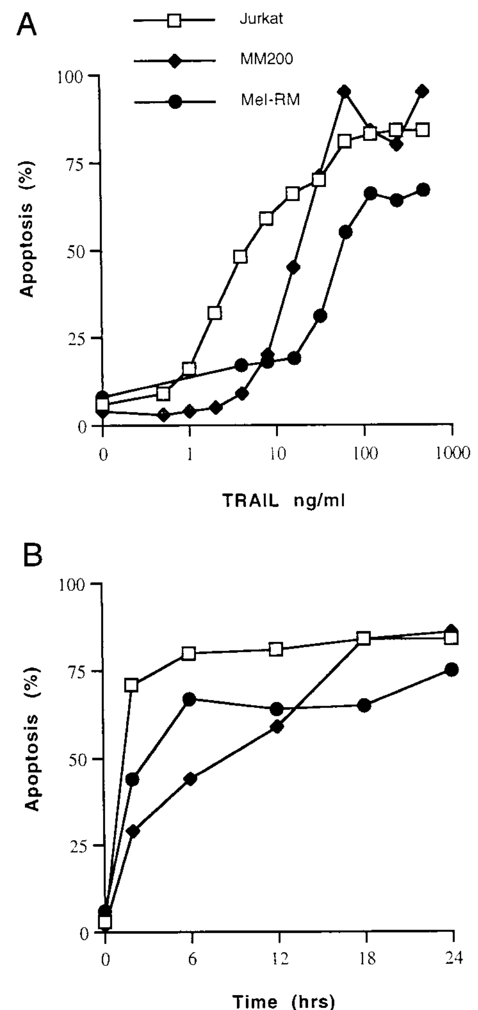

FIGURE 1. Induction of apoptosis in Jurkat and melanoma cells by TRAIL. *A*, Titration of TRAIL against cells from the Jurkat and two melanoma cell lines. *B*, Kinetics of induction of apoptosis by TRAIL at 100 ng/ml against the same target cells.

Results

Induction of apoptosis in melanoma cells by TRAIL

The susceptibility of melanoma cells and the Jurkat T cell line was tested over a range of concentrations of TRAIL. As shown in Figure 1, apoptosis of cells from two melanoma lines was maximal at 100 ng/ml. Jurkat cells appeared more sensitive to apoptosis induced by TRAIL than the melanoma cells. Studies on the kinetics of induction of apoptosis by TRAIL shown in Figure 1*B* indicated that apoptosis was induced rapidly in Jurkat and the Mel-RM cell line and was maximal at 6 h, whereas 18 h was needed for maximal induction of apoptosis in the cells from the MM200 line.

TRAIL induces apoptosis in melanoma cells that are resistant to FasL and other TNF family members

We compared several TNF family members for their ability to induce apoptosis in cells from the Jurkat and MM200 cell lines. As shown in Figure 2, TNF-α, FasL, and TRAIL, but not CD40L, induced apoptosis in Jurkat cells, but only TRAIL induced apoptosis in the MM200 melanoma cells. TRAIL was tested for its

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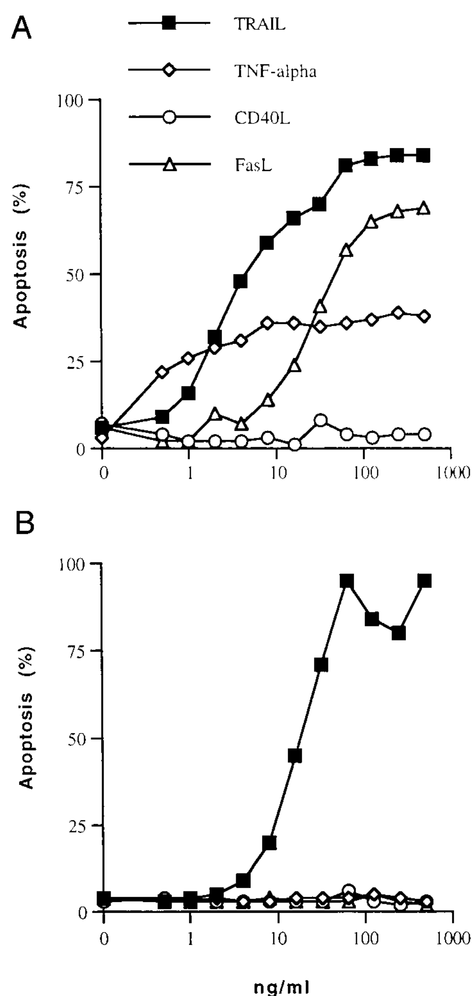

FIGURE 2. *A*, Induction of apoptosis in Jurkat T cells by TRAIL, FasL, and TNF-α. *B*, Induction of apoptosis in melanoma cells from the MM200 cell line by TRAIL but not by FasL, TNF-α, or CD40L. CD40L was active in induction of B7 expression on Daudi cells at concentrations of 10 ng/ml (26).

Table I. *TRAIL-induced apoptosis with cyclohexamide and actinomycin D*

	% TRAIL-Induced Apoptosis[a]	% TRAIL-Induced Apoptosis with Cyclohexamide[b]	% TRAIL-Induced Apoptosis with Actinomycin D
Jurkat	78	—	—
ME4405	61	—	—
MM200	83	—	—
Mel-CV	54	—	—
Mel-RM	76	73	83
Mel-WB	15		—
IgR3	21	44	40
Mel-FH	24	4	54
ME1007	0	—	—
ME10538	2	20	21
Mel-JS	0	0	1

[a] Recombinant TRAIL was added at 100 ng/ml for 18 h.
[b] Cyclohexamide (10 μg/ml) and actinomycin (3 μg/ml) were present throughout culture period. — Indicates not done.

ability to induce apoptosis in a range of melanoma cells that had previously been shown (15) to be resistant to FasL- and TNF-α-induced apoptosis. The results shown in Table I indicate that cells from 4 of 10 melanoma lines (ME4405, MM200, Mel-CV, and Mel-RM) were highly susceptible to TRAIL, and 3 were moderately susceptible (Mel-WB, IgR3, and Mel-FH). Only 3 lines (ME1007, ME10538, and Mel-JS) were resistant to apoptosis induced by TRAIL. Two each of the three cell lines showing moderate or no sensitivity to induction of apoptosis by TRAIL were treated with cyclohexamide or actinomycin D. As illustrated in the table, both of the moderately sensitive lines (IgR3 and Mel-FH) had increased susceptibility to TRAIL-induced apoptosis in the presence of actinomycin D or cyclohexamide. Cells from the resistant line, Mel-JS, remained resistant to TRAIL-induced apoptosis, but the ME10538 line became moderately sensitive. Similar results were obtained in a repeat of the experiment.

TRAIL-induced apoptosis can be inhibited by mAb M180

The specificity of the effects of TRAIL was examined using the mAb M180 to inhibit TRAIL-induced apoptosis. As shown in Figure 3*A*, induction of apoptosis was inhibited by the mAb at concentrations of 1 μg/ml and above. It is noticeable that it was not possible to completely inhibit TRAIL-induced apoptosis of melanoma cells from the MM200 line even at high concentrations of the mAb. The data in Figure 3*B* also show that the mAb at a concentration of 10 μg/ml had limited capacity to neutralize TRAIL, and this was greater in assays against Jurkat ($\equiv$100 ng of TRAIL) than in assays against MM200 ($\equiv$11 ng of TRAIL). Similar results were obtained in a repeat of the experiment.

TRAIL is involved in killing of Jurkat and melanoma target cells by CD4 T cells

We have previously described the cytotoxic activity of four CD4$^+$ T cell clones against autologous and allogeneic melanoma cells (15). In brief, the cytotoxic activity of clones A4C2, C5C5, and 2C4 against autologous melanoma (Mel-FH) was mediated by TCR-$\alpha\beta$ and were MHC class II restricted. The restricting HLA Ag was not identified. The clone C5C4 was not MHC restricted and had different kinetics against Mel-FH. Killing of the allogeneic melanoma Mel-RM was not MHC restricted and was not associated with DNA fragmentation of the nuclei.

These clones were tested for their cytotoxic activity against cells from the autologous and two allogeneic melanoma cell lines and the Jurkat target cell in the presence or absence of M180 mAb (5 μg/ml) against TRAIL. As shown in Figure 4*A*, the killing of Jurkat cells at 4 h by the clones C5C4, C5C5, and 2C4 was markedly inhibited (60–90%) by mAb to TRAIL, and killing by clone A4C2 was inhibited by 37%. In contrast, mAb M3 against Fas at a concentration of 5 μg/ml produced no (A4C2) or only low levels of inhibition (C5C4, C5C5, and 2C4). When the assays were carried $>$16 h, mAbs against TRAIL produced less inhibition of cytotoxicity and mAbs against Fas relatively more inhibition (see Fig. 4*B*) of cytotoxicity by the clones A4C2 and C5C5. This may indicate different kinetics of expression of TRAIL and FasL on the T cells or different kinetics of killing mediated by the ligands. We have shown previously (15) that killing by the 2C4 clone at 16 h may be mediated by TNF, which presumably accounts for the relatively low inhibition produced by MAbs to TRAIL and Fas in the 16-h assay.

Similar experiments were conducted in cytotoxic assays of the CD4 T cell clones against the autologous and allogeneic melanoma cell targets. The data in Table II show that the CTL activity of A4C2 and C5C4 against the autologous melanoma target cells was partially inhibited by mAb M180, whereas M3 against Fas did not inhibit the CTL activity of any of the clones. The cytotoxic activity of all four clones against the Mel-CV line was partially inhibited by the mAb M180, but not by M3 mAb against Fas. Neither mAb inhibited killing of the Mel-RM line. As shown in Table I, Mel-FH was only moderately sensitive to apoptosis induced by TRAIL,

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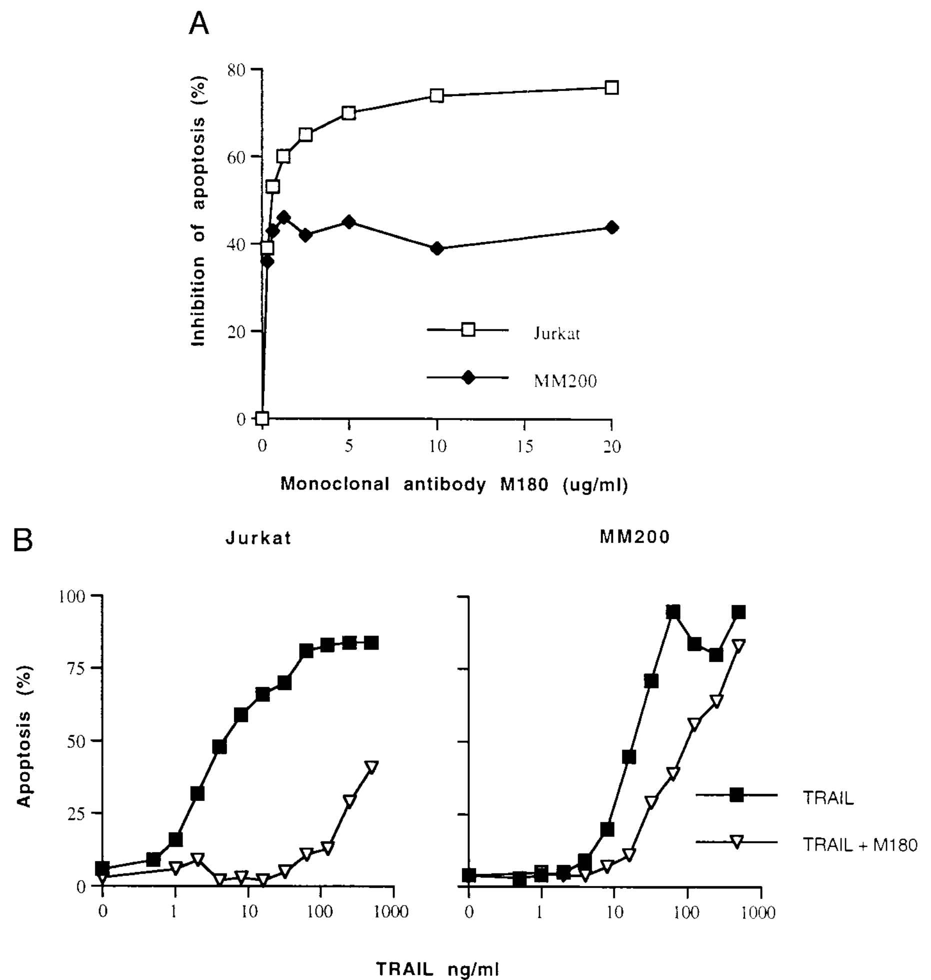

FIGURE 3. *A*, Inhibition of TRAIL (100 ng/ml)-induced apoptosis of Jurkat and MM200 target cells by mAb M180 at different concentrations of the mAb. *B*, Capacity of mAb M180 at 10 μg/ml to inhibit TRAIL in titration of TRAIL against Jurkat and MM200 target cells.

whereas Mel-CV and Jurkat cells were sensitive. This may account for the apparently greater involvement of TRAIL in killing of the Jurkat and Mel-CV target cells by the CD4 clones. These results are representative of two similar studies.

We also tested whether the CD8 CTL clone described previously (16) might also utilize TRAIL or Fas in the killing of melanoma cells from the Mel-CV line. As shown in Table II, specific cytotoxicity at 16 h was 84%, and the percent inhibition produced by M180 was 15%; that produced by the M3 mAb was 4%. Similar degrees of inhibition by the mAbs were seen in a 4-h assay against Mel-CV. Specific cytotoxicity at 4 h was 37%, and inhibition by MAb M180 and M3 was 24 and 14%, respectively.

We examined whether the sensitivity of the target cells to TRAIL (shown in Table I) correlated with their susceptibility to killing by the CD4 T cell clones. The data in Table III indicate that there was a close correlation in that melanoma cells ME4405, Mel-CV, Mel-FH, and Mel-RM, which were sensitive to TRAIL-induced apoptosis, were also sensitive to lysis by the CD4 T cell clones, whereas ME1007 and JS were resistant to killing by both TRAIL and the CD4 T cells. The exception appears to be K562 target cells, which were susceptible to TRAIL (specific cytotoxicity, 20% at 4 h) but were not killed by the CD4 T cells (15).

Discussion

The above results indicate that cells from over half the melanoma lines tested underwent apoptosis in the presence of TRAIL. This occurred at low levels of the ligand and was maximal after 6 to 18 h of culture. These findings were in contrast to the resistance of the melanoma cells to apoptosis induced by FasL and TNF-α, as reported previously (15). It is not clear from the present studies why melanoma cells should be sensitive to TRAIL but not FasL, as the mechanisms involved in the induction of apoptosis by both ligands is thought to be similar. Previous studies have shown that induction of apoptosis by members of the TNF family requires aggregation of their receptors and interaction of their death domains with signal transduction components (3). Aggregation of TRAIL and CD40 in the present studies was facilitated by use of TRAIL or CD40 as leucine zipper fusion proteins. It is possible that TNF-α and FasL were not cross-linked to the same extent, which may account for their inability to induce apoptosis in melanoma cells. However, in previous studies, immobilization of FasL or TNF-α on plastic surfaces did not increase their ability to induce apoptosis of melanoma cells, and cross-linking of mAb M3 against Fas did not induce apoptosis (unpublished data). The membrane-bound rather than the soluble form of FasL was shown to be active in induction of apoptosis of T cells (19), but in previous studies, FasL-expressing CD4 T cells did not induce apoptosis in human melanoma cells (15).

It would therefore appear more likely that the difference in susceptibility of melanoma cells to TRAIL and FasL refects different signaling pathways for induction of apoptosis, e.g., one of the receptors (DR5 or R2) appears to engage the adaptor protein FADD (9) and thereby the protease FADD-like ICE (FLICE) (caspase 8), which is inhibited in many tissues by a protein referred to as FLICE-inhibitory protein (FLIP) (20, 21). This protein was reported as explaining the resistance of melanoma cells to FasL (21).

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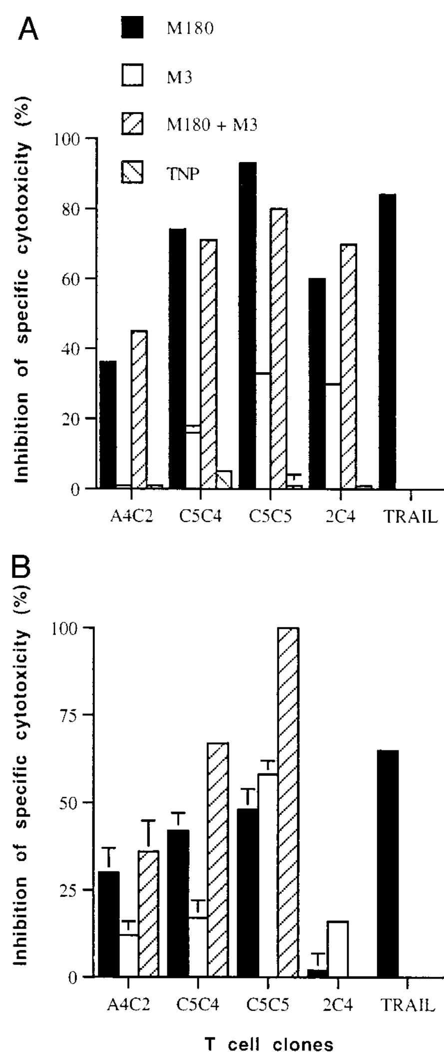

FIGURE 4. Inhibition by mAbs against TRAIL (M180 5 μg/ml) and FasL (M3, 5 μg/ml) of killing of Jurkat T cells by CD4 T cell clones in 4-h ^{51}Cr release assay (*A*). Specific cytotoxicity induced by the T cells A4C2, C5C4, C5C5, and 2C4 was 42, 38, 15, and 10%, respectively. *B*, 16-h ^{51}Cr release assay. Specific cytotoxicity induced by the T cells was 100, 85, 62, and 37%, respectively. The TRAIL-induced killing of Jurkat cells was 90% at 4 h and 92% at 16 h.

In contrast, a second receptor for TRAIL, DR4 or R1, does not appear to mediate its effects through FADD. We hypothesize that melanoma cells may preferentially express R1, and signals from TRAIL may therefore not be inhibited by FLIP. Melanoma cells that are resistant to TRAIL may not express R1 or R2 receptors, or they may express one or both decoy receptors that do not express death domains. Our studies show that some of the melanoma cells that are resistant or partially resistant to TRAIL-induced apoptosis become susceptible after treatment with cyclohexamide or actinomycin D. It is possible these cells have a combination of R1 and R2 type receptors and that production of FLIP or similar proteins are inhibited by cyclohexamide or actinomycin D. Answering these questions is the subject of ongoing studies on the expression of different receptors for TRAIL on melanoma cells.

We have reported previously (15) that several clones of CD4 T cells did not appear to kill melanoma target cells by FasL/Fas interactions, even though previous reports suggested this mechanism appeared important against other target cells (14). In view of the susceptibility of melanoma cells to TRAIL, we examined whether TRAIL interactions might be involved in the killing of

Table II. *Inhibition of percentage of specific cytotoxic activity of CD4$^+$ T cells by mAb to TRAIL and Fas*[a,b,c]

Target Cells	T Cell Clones A4C2 (%)	C5C4 (%)	C5C5 (%)	2C4 (%)	CO (%)
Autol Mel-FH	53	23	65	84	
Autol Mel-FH + M 180 (anti-TRAIL)	40 (25)	8 (65)	60 (8)	82 (2)	
Autol Mel-FH + M3 (anti-Fas)	51 (4)	21 (9)	65 (0)	80 (5)	
Autol Mel-FH + Isotype control	53 (0)	23 (0)	64 (0)	82 (1)	
Allo Mel-RM	81	76	77	66	85
Allo Mel-RM + M180	70 (14)	72 (5)	65 (16)	63 (5)	87 (0)
Allo Mel-RM + M3	82 (0)	76 (0)	72 (0)	66 (0)	89 (0)
Allo Mel-RM + Isotype control	82 (0)	75 (0)	75 (0)	65 (0)	86 (0)
Mel-CV	69	16	34	14	84
Mel-CV + M180	58 (16)	5 (69)	19 (44)	10 (40)	71 (15)
Mel-CV + M3	68 (1)	19 (0)	28 (18)	17 (0)	81 (4)
Mel-CV + Isotype control	68 (1)	18 (0)	33 (1)	16 (0)	84 (0)

[a] M180 and M3 mAbs and isotype control used at 20 μg/ml initially and left in culture for the 16-h assay diluted to 5 μg/ml. Figures in parentheses indicate percentage of inhibition. Autol, autologous; Allo, allogeneic.
[b] In the same assay, specific cytotoxicity induced by FasL and TRAIL on Jurkat T cells was 73 and 85%, respectively. MAb M180 inhibited TRAIL- and FasL-induced specific cytotoxicity by 73 and 9%, respectively. MAb M3 inhibited Fas- and TRAIL-induced specific cytotoxicity by 99 and 0%, respectively.
[c] SEs for all values were <3%. Percentage of inhibition, in the presence of mAb, >5% was significantly different by *t* test of the data.

target cells by CD4 T cells. These studies were made possible by the availability of the M180 mAb, which we and others (9) have shown to have the ability to partially block TRAIL-induced apoptosis. The degree of inhibition produced by the mAbs appeared to differ according to the target cell involved. In studies against the Jurkat T cells, mAb M180 inhibited ~80% of TRAIL-induced apoptosis but <50% of the apoptosis induced in the MM200 melanoma cells. These results may suggest that the receptors for TRAIL on the two cell types have different affinities and that those on the MM200 cells are able to compete more effectively with the mAbs for TRAIL. If this interpretation is correct, the degree of inhibition of CTL activity produced by mAbs against TRAIL may underestimate TRAIL involvement in CTL activity against certain target cells such as the MM200 melanoma cells.

Using the mAb M180 against TRAIL, we were able to show that killing of the Jurkat T cells by three of the CD4 T cell clones was mediated at 4 h almost entirely by TRAIL, with FasL contributing relatively little to the cytotoxicity. The degree of killing induced by FasL appeared to increase in assays conducted over a period of 16 h, which may indicate that TRAIL is expressed early after T cell

Table III. *Cytotoxic activity of CD4 T cells against autologous and allogeneic melanoma target cells*

Cell Line	Specific Cytotoxicity[a] A4C2	C5C4	C5C5	2C4
ME4405	35	58	17	7
Mel-CV	47	62	26	45
Mel-RM	92	83	32	64
Mel-FH	80	36	54	80
ME1007	3	3	0	1
Mel-JS	3	3	0	2
K562	3	1	0	0

[a] Values >6 were significantly (±2 SD) above baseline spontaneous release.

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activation and more transiently than FasL. Further studies are needed on this aspect. Killing of Jurkat T cells by one of the clones (A4C2) appeared to involve cytotoxic mechanisms other than FasL or TRAIL in that the mAb M180 against TRAIL and mAb M3 against Fas inhibited killing by $<50\%$.

TRAIL also appeared to be involved in CD4 T cell-mediated killing of melanoma cells that were sensitive to TRAIL-induced apoptosis. This included cells from the autologous and an allogeneic melanoma cell line. In studies against the autologous melanoma, only two of the clones (A4C2 and C5C4) appeared to mediate their killing by TRAIL. Killing of the autologous melanoma by A4C2 was only partially inhibited by mAbs against TRAIL, which suggested that other cytotoxic mechanisms were involved. This is consistent with our previous studies (15) showing that killing of autologous melanoma cells by the A4C2 clone was partially calcium ion dependent, consistent with perforin granzyme cytotoxic mechanisms, whereas killing by C5C4 was calcium ion independent, consistent with killing by FasL or TRAIL. The mAb against TRAIL also produced only partial inhibition of the cytotoxic activity of the T cell clones against the allogeneic Mel-CV cells, which indicated that other cytotoxic mechanisms were also involved against this target cell.

A second allogeneic melanoma line (Mel-RM) was not killed by Fas or TRAIL. We have shown previously that the CD4 T cell-mediated killing of cells from this line was not MHC restricted and did not involve induction of apoptosis. The actual lytic mechanism remains unknown (15). It is clear from these studies that T cell clones use multiple lytic mechanisms against different target cells, consistent with findings by others (3, 22, 23). Killing of melanoma target cells by MHC class I-restricted CD8 T cells may also involve TRAIL in the early phase of killing in that cytotoxicity against melanoma cells (Mel-CV) by a HLA-A1 restricted clone (16) was partially inhibited after 4 h of culture, but to a lesser extent later in the culture. Further studies to assess the generality of these findings are required.

Further support for the involvement of TRAIL in the killing of target cells by CD4 T cells came from our studies against a range of melanoma target cells. These studies showed a correlation between the susceptibility of the target cells to killing by TRAIL and by the $CD4^+$ T cell clones. Target cells that were resistant to TRAIL-induced apoptosis were also resistant to killing by the CD4 T cell clones. The exception to these findings was the absence of killing of K562 target cells by the CD4 T cell clones even though K562 was found to be sensitive to TRAIL-induced apoptosis (as also reported by others (7)). These findings may indicate that T cell recognition is needed to induce TRAIL expression and in the absence of TCR engagement TRAIL is not expressed. These results are also consistent with the failure to demonstrate TRAIL involvement in the killing of the allogeneic Mel-RM melanoma cells, which did not involve $\alpha\beta$TCR on the CD4 T cell clones (15).

As far as we are aware this is the first study to show that TRAIL may be an important mediator of cell death induced by CD4 T cells. We have previously reported a preponderance of CD4 T cells infiltrating primary melanoma (24) and others have reported an association between infiltration by CD4 T cells and regression of primary melanoma (25). Our present results provide an attractive explanation for these findings and appear to have significance in regard to the therapeutic potential of TRAIL in treatment of patients with melanoma. If the results found here in vitro reflect similar sensitivity of melanoma cells to TRAIL in vivo, this agent may become a valuable additional treatment for control of melanoma.

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