Two molecular mechanisms of T cell–mediated cytotoxicity have recently been defined. Gene knock-out procedures that produced perforin-deficient (1) or granzyme B–deficient (2) mice led to the demonstration of a granule exocytosis–perforin–granzyme B pathway. A Fas pathway had been independently defined through a requirement for Fas at the target cell surface (3, 4). In short-term T cell-mediated cytotoxicity assays both the perforin-based and the Fas-based mechanisms, and no third mechanism, could be detected under all tested circumstances (5). The Fas-based mechanism contributes in particular to antigen-specific T cell–mediated cytotoxicity (3). Because the Fas ligand (Fas-L) (6) is required at the effector cell surface to ensure Fas-based cytotoxicity and because T cells do not normally kill via Fas unless there is specific antigen recognition, we assumed that TCR/CD3 engagement induced the functional availability of this Fas-L (3).

The TCR/CD3 complex includes at the cell surface a module responsible for antigen recognition (TCR) and at least four distinct associated polypeptides (CD3γ, CD3δ, CD3ε, and CD3ζ) that ensure assembly of the complex as well as signal transduction. In the cytoplasmic domain of the CD3 components, an 18 amino acid motif based on a tandem YXXL stretch, the activation receptor homology sequence 1 (ARH-1), carries sufficient structural information to activate both early and late signaling events (reviewed in 7). Pioneering experiments demonstrated that the CD3ζ ARH-1 motif could trigger cytotoxicity but the cytotoxicity mechanism(s) involved were not known at the time (8).

The findings reported here show that TCR/CD3 stimulation of MLC or hybridoma T cells was able to induce Fas-based cytotoxicity, as reported recently elsewhere (9-11). Moreover, in T cell hybridomas, induction could be obtained through the cytoplasmic domain of the CD3 ζ chain and it required the integrity of its ARH-1 motifs. The induction of Fas-based cytotoxicity was dependent on extracellular Ca2+, while its execution was not, and induction required macromolecular synthesis. Depending on the activated T cells, the required Ca2+-dependent step was completed within 1–3 h and an increased expression of the Fas-L message could be demonstrated within the same time frame. Thus, signaling through the TCR/CD3 pathway led to rapid expression of the Fas-L message to ensure Fas-based cytotoxicity upon antigen-specific restimulation.

Materials and Methods

Effector T Cells. For MLCs, spleen cells were obtained from 2–3-month-old C57Bl/6 (H-2b, b) or BALB/c (H-2d, d) mice and processed as indicated (12). On average, 50% of the initial input of responder cells were recovered on day 5. PC60-d11S (d11S) was a subclone of the d10S clone, shown to exert Fas-based cytotoxicity exclusively (3) and previously derived by serial subcloning (3, 4) from the PC60 mouse x rat cytotoxic T hybridoma cell (13). The murine T cell hybridomas L16.24 and T16.19 expressed a CD25ζ chimeric molecule made of the complete human CD25 ecto- and transmembrane domains fused to the complete mouse CD3ζ cyto-
The hybridoma L17.2 expressed a CD25/γ chimeric molecule in which the Y residues at positions Y72, Y111, and Y142 of the CD3γ cytoplasmic segment, i.e., the first Y residues of each of the three CD3γ ρ ARH-1 motifs, had been converted into F residues (14). Flow cytometric analysis of the hybridomas was performed as indicated (15). Effector T cells were tested for cytotoxicity either directly or after a preincubation period.

Preincubation. Effector T cells (5 × 10^6) were preincubated in 2.5 ml of culture medium per well of 6-well plates (Falcon Labware, Oxnard, CA), either as a control in medium alone or in the presence of PI, which is a mixture of PMA (no. P-8139, 2.5 ng/ml; Sigma Chemical Co.) and ionomycin (no. 407952, 0.5 µg/ml; Calbiochem-Behring Corp., San Diego, CA), or after overnight coating of the well with purified antibodies. Coating was performed at 4°C with 5 µg of antibody in 0.5 ml of PBS per well with use of either the 145-2C11 hamster anti-mouse CD3ε antibody (16) or the B1.49.9 mouse anti-human CD25 antibody (0119; Immunotech, Marseille, France). The duration of preincubation on antibody-coated plastic was 1 h for MLC cells or 3 h for T cell hybridomas. In some experiments, preincubation was done in the presence of cycloheximide (10 µg/ml; Sigma Chemical Co.; in this series of experiments, there was only one wash of effector cells after preincubation, which probably led to carry-over of cycloheximide to the cytotoxicity test, which would account for the absence of reversal of the cycloheximide block seen in Fig. 1) or in the presence of actinomycin D (10 µg/ml; Sigma Chemical Co.) or 1 mM EGTA plus 1.5 mM Mg++. To chelate most of the extracellular Ca++ in the presence of an excess of Mg++. Target Cells. Target cells used were either the DBA/2, H-2d T cell lymphoma L1210 cells, expressing low amounts of Fas (3), or derivatives thereof. L1210-3 was obtained by repeated selection of L1210 with Fas-based d11S killer cells. 2 × 10^5 d11S cells were preactivated for 3 h by PMA (2.5 ng/ml) and ionomycin (0.5 µg/ml), incubated for 30 min in the presence of mitomycin C (75 µg/ml), and rinsed 3× in medium. These cells were mixed with 2 × 10^6 L1210 cells in 1 ml of medium, centrifuged for 1 min at 1,200 rpm, and incubated for 4 h. This cell mixture was cultured for 72 h and 5 × 10^6 of the remaining cells (most of which were L1210 cells by morphology) were incubated for another 4 h with 2 × 10^5 d11S cells treated as described above. After 3 days in culture, the cells were cloned by limiting dilution. Cells from the L1210-3 clone had less residual sensitivity to Fas-based cytotoxicity than the L1210 cells used initially (3) but remained sensitive to the perforin-based mechanism (data not shown). The previously described L1210-Fas clone was obtained by transfection of L1210 with a Fas cDNA (3).

Results

TCR/CD3 Induction of Fas-based Cytotoxicity in Activated T Cell Populations. We tested whether engagement of the TCR/CD3 complex induces Fas-based cytotoxicity in MLC cells. Following the techniques used in previous reports (3, 5, 12), we used L1210-Fas target cells (sensitive to both Fas-based and perforin-based mechanisms) in the presence of EGTA-Mg++ (which prevents perforin-based lysis) to assess Fas-based cytotoxicity exclusively. d anti-b MLC cells were preincubated for 1 h either with PI as a positive control or with anti-CD3 antibodies, then tested for cytotoxicity in the presence of EGTA-Mg++. As expected, little (Fig. 1 b) or no (Fig. 1 d) cytotoxicity was detected on control L1210 target cells because L1210 cells are poorly sensitive to Fas-based cytotoxicity and EGTA-Mg++ does not allow perforin-based cytotoxicity. Fas-based cytotoxicity was detected on L1210-Fas target cells (Fig. 1, a and c). Further evidence for a perforin-based mechanism, in addition to preferential lysis of Fas-bearing target cells and to Ca++-independent lysis, was provided by the inhibition by soluble Fas-Fc molecules (6) of cytotoxicity that would account for the absence of reversal of the cycloheximide block seen in Fig. 1) or in the presence of actinomycin D (10 µg/ml; Sigma Chemical Co.) or 1 mM EGTA plus 1.5 mM Mg++. To chelate most of the extracellular Ca++ in the presence of an excess of Mg++. Target Cells. Target cells used were either the DBA/2, H-2d T cell lymphoma L1210 cells, expressing low amounts of Fas (3), or derivatives thereof. L1210-3 was obtained by repeated selection of L1210 with Fas-based d11S killer cells. 2 × 10^5 d11S cells were preactivated for 3 h by PMA (2.5 ng/ml) and ionomycin (0.5 µg/ml), incubated for 30 min in the presence of mitomycin C (75 µg/ml), and rinsed 3× in medium. These cells were mixed with 2 × 10^6 L1210 cells in 1 ml of medium, centrifuged for 1 min at 1,200 rpm, and incubated for 4 h. This cell mixture was cultured for 72 h and 5 × 10^6 of the remaining cells (most of which were L1210 cells by morphology) were incubated for another 4 h with 2 × 10^5 d11S cells treated as described above. After 3 days in culture, the cells were cloned by limiting dilution. Cells from the L1210-3 clone had less residual sensitivity to Fas-based cytotoxicity than the L1210 cells used initially (3) but remained sensitive to the perforin-based mechanism (data not shown). The previously described L1210-Fas clone was obtained by transfection of L1210 with a Fas cDNA (3).

Supplementary figure 1. TCR/CD3 induction of Fas-based T cell-mediated cytotoxicity in MLC cells, its Ca++ dependence and requirement for protein synthesis. d anti-b MLC cells were preincubated for 1 h either with PI (a and b) or on a plastic surface coated with anti-CD3 antibodies (c and d) in the presence of medium (O) EGTA-Mg++ (A), or cycloheximide (C). The subsequent 4-h cytotoxicity test was performed on L1210-Fas or on L1210 target cells in the presence of EGTA-Mg++. The cytotoxicity of these d anti-b cells preincubated without PI or anti-CD3 antibodies (at a ratio of 30:1) was 17% for L1210-Fas and 12% for L1210 target cells. Results are given as percent experimental ^51Cr release minus percent release from target cells alone, which was 9-12%.

Figure 1. TCR/CD3 induction of Fas-based T cell-mediated cytotoxicity in MLC cells, its Ca++ dependence and requirement for protein synthesis. d anti-b MLC cells were preincubated for 1 h either with PI (a and b) or on a plastic surface coated with anti-CD3 antibodies (c and d) in the presence of medium (O) EGTA-Mg++ (A), or cycloheximide (C). The subsequent 4-h cytotoxicity test was performed on L1210-Fas or on L1210 target cells in the presence of EGTA-Mg++. The cytotoxicity of these
induced by PI or by anti-CD3 antibodies (data not shown). Thus, anti-CD3 antibodies engaging the TCR/CD3 complex of MHC cells could trigger Fas-based cytotoxicity.

In addition, induction of Fas-based cytotoxicity could not take place if incubation with anti-CD3 antibodies was done in the presence of EGTA-Mg²⁺ (Fig. 1 c). Thus, TCR/CD3-mediated induction, as opposed to execution (3, 17), of Fas-based T cell–mediated cytotoxicity in activated T cell populations included a Ca²⁺-dependent stage(s). Also, Fas-based cytotoxicity was not induced if preincubation with anti-CD3 antibodies was done in the presence of cycloheximide (Fig. 1 c) or actinomycin D (data not shown), indicating that the synthesis of at least one macromolecular species was required in the effector cell for induction of Fas-based cytotoxic activity.

**TCR/CD3 Induction of Fas-based Cytotoxicity in T Cell Hybridomas.** L16.24 and L17.2 expressed similar amounts of surface CD3, while T16.19 expressed far less surface CD3 (Fig. 2). Accordingly, while cytotoxicity was induced by PI in all three hybridomas, it was induced by anti-CD3e antibodies in L16.24 and L17.2 but not in T16.19 (Fig. 3, a–c). All three hybridomas expressed similar amounts of cell surface human CD25 (Fig. 2). CD25 tagged a CD3ζ cytoplasmic domain, which, however, was wild-type for L16.24 and T16.19 and mutated on the first tyrosine residues of each ARH-1 motif for L17.2. Indeed anti-CD25 antibodies induced cytotoxicity in L16.24 and T16.19 but not in L17.2 (Fig. 3, a–c). These combinations led to a contrasted effect of anti-CD3 and anti-CD25 antibodies on T16.19 and L17.2 (Fig. 3, b and c). Under the various induction conditions used, these three hybridomas significantly lysed L1210-Fas but not L1210-3 cells (Fig. 3, d–f), indicating that the cytotoxicity thus generated was Fas based. These results show that in these hybridomas Fas-based cytotoxicity could be induced via PI, or via the TCR/CD3 complex, or via the CD3ζ chain in isolation. Induction through the latter required the tyrosine motifs previously implicated in signal transduction.

The cytotoxicity by L16.24 hybridoma cells after induction by PI, anti-CD3, or anti-CD25 antibodies was increased if EGTA-Mg²⁺ was added during the cytotoxicity test (Fig. 4, a and b). In marked contrast, it was absent if EGTA-Mg²⁺ was added during induction (Fig. 4, a and c). In this case induction was once again almost completely blocked when cycloheximide or actinomycin D was present (data not shown). Again, this cytotoxicity was essentially (if not only) Fas based because it was much more marked on L1210-Fas than on L1210 (Fig. 4, d–f). These results show that for these hybridoma cells as well, execution of Fas-based cytotoxicity was Ca²⁺ independent but its induction required extracellular Ca²⁺; from another point of view, it also required macromolecular synthesis.

**The TCR/CD3 Complex Signals Increased Expression of the Fas-L.** Fas-L transcripts, which were not or were only barely detectable in nonactivated L16.24, L17.2, and T16.19 T cell hybridomas (Fig. 5), were readily detectable in all of these hybridomas upon a 3-h incubation with PI (Fig. 5 and data not shown). Their appearance was induced by anti-CD3 antibodies in L16.24 and L17.2 much more than in T16.19, and by anti-CD25 antibodies in L16.24 and T16.19 but not in T17.2. Thus, appearance of Fas-L transcripts (Fig. 5) correlated with appearance of Fas-based cytotoxicity (Fig. 3), and they
could be similarly obtained through engagement of the whole TCR/CD3 complex or of an intact CD3 ζ chain.

Discussion

Acquisition of the ability to exert Fas-based cytotoxicity is most probably directly linked to expression of the Fas-L (6), although some contribution of other molecules, perhaps also inducible, cannot be ruled out. Expression of the Fas-L may be due to increased stability of preexisting message and/or to increased transcription. The latter would be consistent with the observed block of induction of Fas-based cytotoxicity in MLC cells and in L16.24 hybridoma cells by cycloheximide or actinomycin D. In a very similar manner, induction by PI of d1OS cytotoxicity could be blocked by cycloheximide and actinomycin D (17). Execution of Fas-based cytotoxicity did not require macromolecular synthesis (17).

Expression of the Fas-L must be tightly controlled because the presence of Fas on a number of tissues within and outside the immune system would make uncontrolled expression quite dangerous (18). The present work shows that the Fas-L, which is not constitutively expressed even on activated lymphocytes, can be swiftly induced on them by restimulation of the TCR/CD3 complex. Activated lymphocytes resulting from a 5-d MLC do not express, or no longer express, detectable levels of the Fas-L (data not shown), and correlative do not spontaneously lyse via the Fas-based pathway unless they are reactivated through their TCR/CD3. This may be obtained with anti-TCR/CD3 antibodies or in a cytotoxicity test upon engagement of the TCR with specific alloantigen-bearing target cells. A 1-h triggering of the TCR/CD3, which is enough to induce Fas-based cytotoxicity in 5-d MLC cells, is not enough to induce it in normal splenic T cells (data not shown); these cells require longer incubations with anti-TCR antibodies (19). Thus, a long or repeated schedule of activation is required to generate Fas-based cytotoxic activity. This may mimic either repeated or prolonged activation by antigen in vivo. In particular, chronic T lymphocyte activation states (such as those seen in autoimmune diseases, or perhaps in some viral diseases) are likely to induce Fas-based cytotoxicity and its eventual physiopathological consequences. The latter may have an effect on the immune system itself, since Fas is involved in its regulation (as shown by the immune dysfunctions in ipr mice; references 20–22), perhaps through Fas-based cytotoxicity (12).

Once expression of the Fas-L is induced, even within the short duration of a 4-h cytotoxicity test, by specific antigen recognition via the TCR, it would lead to Fas-based cytol-
ysis of antigen-relevant, but also eventually of antigen-irrelevant, Fas-bearing target cells. In other words, induction of Fas-based cytotoxicity is antigen-specific, while its execution may not be. This may lead to bystander cell lysis as a function of Fas expression by the bystander cells. Differences in Fas expression may account for some of the discrepancies reported in the literature as to the extent of bystander lysis.

Although the execution of Fas-based cytotoxicity is Ca$^{2+}$ independent, its induction (for instance, within the cytotoxicity test itself following antigen-recognition) requires extracellular Ca$^{2+}$ (Figs. 1 and 4). Also, addition of EGTA-Mg$^{2+}$ to known Fas-based cytotoxicity systems tends to increase $^{51}$Cr release, as shown with preinduced L16.24 hybridoma cells (Fig. 4), for reasons that are not clear to us. Thus, addition of EGTA-Mg$^{2+}$ may tend to both increase and decrease Fas-based cytotoxicity to an unpredictable final level. Clearly, while the existence of EGTA-Mg$^{2+}$-resistant cytotoxicity is a good indication of a Fas-based mechanism, the extent of this EGTA-Mg$^{2+}$-resistant cytotoxicity provides no reliable estimate of the extent of Fas-based cytotoxicity.

TCR/CD3 engagement leads to the sequential activation of protein tyrosine kinase (PTK) src family members, such as p59fyn and p56ck, to the tyrosine phosphorylation of ARH-1 tandem tyrosines, and to the subsequent recruitment and activation of tandem SH2 PTK, such as ZAP70 and p72syk (7). We show here that CD3$^+$ in isolation can induce Fas-based T cell cytotoxicity, provided there is integrity of the T hybridoma cells (Fig. 4). Also, addition of EGTA-Mg$^{2+}$ to known Fas-based cytotoxicity systems tends to increase $^{51}$Cr release, as shown with preinduced L16.24 hybridoma cells (Fig. 4), for reasons that are not clear to us. Thus, addition of EGTA-Mg$^{2+}$ may tend to both increase and decrease Fas-based cytotoxicity to an unpredictable final level. Clearly, while the existence of EGTA-Mg$^{2+}$-resistant cytotoxicity is a good indication of a Fas-based mechanism, the extent of this EGTA-Mg$^{2+}$-resistant cytotoxicity provides no reliable estimate of the extent of Fas-based cytotoxicity.

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