*Brief Definitive Report*

# Fas Transduces Activation Signals in Normal Human T Lymphocytes

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## Summary

The Fas gene encodes a cell surface molecule that is a member of the the nerve growth factor/tumor necrosis factor receptor family of proteins and can mediate programmed cell death (apoptosis) in certain transformed cell lines. To characterize further the biological function of Fas, particularly with regard to its function in normal cells, a panel of monoclonal antibodies (mAbs) was generated against the extracellular portion of human Fas. Some of these mAbs induced apoptosis in transformed cell lines expressing Fas, but only when immobilized on the culture vessel. One of the new Fas mAbs (M38) was used for studies on normal lymphoid cells and found to stimulate the proliferation of purified human T cells and thymocytes when immobilized on culture wells along with CD3 antibody. T cell proliferation induced by Fas mAb was largely interleukin 2 independent and was demonstrated to be due to a direct effect on the precursor T cell. Thus, the data demonstrate that in addition to a role in the induction of apoptosis in certain transformed cell lines, the Fas protein may also play an important role in the activation and proliferation of normal T cells.

Fas (also termed APO-1) was first described as a molecule expressed on the surface of certain cell lines that could mediate programmed cell death, termed apoptosis, when ligated by a specific mAb (1, 2). Little is known, however, about the function of Fas on normal cells. Molecular cloning of the gene encoding human Fas (huFas) revealed it to be a member of a family of related proteins that include the receptors for nerve growth factor and tumor necrosis factor, the CD27, CD30, CD40, 41BB, and OX40 antigens, and the pox virus gene product T2 which encodes a soluble protein capable of binding TNF (3–13). Of considerable interest was the recent demonstration that the autoimmune disease that develops in mice homozygous at the *lpr* gene locus is due to a defect in Fas gene expression (14). Thus, a more complete understanding of the biology of Fas may provide insight into its role in normal immune responses as well as in the generation of autoimmune disease.

For this study we generated a panel of mAbs against huFas and examined the effects of these Fas-specific antibodies on freshly isolated human T lymphocytes that could be induced to express Fas. Our data indicate the existence of a complex set of interactions mediated by Fas that may have important implications regarding the role(s) of this molecule in normal immune responses in addition to its apparent association with autoimmune disease.

## Materials and Methods

*Generation of Fas mAb.* BALB/cJ mice (The Jackson Laboratory, Bar Harbor, ME) were immunized with a purified fusion protein consisting of the extracellular domain of human Fas coupled to the constant region of human IgG1 (huFas.Fc) in Freund's adjuvant. Mice were boosted six times and spleen cells were fused with the murine myeloma 8.653 in the presence of 50% polyethylene glycol/10% DMSO in PBS followed by culture in DMEM/HAT and DMEM/HT selective media. Supernatants from positive wells were tested for the ability to bind biotinylated huFas.Fc in an ELISA and by reactivity to huFas.Fc in Western and dot blots. Hybridomas that produced Abs positive for binding to huFas.Fc but not to human IgG1 were cloned by limit dilution three times. All mAbs were determined to be of the IgG1 isotype and were purified by protein A affinity chromatography.

*Reagents.* The IgM huFas mAb, clone CH-11, was purchased from Medical and Biological Laboratories (Nagoya, Japan). Immobilized CD3 mAb OKT3 was used to costimulate peripheral blood T cells and thymocytes at 1 $\mu$g/ml. PE-conjugated CD4 and CD8 and FITC-conjugated CD25 and CD69 together with PE- and FITC-conjugated isotype matched control antibodies were purchased from Becton Dickinson & Co. (Mountain View, CA). A neutralizing rabbit antiserum generated against human IL-2 was used at a 1:500 dilution, a concentration that could totally inhibit up to 10 ng/ml of IL-2.

*T Cell Costimulation Assay.* T cells were enriched from PBMC by rosetting with 2-aminoethyl isothiouronium bromide hydrobro-

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mide–treated SRBC. Monocytes were depleted by plastic adherence for 1 h at 37°C and the resulting population of cells was >95% $CD3^+$ by flow cytometry. Thymocytes were obtained from infants undergoing corrective cardiac surgery and isolated by Ficoll density centrifugation. Peripheral blood (PB) T cells and thymocytes were cultured at $1 \times 10^5$ per well in 96-well flat-bottomed plates that had been previously coated with mAb as described above. Cultures were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air for 3 d and pulsed with 1 μCi of [$^3$H]TdR for the final 6 h of culture. Wells were then harvested and incorporated radioactivity was determined using a Matrix 96 beta counter (Packard Instrument Company, Inc., Meriden, CT).

*Cytokine Assays.* Cultures for the measurement of IL-2 production were performed in the presence of an IL-2R p55 mAb (2A3) to prevent utilization of IL-2 produced by the T cells. IL-2 levels were measured in a CTLL bioassay (15) using recombinant human IL-2 as a standard. IFN-γ and TNF-α levels were determined by ELISA, as described previously (16).

*Limit Dilution Analysis.* Analysis of the frequency of proliferating human T cell precursors was assessed in microcultures, as described previously using murine T cells (17, 18). T cells were cultured in 60-well Terasaki trays that had been precoated with OKT3 (10 μg/ml) either with or without huFas M38 mAb (10 μg/ml) overnight at 4°C. Cells were cultured in the presence of IL-2 (10 ng/ml) in a total volume of 15 μl at 1–20 cells/well. After 5 d of culture, wells were visually examined for the presence or absence of proliferating T cells using an inverted phase-contrast microscope. A well was scored positive if one or more clusters of at least three blast cells were observed or if the number of blasts present was greater than the input number. Estimates of the frequency of proliferating cell precursors were determined from the Poisson distribution relationship between the number of input cells and the percent negative wells using the minimum $\chi^2$ method (19). Clone size estimates were made by counting the number of cells per well and then adjusting for clonal overlap based upon the Poisson distribution.

*Flow Cytometry.* Cells to be analyzed for expression of T cell activation molecules were first incubated at 4°C in a blocking solution of PBS containing 2% normal rabbit serum and 2% normal goat serum to prevent nonspecific binding of mouse Ig. Cells were washed in FACS® buffer (PBS/1% FCS/0.02% sodium azide) and incubated with CD4-PE or CD8-PE in conjunction with CD25-FITC or CD69-FITC mAb for 30 min at 4°C. Quadrants were set by analysis of cells incubated with PE- and FITC-conjugated isotype matched control antibodies. Flow cytometry was performed using a FACScan® (Becton Dickinson and Co.) and data were collected on $10^4$ viable cells.

## Results and Discussion

*Fas mAbs Costimulate T Lymphocyte Activation.* The two mAbs that initially defined the Fas antigen, namely clone CH-11 and the anti-APO-1 mAbs, were identified based upon their ability to induce death of certain cell lines in vitro (1, 2). Thus, it was of interest to determine whether this was a general characteristic of all Fas-specific Abs or whether Abs selected solely by their ability to bind Fas would have different biological properties. A soluble Fas fusion protein consisting of the extracellular portion of huFas coupled to the Fc domain of human IgG1 (huFas.Fc) was used to immunize mice and generate a panel of mAbs that were reactive with huFas.Fc but not human IgG1 by ELISA (data not shown). Several of these mAbs induced apoptosis in transformed cell lines expressing Fas (such as Jurkat or U937), but only if the mAbs were immobilized on the culture vessel (data not shown).

The ability of our huFas-specific mAb to bind to freshly isolated normal human leukocytes or leukocytes cultured with mitogenic stimuli was assessed by flow cytometry. Although specific binding to neutrophils, monocytes, *Staphylococcus aureus* cowen-activated B cells, and PHA-induced T cell blasts was detected, none of these cell types were induced to undergo cytolysis when cultured with either soluble or immobilized huFas mAb (data not shown). These results support recently published data demonstrating strong expression of Fas by PHA blasts, but no adverse effect on cell viability upon exposure to huFas mAb (20). Given that mAbs against another member of the nerve growth factor receptor (NGFR)/tumor necrosis factor receptor (TNFR) family (namely CD27) have been shown to costimulate T cell proliferation (21), we questioned whether immobilized huFas mAb would costimulate T cells in conjunction with solid-phase CD3 mAb. Some, but not all, of the huFas mAbs were found to be strong costimulators of T cell proliferation with activity equivalent to, or greater than, that of IL-2. For example, huFas M38 costimulated T cell proliferation at concentrations as low as 100 ng/ml (Fig. 1 *A*). Those huFas mAbs that did costimulate T cell proliferation, including CH-11, had this activity only when immobilized and not when added to cultures in solution (Fig. 1 *B*).

Given that activated thymocytes express Fas, we assessed whether these cells could also be activated by huFas Ab. Although results of a recent study suggested that Fas can mediate lysis of normal murine thymocytes in a 4-h $^{51}$Cr-release assay (22), we found that human thymocytes were costimulated to proliferate by immobilized huFas mAbs in the presence of CD3 mAb (Fig. 1 *C*). Thus, it is possible that subsets of thymocytes may respond differentially to signals mediated by Fas. Alternatively, it is also possible that qualitatively different responses (apoptosis or activation) may be induced in thymocytes stimulated through Fas alone when compared with costimulation in the presence of CD3 antibody.

*Ligation of Fas Costimulates Enhanced Expression of T Cell Activation Molecules.* To analyze the effect of Fas mAb on human T cells in more detail we used flow cytometry to determine whether the costimulation of T cells by huFas Ab was accompanied by enhanced expression of T cell activation molecules. Two such molecules, the early activation antigen CD69 and the p55 low affinity chain of the IL-2 receptor CD25, were strongly enhanced on T cells stimulated with huFas M38 plus CD3 mAb compared with CD3 mAb alone (Fig. 2). Enhanced expression of CD25 and CD69 was detected on both $CD4^+$ and $CD8^+$ T cells. Ligation of Fas by immobilized huFas M38 in the presence of CD3 mAb also induced modest increases in expression of the adhesion molecules CD11a (LFA-1), CD18, and CD54 (ICAM-1) (data not shown).

*Role of Cytokines in Fas-mediated Costimulation of T Cells.* Two approaches were used to determine whether the activation of human T cells by Fas mAb was dependent upon soluble cytokine production. First, we assessed whether T cells costimulated with Fas mAb increased their cytokine

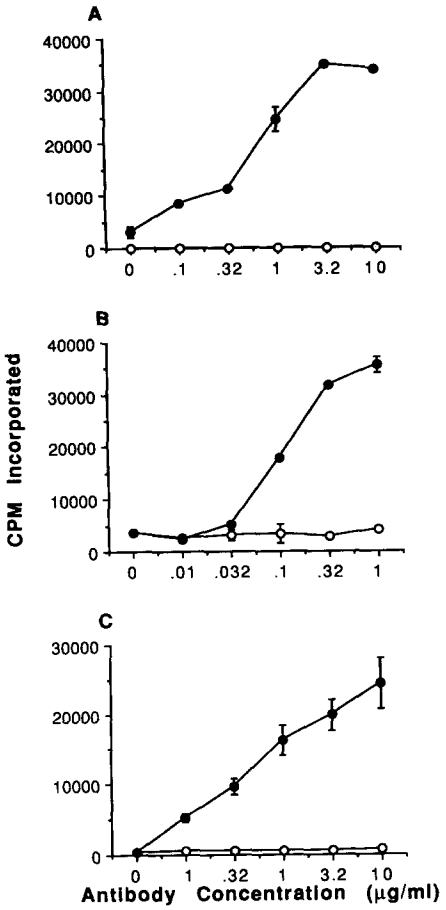


**Figure 1.** Costimulation of T lymphocyte proliferation by huFas mAb. (*A*) PB T cells were cultured with titrated concentrations of immobilized Fas M38 mAb either with (●)or without (○) immobilized CD3 mAb (1 μg/ml) for 3 d and pulsed with [$^3$H]TdR for the final 6 h. (*B*) PB T cells were cultured with immobilized CD3 mAb and titrated concentrations of either soluble (○) or immobilized (●) CH-11 Fas mAb. (*C*) Thymocytes were cultured with titrated concentrations of immobilized huFas mAb in the presence (●) or absence (○) of immobilized CD3 mAb. Data represent mean ± SD of triplicate cultures and are representative of six (*A*), three (*B*), and four (*C*) experiments performed.

**Table 1.** *Fas mAb Costimulates T Cell Cytokine Release*

| mAb Stimulus | IL-2 | IFN-γ | TNF-α |
|---|---|---|---|
| | *U/ml* | *pg/ml* | *pg/ml* |
| Nil | <0.1 | <5 | <5 |
| Fas M38 | <0.1 | <5 | <5 |
| CD3 | 0.8 | 96 | 110 |
| CD3 + Fas M38 | 6.7 | 811 | 1,318 |

Purified T cells were cultured for 48 h with various stimuli and supernatants harvested. Cultures for measuring IL-2 production were performed in the presence of an IL-2R p55 mAb to prevent utilization of IL-2 by the T cells and IL-2 levels were measured by a CTLL bioassay. IFN-γ and TNF-α levels were measured by ELISA. Data are representative of three experiments.

production. T cells stimulated with huFas M38 plus CD3 mAb were found to produce ~10-fold more IL-2, IFN-γ, and TNF-α than cells stimulated with CD3 mAb alone (Table 1). Second, T cells were costimulated with Fas mAb in the presence or absence of a neutralizing IL-2 antiserum. Although this antiserum completely neutralized the effect of exogenous IL-2 on T cell proliferation over a wide range of IL-2 concentrations (Fig. 3 *A*), it had only a minimal effect on Fas mAb costimulation (Fig. 3 *B*).

*Fas mAb Directly Costimulates Single T Cells.* To address the question of whether the T cells were the direct target of action of Fas mAb in T cell activation or whether interactions with accessory cells were required, we used limit dilution analysis in Terasaki microcultures and analyzed the frequency of T cells responsive to Fas mAb. This culture system has been successfully used to analyze the activation requirements for single murine T cells (17, 18). The linear relationship between the number of input cells and the log of the percent negative wells (Fig. 4) indicates that the precursor T cell was the limiting component in these cultures. Although the response varied with different individuals, Fas M38 con-

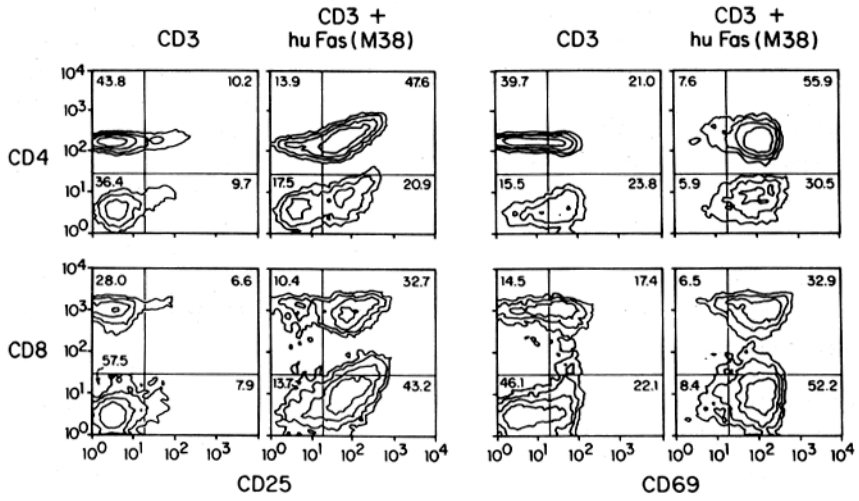


**Figure 2.** Costimulation with huFas M38 enhances CD25 and CD69 expression by CD4$^+$ and CD8$^+$ T cells. PB T cells were stimulated for 48 h with either CD3 mAb alone or CD3 mAb plus huFas M38, and analyzed by two-color flow cytometry using either CD4-PE or CD8-PE together with CD25-FITC or CD69-FITC. Data are representative of three experiments performed.

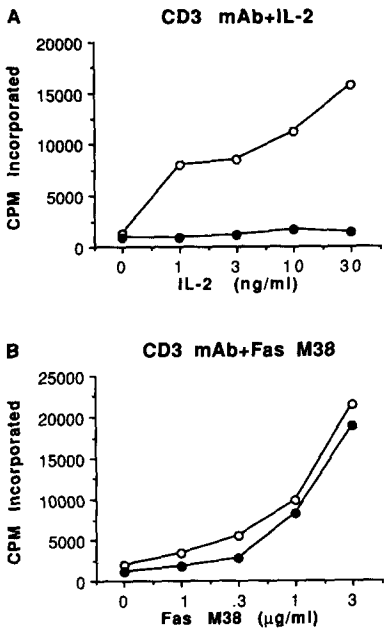


**Figure 3.** Costimulation of T cell proliferation by Fas mAb is largely IL-2 independent. Peripheral blood T cells were cultured for 72 h with CD3 mAb and either IL-2 (*A*) or Fas M38 (*B*). Cells were cultured in the presence (●) or absence (○) of a neutralizing IL-2 antiserum at a 1:500 dilution. Data are representative of four experiments performed.

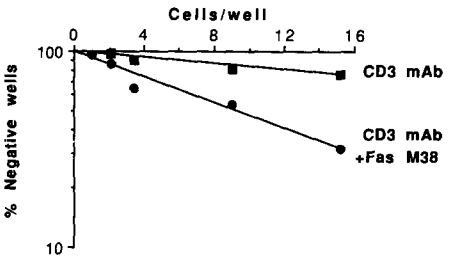


**Figure 4.** Effect of huFas M38 on the frequency of proliferating T cell precursors. Graded numbers of purified T cells were cultured in 15-μl volumes in wells precoated with either CD3 mAb alone or CD3 mAb plus Fas M38 mAb in medium containing 10 ng/ml of IL-2. After 5 d the wells were scored microscopically for proliferating T cell clones.

sistently enhanced the frequency of proliferating T cell clones by a minimum of threefold and increased the average clone size by approximately twofold. Thus, the mean frequency of proliferating T cell clones from four experiments was 16.7% (23.7 cells/clone) with CD3 plus Fas M38 compared with 1.98% (10.7 cells/clone) with CD3 mAb alone. This result indicates that the Fas mAb has a direct costimulatory effect on T cells that does not require the involvement of accessory cells or other cell types. In addition, the data suggest that the effects of Fas mAb observed in high density cultures represent an increase in the frequency of responding T cells as well as an increase in T cell clonal burst size.

The data presented here demonstrate that Fas is capable of not only inducing apoptosis in certain transformed cell lines, but can also be involved in the costimulation of freshly isolated T cells. These findings are reminiscent of the activity of TNF since this cytokine not only induces lysis of certain TNFR-expressing tumor cell lines but can also costimulate freshly isolated T and B lymphocytes (23, 24). Furthermore, although TNF induces lysis of certain monocytic cell lines, it has been reported to inhibit spontaneous apoptosis that occurs when freshly isolated monocytes are cultured in serum-free medium (25).

Fas is expressed on a variety of freshly isolated normal cells, including activated T cells, B cells, monocytes, and neutrophils. However, none of these cell types are induced to undergo apoptosis when cultured with Fas-specific mAb (20, and data not shown). Indeed, at this point only in vitro cultured cell lines have been shown to undergo cytolysis induced by Fas Ab. In normal cells the primary biological function of Fas may not necessarily involve apoptosis, but rather involve cellular activation (as demonstrated here with T cells). Thus, conversion of Fas-mediated signals from activation to apoptotic may be a component of the transformation process and represent a safety mechanism for the immune system to eliminate transformed cells from the host by stimulation through Fas. Alternatively, this signaling conversion may result from repetitive antigenic stimulation, and as such may represent a normal mechanism for controlling lymphocyte expansion/accumulation in vivo. This can be observed in vitro since long-term antigen-reactive (i.e., chronically stimulated) $CD4^+$ T cell clones are lysed when cultured with immobilized Fas mAb (data not shown). It would therefore seem prudent to suggest that the potential role for Fas in cellular activation warrants a reanalysis of the mechanism(s) of generation of autoimmune disease in mice homozygous for the *lpr* gene mutation. The defective expression of Fas in these mice may give rise to an impairment in lymphocyte activation or differentiation as well as a failure to regulate self-reactive T and B cells that in a normal animal would be prevented from accumulating in the periphery by Fas-mediated programmed cell death.

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