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Resistance to Fas-Mediated T Cell Apoptosis in Asthma

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Over activation of CD4+ T cells in the peripheral blood and airway tissues is characteristic of asthma; therefore, we investigated whether activated T cells from asthmatic subjects have altered apoptotic potential through the Fas death receptor. We found that mitogen-stimulated peripheral blood T cells of asthmatic subjects expressed cell surface Fas, but failed to undergo the normal degree of apoptosis after Fas receptor ligation. T cells from asthmatics exhibited normal apoptotic responses to γ-irradiation (dependent on IL-1 converting enzyme family proteases), ceramide, and mitogen challenge, suggesting functional integrity of the apoptotic pathway. Furthermore, the defect in Fas-dependent apoptosis was overcome by prestimulation with allogeneic accessory cells instead of mitogen. Taken together, the findings suggest that selective resistance to Fas-dependent apoptosis reflects altered Ag-driven, accessory cell-dependent signaling and that ineffective activation of Fas signal transduction may contribute to T cell-dependent immunoinflammation in asthma. The Journal of Immunology, 1999, 162: 1717–1722.

Materials and Methods

Human subjects

Healthy nonasthmatic control subjects (6 male and 4 female, aged 20–54 yr) and subjects with asthma (3 male and 14 female, aged 17–64 yr) were recruited using informed consent for a protocol approved by the University Committee for Human Research. Nonasthmatic subjects had no clinical history of airway obstruction, normal forced expiratory volume in 1 s (FEV1)3 (81–129% predicted), and normal airway reactivity to inhaled methacholine (FEV1, PC20 > 16 mg/ml). Asthmatic subjects had a clinical history consistent with intermittent and reversible airway obstruction, mean FEV1 of 85% predicted (range, 42–114%), and hyperreactivity to inhaled methacholine (FEV1, PC20 = 1.51 ± 0.41 mg/ml; range, 0.06–5.40 mg/ml). Two subjects were being treated with inhaled and oral glucocorticoids (GCs) at the time of study. Positive skin test reactivity to a panel of allergens (house dust, trees, grasses, fungi, and dog and cat dander) was present in 14 of the asthmatic subjects and 5 of the control subjects. For GC-withdrawal experiments, six asthmatic subjects were treated with inhaled triamcinolone (1600 μg/day for 30 days) before the first assessment of T cell function. Triamcinolone treatment was discontinued and subjects were monitored for an additional 6 wk or until peak expiratory flow had decreased by 20% at which time a second assessment of T cell function was obtained.

T cell culture

PBMCs were isolated using Ficoll-Hypaque (Pharmacia, Piscataway, NJ), and placed at 5 × 10^6 cells/ml/10-mm well in RPMI 1640 supplemented with 7.5% FBS, l-glutamine, nonessential amino acids, sodium pyruvate, 2-ME, and penicillin/streptomycin. Isolated cells exhibited 100% viability by trypan blue exclusion and no detectable contamination by granulocytes or erythrocytes. The protein form of PHA (PHA-P) (10 μg/ml) was added to the medium on day 0, and IL-2 (50 U/ml) was added on day 1 and then every 3 days. Cell cultures were maintained at a density of 1–2 × 10^6 cells/ml and were harvested at weekly intervals, each 3 days after the last addition of IL-2. For allogeneic stimulation, PBMCs (5 × 10^6/ml) were stimulated with γ-irradiated (20 Gy) allogeneic PBMCs (1 × 10^6 cells) from other nonasthmatic or asthmatic subjects. The next day cell cultures were diluted with medium (1:1, v/v) containing IL-2 and maintained for 2 wk as described above.

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Fas activation

T cells were placed into 10-mm wells (3 × 10^5 cells/0.5 ml/well) with medium alone or with media containing 2 µg/ml of mouse anti-Fas IgM-mAb CH-11 (Upstate Biotechnology, Lake Placid, NY) or IgGl-mAb DX-2 (PharMingen, San Diego, CA) as well as control IgM- or IgGl-mAb for 18 h at 37°C. For IgGl mAbs, wells were precoated with goat anti-mouse IgG.

Apoptosis assays

For flow cytometry, cell samples were resuspended in PBS containing 50 µg/ml propidium iodide (PI; Boeringer Mannheim, Indianapolis, IN) for 15 min to achieve maximal staining and then were analyzed for cell size (forward-angle light scatter), density (side-angle light scatter), and membrane integrity (PI exclusion) using an Epics Elite (Coulter, Hialeah, FL) or FACSCalibur (Becton Dickinson, Mountain View, CA) flow cytometer as described previously (12, 13). PI staining vs forward-angle light scatter was used to calculate the percentage of live cells in each sample based on 30,000 events. Phosphatidylserine externalization was detected by labeling cells with 1-palmitoyl-[6-[7-nitro-2–1,3-benzoxadiazol-4-yl]amino]caproyl]-sn-glycero-3-phosphoserine (NBD-PS; Avanti Polar Lipids, Alabaster, AL) and monitoring the level of this fluorescent phospholipid analogue on the cell surface by flow cytometry as described previously (14). To assess nuclear morphology, 1 × 10^6 cells were spun onto a microscope slide, fixed in methanol, and incubated with PBS containing Hoechst dye no. 33342 (10 µg/ml, Molecular Probes, Eugene, OR) for 10 min at 25°C. Slides were rinsed in PBS and mounted in Vectashield (Vector Laboratories, Burlingame, CA) for fluorescence microscopy. To assess DNA fragmentation, cells (2 × 10^5 cells/condition) were lysed, and the DNA was subjected to electrophoresis in a 1.8% agarose gel containing ethidium bromide as described previously (15).

T cell phenotyping

Levels of Fas and other cell surface proteins were determined by flow cytometry using T cells cultured from nonasthmatic and asthmatic subjects. For Fas levels, cell samples (2 × 10^5 cells/condition) were incubated with anti-Fas mAb DX-2 (2 µg) followed by FITC-labeled goat anti-mouse Ab (1:150) or phycoerythrin (PE)-conjugated anti-Fas mAb (Becton Dickinson) in PBS containing 0.2% BSA and 0.01% sodium azide for 20 min at 4°C. For other T cell markers, cells were labeled with mAbs to CD2, CD3, CD4, CD8, CD25, CD45RO, or HLA-DR either as FITC- or PE-conjugated or as unconjugated followed by secondary labeled Ab (Becton Dickinson). Labeled cells were fixed in 1% paraformaldehyde and analyzed by flow cytometry to determine the percentage of positive cells in each sample based on 10,000 events in live cell gate/condition and corrected for background detected with isotype-matched control for primary mAb.

Results

Asthmatic and healthy nonasthmatic control subjects donated PBMCs that were subjected to Fas activation and flow cytometric analysis of apoptosis. Freshly isolated PBMCs, incubated with saturating concentrations of anti-Fas mAb, did not exhibit increased apoptosis; this is consistent with the requirement of the Fas system for precisely coordinated and prolonged T cell activation (16). Thus, in normal subjects and in asthmatic subjects with stable disease, the percentage of activated T cells is low (5–7) and the percentage with the precise activation characteristics required for Fas sensitivity is likely even lower. In fact, even in experiments with cells obtained by bronchoalveolar lavage, we observed only minimal Fas responsiveness of freshly isolated T cells from normal or asthmatic subjects (M.O. and M.J.H., unpublished observations). Each of these results (no significant Fas responsiveness in freshly isolated blood T cells and minimal responsiveness in freshly isolated bronchoalveolar T cells) has also been reported by others using normal and sarcoidosis subjects (17). Apparently, apoptosis of a minor Fas-sensitive T cell population is not readily detected in a freshly isolated and therefore heterogeneous PBMC or bronchoalveolar cell preparation. Accordingly, we established primary T cell cultures stimulated initially with the T cell mitogen PHA and maintained in IL-2-containing media and then subjected the cultures at weekly intervals to anti-Fas mAb treatment and assessment of apoptosis by cell size, density, and dye exclusion during DNA fragmentation, cells (2 × 10^5 cells/condition) were lysed, and the DNA was subjected to electrophoresis in a 1.8% agarose gel containing ethidium bromide as described previously (15).

\begin{align*}
\text{Values for live cells were derived from the lower right quadrant of cytograms. Values for Fas-inducible cell death } &= \text{[(no. live cells with no mAb } - \text{ no. live cells with mAb) } / \text{no. live cells with no mAb)] and represent mean } \pm \text{ SE for 10 subjects/group } (*, p < 0.05). \\
\text{Levels of live cells } (left \text{ graph}) \text{ and corresponding increase in cell death above background } (right \text{ graph}) \text{ in T cell cultures from nonasthmatic (NA) and asthmatic (A) subjects after treatment with no mAb (open bars), control mAb (shaded bars), or anti-Fas mAb (filled bars). }
\end{align*}

FIGURE 1. Defective Fas-mediated apoptosis in activated T cell cultures from asthmatic subjects assessed by PI staining and flow cytometry. a, Representative cytograms from a nonasthmatic control and an asthmatic subject. T cell cultures were established from PHA-stimulated PBMCs, maintained in IL-2 for 2 wk, and then treated with anti-Fas or isotype-matched control mAb. Cell samples were then resuspended in PBS containing PI and analyzed for red fluorescence vs forward-angle light scatter. Quadrant settings were based on cellular autofluorescence, and the value in each quadrant represents the percentage of cells in that quadrant (lower right quadrant contains live cells). b, Levels of live cells (left graph) and corresponding increase in cell death above background (right graph) in T cell cultures from nonasthmatic (NA) and asthmatic (A) subjects after treatment with no mAb (open bars), control mAb (shaded bars), or anti-Fas mAb (filled bars). Values for live cells were derived from the lower right quadrant of cytograms. Values for Fas-inducible cell death = [(no. live cells with no mAb } - \text{ no. live cells with mAb) } / \text{no. live cells with no mAb)] and represent mean } \pm \text{ SE for 10 subjects/group } (*, p < 0.05). \\
\text{c, Levels of live cells } (left \text{ graph}) \text{ and corresponding increases in cell death } (right \text{ graph}) \text{ in T cell cultures from asthmatic subjects with } (+) \text{ and without } (-) \text{ GC treatment using the same methods. Values represent mean } \pm \text{ SE for six subjects.}
flow cytometry. In T cell cultures from normal subjects, maximal levels of Fas-dependent cell death were achieved at 2 wk, and Fas-dependent apoptosis was accompanied by characteristic decreases in cell size and dye exclusion as assessed by flow cytometry (Fig. 1, a and b). By contrast, T cell cultures from asthmatic subjects exhibited an invariant defect in Fas-dependent apoptosis throughout the culture period of 4 wk (Fig. 1, a and b).

Fas resistance in asthma was not dependent on treatment with GCs (because only some subjects were using these agents at the time of study). Furthermore, removal from treatment under a standardized GC-withdrawal protocol did not render the cells sensitive to Fas-mediated apoptosis (Fig. 1c). Taken together, these data indicate that the observed resistance to Fas-induced cell death is a stable phenotype in cultures of T cells from asthmatics and is not dependent on concomitant treatment with GCs. Initial data also indicated that Fas resistance did not appear to depend on atopy (because atopic and nonatopic subjects were present in nonasthmatic and asthmatic groups). Thus, we detected Fas resistance in three of three nonatopic asthmatic subjects. However, a larger sample size will be required to be certain that Fas resistance in asthma is not dependent on atopy.

Fas resistance in asthma was also observed when apoptosis was determined by additional assays of apoptosis. Thus, assays of phosphatidylserine externalization (using a phospholipid analogue and flow cytometry), nuclear morphology (using a DNA-binding dye and fluorescence microscopy), and DNA degradation (using gel electrophoresis), each confirmed that Fas-dependent T cell apoptosis was decreased in T cells from asthmatic vs control subjects (Fig. 2 and data not shown).

To begin defining the nature of the defect in Fas signaling, cultured-T cells were also subjected to analysis of cell surface levels of Fas receptor. We found no significant difference between the mean levels of Fas+ T cells or the mean level of Fas/T cell in cultured-T cell populations from nonasthmatic vs asthmatic subjects (Fig. 3 and data not shown). In particular, asthmatic subjects with normal levels of Fas still exhibited a marked decrease in Fas-dependent apoptosis. Asthmatic subjects had 23% lower levels of Fas+ T cells during treatment with GCs, but this treatment (as noted above) was also unrelated to Fas resistance. The poor correlation between Fas-dependent apoptosis with Fas levels is consistent with the observation that Fas levels are not predictive of biologic responsiveness (18).

FIGURE 2. Defective Fas-mediated apoptosis in activated T cell cultures from asthmatic subjects assessed with a phospholipid analogue and flow cytometry to detect: (a) phosphatidylserine externalization, (b) a DNA-binding dye and photomicroscopy to detect altered nuclear morphology, and (c) gel electrophoresis to detect DNA fragmentation. a, Representative cytograms from a nonasthmatic control and an asthmatic subject. T cell cultures were established and treated with anti-Fas or isotype-matched control mAb as described in Fig. 1. Cell samples were then resuspended in PBS containing NBD-PS and analyzed for fluorescence vs forward-angle light scatter. Quadrant settings were based on cellular autofluorescence, and the value in each quadrant represents the percentage of cells in that quadrant (upper right quadrant contains NBD-PS+ live cells). b, Representative photomicrographs from a control and an asthmatic subject. T cell cultures were again treated with anti-Fas or control mAb, and cell samples were prepared by cytocentrifugation and then stained with DNA-binding fluorescent dye (Hoechst 33342). Arrows indicate condensed and fragmented nuclei (apoptotic bodies). c, Representative gel electrophoresis from a control and an asthmatic subject. T cell cultures were untreated (no Ab) or treated with control mAb (IgM) or anti-Fas mAb (Fas Ab), and cellular DNA for each condition was subjected to electrophoresis in a 1.8% agarose gel containing ethidium bromide. Lane 1 in each gel contains DNA size standards (Std). All examples are representative of at least three subjects.
To further determine whether altered ability of asthmatic T cells to undergo Fas-mediated apoptosis is due to improper T cell activation or the selection of an abnormal subset of T cells, we analyzed the cell surface expression of activation-associated and phenotypic T cell markers. Expression of other activation markers as well as T cell phenotype was similar for asthmatic and nonasthmatic subjects (Fig. 3). In particular, we found the usually low percentage of CD4+CD8+ T cells in asthmatic and nonasthmatic subjects in freshly isolated (19), and cultured T cells (Fig. 3), in contrast to the increase in double-negative T cells found in association with Fas mutations (10, 11).

The observed defect in T cell apoptosis appeared independent of mitogen-stimulated T cell proliferation and specific for the Fas-dependent pathway. Thus, initial PHA stimulation causes equivalent proliferative responses in T cell cultures from asthmatic and nonasthmatic subjects (data not shown). In addition, other inducers of apoptosis such as γ-irradiation caused the same degree of apoptosis in T cells cultured from asthmatic and nonasthmatic subjects (data not shown). Ceramide-induced apoptosis was not prevented by z-VAD-FMK or z-DEVD-FMK (Fig. 4), consistent with observations by others (23) and us (24) for cell lines. Taken together, Fas resistance in asthmatic vs nonasthmatic subjects, we found that ceramide treatment resulted in a similar degree of apoptosis for T cells taken from both groups of subjects (Fig. 4). Concentration-response relationships for ceramide-induced apoptosis (using 1–100 μM of C2-ceramide) were also similar for T cells taken from asthmatic and control subjects (data not shown). Ceramide-induced apoptosis was not prevented by z-VAD-FMK or z-DEVD-FMK (Fig. 4), consistent with observations by others (23) and us (data not shown) using T cell lines. Taken together, Fas resistance in asthmatic T cells did not appear to be due to T cell defects in Fas expression, caspase activities, or ceramide-driven events and so more likely may represent a defect in initial triggers leading to Fas activation.

Accordingly, we tested whether initial mitogen stimulation of T cells by PHA in the presence of accessory cells and the consequent defect in Fas-triggered apoptosis indicated an underlying defect in accessory cell action. Initial evidence of this possibility was obtained when rechallenge with PHA (2 wk later when accessory cells were depleted; Ref. 3) caused equivalent apoptosis in T cells cultured from asthmatic and nonasthmatic subjects (Fig. 5).
greater potency of nonself over mitogenic stimulation. c, consistent with allogeneic over PHA stimulation (Fig. 5).

We also note that asthma is characterized by a relative increase in Fas and a marked increase in IL-2R (CD25) expression and an apoptotic response similar to normal subjects (data not shown). The defect in Fas-dependent apoptosis in asthmatic subjects may be due to altered T cell or accessory cell capacity. Based on these observations, Fas resistance in asthmatic subjects may be due to altered T cell or accessory cell capacity.

Restoring Fas-mediated apoptosis in T cell cultures from asthmatic subjects by activation with allogeneic accessory cells. Based on these observations, Fas resistance in asthmatic subjects may be due to altered T cell or accessory cell capacity. Based on these observations, Fas resistance in asthmatic subjects may be due to altered T cell or accessory cell capacity.

**Discussion**

The present data demonstrate that asthma is associated with defective activation of T cell apoptosis through the Fas death receptor. The defect in Fas-dependent apoptosis appears specific and distinct because: 1) other activators of apoptosis (PHA and γ-irradiation) act normally; 2) ICE activation and ceramide action appear normal; 3) and the defect is overcome by T cell stimulation with allogeneic accessory cells. Based on these observations, Fas resistance in asthmatic subjects may be due to altered T cell or accessory cell capacity for activation of Fas-dependent events. Thus, subtle T cell alterations in Fas function, signaling (by Daxx, or FADD-dependent pathways (24), or dampening (by Bcl-xL (25), FAP-1 (26), sentrin (27), viral (28) or nonviral (29, 30) FLIPs) as well as a defect in accessory cell capacity to activate Fas-dependent events may underlie the observed Fas resistance in asthmatic subjects. Other deficiencies in accessory cell function (e.g., decreased IL-12 production; Ref. 31) may support a more general but distinct defect in accessory cell regulation of T cell function in asthma. In that context, asthma may share a propensity for decreased IL-12 production and a Th2-cytokine profile (32) but does not manifest preferential expansion of CD4+ CD8+ T cells or autoimmunity, all abnormalities that are characteristic of genetic defects in Fas or Fas ligand (1, 10, 11). We do not yet have direct evidence that resistance to Fas-mediated apoptosis in cultured T cells precisely reflects the T cell behavior in vivo. However, any defect that limits T cell apoptosis in asthma could serve to selectively amplify immune cell accumulation at an airway site in which Ag-specific T cells are then rendered competent for unrestricted autocrine and paracrine activities. The manifestation of the defect in the airway likely relates to this site as one that is commonly exposed to allergen and even normally operates at a lower level of efficiency to protect against allergic sensitization (33).

In addition to Fas-induced apoptosis, we note that T cell cultures from normal control and asthmatic subjects exhibit a significant basal level of cell death (based on PI staining). At least some of this basal effect is due to nonspecificity of PI staining. These characteristics of T cell culture and PI staining lead us and others to use a definition of specific cell death based on the absence of PI staining and normal forward-angle light scatter as well as the analysis of data for percentage increases in cell death above background levels (as noted above and in Ref. 12). The basis for basal cell death during culture is uncertain, but there is no evidence that it is mediated by the Fas system. Thus, Fas-mediated death normally requires expression of Fas ligand, and ligand is nearly undetectable under these control culture conditions (see Fig. 5).

The precise molecular basis for resistance to Fas-mediated apoptosis in asthma remains uncertain, but our results address several possibilities. Thus, as noted above, it appears that decreased levels of Fas receptor do not account for resistance, but an alteration in receptor signaling remains a possible mechanism. Other work suggests that Fas signaling may differ when triggered by anti-Fas mAb vs the different forms of Fas ligand (34). In that regard, our initial experiments indicate the same profile of apoptotic responsiveness with soluble Fas ligand as with anti-Fas mAb in T cell cultures from nonasthmatic and asthmatic subjects, (S.J. and M.J.H., unpublished observations). However, it is possible that membrane and soluble forms of Fas ligand may exert differential effects on Fas signaling in peripheral blood T cells (34), and this possibility as well as other features of the Fas receptor complex still need to be defined.

Nonetheless, the capacity of Fas ligand to trigger T cell death implies a role for the Fas/Fas ligand system in controlling the endogenous T cell response to allergen stimulation. In the context of the asthma, it would therefore be useful to analyze Fas-mediated apoptosis in response to naturally occurring allergens in addition to the present use of more generic T cell activation by PHA or al-loantigen. An assessment of allergen-induced apoptosis would require isolation of allergen-specific T cell clones from asthmatic subjects as well as from a control group without asthma, such as those with allergic rhinitis. Our initial results suggest that resistance to Fas-mediated apoptosis may be lost during long-term culture and repeated stimulation required for T cell cloning (S.J. and M.J.H., unpublished observations), but additional work will be needed to more fully address this question. In that same context, we also note that asthma is characterized by a relative increase in...
CD4+ T cells with a Th2-type cytokine profile (6). More recent work with highly polarized CD4+ T cell clones, derived from transgenic mice, provides evidence of preferential Fas-mediated Ag-stimulated apoptosis in Th1 vs Th2 effector subsets (35). Thus, a skewed Th2-type profile in asthma may also reflect the observed defect in T cell apoptosis because T cells from asthmatics are in an environment in which the Fas system is less active and so may preferentially eliminate the more susceptible Th1 cells. As was the case for allergen stimulation experiments, preferential sensitivity of Th subsets may also be best addressed by generating T cell clones with a homogenous Th1 or Th2 cell phenotype.

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References


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