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Fas-Based d10S-Mediated Cytotoxicity Requires Macromolecular Synthesis for Effector Cell Activation but not for Target Cell Death

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Fas-based d10S-mediated cytotoxicity requires macromolecular synthesis for effector cell activation but not for target cell death

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SUMMARY

Two main mechanisms seem at play in T cell-mediated cytotoxicity, a process in which target cell death often follows an apoptotic cell death pattern. One of these involves Fas at the target cell surface and a Fas ligand at the effector cell surface. This allowed us to reinvestigate the long-standing question of macromolecular synthesis requirement in T cell-mediated cytotoxicity, using the d10S model cell line which is cytotoxic apparently only via the Fas molecularly defined mechanism. We showed, first, that induction of cytotoxic activity of effector cells, obtained by preincubating these effector cells with a phorbol ester and a calcium ionophore, could be inhibited by macromolecular synthesis inhibitors (cycloheximide, actinomycin D, DRB). We then investigated whether macromolecular synthesis was required, when effector and target cells were mixed, to obtain target cell death. Preincubating already activated effector cells for 30 min with macromolecular synthesis inhibitors, then adding target cells and performing the ^{51}Cr release cytotoxicity test in the presence of these inhibitors, did not significantly decrease subsequent target cell death, indicating that already activated effector cells could kill without further requirement for macromolecular synthesis. In addition, target cell preincubation for up to 3 h in the presence of one of these inhibitors did not decrease cell death. The high sensitivity of mouse thymocytes to this type of cytotoxicity enabled us to devise the following experiment. As previously shown by others, thymocyte death induced by dexamethasone (DEX) could be blocked by coincubation with cycloheximide (CHX). Such DEX-treated CHX-rescued thymocytes, the survival of which was an internal control of efficiency of protein synthesis inhibition, were then subjected to effector cells in the presence of CHX, and were shown to die. Thus, there is no requirement for macromolecular synthesis at the target cell level in this variety of apoptotic cell death. Altogether, in this defined mechanism of T cell-mediated cytotoxicity, macromolecular synthesis is required for d10S effector cell activation, but not for lysis by already activated effector cells nor for target cell death.

1. INTRODUCTION

The main criteria of apoptosis (Kerr *et al.* 1972), one of several ways used by a cell to die, are morphological alterations such as cytoplasmic and nuclear condensation/fragmentation, and often DNA fragmentation and a requirement for macromolecular synthesis (MMS) (reviewed by Cohen 1991; Ellis *et al.* 1991; Golstein *et al.* 1991; Kerr & Harmon 1991; Lockshin & Zakeri 1991). MMS in the context of this report should be regarded as mRNA and protein synthesis. Several distinct types of cell death may exist under the collective concept of apoptosis. They might be distinguished according to their faithfulness to the above-mentioned criteria (see discussion in Golstein *et al.* 1991). Thus, sometimes a morphologically apoptotic cell death does not require MMS (see below), or is facilitated if MMS is inhibited (Ruff & Gifford 1981), or can even be triggered by MMS inhibitors (Searle *et al.* 1975; Martin *et al.* 1990; Cohen 1991). The target cell death induced by cytotoxic T cells (Berke 1989; Tschopp & Nabholz 1990; Young *et al.* 1990; Duke 1991; Golstein *et al.* 1991; Podack *et al.* 1991), considered apoptotic on the basis of morphologic criteria and DNA fragmentation, was reported not to require MMS (Duke *et al.* 1983). More recent results, however, suggested that target cell MMS may be required in certain cases or circumstances of T cell-mediated cytotoxicity (Landon *et al.* 1990; Zychlinsky *et al.* 1991). These apparent discrepancies might be due, in particular, to the fact that different mechanisms of T cell-mediated cytotoxicity (Duke 1991) were considered.

Recently, we showed (Rouvier *et al.* 1993) that most if not all of the calcium-independent component of T cell-mediated cytotoxicity involved the death-transducing Fas molecule (Yonehara *et al.* 1989; Trauth *et al.* 1989) at the target cell surface. This Fas-based mechanism was first detected using d10S model cytotoxic cells (Rouvier *et al.* 1993), but was

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not limited to these. It could be shown to account, in particular, for the calcium-independent component of cytotoxicity by populations of alloimmune peritoneal exudate lymphocytes (Rouvier *et al.* 1993) and of mixed leukocyte culture cells, and by several cytotoxic T cell clones (M.-F. Luciani & P. Golstein, unpublished observations). This mechanism is defined in molecular terms by a requirement for Fas at the target cell surface (Rouvier *et al.* 1993) and by a requirement for the Fas ligand (Suda *et al.* 1993) at the effector cell surface. In parallel, a perforin-based mechanism, detected some years ago (Henkart 1985; Podack 1985), was formally demonstrated as a major mechanism of T cell-mediated cytotoxicity through the use of perforin knock-out mice (D. Kägi & H. Hengartner, personal communication).

In this study, we reinvestigate the requirement for MMS in the Fas-based molecularly defined system of T cell-mediated cytotoxicity. We took advantage of the availability of prototypic effector cells (d10S) exerting cytotoxicity apparently only via the Fas-dependent mechanism, and of the sensitivity of thymocytes in this system, allowing their use in cell-death-inhibition internally controlled experiments. We thus demonstrated, first, that there is MMS requirement for d10S effector cell activation, and, second, that there is no significant requirement for MMS at the actual killing stage, and especially in dying target cells, in this mechanism of T cell-mediated cell death.

2. MATERIALS AND METHODS

(*a*) *Culture conditions, cells and reagents*

All incubation and culture procedures were done at 37°C in a water-saturated 7% CO_2 atmosphere, in RPMI 1640 or DME medium (Gibco Bio-Cult, Glasgow, U.K.) enriched with 5% foetal calf serum (FCS, Biological Industries, Israel). Target cells were either YAC tumour cells or thymocytes freshly explanted from 6–8 week-old C57Bl/6 mice.

PC60 cells (Conzelmann *et al.* 1982), a hybridoma between a mouse cytotoxic T cell clone with anti-male D^b specificity (Von Boehmer *et al.* 1979) and a derivative from the rat T lymphoma W/Fu (C58NT)D, and all PC60-derived (PC60-d) cells including PC60-d10S cells (d10S for short) were grown and cloned in Dulbecco medium (Gibco Biocult) enriched with 5% FCS. Cloning was done by limiting dilution in flat-bottomed wells of 96-well tissue culture microplates (C.E.B., France). Most of the PC60-d clones were derived from wells having received an average number of 0.3 cells per well. After sufficient growth, cloned cells were transferred to wells of 24-well tissue culture plates (Costar, Cambridge, Massachusetts) and later to tissue culture flasks (Falcon, Becton Dickinson, Lincoln Park, New Jersey). These PC60-d cloned cells were then induced to check their cytotoxic potential. Induction of cytolysis was by addition, at the beginning of the cytotoxicity test or in preincubation experiments, of a mixture of phorbol myristic acetate (PMA; Sigma, Saint-Louis, Missouri; final concentration 10 ng ml^{-1}) and of the Ca^{2+} ionophore ionomycin (Calbiochem, San Diego, California; final concentration 3 µg ml^{-1}). d10S cells activated by preincubation for 3 h with PMA and ionomycin are denoted d10S PI. The protein synthesis inhibitor cycloheximide (CHX) and the RNA synthesis inhibitors actinomycin D and dichlororibofuranosylbenzimidazole (DRB) were all from Sigma.

(*b*) *Cell-mediated cytotoxicity tests*

^{51}Cr release tests were carried out in V-shaped wells of 96-well microtitre plates with ^{51}Cr-labelled target cells (either 10^4 YAC or 10^5 thymocytes) and effector cells at the indicated ratios in a total volume per well of 200 µl of RPMI medium with 10% FCS. The plates were centrifuged (200 *g*, 2 min) and incubated for 4 h unless specified otherwise. After another centrifugation, 100 µl aliquots of supernates were assayed for radioactivity. The fraction of the total radioactivity released was then calculated, and the results were expressed as % experimental ^{51}Cr release minus % ^{51}Cr release from target cells alone, except for the experiment in figure 3*d*.

Some experiments with thymocytes (figure 3*a–c*) were assayed using a trypan blue exclusion test. Somewhat erratic results in preliminary experiments were found to be due to variations of thymocyte staining with time after addition of trypan blue to the thymocyte suspensions. In the experiments presented in this report, trypan blue was added at the indicated times to the cell suspensions, which were kept for a further period of time of 20 to 40 min at room temperature and only then microscopically assessed for cell death; this resulted in the presence of two unambiguously distinct types of cells, 'white' with contrasted edges considered living, and blue at various stages of disintegration. Because the number of cells having died in a culture may exceed the number of remaining blue cells, only living cells were counted. The results are expressed as % surviving cells (concentration of living cells in experimental group/initial concentration of living cells ×100).

3. RESULTS

(*a*) *Preliminary considerations*

The inducible cytotoxic T cell hybridoma PC60 (Conzelmann *et al.* 1982), obtained from M. Nabholz, was serially subcloned, with systematic passaging of the clones that after induction were most cytotoxic against ^{51}Cr-labelled YAC target cells. A clone obtained after the tenth serial subcloning, called d10S, was used for the experiments described in this report. After induction with a mixture of PMA and ionomycin, cytotoxicity by d10S cells has many characteristics of a calcium-independent MHC-unrestricted T cell cytotoxicity, and leads to DNA fragmentation in YAC target cells (not shown). d10S PI cells efficiently kill both YAC target cells and thymocytes, the latter at very low ratios of effector to target cells ((Rouvier *et al.* 1993); table 1).

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Table 1. *Inhibition by cycloheximide of d10S effector cell activation by a mixture of PMA and ionomycin*

			cytotoxic activity of d10S cells preincubated with PI[a]					
cytotoxicity test[b]			without CHX			with CHX		
targets	CHX in test	E : T	3	1	0.3	3	1	0.3
YAC	−		79[c]	68	26	33	36	9
	+		77	51	22	11	5	5
		E : T	0.3	0.1	0.03	0.3	0.1	0.03
thymocytes	−		32	31	15	19	18	13
	+		46	21	9	14	8	3

[a] Effector d10S cells were preincubated for 3 h in the presence of PMA and ionomycin (PI) either with or without cycloheximide (CHX) at a final concentration of 10 μg ml^{-1}, and washed by centrifugation.
[b] The 4 h cytotoxicity test was run at the indicated E : T (effector : target cell) ratio, in the presence of PI, and with or without cycloheximide.
[c] Results expressed as % experimental ^{51}Cr release minus % ^{51}Cr release of target cells alone (7–8 for YAC, 14–16 for thymocytes).

Importantly, all of the detectable cytotoxicity of d10S PI, at least when tested against thymocytes and against Fas-transfected L1210 tumour cells, requires the presence of Fas on the target cell surface (Rouvier *et al.* 1993); this is most probably also true for the equally calcium-independent (Rouvier *et al.* 1993) cytotoxicity of d10S PI tested against YAC tumour target cells. Using d10S PI as effector cells, in particular with thymocytes as target cells, seems therefore to be the best means towards exploring an isolated molecular mechanism of cell-mediated cytotoxicity.

(*b*) *Induction of d10S cytolytic activity by PMA and ionomycin requires macromolecular synthesis*

Table 1 shows that d10S activation by incubation for 3 h with PI before the cytotoxicity test led to less cytotoxicity if this preincubation was performed in the presence of CHX. Because of the reversibility of the CHX effect, the inhibition of induction of cytolysis was more marked if CHX was present, not only during preincubation, but also during the cytotoxicity test itself (table 1). d10S activation by PI was significantly inhibited by CHX at concentrations as low as 0.1 μg ml^{-1} (figure 1*a*, dotted line), and also by actinomycin D and DRB (figure 1*b*,*c*, dotted lines). The inhibition of activation was most probably related to inhibition of MMS, as it occurred using each of three drugs known to interfere with distinct steps of MMS, and at low concentrations of these drugs, in particular of CHX. d10S activation with PI thus most probably required MMS. Interestingly, after 3 h in PI the effector cells had similar cytolytic activity whether the cytotoxicity test was performed in the presence or in the absence of CHX (table 1), i.e. the PI-activated effector cells had synthesized by then all the macromolecules required to kill.

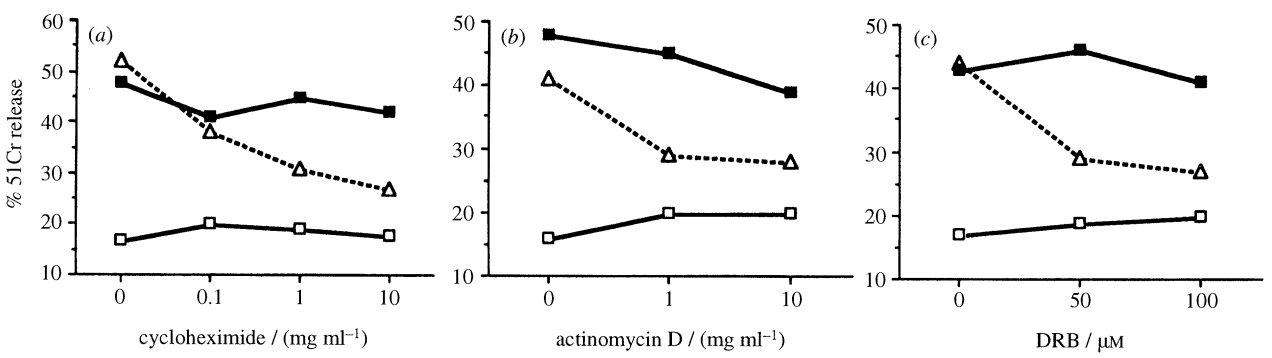

Figure 1. The lack of effect on cytotoxicity of inhibitors of MMS added at the beginning of the cytotoxicity test. In three separate experiments, cycloheximide (*a*), actinomycin D (*b*) or DRB (*c*) at the indicated final concentrations were added to d10S effector cells, preactivated (filled squares) or not (open squares) with a mixture of PMA and ionomycin. After 30 min of incubation at 37°C, YAC target cells were added and the cytotoxicity test proceeded for 4 h. The effector : target cell ratio was 1 : 1 in all cases. As a positive drug efficiency control, the same concentrations of inhibitors were used with non-preactivated d10S cells, and PMA and ionomycin were added directly to the cytotoxicity test; under these conditions the inhibitors affected activation, resulting in less cytotoxic activity generated during the test (dotted line). The cytotoxicity is expressed as % ^{51}Cr release as a function of inhibitor concentration. Spontaneous ^{51}Cr release was 10–20%.

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(c) *Lysis of target cells by PI-activated d10S effector cells requires macromolecular synthesis neither at the effector nor at the target cell level*

In the experiments described in figure 1, already activated effector cells (d10S PI) were incubated for 30 min in the presence of the inhibitors before the beginning of the cytotoxicity test, which was performed also in the presence of these inhibitors. As in the experiment described in table 1, this led to little or no significant decrease in cytotoxicity, indicating that already activated effector cells did not require MMS to lyse target cells. Because target cells were lysed to approximatlely the same degree, whether or not CHX was present in the cytotoxicity test, the experiments described in table 1 and in figure 1*a* also suggested that the target cells possessed all the proteins required to die. The lack of any significant decrease of cytotoxicity when either actinomycin D or DRB were added at the beginning of the cytotoxicity test (figure 1*b*,*c*) suggested that no RNA synthesis was required at the dying target cell level. These conclusions still applied when YAC or thymocyte target cells were preincubated for 30 min in the presence of CHX or of actinomycin D before the cytotoxicity test (figure 2). In fact, preincubation of YAC target cells with actinomycin D for as long as 3 h before the cytotoxicity test did not significantly decrease their sensitivity to d10S PI (not shown).

Although many of the experiments above were controlled in terms of drug activity (as the drugs were shown in the same experiments to inhibit effector d10S cell activation), they were not, however, internally controlled for efficiency of macromolecular synthesis inhibition at the target cell level. To this end, we took advantage of the observation that effector d10S PI cells could lyse not only YAC target cells, but also thymocytes, the latter moreover at very low ratios of effector to target cells (Rouvier *et al.* 1993; table 1, figure 2*b*,*c*). Thymocytes could also be lysed by DEX in a classical *in vitro* apoptosis system (Wyllie 1980).

As shown before (Cohen & Duke 1984; Wyllie *et al.* 1984), thymocytes undergoing apoptotic death after 10–20 h of incubation with DEX (figure 3*a*) did not die if this incubation included CHX (figure 3*b*), showing that protein synthesis by the dying cells is required for death to occur in this case. Thymocytes treated with DEX and protected by addition of CHX were processed after overnight incubation in three different ways. When washed and resuspended in medium without CHX, they began to die, thus reflecting a long-term death signal given by DEX. This was shown in both a trypan blue viability assay (figure 3*c*) and a ^{51}Cr release assay (figure 3*d*). When resuspended in the presence of CHX, they did not die (figure 3*c*,*d*), thus reflecting the MMS requirement of DEX-induced apoptotic death and the efficiency of CHX to block this MMS. When d10S PI effector cells were added to these CHX-blocked thymocytes, the thymocytes died (figure 3*d*). The latter results, obtained in each of three similar experiments, showed that thymocytes can be killed by d10S PI effector cells even when the protein molecules whose synthesis is required for DEX-induced apoptosis of the same thymocytes are demonstrably not synthesized. A reservation to these conclusions would stem from the possibility, which we do not consider very likely, that effector cells would lyse with similar efficiency, but through a different mechanism, untreated thymocytes and thymocytes treated with DEX and CHX.

4. DISCUSSION

Studies on MMS requirements of T cell-mediated cytotoxicity, undertaken to provide indications as to the mechanism(s) of the latter, have included both effector and target cell preincubation or coincubation

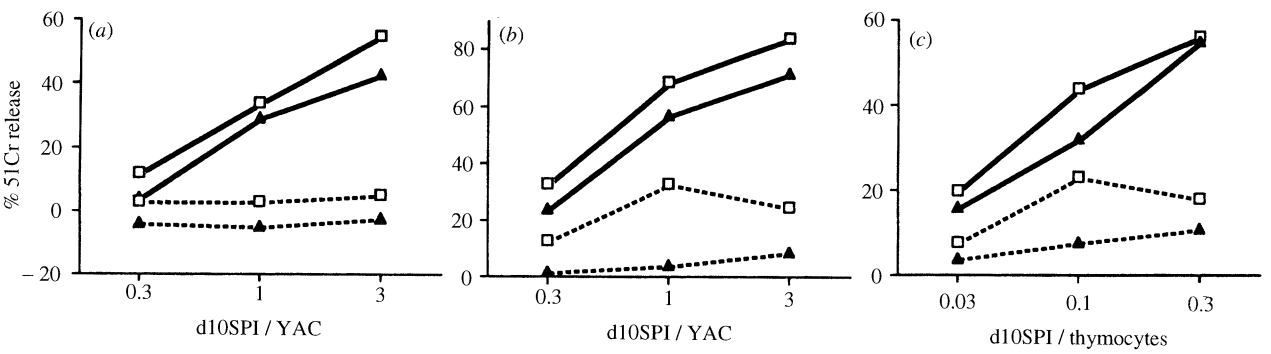

Figure 2. The lack of effect on cytotoxicity of target cell preincubation with inhibitors of MMS. In a first experiment (*a*), YAC target cells were incubated for 30 min at 37°C before the cytotoxicity test with medium alone (open squares) or with actinomycin D at a final concentration of 5 μg ml^{-1} (filled triangles). In a second experiment, YAC cells (*b*) or thymocytes (*c*) were incubated similarly with medium alone (open squares) or with cycloheximide at a final concentration of 10 μg ml^{-1} (filled triangles). Target cells preincubated with a drug were washed (to eliminate released ^{51}Cr) and were re-exposed to the same drug at the same final concentration during the 4 h cytotoxicity test. Effector cells were d10S-preactivated by incubation with PMA and ionomycin. As a positive drug efficiency control, this preactivation was performed in the presence of the corresponding drug (dotted lines) or in its absence (full lines). The cytotoxicity is expressed as % ^{51}Cr release as a function of effector to target cell ratio. Spontaneous ^{51}Cr release was 7–17%.

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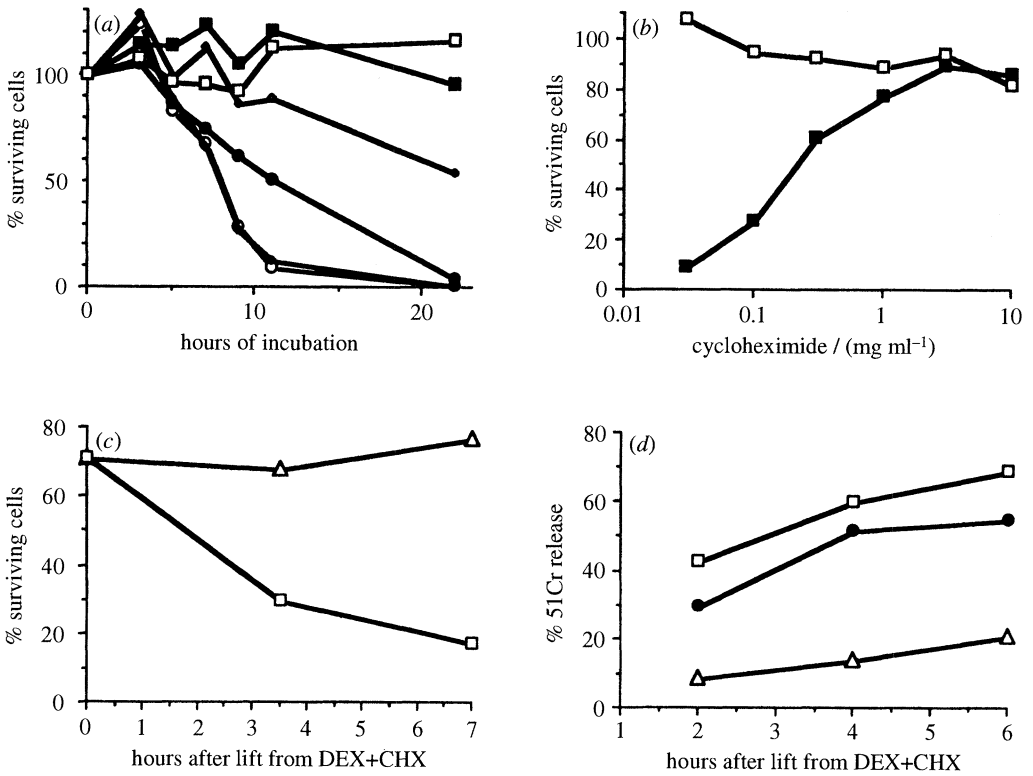

Figure 3. Thymocytes rescued by cycloheximide from dexamethasone-induced death could be lysed by d10S PI cells. (*a*) % surviving thymocytes after incubation for various lengths of time with either medium alone (open squares), or tenfold increasing concentrations of dexamethasone up to 10^{-6} M (open circles). (*b*) % surviving thymocytes after overnight incubation with medium (open squares) or 10^{-6} M dexamethasone (filled squares) and various concentrations of cycloheximide. Thymocytes thus incubated overnight with both dexamethasone (10^{-6} M) and cycloheximide (10 μg ml^{-1}) were washed and reincubated either in medium alone (open squares) or with cycloheximide (10 μg ml^{-1}; open triangles); death of cycloheximide-unprotected, but not of cycloheximide-protected thymocytes, was followed with time, in separate experiments, either as % surviving cells (*c*) or in a ^{51}Cr release test (*d*). Addition of d10S PI to cycloheximide-protected thymocytes, at a ratio of 0.1 : 1, resulted in significant lysis of these thymocytes (*d*, filled circles). Results are given as uncorrected % ^{51}Cr release.

with inhibitors of MMS (Brunner *et al.* 1968; Thorn & Henney 1976; Landon *et al.* 1990; Zychlinsky *et al.* 1991). The variability of the results thus obtained may have originated from the use of different effector cells, different stages of activation of these, different target cells, different concentrations of inhibitors, or from the initially unsuspected heterogeneity of the mechanisms of T cell-mediated cytotoxicity under investigation. The availability of a T cell-mediated cytotoxicity system lysing via a defined mechanism through target-cell-surface Fas (Rouvier *et al.* 1993) enabled us to re-evaluate in this system the MMS requirements (and may be of use to investigate other parameters of T cell-mediated cytotoxicity). We reached two conclusions: in our d10S cell model system, MMS is required for the induction of cytotoxic activity, and is not required at the actual killing stage nor for the death of the target cell.

Effector d10S cells were activated with a mixture of PMA and ionomycin, an approach known to induce in T killer cells significant levels of cytotoxic activity (Russell 1986; Lancki *et al.* 1987). For d10S cells this activation required MMS for a period of 3 h (this paper), and led to an increased transcription of Fas ligand message (Suda *et al.* 1993). The MMS-requiring activation of d10S cells and the Ca^{2+}-independence of their cytotoxicity (Rouvier *et al.* 1993) are very reminiscent of recently described characteristics of several CD4$^+$ cytotoxic cell clones (Shih & Bollom 1990; Strack *et al.* 1990; Tite 1990; Ju 1991; Abrams & Russell 1991; Grogg *et al.* 1992; Ozdemirli *et al.* 1992). Among these, some seemed to use a TNF-based mechanism of lysis. Some others behaved in a way suggesting, by comparison with our results, the possibility that they use a Fas-dependent mechanism. For instance, CD4 Th1 clones could be activated by PMA and ionomycin, which required MMS, and then exerted cytotoxicity in a calcium-independent manner (Ozdemirli *et al.* 1992). In addition, in some other systems cytotoxic activity could be induced rather rapidly, as in human blood T cells (Azuma *et al.* 1993).

As discussed before (Rouvier *et al.* 1993), Fas transduction of target cell death implies that the effector cell expresses a functional Fas ligand. MMS may be required, not only for the synthesis of enough of this ligand (Suda *et al.* 1993), but also for the synthesis or the functional availability of any molecule (such as LFA-1, not shown) other than Fas but also required for lysis by d10S cells. Once activated, the d10S cells did not require MMS to induce the death of their target cells; one might speculate that metabolic requirements at the effector cell level may then be minimal. The observation that formaldehyde-fixed

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activated d10S cells did not exhibit any cytotoxic activity (V. Depraetere, unpublished data) prevents any firm conclusions being drawn.

Cytotoxicity by already activated d10S did not require MMS in the dying target cells, nor was it increased in the presence of MMS inhibitors. Cell death in this case is Fas-transduced (Rouvier *et al.* 1993). Fas-transduced cell death occurring upon engagement of Fas with antibodies has been reported to increase upon inhibition of MMS (Yonehara *et al.* 1989; Itoh *et al.* 1991). Fas-based cell-mediated lysis may not be equivalent to anti-Fas antibody-mediated cell death: cytotoxic T cells may contribute factors other than the Fas ligand. Another, not unlikely possibility is that the nature of the Fas-bearing target cells conditions the effect of MMS inhibitors. These possibilities could be tested by investigating in parallel, on the same Fas-bearing cells, the effect of these antibodies and of activated d10S cells.

As discussed before (Golstein *et al.* 1991), T cell-induced target cell death shares with classical models of apoptosis characteristic morphological features (Sanderson 1976; Don *et al.* 1977; Liepins *et al.* 1977; Matter 1979) and DNA fragmentation (Russell *et al.* 1982; Russell 1983; Duke *et al.* 1983; Cohen *et al.* 1985; Schmid *et al.* 1986; but see Zychlinsky *et al.* 1991). The lack of effect of MMS inhibitors on T cell-mediated cell death is one of the features making this type of apoptosis apparently different from several others. In the present series of experiments this could be demonstrated using the same dying cells: the d10S cell-mediated death of thymocytes did not require the synthesis of any of the molecules whose synthesis was required for the DEX-induced apoptotic death of the same thymocytes. Moreover, although under certain conditions DEX protects cells from antibody-mediated, TCR/CD3-transduced cell death (Zacharchuk *et al.* 1990; Iseki *et al.* 1991; Iwata *et al.* 1991), or from TNF-mediated, TNF-R-transduced cell death (Beyaert *et al.* 1990), it clearly did not protect thymocytes from d10S-mediated, Fas-transduced cell death. It could be that in some cases T cell-mediated 'apoptosis' mechanistically differs, more than just by the initial death signal transduction pathway, from other types of apoptotic death.

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