

## Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas

Thomas Brunner\*, Rona J. Mogil\*, Drake LaFace\*, Nam Jin Yoo\*, Artin Mahboubi\*, Fernando Echeverri\*, Seamus J. Martin\*, Walker R. Force†, David H. Lynch‡, Carl F. Ware† & Douglas R. Green\*

\* Division of Cellular Immunology, La Jolla Institute for Allergy and Immunology, 11149 North Torrey Pines Road, La Jolla, California 92037, USA  
 † Division of Biomedical Sciences, University of California, Riverside, California 92521, USA  
 ‡ Department of Immunobiology, Immunex Research and Development Corp., Seattle, Washington 98101, USA

A NUMBER of murine T-cell hybridomas undergo apoptosis within a few hours of activation by specific antigens, mitogens, antibodies against the T-cell antigen receptor, or a combination of phorbol ester and calcium ionophore<sup>1-3</sup>. This phenomenon has been extensively studied as a model for clonal deletion in the immune system, in which potentially autoreactive T cells eliminate themselves by apoptosis after activation, either in the thymus<sup>4</sup> or in the periphery<sup>5</sup>. Here we show that the Fas/CD95 receptor, which can transduce a potent apoptotic signal when ligated<sup>6,7</sup>, is rapidly expressed following activation of T-cell hybridomas, as is its functional, membrane-bound ligand<sup>8</sup>. Interference with the ensuing Fas/Fas-ligand interaction inhibits activation-induced apoptosis. Because T-cell receptor ligation can induce apoptosis in a single T hybridoma cell, we suggest that the Fas/Fas-ligand interaction can induce cell death in a cell-autonomous manner.

To address the possible role of Fas/Fas-ligand interactions in activation-induced apoptosis in T-cell hybridomas, we examined the expression of Fas and Fas-ligand in the hybridoma line A1.1, which rapidly undergoes apoptosis following activation<sup>1,3</sup>. A1.1 cells do not express cell-surface Fas unless they are activated by T-cell receptor ligation (Fig. 1a). Expression of the cell-surface Fas molecule was readily detectable at 4 h post-activation. Furthermore, expression of Fas messenger RNA after activation in these cells was optimal at ~2 h post-activation, as determined

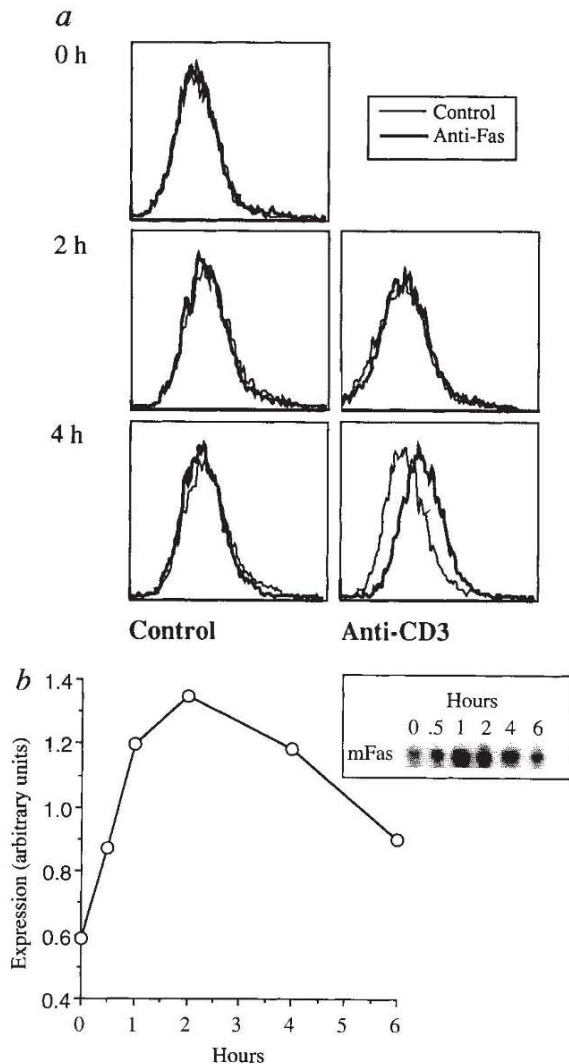


FIG. 1 Activation of T hybridoma cells induces expression of Fas. *a*, Detection of cell-surface Fas by immunofluorescence. *b*, Detection of Fas mRNA by RNase protection.

METHODS. A1.1 murine T hybridoma cells were plated ( $5 \times 10^5$  cells  $\text{ml}^{-1}$ ) on untreated or anti-CD3 (145-2C11) treated tissue culture plastic for the indicated time. For immunofluorescence, cells were washed in PBS containing 0.2% BSA and 0.02% sodium azide (wash buffer). Cells ( $5 \times 10^5$  cells  $\text{ml}^{-1}$ ) were incubated at room temperature in wash buffer containing  $2.5 \mu\text{g ml}^{-1}$  normal mouse immunoglobulin for 15 min, after which anti-Fas antibody (Jo2; Pharmingen) was added at  $2.5 \mu\text{g ml}^{-1}$  and cells were incubated for 30 min at  $4^\circ\text{C}$ . Cells were washed and resuspended in phycoerythrin (PE)-conjugated goat anti-hamster IgG,  $2.5 \mu\text{g ml}^{-1}$  (Accurate, Westbury, NY) for 30 min at  $4^\circ\text{C}$ . Cells were washed once and resuspended in PBS containing 1% formaldehyde and analysed on a FACScan (Becton Dickinson). For RNase protection, labelled RNA transcripts were prepared from murine Fas and actin cDNA cloned into Bluescript and assayed using a commercial system (Ambion). The densitometer scan of the mouse Fas signal (inset) was standardized against the actin signal to determine expression (arbitrary units).

by RNase protection (Fig. 1b) and northern hybridization (data not shown).

Similarly, functional Fas-ligand is induced following activation of A1.1 cells. The cells were examined for their ability to kill a Fas<sup>+</sup> target-cell line. Activation of A1.1 cells by T-cell receptor ligation (Fig. 2a) or phorbol ester and ionomycin (data not shown) induced DNA fragmentation in Fas-transfected L1210 cells<sup>9</sup>, but not in the Fas<sup>low</sup> parental line. Similarly, activated A1.1 cells induced DNA fragmentation in a Fas<sup>+</sup> P815 cell line and in Fas<sup>+</sup> Jurkat cells (data not shown). Another T-

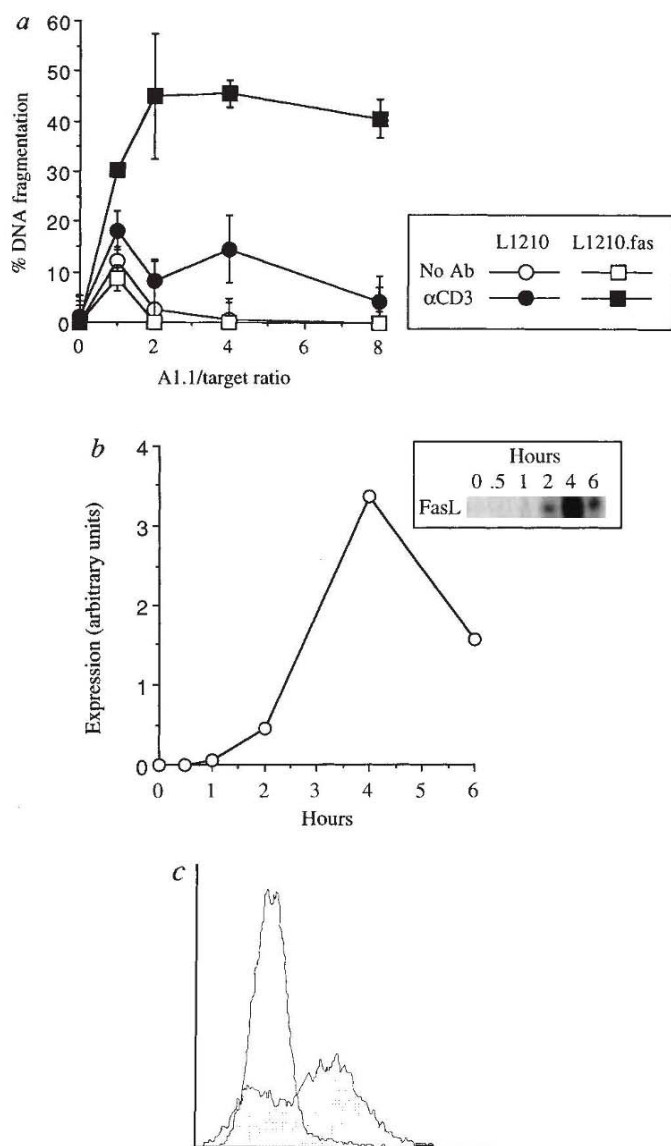


FIG. 2 Activation of T hybridoma cells induces expression of Fas-ligand. a, Induction of cytotoxic activity against Fas<sup>+</sup> target cells. b, Detection of Fas-ligand mRNA by RNase protection. c, Detection of cell-surface Fas-ligand.

**METHODS.** For assessment of activation-induced cytotoxicity, Fas<sup>low</sup> L1210 or Fas<sup>+</sup> L1210 cells<sup>9</sup> were labelled for 2 h with 5  $\mu\text{Ci ml}^{-1}$  [<sup>3</sup>H]thymidine (79 Ci mmol<sup>-1</sup>; Amersham) at 37 °C in RPMI/5% FCS. Cells were washed twice with HBSS and resuspended in RPMI/5% FCS. The target cells ( $2 \times 10^4$ ) were combined with the indicated ratio of A1.1 hybridoma cells in a total volume of 200  $\mu\text{l}$  per well in RPMI/5% FCS to untreated or anti-CD3-coated 96 well plates and incubated at 37 °C for 8 h. Unfragmented labelled high-molecular-weight DNA was collected by filtration through glass fibre filters (Pharmacia) and counted in a liquid scintillation counter<sup>29</sup>. Data are expressed as per cent DNA fragmentation:  $100 \times (1 - \text{c.p.m. in experimental group per c.p.m. of unstimulated targets only}) \pm \text{s.e.m.}$  Each data point represents the mean of quadruplicates of a representative experiment. For RNase protection, A1.1 T hybridoma cells were activated as for Fig. 1 and analysed using labelled RNA transcripts prepared from murine Fas-ligand<sup>8</sup> and actin cDNA cloned into Bluescript. The Fas-ligand signal was densitometered and standardized against the actin signal (arbitrary units, not comparable to Fig. 1). A chimaeric Fas-Fc protein was used to detect cell-surface Fas-ligand, produced as follows. A cDNA encoding the extracellular domain of Fas was isolated by PCR from the human T cell hybridoma II-23.D7. This fragment was ligated in-frame to a 710-bp cDNA fragment encoding the hinge, CH2 and CH3 domains of human IgG1 and subcloned into pCD302. For construction of the baculovirus transfer vector, a double-stranded linker was designed with an internal EcoRI site and a KpnI overhang with the sequence 5'-GAGAA-TTCGGGTAC-3' and for the bottom strand 5'-CCGAATTCTC-3'. The bottom strand was treated with T4 polynucleotide kinase before annealing the linker and subsequent ligation to the Fas-Fc KpnI/NotI fragment previously excised from pCD302. The linker added a 5'EcoRI site to the Fas-Fc fragment which allowed ligation into the EcoRI/NotI site of the baculovirus transfer vector, pVL1392 (In Vitrogen). Tn5B1-4 cells were infected with recombinant baculovirus in serum-free medium and Fas-Fc purified as described<sup>11</sup>. The resulting protein resolved as a single band by SDS-PAGE. Detection of cell-surface Fas-ligand was as follows. A1.1 cells were activated on anti-CD3-coated plastic for 4.5 h, washed and stained with Fas-Fc (25  $\mu\text{g ml}^{-1}$  for 30 min at 4 °C). Cells were washed and incubated with biotinylated rabbit anti-human IgG (Dako, Carpinteria, CA) for 30 min at 4 °C, washed, and incubated with a streptavidin-tricolour fluorescent reagent (Caltag) for 30 min at 4 °C. Cells were again washed, fixed with 1% formaldehyde in PBS and analysed using a FACScan. There was no staining in the absence of Fas-Fc (data not shown). Control (unstimulated) A1.1 cells are shown in white, activated cells in grey.

cell hybridoma, 2B4, which undergoes activation-induced apoptosis, also kills Fas<sup>+</sup> targets upon activation (data not shown). In contrast, unactivated A1.1 or 2B4 cells did not kill any of the targets tested. Thus, activation of A1.1 induces functional Fas-ligand, which in turn induces apoptosis in Fas<sup>+</sup> target cells. Activation-induced expression of Fas-ligand was confirmed using RNase protection, in which expression of Fas-ligand was rapidly induced following activation of A1.1 cells (Fig. 2b). Optimal expression was detected 4 h post-activation with anti-CD3. Activation with phorbol myristylacetate (PMA) plus ionomycin also induces expression of Fas-ligand, as detected by RNase protection (data not shown).

To detect Fas-ligand on the surface of activated T hybridoma cells, we used a fusion protein composed of human Fas and a human immunoglobulin constant region, Fas-Fc. A similar chimaeric protein was used in the purification and characterization of Fas-ligand<sup>8</sup>. Human Fas binds murine Fas-ligand<sup>10</sup>, so we expected this protein to detect Fas-ligand on the murine T hybridoma cells. Unactivated A1.1 T hybridoma cells did not stain with Fas-Fc, but staining was significant with cells that had been stimulated for 4.5 h with anti-CD3 (Fig. 2c). Similar expression was seen following activation with PMA plus ionomycin (data not shown). No staining was detected in either the unstimulated or activated cells when the secondary reagents (biotin-anti-human immunoglobulin plus fluorescent avidin) were used without Fas-Fc protein (data not shown). Thus,

although unstimulated A1.1 T hybridoma cells are negative for Fas-ligand function, mRNA or surface staining, all three are rapidly induced by activation of the cells by T-cell-receptor ligation or by phorbol ester plus ionomycin.

The expression of both Fas and Fas-ligand following activation suggested that the interaction of these molecules might be the basis for activation-induced apoptosis. To test this idea, the human Fas-Fc protein was used as a competitive inhibitor of Fas/Fas-ligand interactions<sup>10</sup>. We found that Fas-Fc effectively inhibited activation-induced apoptosis in A1.1 cells (Fig. 3), as well as in two other commonly used T cell hybridomas, DO11.10 (data not shown) and 2B4. The Fas-Fc construct inhibited the apoptotic appearance (Fig. 3a, b), the loss of intact nuclear DNA (Fig. 3c), and the DNA fragmentation associated with apoptosis (Fig. 3d). Although Fas-Fc blocked activation-induced apoptosis, production of interleukin-2 following activation was unaffected by the presence of the inhibitor (data not shown). In contrast to Fas-Fc, another fusion protein composed of the p60 receptor for tumour-necrosis factor (TNF) and the human immunoglobulin constant region (TNFR60-Fc)<sup>11</sup> had no effect on activation-induced apoptosis (Fig. 3a-d). The effects of Fas-Fc versus TNFR-Fc on activation-induced apoptosis 2B4 cells (Fig. 3c) was also observed in A1.1 cells (data not shown). Furthermore, a murine Fas-Fc chimaeric protein<sup>12</sup> likewise inhibited activation-induced apoptosis in A1.1 cells (data not shown).

In addition to apoptosis, activation of T-cell hybridomas induces inhibition of DNA synthesis and cell-cycle arrest<sup>13</sup>. We examined whether Fas-Fc would interfere with this activation-induced effect. As expected, DNA synthesis was inhibited by >60% in activated versus control cultures<sup>13</sup> (data not shown). However, whereas Fas-Fc blocked activation-induced cell death in this experiment, DNA synthesis remained inhibited to the same extent as in the activated cultures without Fas-Fc. Thus, unlike activation-induced apoptosis, activation-induced cell-cycle arrest in T-cell hybridomas does not seem to depend on a Fas/Fas-ligand interaction.

Activation of T-cell hybridomas induces expression of both Fas and Fas-ligand on the cell surface and the subsequent interaction induces apoptosis. This explains why, when activated and non-activated T-cell hybridomas are mixed, only the activated cells die<sup>2,3,13</sup>. The cells that had not been activated do not express Fas and therefore cannot receive the apoptotic signal induced by Fas-ligand. This leaves the question of whether activation-induced apoptosis is 'fratricide', in which an activated cell bearing Fas-ligand kills neighbouring activated cells bearing Fas, or 'suicide', in which these molecules can interact to induce apoptosis on a single activated cell.

We addressed this question directly by examining the fate of single cells on which the T-cell receptor was ligated. We saw no effect of cell density on the induction of apoptosis in A1.1 cells plated on anti-CD3-coated plastic (Fig. 4a). The frequency of activation-induced apoptosis among wells containing only a

single cell was not significantly different from that of wells containing cells that could potentially interact. Previous studies using larger number of cells had also suggested that activation-induced apoptosis proceeded independently of cell density<sup>14</sup>. Furthermore, we found that Fas-Fc interfered with activation-induced apoptosis in single cells (Fig. 4b). Thus, activation-induced apoptosis, proceeding through a Fas/Fas-ligand interaction, is cell autonomous and the requisite Fas/Fas-ligand interaction can proceed on a single activated cell.

It is not clear how this Fas/Fas-ligand interaction occurs on a single cell. Although production of a soluble Fas-ligand would account for this effect, we detected no Fas-ligand activity in fivefold concentrated supernatants of activated A1.1 cells using Fas<sup>+</sup> L1210 cells as targets (data not shown). Alternatively, membrane-bound ligand and receptor might come into contact during movements of the cell membrane (contact of microvilli, for example), although the possibility that this occurs within the cell is unlikely because addition of competitor (Fas-Fc) blocks activation-induced cell death.

Activation-induced apoptosis in T-cell hybridomas depends on macromolecular synthesis<sup>2,3,14</sup>, consistent with the need for *de novo* synthesis of Fas and Fas-ligand. It is inhibited by cyclosporin A<sup>1,15</sup>, glucocorticoids<sup>16,17</sup> and retinoids<sup>18,19</sup>, which might inhibit expression of the interacting molecule(s) after activation. Activation-induced apoptosis also depends on the Myc/Max heterodimer<sup>20,21</sup> and the Nur77 steroid receptor<sup>22,23</sup>, which are transcriptional regulators that might control expression of Fas

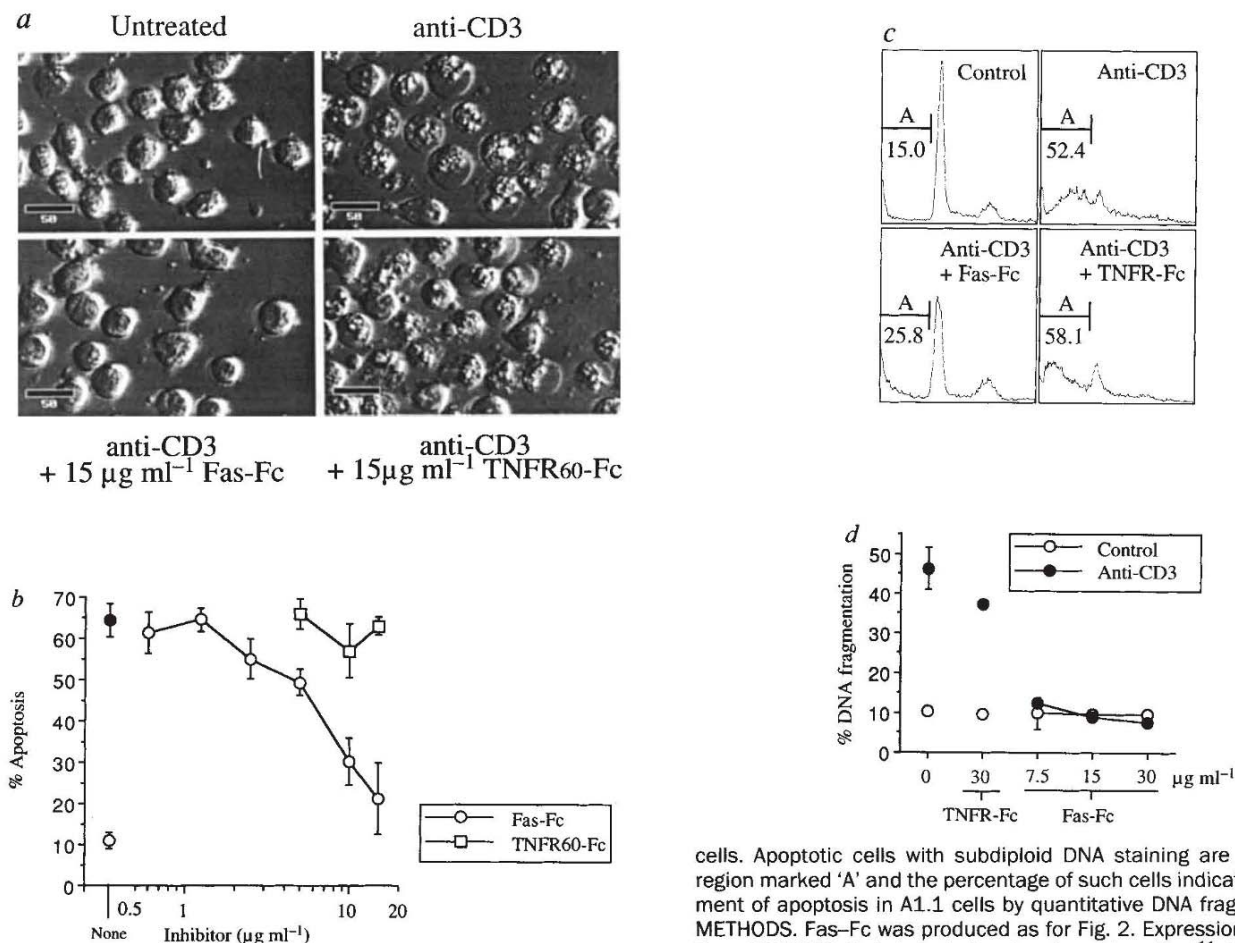


FIG. 3 Activation-induced apoptosis in T hybridoma cells is inhibited by a Fas-Fc chimaeric protein. *a*, Morphological appearance of A1.1 cells activated on anti-CD3-coated plastic in the presence or absence of Fas-Fc (15 µg ml<sup>-1</sup>) or TNFR60-Fc<sup>11</sup> (15 µg ml<sup>-1</sup>). The scale bar depicts arbitrary units. *b*, Assessment of apoptosis in A1.1 cells by acridine orange/ethidium bromide fluorescence staining. *c*, Assessment of apoptosis in 2B4 cells by propidium iodide staining of permeabilized

cells. Apoptotic cells with subdiploid DNA staining are shown in the region marked 'A' and the percentage of such cells indicated. *d*, Assessment of apoptosis in A1.1 cells by quantitative DNA fragmentation. METHODS. Fas-Fc was produced as for Fig. 2. Expression and purification of TNFR60-Fc in baculovirus has been described<sup>11</sup>. For morphological assessment of apoptosis, cells were photographed directly (*a*) or examined under ultraviolet light after staining with acridine orange/ethidium bromide<sup>30</sup>. Apoptotic cells showed extensive membrane blebbing, condensed chromatin and nuclear fragmentation. Permeabilized 2B4 cells were stained with propidium iodide for cell-cycle analysis<sup>30</sup>. A1.1 cells labelled 16 h earlier with <sup>3</sup>H-thymidine were used for quantitative DNA fragmentation<sup>20,30</sup>.

and/or Fas-ligand following activation. Many observations on activation-induced apoptosis in T-cell hybridomas need to be re-evaluated in terms of Fas and Fas-ligand expression.

Activation of proteases has been implicated in activation-induced apoptosis<sup>24</sup> and we have found that an intracellular protease substrate is cleaved rapidly during both activation- and Fas-induced apoptosis (S.J.M. and D.R.G., unpublished), suggesting that proteases act downstream of Fas ligation. Protease activation of cyclin-dependent kinases is required for at least one form of apoptosis<sup>25</sup> and activation-induced apoptosis also depends upon such kinases<sup>26</sup>. Thus, Fas ligation, protease activation, and cyclin-dependent kinase function might act in series to mediate activation-induced apoptosis.

A system has been described in which stimulation of previously activated and rested T cells induces apoptosis<sup>27</sup>, an effect that depends on functional Fas and Fas-ligand as it is not evident in T cells from Fas-defective *lpr* mice; neither is peripheral deletion of T cells following antigen stimulation *in vivo*<sup>28</sup> (R.J.M. *et al.*, submitted), so this process might also involve an activation-induced Fas/Fas-ligand interaction. Using a previously described method<sup>12</sup>, we observed that Fas-Fc blocks apoptosis induced by activation of the Th1-type CD4<sup>+</sup> T cell line RA-8: apoptosis was >60% after 18 h culture on anti-TCR $\beta$ -coated plastic (~10% in uncoated plates), and this activation-induced

apoptosis was reduced to ~24% in the presence of Fas-Fc (data not shown). Thus, in addition to its role in T-cell hybridomas, a Fas/Fas-ligand interaction seems to mediate activation-induced apoptosis in cases involving non-transformed T cells, supporting a possible immunoregulatory role for apoptosis induced by Fas during normal immune responses.

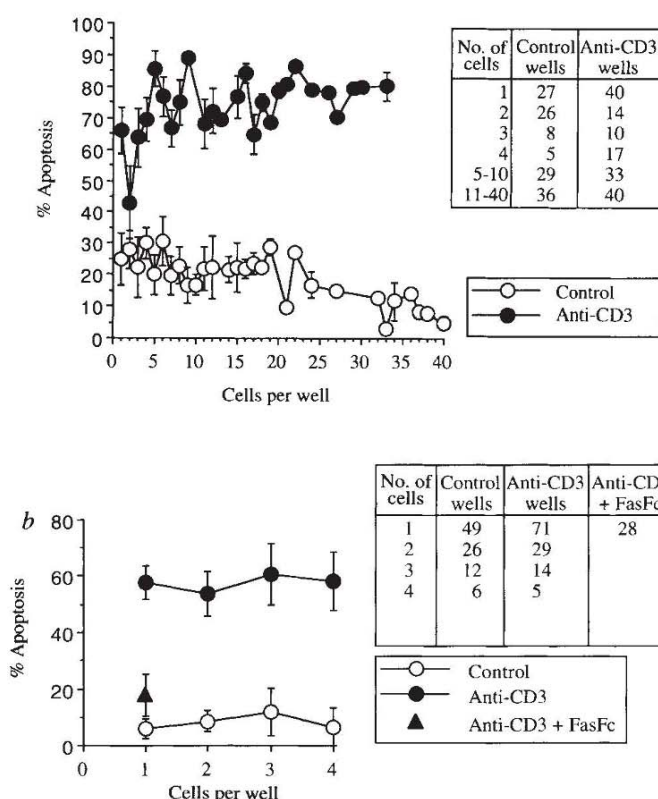


FIG. 4 Activation-induced apoptosis in T hybridoma cells is not density-dependent. *a*, Activation-induced apoptosis in A1.1 cells at a range of cell densities. *b*, Inhibition by Fas-Fc of activation-induced apoptosis in single A1.1 cells.

**METHODS.** A1.1 T hybridoma cells were plated in limiting numbers ranging from 1–40 cells per well in 10  $\mu$ l in untreated or anti-CD3-coated tissue culture microwells. Cells were cultured overnight, stained with acridine orange/ethidium bromide<sup>30</sup> *in situ* and viewed under ultraviolet on an inverted microscope. The total numbers of normal and apoptotic cells in each well were counted: the number of wells with a given number of cells is shown for each condition (inset). For inhibition with Fas-Fc, cells were plated in microwells at ~1 cell per well and counted under the microscope; Fas-Fc was added (25  $\mu$ g ml<sup>-1</sup>) to some anti-CD3-coated wells containing single cells. Wells were checked for apoptosis after culture.

- Shi, Y., Sahai, B. M. & Green, D. R. *Nature* **339**, 625–626 (1989).
- Ucker, D. S., Ashwell, J. D. & Nickas, G. J. *Immun.* **143**, 3461–3469 (1989).
- Shi, Y. *et al.* *J. Immun.* **144**, 3326–3333 (1990).
- Smith, C. A., Williams, G. T., Kingston, R., Jenkinson, E. J. & Owen, J. J. T. *Nature* **337**, 181–184 (1989).
- Kawabe, Y. & Ochi, A. *Nature* **349**, 245–248 (1991).
- Yonehara, S., Ashii, A. & Yonehara, M. *J. exp. Med.* **169**, 1747–1756 (1989).
- Trauth, B. C. *et al.* *Science* **245**, 301–305 (1989).
- Suda, T., Takahashi, T., Golstein, P. & Nagata, S. *Cell* **75**, 1169–1178 (1993).
- Rouvier, E., Luciani, M. F. & Golstein, P. *J. exp. Med.* **177**, 195–200 (1993).
- Ramsdell, F. *et al.* *Eur. J. Immun.* **24**, 928–933 (1994).
- Crowe, P. D., VanArsdale, T. L., Walter, B. N., Dahms, K. M. & Ware, C. F. *J. Immun. Meth.* **168**, 79–89 (1994).
- Ramsdell, F. *et al.* *Int. Immun.* **6**, 1545–1553 (1994).
- Ashwell, J. D., Cunningham, R. E., Noguchi, P. D. & Hernandez, D. J. *exp. Med.* **165**, 173–194 (1987).
- Green, D. R., Bissonnette, R. P., Glynn, J. M. & Shi, Y. *Sem. Immun.* **4**, 379–388 (1992).
- Mercep, M., Noguchi, P. D. & Ashwell, J. D. *J. Immun.* **142**, 4085–4092 (1989).
- Zacharchuk, C. M., Mercep, M., Chakroborty, P. K., Simons, S. S. & Ashwell, J. D. *J. Immun.* **145**, 4037–4045 (1990).
- Iwata, M., Hanaoka, S. & Sato, K. *Eur. J. Immun.* **21**, 643–648 (1991).
- Iwata, M., Mukai, M., Nakai, Y. & Iseki, R. *J. Immun.* **149**, 3302–3308 (1991).
- Yang, Y., Vacchio, M. S. & Ashwell, J. D. *Proc. natn. Acad. Sci. U.S.A.* **90**, 6170–6174 (1993).
- Shi, Y. *et al.* *Science* **257**, 212–214 (1992).
- Bissonnette, R. P., McGahon, A. & Green, D. R. *J. exp. Med.* **180**, 2413–2418 (1994).
- Liu, Z.-G., Smith, S. W., McLaughlin, K. A., Schwartz, L. M. & Osborne, B. A. *Nature* **367**, 281–284 (1994).
- Woronicz, J. D., Calnan, B., Ngo, V. & Winoto, A. *Nature* **367**, 277–281 (1994).
- Sarin, A., Adams, D. H. & Henkart, P. A. *J. exp. Med.* **178**, 1693–1700 (1993).
- Shi, L. *et al.* *Science* **263**, 1143–1145 (1994).
- Fotadar, R. *et al.* *Molec. cell. Biol.* (in the press).
- Russell, J. H., Rush, B., Weaver, C. & Wang, R. *Proc. natn. Acad. Sci. U.S.A.* **88**, 4409–4413 (1993).
- Singer, G. G. & Abbas, A. K. *Immunity* **1**, 365–371 (1994).
- Matzinger, P. *J. Immun. Meth.* **145**, 185–192 (1991).
- McGahon, A. J. *et al.* in *Cell Death* (eds Schwartz, L. M. & Osborne, B. A.) (Academic, Orlando, in the press).

**ACKNOWLEDGEMENTS.** T.B. and R.J.M. contributed equally to this work. Supported by grants from the US NIH; S.J.M. is a Wellcome Trust Fellow and T.B. is a fellow of the Swiss NSF. We thank W. Nishioka, A. McGahon, F. Ramsdell and A. Fotadar for discussion, P. Crowe and B. Walter for purification of TNFR-Fc and Fas-Fc, and S. Nagata and P. Golstein for reagents.