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Human Lymphocyte Apoptosis after Exposure to Influenza A Virus

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Infection of humans with influenza A virus (IAV) results in a severe transient leukopenia. The goal of these studies was to analyze possible mechanisms behind this IAV-induced leukopenia with emphasis on the potential induction of apoptosis of lymphocytes by the virus. Analysis of lymphocyte subpopulations after exposure to IAV showed that a portion of $CD3^+$, $CD4^+$, $CD8^+$, and $CD19^+$ lymphocytes became apoptotic (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling positive). The percentage of cells that are infected was shown to be less than the percentage of apoptotic cells, suggesting that direct effects of cell infection by the virus cannot account fully for the high level of cell death. Removal of monocytes-macrophages after IAV exposure reduced the percent of lymphocytes that were apoptotic. Treatment of virus-exposed cultures with anti-tumor necrosis factor alpha did not reduce the percentage of lymphocytes that were apoptotic. In virus-exposed cultures treated with anti-FasL antibody, recombinant soluble human Fas, Ac-DEVD-CHO (caspase-3 inhibitor), or Z-VAD-FMK (general caspase inhibitor), apoptosis and production of the active form of caspase-3 was reduced. The apoptotic cells were Fas-high-density cells while the non-apoptotic cells expressed a low density of Fas. The present studies showed that Fas-FasL signaling plays a major role in the induction of apoptosis in lymphocytes after exposure to IAV. Since the host response to influenza virus commonly results in recovery from the infection, with residual disease uncommon, lymphocyte apoptosis likely represents a part of an overall beneficial immune response but could be a possible mechanism of disease pathogenesis.

Influenza virus has been shown to induce apoptosis in tissue culture cells (18, 43) and in peripheral blood monocytes (14, 19). A depletion of lymphocytes due to apoptosis has also been described in mice infected with a highly virulent influenza A virus (IAV) (H5N1) isolated from humans (46). The immunopathological mechanisms and the role played by the virus infection of leukocytes with respect to disease pathology in general and leukocyte death in particular have not been elucidated. An early lymphopenia has been described in IAV-infected patients (7, 10, 24), and inoculation of humans with IAV has been shown to cause a decrease in both T- and B-cell numbers during illness (7, 10). In the experimental infections, volunteers developed a severe T-cell lymphopenia and a moderate B-cell lymphopenia even though seroconversion occurred in 90% of the volunteers, suggesting that T- and B-cell functions were preserved (10, 12). This observed lymphopenia could be the result of cell migration from the circulation and/or cell death caused by necrosis or by apoptosis or through suppression of hematopoeisis.

Fas and FasL have been shown to play a role in the induction of apoptosis of activated mature T cells at the culmination of an immune response (21, 32) and in the killing of virus-infected or neoplastic cells by cytotoxic T cells (48). One of the best-characterized death receptors, Fas (CD95) is a 48-kDa transmembrane glycoprotein belonging to the tumor necrosis factor (TNF) receptor family (29, 31, 32). Fas has been shown to be involved in the induction of apoptosis when cross-linked with anti-Fas antibodies (21, 49) or Fas ligand (FasL) (42). FasL is a 40-kDa TNF family member protein that induces apoptosis by binding to Fas, its cell surface receptor. FasL expression on cytotoxic T cells can induce cytolysis of target cells expressing Fas (26, 42). Resting monocytes-macrophages express a low level of Fas receptor but no FasL. Once activated, these cells express increased Fas as well as FasL, which is rapidly expressed after mobilization from presynthesized stores (26). It has been suggested that monocytes-macrophages can trigger apoptosis in other types of cells by regulated expression of FasL on their cell surface and by release of soluble FasL (5).

Apoptosis signal transduction and induction is associated with the coordinated action of a series of caspases (aspartate-specific cystein proteases) (13, 23, 40, 45). Following binding of Fas to FasL, trimerization of Fas recruits the Fas-associated death domain (FADD) through interactions of Fas and FADD. This step is followed by caspase-8 binding, and interactions between FADD and caspase-8 result in the activation of caspase-8. Activation of caspase-8 initiates the activation of a cascade of caspases including caspase-3 (22, 23, 28). Caspase-3 activities have been shown to control both the cytoplasmic and nuclear events associated with Fas-mediated apoptosis (51).

In this study we analyzed apoptosis and expression of Fas (CD95), FasL, and the active form of caspase-3 by peripheral blood mononuclear leukocytes (MNL) that were exposed to IAV. We determined that apoptosis does occur in cells exposed to IAV, and we present data suggesting a role for Fas-FasL-mediated induction of apoptosis in peripheral blood lymphocytes.

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MATERIALS AND METHODS

Virus stocks. Influenza A/AA/Marton/43 (H1N1) virus was grown in allantoic cavities of 10-day-old embryonated hen's eggs. The allantoic fluid was pooled after collection and frozen at −70°C until titered to 10^7 or 10^8 when assayed in Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection, Rockville, Md.) or used for exposure of MNL (37). For sham exposures, allantoic fluid from uninfected eggs was collected, pooled, and frozen at −70°C until used. Influenza A virus strains A/Bethesda/85 (H3N2) (wild type, termed wt A/Bethesda) and A/Ann Arbor/6/60 × A/Bethesda/85 (cold adapted, termed ca A/Bethesda) were a kind gift of Brian Murphy (National Institute of Allergy and Infectious Diseases, Bethesda, Md.). Heat-inactivated A/AA/Marton/43 was prepared by incubation for 1 h at 56°C.

Collection of MNL and exposure to virus. Informed consent for withdrawal of blood was obtained from all donors. Donors ranged in age from 18 to 45 years. Equal numbers of male and female subjects were used as volunteer donors. It was expected that all donors had experienced past in vivo exposure to IAV.

MNL were obtained from the peripheral blood of healthy human volunteers by Ficoll-Hypaque sedimentation (Pharmacia) (3). Cells were counted and viability was determined (i) by the ability to exclude trypan blue under light microscopy or (ii) by the use of propidium iodide with analysis by fluorescent microscopy and flow cytometry.

MNL were exposed or sham exposed to influenza virus at a multiplicity of infection of 1 for 1 h at 37°C in serum-free RPMI 1640 supplemented with 2 mM glutamine, 100 U of penicillin G, and 100 μg of streptomycin/ml (37). For sham exposures, cells were exposed to a volume of chicken allantoic fluid equal to that used for virus infections. After 1 h of exposure or sham exposure to virus, the MNL or purified subpopulations of cells were washed in warm medium, centrifuged, and reincubated at 37°C in medium supplemented with 10% heat-inactivated defined fetal calf serum (Hyclone).

Monocytes-macrophages were depleted from cultures by adherence after 1 h of serum-free cell culture followed by staining of residual monocytes-macrophages with anti-CD14 antibody and gating for cell sorting on CD14-negative cells. The sorted cells were collected, resuspended in warm culture medium, and reincubated at 37°C in medium supplemented with 10% heat-inactivated defined fetal calf serum (Hyclone).

Flow cytometry analyses. Acquisition, cell sorting, and analysis were done using a FACSort (Becton Dickinson). For analyses of lymphocytes or monocytes-macrophages, forward versus side light scatter was used for gating. Sorting based on cell phenotype was performed by gating on the fluorescent population to be collected, or for monocyte-macrophage depletion experiments, gates for cell collection were set after CD14 staining (with gating on unstained, non-$CD14^+$ cells). Acquisition and analysis were performed using Cellquest software (Becton Dickinson). Calibration of the equipment for validation of the logarithmic linearity required for the estimation of the number of molecules of CD95 was accomplished by using Spherotech Rainbow particles (Spherotech). Quantitation of surface molecule expression was done using Quanticalc Software (Becton Dickinson).

Staining for analyses of cell phenotype. Phenotypes of cells were determined by using monoclonal antibodies (MAbs) to identify monocytes-macrophages ($CD14^+$) or $CD3^+$, $CD4^+$, $CD8^+$, and $CD19^+$ lymphocyte subsets (Becton Dickinson). Antibodies were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll protein (PerCP), and corresponding immunoglobulin G (IgG)-matched isotype control antibodies were used to set baseline values for analysis markers. For surface staining, appropriate concentrations of a single MAb, combinations of multiple MAbs, or IgG-matched control antibodies were mixed with cells and treated as described by the manufacturer (Becton Dickinson or BD Pharmingen). After fixation in 2% paraformaldehyde (PAF), cells were stored at 4°C until fluorescent microscopy and/or flow cytometric analysis was performed.

Analyses of cell apoptosis. Apoptosis was determined by quantitation of DNA strand breaks using the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay (In Situ Cell Death kit; Boehringer Mannheim). The cell phenotype or activation state was established by cell surface labeling with MAbs prior to the TUNEL reaction. After being stained, cells were fixed in 2% PAF and permeabilized for 10 min using 0.6% *n*-octyl glucopyranoside (Sigma Chemical Co.). The TUNEL reaction was carried out as described in the manufacturer's instructions. In a subset of experiments, the apoptosis was confirmed after cell sorting by demonstration of DNA fragmentation resulting in the presence of bands of 180 bp or multiples thereof, forming a characteristic ladder effect on gel electrophoresis (15).

Analyses of viral protein production. Cells were stained for surface expression of CD3, CD4, or CD8 prior to staining for viral protein expression. After fixation in 2% PAF, the cells were permeabilized for 10 min in 0.6% *n*-octyl glucopyranoside (Sigma Chemical Co.). Expression of influenza virus proteins was determined by indirect immunofluorescent staining using goat anti-H1 hemagglutinin, anti-N1 neuraminidase, and anti-M polyclonal antisera (National Institutes of Health [NIH] reference reagents). Cells were incubated in 20 μl of a 1:500 dilution of goat anti-influenza virus antibodies for 1 h. After this step cells were washed with Dulbecco's phosphate-buffered salt solution (DPBS) and stained with 20 μl of a 1:500 dilution of FITC-conjugated rat anti-goat IgG antibody (Organon Teknika) for 1 h.

Analyses of Fas, FasL, and active caspase-3 expression. The expression of membrane-bound Fas (CD95) and FasL by lymphocytes and monocytes was measured by flow cytometry of sham-exposed and virus-exposed MNL. Cells were then stained for Fas or FasL expression. Antibody-binding capacity can be used to determine values of antigen density on the cell surface, since the binding capacity can be related directly to the level of antigen expression on the surface of a cell (9). Quantitative analyses using this assay were performed to evaluate the expression of CD95 by $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes. For the quantitation estimates of cell surface molecules, 10^6 MNL were incubated with 20 μl of either anti-CD3, -CD4, or -CD8 conjugated to PerCP (BD Pharmingen) and 20 μl of anti-CD95 conjugated to PE (Becton Dickinson). Antibody labeling was performed for 0.5 h at 4°C. Cells prepared for quantitations of surface molecules on $CD4^+$ and $CD8^+$ lymphocytes were analyzed immediately after staining without fixation of cells. Cells prepared for analysis of CD95 expression (percent positive) and apoptosis were fixed in 2% PAF. The percent apoptotic cells for $CD3^+$ $CD95^+$ lymphocytes was determined by using the TUNEL assay.

Quantitation of immunofluorescence intensity was used to estimate the average number of molecules expressed on cells (8, 16). Calibration of the equipment was done using Spherotech Beads (20). Fluorescence quantitation was done using the Quantiquest system (Becton Dickinson). Samples were gated on lymphocytes by using forward and side scatter gates, and then cursors were set to measure the median relative fluorescence intensity (RFI) of the cell population under study (20). For the median RFI on $CD3^+$, $CD4^+$, or $CD8^+$ T-cell measurements, a light scatter gate was combined with a second gate set on cells positive for CD3, CD4, or CD8 and CD95. Expression of CD95 molecules on $CD3^+$, $CD4^+$, or $CD8^+$ cells was measured by gating on both dim and brightly staining cells.

For FasL staining, 20 μl of a 1:100 dilution of anti-FasL antibody (clone NOK-1; BD Pharmingen) was added to appropriate samples. After a 0.5-h incubation, cells were washed with DPBS and stained with 20 μl of a 1:500 dilution of FITC-conjugated goat anti-mouse $F(ab)_2$ antibody (Organon Teknika) for 0.5 h. Cells were analyzed by fluorescent microscopy and flow cytometry without fixation.

In a set of experiments after 1 h of sham exposure or exposure to IAV, 1 μg of soluble recombinant human Fas/ml (6) or 1 μg of azide-free neutralizing anti-FasL antibody (clone NOK-1)/ml was added to cultures of MNL to determine the influence of Fas-FasL signaling on production of the active form of caspase-3 or the induction of apoptosis after IAV exposure. Purified isotype-matched control antibodies (mouse IgG1, clone MOPC-21) were added to sham- and virus-exposed cultures and had no effect on caspase-3 production or apoptosis induction. To assess the role of Fas-FasL-triggered caspase activation in IAV exposure-induced apoptosis, each of the inhibitors Z-VAD-FMK (general caspase inhibitor) (20 μM), AC-DEVD-CHO (caspase-3 inhibitor) (20 μM), and the control inhibitor Z-FA-FMK (20 μM) was added to a set of MNL cultures (17, 39). The caspase-3 level was evaluated by staining 10^6 sham-exposed or virus-exposed MNL with 20 μl of PerCP-conjugated anti-CD3 antibody (44). After 30 min the cells were washed and fixed in 2% PAF for 2 h. The $CD3^+$-stained cells were permeabilized for 10 min using 0.6% *n*-octyl glucopyranoside (Sigma Chemical Co.) and then stained for caspase-3 expression with 20 μl of FITC-conjugated rabbit anti-active caspase-3 MAb (clone C-92-605) (BD Pharmingen). After 30 min the cells were washed and analyzed immediately. The percent apoptotic cells for all cell treatments was determined by using the TUNEL assay.

In another subset of experiments, after 1 h of sham exposure or exposure to IAV, 1 μg each of stimulatory anti-human Fas antibody (clone DX2), agonistic anti-FasL antibody (clone G247-4), anti-TNF-α antibody (clone Mab1), or anti-ICAM-1 (clone 84H10) (AMAC Inc.) antibody/ml was added to cultures of MNL to determine the influence of Fas-FasL, TNF-α, or cell clustering and cell-cell interactions, respectively, on induction of apoptosis in $CD4^+$ or $CD8^+$ lymphocytes. Emetine (10^{-5} M) (Sigma Chemical Co.) was also added to a set of MNL cultures 3 h (to allow synthesis of viral proteins) after sham exposure or exposure to IAV. Cells were harvested after 24 h, washed, stained with anti-CD4 or -CD8 PerCP-conjugated antibodies, and then fixed with 2% PAF. The percent apoptotic cells for these cell treatments was determined using the TUNEL assay.

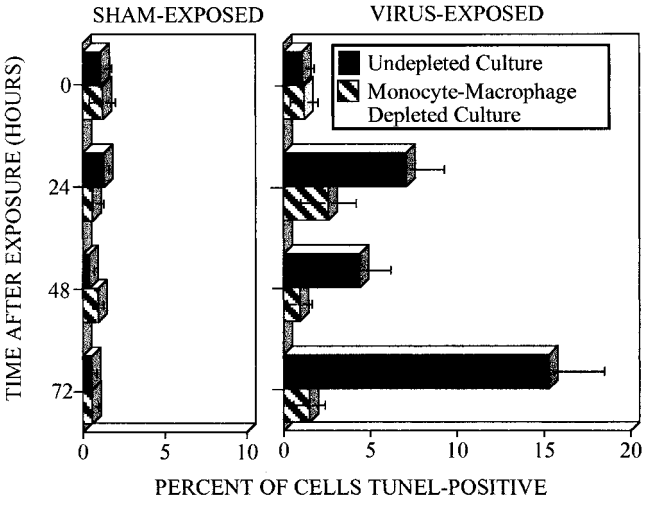

FIG. 1. Role of monocytes-macrophages in induction of $CD3^+$ lymphocyte apoptosis. MNL were sham exposed or exposed to virus, and after 1 h cultures were sham depleted (solid bars) or depleted (hatched bars) of monocytes-macrophages. Results from five experiments are presented as the mean percentage of apoptotic ($TUNEL^+$) $CD3^+$ cells ± SD. Data from 10,000 $CD3^+$ cells were collected for each sample.

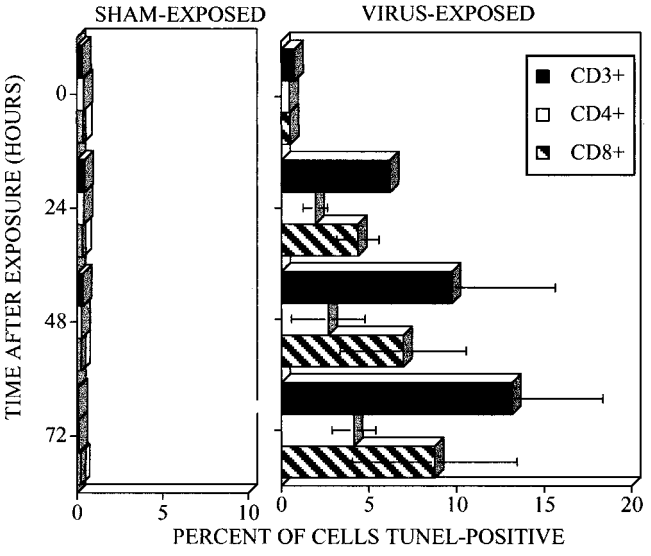

FIG. 2. Apoptosis-related cell death of sham-exposed and virus-exposed $CD3^+$ (solid bars), $CD4^+$ (open bars), and $CD8^+$ (hatched bars) lymphocytes. Results from five experiments are presented as mean percentage of apoptotic ($TUNEL^+$) cells ± SD. Data from 10,000 $CD3^+$, $CD4^+$, or $CD8^+$ cells were collected for each sample.

Statistical analyses. Results are expressed as the mean ± the standard deviation (SD) for the stated number of experiments. The statistical significance of differences in the means was calculated using Student's *t* test performed by EXCEL software (Microsoft, Inc., Bothwell, Wash.). Mean differences in the values were considered significant when P was less than 0.05.

RESULTS

Both monocytes-macrophages (47% ± 6%) and lymphocytes (7% ± 2%), identified by forward and side light scatter properties, expressed viral proteins 24 h after exposure. The percentage of monocytes-macrophages positive for the production of viral proteins was slightly higher at 48 and 72 h after exposure but there was no significant increase in the percentage of total lymphocytes positive for the production of viral proteins at the later time points. Of the $CD3^+$ T cells, 4.6% ± 0.87% at 24 h and 5.1% ± 0.4% at 48 h were positive for the production of viral proteins. Among the lymphocyte subsets, $CD3^+$, $CD4^+$, $CD8^+$, and $CD19^+$ cells were all shown to produce viral proteins after IAV exposure (data not shown).

Lymphocyte apoptosis. To detect apoptosis in lymphocytes, $CD3^+$, $CD4^+$, $CD8^+$, and $CD19^+$ cells were sorted (99 to 100% pure for the labeled cell type) and DNA fragmentation was assayed using agarose gel electrophoresis of DNA extracted from cells 24 h after sham exposure or virus exposure. Characteristic DNA ladders were seen only in $CD3^+$, $CD4^+$, $CD8^+$, and $CD19^+$ virus-exposed cells and never in sorted cells from sham-exposed cultures (data not shown). The TUNEL assay was then used to detect DNA strand breaks indicative of apoptosis on a single-cell basis.

Measurable levels of apoptosis were seen in $CD3^+$ T cells after exposure to virus but not in sham-exposed cells harvested at 24 h ($P = 0.03$), 48 h ($P = 0.0016$), or 72 h ($P = 0.009$). The percentage of $CD3^+$ cells that were apoptotic was reduced in virus-exposed, monocyte-macrophage-depleted cultures at 24 h ($P = 0.069$) and was significantly reduced at 48 ($P = 0.017$) and 72 h ($P = 0.004$) (Fig. 1). If monocytes-macrophages were removed 1 h after exposure to virus and then added back 24 h later, levels of apoptosis seen in $CD3^+$ cells increased from levels that were seen for depleted cultures but did not reach the level seen for undepleted MNL cultures (data not shown).

Analysis of lymphocyte subsets showed that apoptosis was seen in $CD3^+$, $CD4^+$, $CD8^+$ (Fig. 2), and $CD19^+$ (data not shown) cells. Sham-exposed $CD3^+$, $CD4^+$, $CD8^+$, and $CD19^+$ cells exhibited levels of staining similar to that seen for the TUNEL-negative assay controls (less than 1% positive). Significant increases in the percentage of apoptotic cells in virus-exposed compared to sham-exposed cell cultures were seen at 24 h for $CD3^+$ ($P = 3.69 \times 10^{-5}$), $CD4^+$ ($P = 0.00053$), and $CD8^+$ ($P = 1.85 \times 10^{-5}$) cells and at 48 h for $CD3^+$ ($P = 0.003$), $CD4^+$ ($P = 0.022$), and $CD8^+$ ($P = 0.001$) T-cell subpopulations. A significantly higher percentage of $CD8^+$ cells was seen to be TUNEL positive after exposure to virus than similarly treated $CD4^+$ cells ($P = 0.009$ and $P = 0.030$ at 24 and 48 h, respectively) (Fig. 2).

Measurement and quantitation of CD95. Surface Fas (CD95) expression was examined for gated populations of $CD4^+$ cells and $CD8^+$ cells. There was no statistically significant increase in expression of CD95 by virus-exposed $CD4^+$ cells compared to sham-exposed cells at any of the time points measured (Fig. 3A). The percentage of virus-exposed $CD8^+$ cells expressing CD95 increased rapidly after exposure to virus (Fig. 3B) and was significantly different from that of sham-exposed cells at 24 h ($P = 0.022$), 48 h ($P = 0.001$), 72 h ($P = 0.037$), and 96 h ($P = 0.047$). The average number of molecules of CD95 per cell was quantitated before and after exposure to IAV. There was a significant decrease in expression of CD95 by virus-exposed $CD4^+$ cells at 12 h ($P = 0.046$) but levels were increased on virus-exposed compared to sham-exposed $CD4^+$

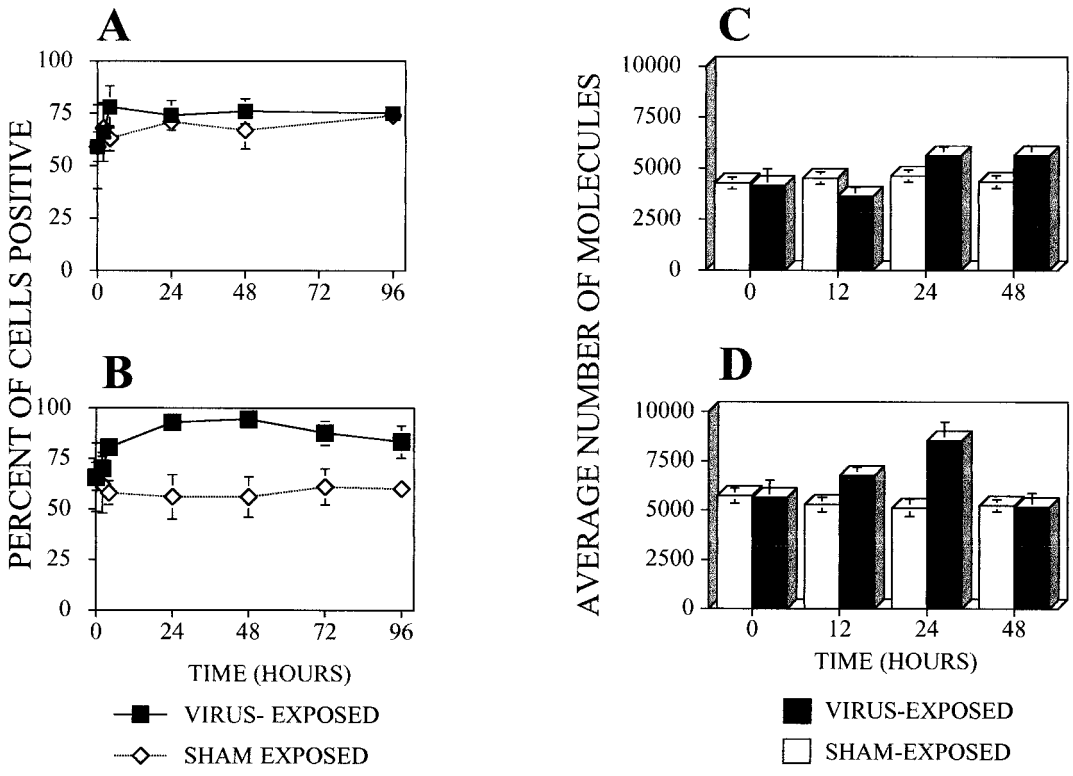

FIG. 3. CD95 expression by lymphocytes after sham exposure (open symbols and bars) or exposure to virus (solid symbols and bars). Results from five experiments are presented as the mean percentage of $CD4^+$ (A) or $CD8^+$ (B) cells that were CD95 positive ± SD and as the mean number of molecules of CD95 expressed on the surface of the $CD4^+$ (C) and $CD8^+$ (D) cells. For each time point, the data from 10,000 $CD4^+$ or $CD8^+$ cells were collected.

cells at 24 h ($P = 0.041$) and 48 h ($P = 0.033$) (Fig. 3C). The expression of CD95 increased significantly on virus-exposed compared to sham-exposed $CD8^+$ cells at 12 h ($P = 0.048$) and 24 h ($P = 0.001$) (Fig. 3D). The differences in expression of CD95 by virus-exposed $CD4^+$ versus $CD8^+$ cells at 12 h ($P = 0.038$) and 24 h ($P = 0.00046$) were significant.

The average number of molecules (density) of CD95 on virus-exposed $CD3^+$ TUNEL-positive and $CD3^+$ TUNEL-negative lymphocytes was determined. The density of CD95 on $CD3^+$ TUNEL-positive compared with $CD3^+$ TUNEL-negative lymphocytes was increased significantly at 24 h ($P = 0.00039$) (Fig. 4).

FasL expression by virus-exposed cells and influence of anti-Fas and anti-FasL antibodies. FasL expression by virus-exposed MNL was increased compared to sham-exposed MNL (Fig. 5). The majority of FasL was expressed on monocytes-macrophages within the MNL, but small increases were noted in the lymphocyte population as well (gating was done on $CD3^+$ cells; data not shown) ($P = 0.0005$) at 24 h.

The production of active caspase-3 was measured in $CD3^+$ lymphocytes (Fig. 6A and B). Significantly more caspase-3 was produced by IAV-exposed MNL cultures ($P = 3.9 \times 10^{-5}$) than sham-exposed cultures (Fig. 6B). In IAV-exposed MNL the levels of caspase-3 production were significantly decreased in cultures treated with anti-FasL antibody ($P = 0.002$) and soluble recombinant human Fas ($P = 0.001$), as well as with the caspase inhibitors Z-VAD-FMK ($P = 2.3 \times 10^{-5}$) and AC-DEVD-CHO ($P = 1.48 \times 10^{-5}$). Sham-exposed cultures exhibited minimal levels of caspase-3 production (<1.4%), and treatment of these cultures with anti-FasL antibody, soluble recombinant human Fas, or the caspase inhibitors Z-VAD-

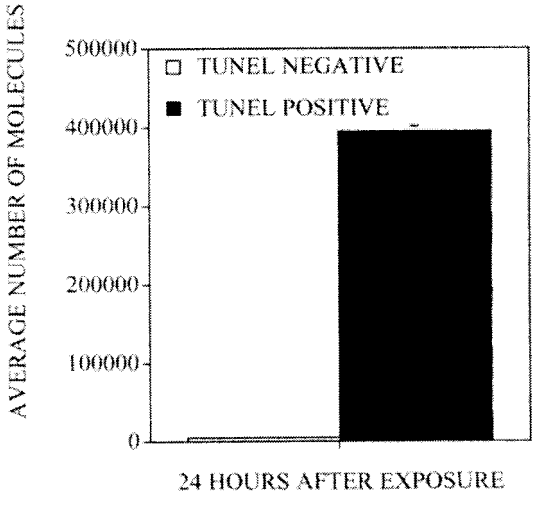

FIG. 4. Level of CD95 density (average number of molecules per cell) for TUNEL-negative (open bar) and TUNEL-positive (solid bar) $CD3^+$ lymphocytes 24 h after exposure to IAV. The data from 20,000 $CD3^+$ cells were collected.

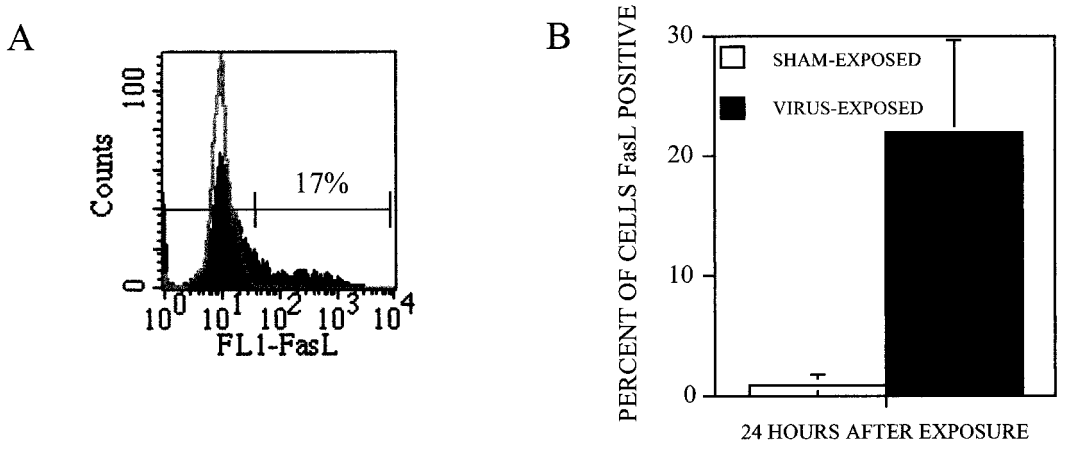

FIG. 5. (A) Data from one representative experiment showing FasL expression in IAV-exposed MNL (solid histogram) and sham-exposed MNL (grey line). For each sample, data from 10,000 cells were collected. (B) FasL expression by MNL after sham exposure (open bar) or exposure to virus (solid bar). Results from five experiments are presented as the mean percentage of cells that expressed FasL ± SD. For each sample, data from 10,000 cells were collected.

FMK and AC-DEVD-CHO did not significantly increase or decrease the level of caspase-3 (Fig. 6B). Treatment of sham-exposed cultures with the control inhibitor Z-FA-FMK (mean, 1.05 ± 0.11) did not increase or decrease the level of caspase-3 production. Treatment of IAV-exposed cultures with the control inhibitor Z-FA-FMK did not result in a decrease in caspase-3 production (mean, 29.46 ± 11.8).

The sensitivity of $CD3^+$ lymphocytes from virus-exposed cultures to Fas-FasL-induced apoptosis was evaluated through the addition of neutralizing anti-FasL antibody, soluble recombinant Fas, or the caspase inhibitors AC-DEVD-CHO and Z-VAD-FMK to sham-exposed and influenza virus-exposed MNL cultures. In IAV-exposed MNL, the levels of apoptosis were significantly decreased in cultures treated with soluble recombinant human anti-FasL antibody ($P = 0.0007$) or soluble recombinant human Fas ($P = 0.015$), as well as with the caspase inhibitors Z-VAD-FMK ($P = 0.008$) and AC-DEVD-CHO ($P = 0.017$). Sham-exposed cultures exhibited minimal levels of apoptosis (<1.25%), and treatment of the sham-exposed cultures with the reagents listed above did not significantly increase or decrease the level of apoptosis (Fig. 6A and C). Treatment of sham-exposed cultures with the control inhibitor Z-FA-FMK did not result in an increase or decrease in the level of apoptosis (mean, 1.2 ± 0.45). Treatment of IAV-exposed cultures with the control inhibitor Z-FA-FMK did not result in a decrease in the level of apoptosis (mean, 10.26 ± 3.2).

The level of apoptosis in $CD8^+$ T cells exposed to IAV was enhanced significantly ($P = 0.027$) by the addition of anti-Fas antibodies (Fig. 7) but $CD4^+$ cells were not affected ($P = 0.20$). Blocking of FasL-Fas interactions through the addition of anti-FasL antibodies inhibited apoptosis of $CD8^+$ cells ($P = 0.012$). Addition of emetine also reduced the percentage of apoptotic virus-exposed $CD8^+$ cells ($P = 0.011$) (Fig. 7). $CD4^+$ cells did exhibit a significant decrease in apoptotic cells after treatment with emetine ($P = 0.02$) but not with anti-Fas ($P = 0.2$) or anti-FasL ($P = 0.06$) antibodies. Purified isotype-matched control antibodies were added to sham- and virus-exposed cultures and had no effect on caspase-3 production or apoptosis induction.

Evaluation of apoptosis in inactivated IAV and other strains of IAV. Significant increases in the percentage of TUNEL-positive cells in virus-exposed compared to sham-exposed $CD3^+$ cells were seen after exposure to IAV strains A/AA/Marton/43, wt A/Bethesda ($P = 0.0003$), and ca A/Bethesda ($P = 0.0059$) (Fig. 8). A significant decrease in the percentage of TUNEL-positive cells was observed in the inactivated virus-exposed cells compared to the infectious virus-exposed $CD3^+$ cells. There was also a significant decrease in the percentage of TUNEL-positive $CD3^+$ cells exposed to the cold-adapted virus vaccine strain ca A/Bethesda ($P = 0.024$) compared to the wild-type strain wt A/Bethesda.

DISCUSSION

Previous studies have described a lymphopenia occurring after natural infection with influenza A virus or administration of attenuated influenza A virus vaccine (7, 10, 12, 24). The mechanism of this leukopenia, and specifically the role played by viral infection of lymphocytes, has not been determined. If redistribution of lymphocytes alone were responsible for the IAV-induced lymphopenia, an intact functional activity of the remaining circulating lymphocytes would be expected. Previous studies by our laboratory demonstrated that leukocytes exposed to IAV showed proliferation in response to the virus but concomitant depression of responses to mitogen stimulation (37). The decreased activation that could be induced by the presence of mitogen suggests a loss of functional activity. It is possible that nonvirus-directed responses are suppressed in part due to the induction of apoptosis as a result of IAV exposure. Apoptosis plays a crucial role in the regulation of leukocyte numbers and in the regulation of immunological response to virus infection (2, 47, 50).

Apoptosis of lymphocyte subsets. An important step in understanding the pathogenesis of IAV-induced leukopenia would be to determine the role played by apoptosis in the

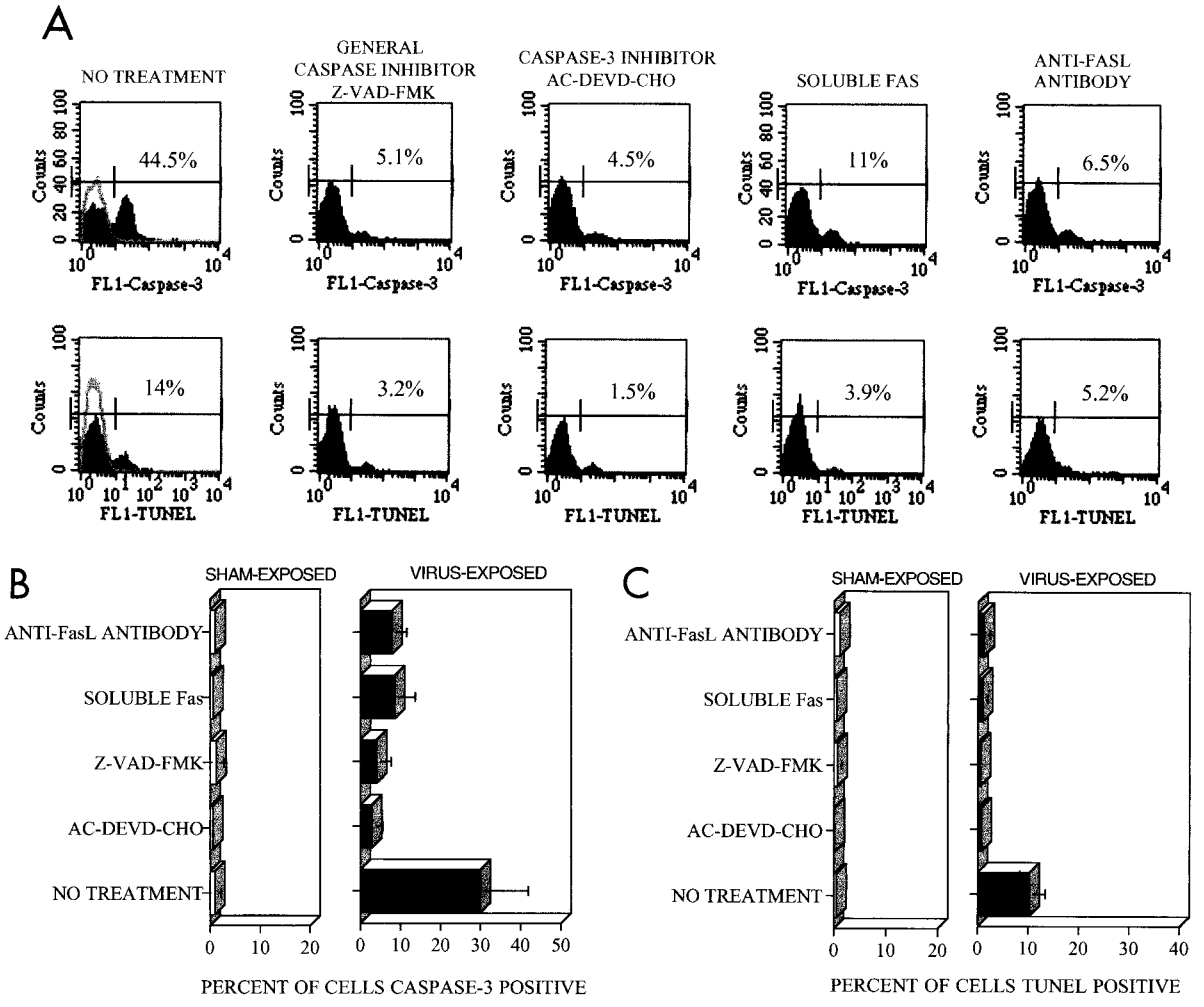

FIG. 6. (A) Data from one representative experiment showing production of the active form of caspase-3 (upper row of histograms) or the induction of apoptosis (lower row of histograms) in IAV-exposed $CD3^+$ cells (solid histograms). The sham-exposed caspase-3 level or percent apoptosis is shown as the gray line in the far left histogram of each row. The effect of treatment of IAV-exposed cells with recombinant soluble human Fas, anti-FasL antibody, the general caspase inhibitor Z-VAD-FMK, or the caspase-3 inhibitor AC-DEVD-CHO are shown. Treatment of sham-exposed $CD3^+$ cells with recombinant soluble human Fas, anti-FasL antibody, the general caspase inhibitor Z-VAD-FMK, or the caspase-3 inhibitor AC-DEVD-CHO produced levels of caspase-3 less than 1.3% and levels of apoptosis less than 1%. For each sample, data from 10,000 $CD3^+$ cells were collected. (B) Active caspase-3 production in $CD3^+$ sham-exposed (open bars; left graph) and virus-exposed (solid bars; right graph) lymphocytes after treatment with anti-FasL antibody, recombinant soluble human Fas, the general caspase inhibitor Z-VAD-FMK, or the caspase-3 inhibitor AC-DEVD-CHO. Results represent the mean percentage of apoptotic cells ± SD from five experiments, showing the effect of the above treatments. For each sample, data from 10,000 $CD3^+$ cells were collected. (C) Apoptosis of $CD3^+$ sham-exposed (open bars; left graph) and virus-exposed (solid bars; right graph) lymphocytes after treatment with anti-FasL antibody, recombinant soluble human Fas, the general caspase inhibitor Z-VAD-FMK, or the caspase-3 inhibitor AC-DEVD-CHO. Results represent the mean percentage of apoptotic cells ± SD from five experiments, showing the effect of the above treatments. For each sample, data from 10,000 $CD3^+$ cells were collected.

depletion of lymphocytes. Influenza virus has been shown to induce apoptosis in a number of cell types, including peripheral blood monocytes-macrophages (18), and in avian cell lines (43). In the present studies, lymphocyte apoptosis was detected as early as 1 day after exposure to virus. Analysis of cell phenotypes showed that induction of apoptosis occurred in virus-exposed but not sham-exposed $CD3^+$, $CD4^+$, and $CD8^+$ cells. Significantly more $CD8^+$ cells than $CD4^+$ cells were apoptotic at both 24 and 48 h after exposure to virus. The percentage of $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes positive for apoptosis was significantly reduced in virus-exposed but monocyte-macrophage-depleted cultures. In cultures in which monocytes-macrophages were removed 1 h after exposure to virus and then added back 24 h later, levels of apoptosis were seen to increase from those of depleted cultures but did not reach the levels seen in undepleted MNL cultures. These data suggest that a major mechanism of apoptosis in these cells must involve cell-cell interactions between monocytes-macrophages and $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes.

Role of activation and Fas-FasL in induction of apoptosis after exposure to virus. The Fas-FasL system is recognized as a major pathway for the induction of apoptosis in cells. For example, in a process referred to as activation-induced cell death, activation of T cells can result in apoptotic death me-

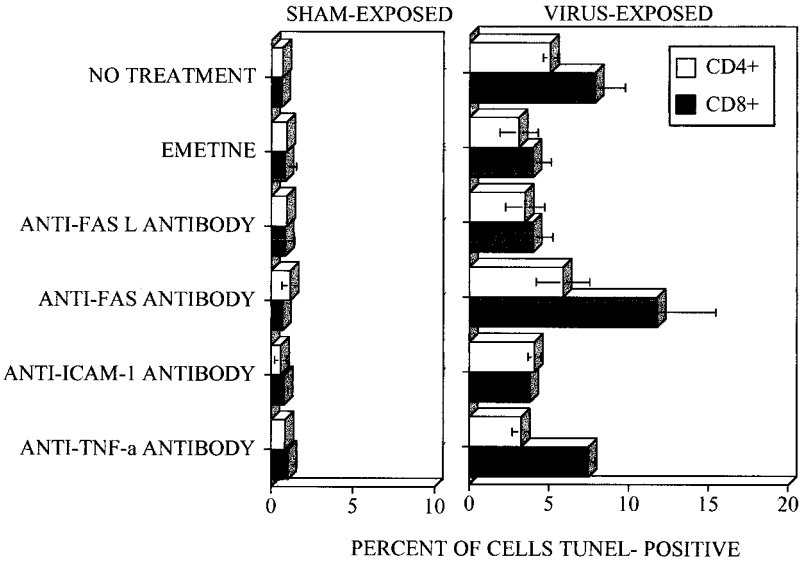

FIG. 7. Apoptosis of sham-exposed and virus-exposed $CD4^+$ (open bars) and $CD8^+$ (solid bars) lymphocytes after treatment with emetine, anti-Fas antibody, anti-FasL antibody, anti-TNF-α antibody, or anti-ICAM-1 antibody. Results represent the mean percentage of apoptotic cells ± SD from five experiments, showing the effect of emetine (10−5 M), anti-Fas antibody, and anti-FasL antibody, or from three experiments, showing the effect of anti-TNF-α antibody or anti-ICAM-1 antibody treatment on the levels of apoptosis in $CD4^+$ and $CD8^+$ cells. For each sample, data from 10,000 $CD4^+$ or $CD8^+$ cells were collected.

diated by Fas (CD95)-FasL interactions. A possible role for Fas-mediated activation-induced cell death in $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes after exposure to influenza virus warranted consideration.

An increase in the number of Fas-expressing $CD4^+$ and, especially, $CD8^+$ cells was observed after exposure to virus. It may not be the expression of Fas but the quantity of Fas expressed that controls the induction of apoptosis in activated cells. The density (average number of molecules) of Fas expression also increased on both $CD4^+$ and $CD8^+$ lymphocytes after exposure of the MNL cultures. The combination of the percent $CD8^+$ cells expressing Fas plus the density of Fas expression by those cells was most clearly evident. In a comparison between the average number of Fas molecules expressed on $CD8^+$ and on $CD4^+$ cells, significant differences were noted at 12 h ($P = 0.0038$) and 24 h ($P = 0.00046$) after exposure to influenza virus. The increase in the density of Fas on $CD3^+$ cells did correlate with the increase in apoptosis in those cells. The highest density of Fas was seen on $CD3^+$ TUNEL-positive cells (mean, 39,487 ± 6,564 molecules; $P = 0.00039$) compared to $CD3^+$ TUNEL-negative cells (mean, 4,838 ± 2,227).

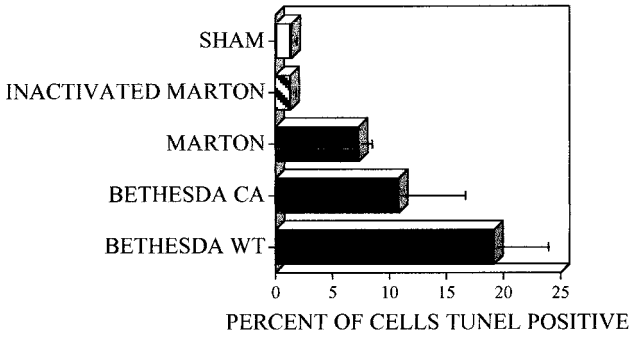

FIG. 8. Apoptosis of sham-exposed (open bar), heat-inactivated virus-exposed (hatched bar), and virus-exposed (solid bars) $CD3^+$ lymphocytes. MNL were exposed to influenza A virus strains A/AA/Marton/43 (H1N1), heat-inactivated A/AA/Marton/43, wild-type A/Bethesda/85 (H3N2) (termed wt A/Bethesda), or cold-adapted A/Ann Arbor/6/60 × A/Bethesda/85 (termed ca A/Bethesda) for 24 h. Results represent the mean percentage of apoptotic cells ± SD from five experiments. For each sample, data from 10,000 $CD3^+$ cells were collected.

The expression of FasL on cells is strictly regulated and thought to be the triggering event in the induction of Fas-FasL-mediated apoptosis. Monocytes-macrophages ($CD14^+$ cells) were the major cell type expressing FasL but $CD3^+$ T cells also exhibited an upregulation in FasL expression after exposure to virus. Although levels of Fas and FasL were shown to increase after exposure to IAV, the actual role of Fas-FasL signaling in the induction of apoptosis was not clear. Caspase-3 has been shown to mediate Fas-FasL induction of apoptosis (51). Levels of caspase-3 increased significantly after exposure to IAV. Neutralizing FasL antibody, recombinant soluble human Fas, and the caspase inhibitors Z-VAD-FMK (a general caspase inhibitor) and AC-DEVD-CHO (a caspase-3-specific inhibitor) reduced the production of active caspase-3 and the induction of apoptosis in IAV-exposed $CD3^+$ lymphocytes. These data support the involvement of Fas-FasL in the induction of apoptosis in lymphocytes after exposure to influenza virus.

Fas-FasL-mediated killing has been shown to be sensitive to inhibition of protein synthesis by emetine (4, 25) although this treatment is fairly nonspecific. A significant decrease in apoptotic $CD8^+$ cells was seen after treatment with emetine as well as in cells treated with anti-FasL antibody. Although not statistically significant, $CD4^+$ cells also exhibited a decrease in apoptosis after treatment with emetine ($P = 0.63$) or anti-FasL antibody ($P = 0.69$). In general, however, removal of mono-

cytes-macrophages was more effective in reducing the percentage of apoptotic cells than was inhibition of protein synthesis using emetine.

Induction of apoptosis by exposure to heat-inactivated virus and other strains of IAV. It is not clear at this time what component of influenza virus induces apoptosis. UV-inactivated virus does not induce apoptosis (27), nor does heat-inactivated influenza virus (Fig. 8), although heat-inactivated IAV does activate lymphocytes (11, 36). To understand the role that apoptosis may play in the pathogenesis of influenza virus infection, we exposed human MNL to wild-type as well as heat-inactivated and IAV cold-adapted vaccine strains of influenza virus. The cold-adapted strain of virus contains the wild-type neuraminidase and hemagglutinin genes but derives all other components of their genome from cold-adapted strains of virus.

All of the influenza infectious viruses induced significant levels of apoptosis compared to sham-exposed cells. The wt A/Bethesda virus induced significantly higher levels of apoptosis than the ca A/Bethesda virus strain. The ca A/Bethesda strain has been successfully used as a vaccine strain and has not been associated with induction of fever or other clinical symptoms (41). These data suggest that the induction of apoptosis may be associated with strain virulence, since this attenuated ca virus induced less apoptosis than was seen in cells exposed to either the A/AA/Marton/43 or wt A/Bethesda viruses.

Future studies regarding induction of apoptosis by exposure to virus. Since the host response to influenza virus commonly results in recovery from the infection, with residual disease uncommon, lymphocyte apoptosis likely represents a part of an overall beneficial immune response (35). The role that apoptosis plays in immune regulation and, potentially, in limitation of the pathology that would be related to the response to viral challenge warrants further investigation.

Lymphocyte depletion via apoptosis after exposure to IAV could be the result of virus-induced cytokine stimulation, viral induction of Fas, or other cell-virus interactions. Further studies will concentrate on the abilities of specific IAV-induced cytokines or viral proteins to function as triggers of apoptosis. IAV is an effective inducer of cytokines such as gamma interferon and TNF-α (30), and both factors have been shown to increase Fas and FasL expression on T cells (1) and induce apoptosis (38). A number of IAV gene products warrant consideration as possible triggers of apoptosis. Differential induction of apoptosis has been seen in MDCK and U-937 cells exposed to IAV strains of differing virulence (27). Clone 7a (virulent for humans and ferrets) induced more apoptosis than A/Fiji (attenuated for both species), and the ability of these clones to induce apoptosis was correlated with the differences in the amounts of neuraminidase activity in the two strains (33). In this same study treatment with anti-neuraminidase compounds was shown to abrogate apoptosis in MDCK cells (33). IAV nonstructural protein nucleoprotein NS1 and NP are also possible candidates as triggers of apoptosis, and both are produced early in infection (34). The NS1 of A/Duck/Alberta/35/76 (H1N1) has 50% homology to regions of the Fas antigen (18), which could support a role for NS1 in induction of apoptosis. Coexpression of Bcl-2 has been shown to alter apoptosis, possibly through inhibition of localization of NS1 and NP from the nucleus to the cytoplasm (18). Although production of either NA, NP, or NS1 may play a role in induction of apoptosis, other as-yet-undetermined viral gene products or intracellular processes must also be involved and warrant investigation.

REFERENCES

1. **Arnold, R., M. Seifert, K. Asadullah, and H. D. Volk.** 1999. Crosstalk between keratinocytes and T lymphocytes via Fas/Fas ligand interaction: modulation by cytokines. J. Immunol. **162:**7140–7147.
2. **Borthwick, N. J., M. Bofill, I. Hassan, P. Panayiotidis, G. Janossy, M. Salmon, and A. N. Akbar.** 1996. Factors that influence activated CD8+ T-cell apoptosis in patients with acute herpesvirus infections: loss of costimulatory molecules CD28, CD5 and CD6 but relative maintenance of Bax and Bcl-X expression. Immunology **88:**508–515.
3. **Boyum, A.** 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand. J. Clin. Lab. Investig. Suppl. **97:**77–89.
4. **Brossart, P., and M. J. Bevan.** 1996. Selective activation of Fas/Fas ligand-mediated cytotoxicity by a self peptide. J. Exp. Med. **183:**2449–2458.
5. **Brown, S. B., and J. Savill.** 1999. Phagocytosis triggers macrophage release of Fas ligand and induces apoptosis of bystander leukocytes. J. Immunol. **162:**480–485.
6. **Cheng, J., T. Zhou, C. Liu, J. P. Shapiro, M. J. Brauer, M. C. Kiefer, P. J. Barr, and J. D. Mounts.** 1994. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. Science **263:**1759–1762.
7. **Criswell, B. S., R. B. Couch, S. B. Greenberg, and S. L. Kimzey.** 1979. The lymphocyte response to influenza in humans. Am. Rev. Respir. Dis. **120:** 700–704.
8. **Davis, K. A., B. Abrams, S. B. Iyer, R. A. Hoffman, and J. E. Bishop.** 1998. Determination of CD4 antigen density on cells: role of antibody valency, avidity, clones, and conjugation. Cytometry **33:**197–205.
9. **Denny, T. N., D. Stein, T. Mui, A. Scolpino, and B. Holland.** 1996. Quantitative determination of surface antibody binding capacities of immune subsets present in peripheral blood of healthy adult donors. Cytometry **26:**265–274.
10. **Dolin, R., D. D. Richman, B. R. Murphy, and A. S. Fauci.** 1977. Cell-mediated immune responses in humans after induced infection with influenza A virus. J. Infect. Dis. **135:**714–719.
11. **Ettensohn, D. B., and N. J. Roberts, Jr.** 1983. Human alveolar macrophage support of lymphocyte responses to mitogens and antigens: analysis and comparison with autologous peripheral-blood-derived monocytes and macrophages. Am. Rev. Respir. Dis. **128:**516–522.
12. **Faguet, G.** 1981. The effect of killed influenza virus vaccine on the kinetics of normal human lymphocytes. J. Infect. Dis. **143:**252–258.
13. **Fernandes-Alnemri, T., G. Litwack, and E. S. Alnemri.** 1995. Mch2, a new member of the apoptotic Ced-3/Ice cysteine protease gene family. Cancer Res. **55:**2737–2742.
14. **Fesq, H., M. Bacher, M. Nain, and D. Gemsa.** 1994. Programmed cell death (apoptosis) in human monocytes infected by influenza A virus. Immunobiology **190:**175–182.
15. **Gavrieli, Y., Y. Sherman, and S. A. Ben Sasson.** 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. **119:**493–501.
16. **Gratama, J. W., J. L. D'Hautcourt, F. Mandy, G. Rothe, D. Barnett, G. Janossy, S. Papa, G. Schmitz, and R. Lenkei.** 1998. Flow cytometric quantitation of immunofluorescence intensity: problems and perspectives. European Working Group on Clinical Cell Analysis. Cytometry **33:**166–178.
17. **Gregoli, P. A., and M. C. Bondurant.** 1999. Function of caspases in regulating apoptosis caused by erythropoietin deprivation in erythroid progenitors. J. Cell. Physiol. **178:**133–143.
18. **Hinshaw, V. S., C. W. Olsen, N. Dybdahl-Sissoki, and D. Evans.** 1994. Apoptosis: a mechanism of cell killing by influenza A and B viruses. J. Virol. **68:**3667–3673.
19. **Hofmann, P., H. Sprenger, A. Kaufmann, A. Bender, C. Hasse, M. Nain, and D. Gemsa.** 1997. Susceptibility of mononuclear phagocytes to influenza A virus infection and possible role in the antiviral response. J. Leukoc. Biol. **61:**408–414.
20. **Hultin, L. E., J. L. Matud, and J. V. Giorgi.** 1998. Quantitation of CD38 activation antigen expression on CD8+ T cells in HIV-1 infection using CD4 expression on CD4+ T lymphocytes as a biological calibrator. Cytometry **33:**123–132.
21. **Ju, S. T., D. J. Panka, H. Cui, R. Ettinger, M. el Khatib, D. H. Sherr, B. Z. Stanger, and A. Marshak-Rothstein.** 1995. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature **373:**444–448.
22. **Juo, P., C. J. Kuo, J. Yuan, and J. Blenis.** 1998. Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. Curr. Biol. **8:**1001–1008.

23. **Kumar, S.** 1999. Mechanisms mediating caspase activation in cell death. Cell Death Differ. **6:**1060–1066.
24. **Lewis, D. E., B. E. Gilbert, and V. Knight.** 1986. Influenza virus infection induces functional alterations in peripheral blood lymphocytes. J. Immunol. **137:**3777–3781.
25. **Linsley, P. S., J. Bradshaw, M. Urnes, L. Grosmaire, and J. A. Ledbetter.** 1993. CD28 engagement by B7/BB-1 induces transient down-regulation of CD28 synthesis and prolonged unresponsiveness to CD28 signaling. J. Immunol. **150:**3161–3169.
26. **Mincheff, M., D. Loukinov, S. Zoubak, M. Hammett, and H. Meryman.** 1998. Fas and Fas ligand expression on human peripheral blood leukocytes. Vox Sang. **74:**113–121.
27. **Morris, S. J., G. E. Price, J. M. Barnett, S. A. Hiscox, H. Smith, and C. Sweet.** 1999. Role of neuraminidase in influenza virus-induced apoptosis. J. Gen. Virol. **80:**137–146.
28. **Muzio, M.** 1998. Signalling by proteolysis: death receptors induce apoptosis. Int. J. Clin. Lab. Res. **28:**141–147.
29. **Nagata, S., and P. Golstein.** 1995. The Fas death factor. Science **267:**1449–1456.
30. **Nichols, J. E., T. F. Fitzgerald, and N. J. Roberts, Jr.** 1993. Human macrophage responses to vaccine strains of influenza virus: synthesis of viral proteins, interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha. Vaccine **11:**36–42.
31. **Oehm, A., I. Behrmann, W. Falk, M. Pawlita, G. Maier, C. Klas, M. Li-Weber, S. Richards, J. Dhein, and B. C. Trauth.** 1992. Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. J. Biol. Chem. **267:**10709–10715.
32. **Owen-Schaub, L. B., S. Yonehara, W. L. Crump III, and E. A. Grimm.** 1992. DNA fragmentation and cell death is selectively triggered in activated human lymphocytes by Fas antigen engagement. Cell. Immunol. **140:**197–205.
33. **Price, G. E., H. Smith, and C. Sweet.** 1997. Differential induction of cytotoxicity and apoptosis by influenza virus strains of differing virulence. J. Gen. Virol. **78:**2821–2829.
34. **Privalsky, M. L., and E. E. Penhoet.** 1981. The structure and synthesis of influenza virus phosphoproteins. J. Biol. Chem. **256:**5368–5376.
35. **Roberts, N. J., Jr.** 1988. The concept of immunofocusing illustrated by influenza virus infection. Rev. Infect. Dis. **10:**1071–1074.
36. **Roberts, N. J., Jr., M. E. Diamond, R. G. Douglas, Jr., R. L. Simons, and R. T. Steigbigel.** 1980. Mitogen responses and interferon production after exposure of human macrophages to infectious and inactivated influenza viruses. J. Med. Virol. **5:**17–23.
37. **Roberts, N. J., Jr., and J. E. Nichols.** 1989. Regulation of proliferation after influenza virus infection of human mononuclear leukocytes. J. Med. Virol. **27:**179–187.
38. **Sarin, A., M. Conan-Cibotti, and P. A. Henkart.** 1995. Cytotoxic effect of TNF and lymphotoxin on T lymphoblasts. J. Immunol. **155:**3716–3718.
39. **Schrantz, N., D. A. Blanchard, M. T. Auffredou, S. Sharma, G. Leca, and A. Vazquez.** 1999. Role of caspases and possible involvement of retinoblastoma protein during TGFbeta-mediated apoptosis of human B lymphocytes. Oncogene **18:**3511–3519.
40. **Slee, E. A., C. Adrain, and S. J. Martin.** 1999. Serial killers: ordering caspase activation events in apoptosis. Cell Death Differ. **6:**1067–1074.
41. **Snyder, M. H., M. L. Clements, R. F. Betts, R. Dolin, A. J. Buckler-White, E. L. Tierney, and B. R. Murphy.** 1986. Evaluation of live avian-human reassortant influenza A H3N2 and H1N1 virus vaccines in seronegative adult volunteers. J. Clin. Microbiol. **23:**852–857.
42. **Suda, T., T. Takahashi, P. Golstein, and S. Nagata.** 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell **75:**1169–1178.
43. **Takizawa, T., S. Matsukawa, Y. Higuchi, S. Nakamura, Y. Nakanishi, and R. Fukuda.** 1993. Induction of programmed cell death (apoptosis) by influenza virus infection in tissue culture cells. J. Gen. Virol. **74:**2347–2355.
44. **Tanaka, M., T. Suda, T. Takahashi, and S. Nagata.** 1995. Expression of the functional soluble form of human fas ligand in activated lymphocytes. EMBO J. **14:**1129–1135.
45. **Thornberry, N. A.** 1998. Caspases: key mediators of apoptosis. Chem. Biol. **5:**R97–R103.
46. **Tumpey, T. M., X. Lu, T. Morken, S. R. Zaki, and J. M. Katz.** 2000. Depletion of lymphocytes and diminished cytokine production in mice infected with a highly virulent influenza A (H5N1) virus isolated from humans. J. Virol. **74:**6105–6116.
47. **Walsh, C. M., A. A. Glass, V. Chiu, and W. R. Clark.** 1994. The role of the Fas lytic pathway in a perforin-less CTL hybridoma. J. Immunol. **153:**2506–2514.
48. **Williams, G. T.** 1991. Programmed cell death: apoptosis and oncogenesis. Cell **65:**1097–1098.
49. **Yonehara, S., A. Ishii, and M. Yonehara.** 1989. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J. Exp. Med. **169:**1747–1756.
50. **Zanussi, S., C. Simonelli, M. D'Andrea, C. Caffau, M. Clerici, U. Tirelli, and P. DePaoli.** 1996. CD8+ lymphocyte phenotype and cytokine production in long-term non-progressor and in progressor patients with HIV-1 infection. Clin. Exp. Immunol. **105:**220–224.
51. **Zheng, T. S., S. F. Schlosser, T. Dao, R. Hingorani, I. N. Crispe, J. L. Boyer, and R. A. Flavell.** 1998. Caspase-3 controls both cytoplasmic and nuclear events associated with Fas-mediated apoptosis in vivo. Proc. Natl. Acad. Sci. USA **95:**13618–13623.