 Cytometry 35:80–90 (1999)

Rapid Communication

Comparison of Multiple Assays for Kinetic Detection of Apoptosis in Thymocytes Exposed to Dexamethasone or Diethylstilbesterol

K.J. Donner, K.M. Becker, B.D. Hissong, and S. Ansar Ahmed*

Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, Virginia

Received 15 June 1998; Revision Received 5 August 1998; Accepted 22 August 1998

Background: Techniques to measure apoptosis are used to study a wide spectrum of conditions, from acquired immune deficiency syndrome (AIDS) to cancer to autoimmune diseases. Therefore, a critical comparison of common assays for apoptosis is warranted.

Methods: The kinetics of apoptosis induction in dexamethasone (DEX)-exposed thymocytes was examined after 2, 4, 8, 12, 26–28, and 48–50 h of culture. An additional aim was to ascertain whether a similar thymic atrophy-inducing hormone, diethylstilbestrol (DES), also directly induces thymocyte apoptosis. Apoptosis was evaluated by flow cytometric examination of cells stained with propidium iodide (PI), 7-aminoactinomycin D (7-AAD), or fluorescein isothiocyante (FITC)-annexin; by forward- and side-scatter (FS, SS) analysis, cell-size analyzer; and through cytopathologic examination.

Results: After 4 h of DEX exposure, apoptosis was evident by 7-AAD, annexin, and cytopathological assays, but no cells with sub-diploid DNA content were evident by PI analysis. Maximal apoptosis was evident by all the above flow cytometric techniques at 12 h after DEX exposure. The 7-AAD and annexin assays, which allow discrimination between early apoptosis and late apoptosis/necrosis, were comparable and identified similar apoptotic populations. Appearance of a $FS^{low}/SS^{increased}$ population was evident only after 12 h of DEX exposure. Apoptosis could not be detected by any of the above assays in thymocytes exposed to various doses of DES.

Conclusion: Two of the six assays, 7-AAD and annexin, were similar in detecting apoptosis at an early kinetic time point. Results of both assays were comparable at all time points studied. Our studies imply that DEX and DES induce thymic atrophy, in vivo, by different mechanisms. Cytometry 35:80–90, 1999. © **1999 Wiley-Liss, Inc.**

Key terms: diethylstilbestrol; dexamethasone; apoptosis; in vitro; hormone; propidium iodide; 7-aminoactinomycin D; annexin V

Cell death can occur by at least two different pathways: apoptosis and necrosis. Apoptosis, a Greek term to portray the ''falling of leaves'' was initially described by Kerr and associates as a form of cell death (1). Apoptosis is distinguished from necrosis by unique cytological, biochemical, and molecular features. Nuclear features of apoptosis include chromatin condensation and DNA fragmentation, and changes at the membrane level are also evident in apoptotic cells. Phosphatidylserine, a negatively charged phospholipid, translocates from the inner to the outer leaflet of the membrane and becomes accessible to the external milieu. This presumably is an early event in the apoptotic death pathway. Apoptotic cells generally shrink in size (hence, apoptosis was initially referred to as ''shrinkage necrosis''), whereas necrotic cells show swelling. Apoptosis induces activation of specific endonucleases that fragment the DNA and neatly pack the fragmented DNA into apoptotic bodies for elimination by macrophages, thus abrogating inflammatory reactions. In sharp contrast, a consequence of necrosis is the induction of inflammation. Cell death by apoptosis (unlike necrosis) involves the activation of specific genes in a highly regulated and ordered fashion, hence the term ''programmed cell death'' is interchangeably used.

Contract grant sponsor: National Institutes of Health, National Institute of Environmental Health Sciences; Contract grant number: 1R01-ES08043.

*Correspondence to: Dr. S. Ansar Ahmed, Center for Molecular Medicine and Infectious Diseases, # 1410, Prices Fork Road, VA-MD Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24061.

E-mail: ansrahmd@vt.edu

Developmental biological studies initially noted that the resorption of tails of tadpoles and the loss of webbing of forelimbs are due to apoptosis. Since then, observations of apoptosis have been extended to many mammalian situations with profound physiological and pathological implications. For example, apoptosis is involved in the ordered replacement of aged cells in the tissues of the body (obviating either excess or diminished numbers of cells) without provoking a potentially fatal inflammatory reaction (2). In the immune system, thymocytes with strong affinity for self-antigens or with defective binding to major histocompatibility complex (MHC) molecules are deleted in the thymus (negative selection) by apoptosis (2). Cell death of target cells mediated by cytotoxic cells also involves apoptosis (2). It has also become apparent that defects in apoptosis have pathological consequences. Failure of apoptosis in autoreactive thymocytes can launch an untoward autoimmune attack, and impairment of apoptosis has been noted in cancer (reviewed in 3). In contrast, accelerated apoptosis has been observed in acquired immune deficiency syndrome (AIDS) and other immunodeficiency diseases (4). It is clear from many studies that apoptosis is involved in a wide spectrum of conditions. Therefore, a critical evaluation of the assays for apoptosis is warranted.

Initial studies used DNA fragmentation assays as a ''benchmark'' to detect apoptosis. Fragmented DNA, often regarded as the hallmark of apoptosis (1,5–7), is visualized by gel electrophoresis as DNA laddering (5,8) or in situ nick translation (ISNT-dUTP) assays (9,10). However, DNA laddering is not always indicative of apoptosis (11). Furthermore, DNA fragmentation assays necessitate the disruption of cells, and therefore this method is an inappropriate assay for phenotyping apoptotic cells (12). The use of DNA-binding dyes and fluorochrome-labeled annexin V in flow cytometric assays is becoming increasingly popular. Because many techniques to detect apoptosis differ in sensitivity, reliability, cost, simplicity, and the ability to identify apoptosis at an early stage, it is imperative to perform kinetic studies and compare common assays for apoptosis. We had two objectives for the present studies. The first was to monitor and quantify kinetic apoptotic changes in thymocytes exposed to a synthetic corticosteroid hormone, dexamethasone (DEX). The six selected assays represented changes at the membrane level by staining phosphatidyl-serine (FITC-annexin V), in the membrane, resulting in staining of DNA with 7-aminoactinomycin D (7-AAD), at the nucleic acid level (propidium iodide [PI] staining of cells with hypodiploid DNA content), in the cytology (blebbing, apoptotic bodies) and in the cell size or granularity analysis (forward/side-scatter analysis and Cell Counter and Analyzer System). The second objective was to determine whether diethylstilbestrol (DES), another synthetic thymic atrophy-inducing hormone, directly induces apoptosis of thymocytes analogous to DEX.

MATERIALS AND METHODS

Mice

Seven- to nine-month-old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were housed individually in rooms maintained at standard 14 h of light/10 h of dark and fed a commercial pellet diet and water ad libitum. All experimental animals were cared, maintained, and terminated (by cervical dislocation) in accordance with Virginia Polytechnic Institute and State University guidelines.

DEX Solutions

A 1 μg/ml working solution of DEX (Sigma Chemical Co., St. Louis, MO) was made in phenol red–depleted RPMI-1640 complete medium containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine (Mediatech, Herndon, VA), 50 IU/ml penicillin (Mediatech), and 50 μg/ml streptomycin (Mediatech). Phenol red–depleted media was used because it has been shown to have estrogenic properties (13,14). We have recently shown that 1 μg/ml of DEX, in vitro, induces apoptosis of thymocytes as assessed by flow cytometry using PI staining and by microscopic evaluation of ethidium bromide/acridine orange–stained cells (15).

DES Solutions

DES (0.2684 g; ICN Biomedicals, Aurora, OH) was dissolved in 10 ml of absolute ethanol to obtain a working solution of 10^{-1} M DES. One milliliter of 10^{-1} M DES solution was added to 3 ml of ethanol and 6 ml of phenol red–depleted incomplete medium (without FBS and nonessential amino acids) to make a 10^{-2} M DES solution. Tenfold serial dilutions were made in phenol red–depleted RPMI-1640 complete medium to obtain 10^{-4} M (0.04% ethanol final concentration) to 10^{-9} M (0.0000004% ethanol final concentration) solutions of DES. Various doses of DES were used to ascertain whether this hormone induces apoptosis of thymocytes only at a certain dose. Controls included cells cultured in medium with and without ethanol. To exclude any effects of minute quantities of ethanol, cells were cultured in medium containing ethanol, which was at a concentration comparable to the diluent ethanol in 10^{-4} M and 10^{-7} M DES.

Isolation of Thymocytes

Immediately after the mice were sacrificed via cervical dislocation, thymuses were removed, pooled, and isolated in cold, phenol red–depleted RPMI-1640 complete medium under sterile conditions, as described in earlier studies (16). The cell suspension was washed once at 200 *g* for 5–8 min at 4°C and resuspended in 10 ml of complete medium. Cell numbers and viability were assessed by trypan blue exclusion and were adjusted to 5×10^6 cells/ml. All thymocyte preparations had viability >99%.

Cell Culture

One hundred microliters of thymocytes, at a concentration of 5×10^6 cells/ml (5×10^5 cells/well), were plated

in Corning Cell Wells™ 96-well round-bottom tissue-culture plate (Corning, NY). One hundred microliters of either DES or DEX in complete medium was added to each well to bring the final volume to 200 µl. Control cultures consisted of 100 µl complete medium with and without ethanol. Cells were cultured at 37°C, 5% CO_2 in a Nuaire water-jacketed CO_2 incubator (Plymouth, MN). Cells were analyzed at 2, 4, 8, 12, and 24–26 hours after culture. In some studies, cells were also analyzed at 48–50 h of culture.

Flow Cytometric Analysis

Cells were processed on a Coulter Epics XL/MXL flow cytometer (Hialeah, FL), and data were analyzed with the Immuno-4 software program.

Propidium iodide staining. For PI staining, 5×10^5 cells were centrifuged at 1000 *g* for 5 min and resuspended in 250 µl of Vindelov's PI medium (1.21 g of Tris base, 584 mg of NaCl, 10 mg of RNAse, 50.1 mg of PI, 1 ml of Nonidet P-40, pH 8.0, in 1 l final volume). The RNAse in Vindelov's PI medium prevented the binding of PI to RNA. Cells were then mixed with 250 µl of standard azide buffer (1 l: 10 g of FA Bacto buffer, 10 ml of 10% NaN_3, 10 ml of heat-inactivated FBS, 50.1 mg of PI, 970 ml of distilled H_2O, pH 7.2) (15,17). Cells were kept at 4°C and measured by the flow cytometer on FL-3 (red fluorescence channel) after 24 h. Apoptotic cells were identified as sub-G_0/G_1 (hypodiploid) peak.

7-AAD staining. A working solution of 0.02 mg/ml of 7-AAD (Molecular Probes, Eugene, OR) in (FACS) buffer (phosphate-buffered saline [PBS] with 2% FBS, 0.1% sodium azide) was prepared and kept at 4°C in the dark until use. Cell samples at each time point were centrifuged at 200 *g* for 5–8 min at 4°C and resuspended in 100 µl of either cold PBS or FACS buffer, and then 7-AAD (20 µg/100 µl) was added as per the procedure of Schmid et al. (18). Cells were incubated for no more than 30 min at 4°C in the dark and then analyzed by a flow cytometer. Control samples consisted of cells without 7-AAD, and their apoptotic percentages were subtracted as background. Cells were visualized in FL-3 as 7-AAD^{dull}, 7-$AAD^{intermediate}$, and 7-AAD^{bright} and denoted as live, early apoptotic, and late apoptotic/necrotic cells, respectively, as previously reported (18).

Annexin V staining. At each particular time point, cells were pelleted at 200 *g* for 5 min, washed in PBS, and resuspended in 200 µl of 1× binding buffer (Clonetech Laboratories, Palo Alto, CA). Staining and analysis were done as per manufacturer's instructions. Five microliters of fluorescein isothiocyanate (FITC)-annexin V (final concentration: 1 µM/ml; ApoAlert, Clonetech Laboratories) and 5 µl of PI were added to each cell suspension. Annexin V fluorescence emission was detected in the FL-1 (green fluorescence) channel, and PI was detected in FL-2 (orange fluorescence). PI staining is a dye-exclusion assay that discriminates between cells with intact membranes (PI^-) and permeabilized membranes (PI^+) (19).

Cells were incubated at room temperature for 5–15 min in the dark and subsequently analyzed by flow cytometry. Controls consisted of sample without annexin and were subtracted from test samples as background. Cell populations that were annexin V^-/PI^- were regarded as live cells, whereas populations that were annexin V^+/PI^- were considered apoptotic (likely early apoptotic populations). Dead, necrotic, or late-stage apoptotic cell populations were represented by annexin V^+/PI^+ (19).

Simultaneous 7-AAD and FITC-annexin V staining. Thymocytes were cultured in media for 12 h, a time at which early (and late) apoptosis is evident by both 7-AAD and FITC-annexin V labeling. Cells were washed with PBS, resuspended in 200 µl of 1× annexin binding buffer, and stained with 5 µl of FITC-annexin V (1 µM/ml). After 15 min at room temperature, cells were washed with PBS and resuspended in 100 µl of FACS buffer containing 7-AAD (20 µg/100 µl). Cells were incubated for no more than 15 min at 4°C in the dark and then visualized flow cytometrically. Three subsets of cells, based on intensity of staining with 7-AAD, were identified. Subsequently, each subset was gated to visualize for FITC-annexin V positivity. Conversely, FITC-annexin V^+ cells were backgated for 7-AAD staining intensity.

Forward- and Side-scatter analysis. At each time point, 100 µl of cells was also subjected to forward-scatter (FS) and side-scatter (SS) analysis as an approximate indicator of cell size and cellular granularity and complexity. It is well established that cells undergoing apoptosis have decreased cell size (FS) and increased granularity (SS) (20–23).

In selected studies, changes in FS/SS profiles were compared with 7-AAD staining. Thymocytes were cultured for 4 h (a time at which early apoptosis was evident by 7-AAD) and 12 h (a time at which a $FS^{low}/SS^{increased}$ subpopulation was evident by FS/SS analysis). Each subset stained with 7-AAD was analyzed for FS/SS profile. Furthermore, the $FS^{low}/SS^{increased}$ subpopulation was gated and analyzed for 7-AAD positivity.

Cell Counter and Analyzer

Twenty microliters of control and DEX- or DES-exposed cells were added to 10 ml of sterile filtered PBS (pH = 7.2) and analyzed with CASY-1 Cell Counter and Analyzer System (Scharfe System GmbH, Reutlingen, Germany). This system is designed to analyze three 400-µl aliquots of sample per run. The data from the three aliquots are presented as a histogram based on the relative diameter or volume of the cells counted. To ensure the validity of the cell counts, each sample was run in duplicate. Consistent replication between duplicates was seen. Data from both runs were used when calculating the percentage of thymocytes in the main peak of the histogram (4.9–6.2 µm).

Cytopathological Identification of Apoptosis

Four hundred microliters of thymocytes cultured at 5×10^5 cells/well from each treatment (two representative wells combined) were centrifuged at 350 *g* for 6 min (IEC Centra-7R, International Equipment, Needham Heights, MA) and resuspended in 400 µl of incomplete phenol

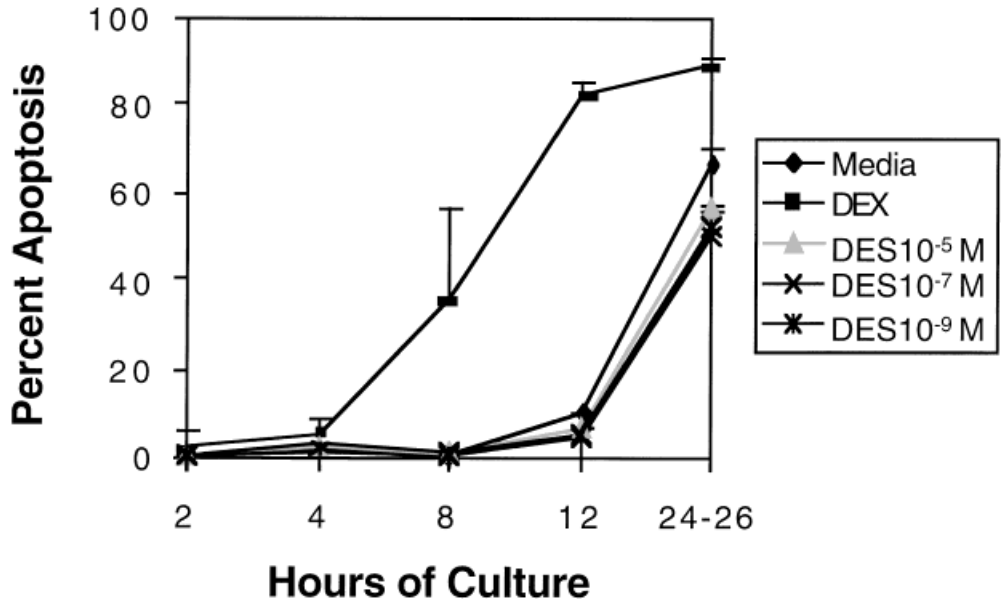

FIG. 1. Thymocytes from C57BL/6 mice were cultured in medium, DEX, or DES at 10^{-5} M, 10^{-7} M, and 10^{-9} M concentrations for 2, 4, 8, 12, and 24–26 h. At each time point, PI staining was performed as described in Materials and Methods. Apoptotic cells were identified as sub-G_0/G_1 (hypodiploid) peak. Data are means ± S.E. and represent three experiments. Apoptosis of DEX-treated thymocytes was statistically significant compared with medium-treated thymocytes ($P \leq 0.01$).

red–depleted RPMI medium with 2% bovine serum albumin (BSA) and 0.1% sodium azide. Cytospin preparations were centrifuged at low speed, approximately 17 *g*, for 7 min in a Cyto-Tek centrifuge (Miles Scientific, Elkart, IN). The slides were stained immediately with modified Wright stain (Sigma Diagnostics, St. Louis, MO). Apoptotic cells were identified on the basis of nuclear condensation, blebbing, and cell shrinkage. Two hundred cells were counted under oil at ×125 magnification, and the percentage of apoptotic thymocytes was calculated.

Statistics

SAS 6.10 software for Macintosh (SAS Institute Inc., Cary, NC) was used for data analysis. The data from each assay for apoptosis were analyzed to determine whether cultures exposed to DEX or any of the DES treatments were statistically different from control cultures. The general linear model procedure allowed the comparison of each treatment to all other treatments to determine whether a statistical difference was evident. For the 7-AAD, annexin-V, and PI assays, the 2- to 26-h data were analyzed. The 2- to 50-h data from the cell size analyzer and the 2- to 12-h data from the cytospin preparations were analyzed.

RESULTS

Analysis of Hypodiploid DNA Content in Hormone-Exposed Thymocytes by PI Staining

After 2 h of culture, thymocytes treated with medium, DEX, or DES showed no evidence of apoptosis as assessed by sub-G_0/G_1 subpopulations. Thymocytes cultured for 4 h with DEX showed a marginal increase in apoptosis (2.9%) compared with those in medium (0.6%). After 8 h of incubation, cells cultured with DEX had a higher percentage of apoptosis than controls (DEX versus media: 36% versus 1%) (Fig. 1). At 12 h, DEX-cultured thymocytes continued to show more induction of apoptosis than those in medium. By 24–26 h of culture, the percentage of apoptotic cells for DEX-treated thymocytes remained higher than those in medium. Importantly, cells cultured with various doses of DES, unlike DEX, had apoptotic percentages comparable to those in medium at all time points.

Analysis of Hormone-Induced Apoptosis of Thymocytes by 7-AAD Assay

Kinetic studies showed that apoptosis was evident in thymocyte populations, albeit a small percentage (average 4%), at as early as 2 h of culture in all samples. However, at this early time point, cells exposed to DEX or DES were not different from those cultured in medium only. After 4 h, apoptosis was detected in thymocytes cultured with DEX by all three indicators: early apoptosis (7-AAD^{intermediate}, 14.8 ± 6.5% versus 7.7 ± 2.5% for DEX and medium, respectively), late apoptosis/necrosis (7-AAD^{bright}, 7.9 ± 5.8% versus 5.7 ± 2.5%), and total dying/dead (7-AAD$^{bright + intermediate}$, 22.7 ± 10.6% versus 13.4%), (Fig. 2). At this 4-h time point, apoptotic cell percentages were approximately evenly distributed between 7-AAD^{intermediate} and 7-AAD^{bright} for all three types of cultured cells (DES, DEX, and medium). For example, in cells cultured with medium, 7.7% of cells were 7-AAD^{intermediate} and 5.7% of cells were 7-AAD^{bright}. After 8 h, thymocytes cultured in DEX had increased total apoptosis (1.7–2.9 times) compared with cells incubated in medium. The vast majority of thymocytes cultured in DEX at this time period were detected in the early apoptosis stage (7-AAD^{intermediate}). Twelve hours after culture, DEX-exposed thymocytes

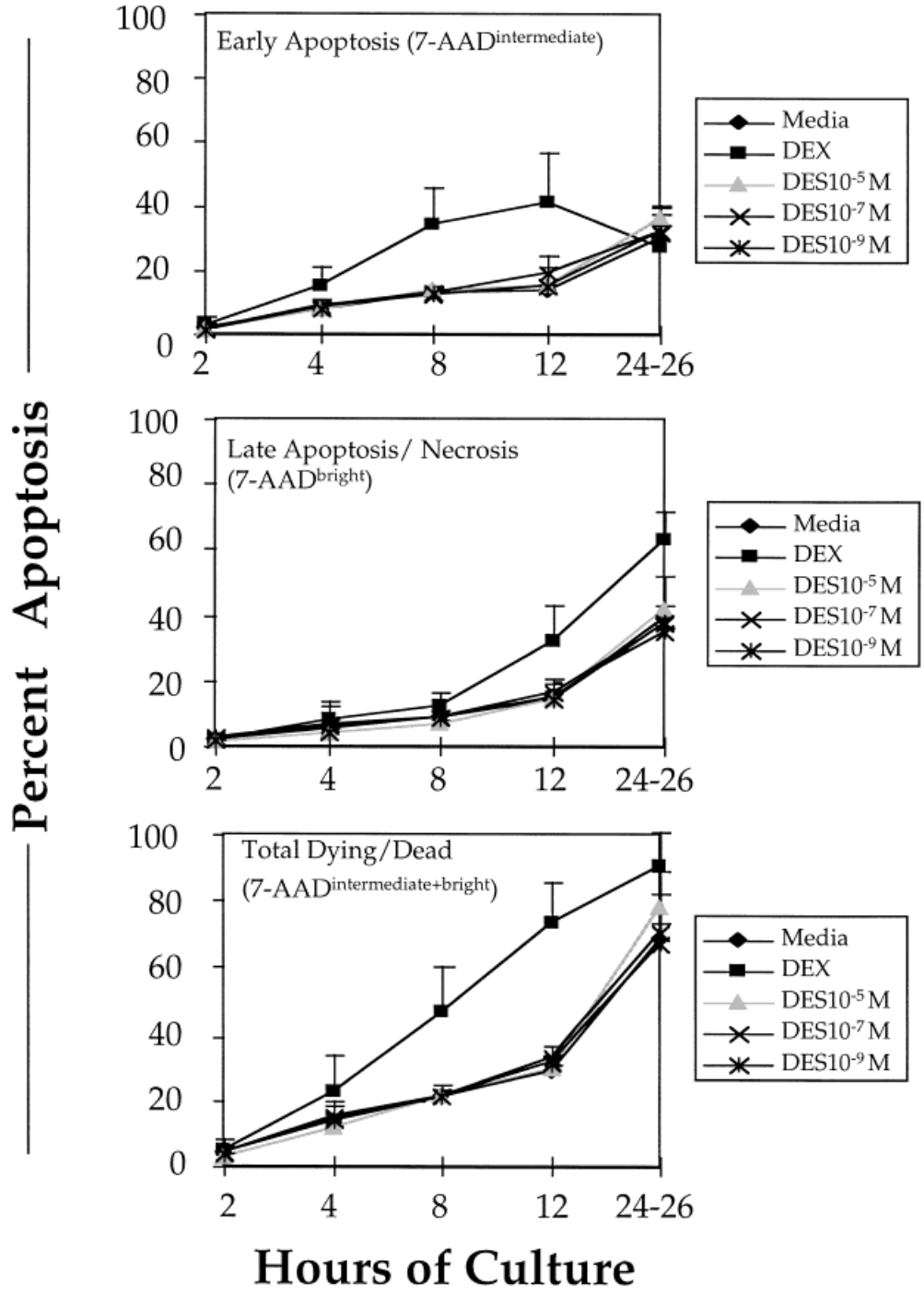

FIG. 2. Thymocytes from C57BL/6J mice were cultured in medium, DEX, or DES at 10^{-5} M, 10^{-7} M, and 10^{-9} M concentrations for 2, 4, 8, 12, and 24–26 h. At each time point, 7-AAD was added to individual samples and analyzed flow cytometrically as described in Materials and Methods. Cells detected as 7-AAD^{intermediate}, 7-AAD^{bright}, and 7-AAD$^{intermediate\ +\ bright}$ were denoted to be early apoptotic, late apoptotic/necrotic, and total apoptotic cells, respectively. Data are means ± S.E. The 2- and 8-h time points represent the means of three experiments. The 4, 12, and 24- to 26-h time points represent five experiments. Apoptosis of DEX-treated thymocytes was statistically significant compared with medium-treated thymocytes ($P \leq 0.01$).

yielded a 2.5-fold increase in apoptotic cells compared with controls, and by 24–26 h, control thymocytes (cultured in medium only) had undergone marked apoptosis. Cultures with various doses of DES were not different from the medium-treated control at any time point studied. During 24–26 h of culture, thymocytes cultured in medium only or DES continued to show apoptotic percentages evenly distributed between early and late apoptosis. However, thymocytes treated with DEX demonstrated a significantly greater percentage of cells in late apoptotic/

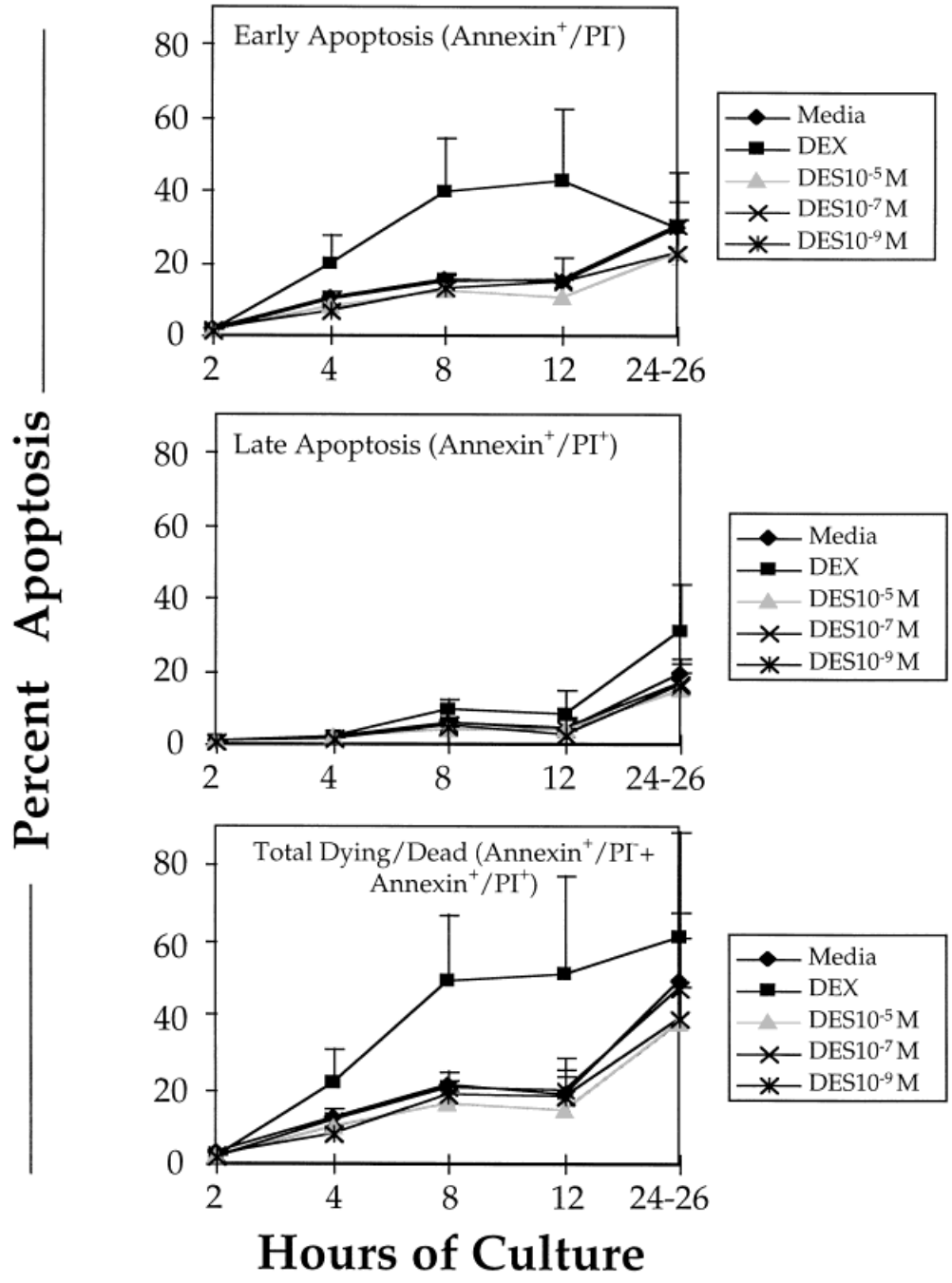

FIG. 3. Thymocytes from C57BL/6 mice were cultured in medium, DEX, or DES at 10^{-5} M, 10^{-7} M, and 10^{-9} M concentrations for 2, 4, 8, 12, and 24–26 h. At each time point, FITC-annexin-V/PI was added to individual samples and analyzed flow cytometrically as described in Materials and Methods. Cells that were annexin$^+$/PI$^-$ or annexin$^+$/PI$^+$ were denoted positive for apoptosis and late apoptotic/necrotic or dead cells, respectively. Data are means ± S.E. The 2- and 8-h time points represent the means of three experiments. The 4, 12, and 24- to 26-h time points represent the means of five experiments. Apoptosis of DEX-treated thymocytes was statistically significant compared with medium-treated thymocytes ($P \leq 0.01$).

necrotic stage than those in the early apoptotic stage. By 48 h, all three thymocyte cell-cultures (DES, DEX, and medium only) had limited viability; the percentage of live cells was <10% (data not shown). At this 48-h time point, the great majority of thymocytes undergoing apoptosis in all three cultures was detectable in the late apoptotic stage, 7-AAD^{bright}.

Analysis of Hormone-Induced Apoptosis of Thymocytes by Annexin-V Assay

At 2 h, a small percentage (2–3%) of apoptotic thymocyte populations were detectable in all cultures (Fig. 3). DEX-treated thymocytes at 4 h of culture had a higher percentage of early apoptotic (annexin$^+$/PI$^-$) cells (DEX 19.6%, medium 10.3%) and total apoptotic (annexin$^+$/PI$^-$

plus annexin$^+$/PI$^+$) cells (DEX 21.6%, medium 12.0%). After 8 h of culture, thymocytes treated with DEX had a 2.6-fold increase in early apoptotic and 2.4-fold increase in total annexin$^+$ cells compared with medium-treated cells. Thymocytes exposed to DEX continued to have increased induction of both early (2.8-fold difference) and total (2.7-fold annexin$^+$) apoptosis at 12 h compared with controls. At 24–26 h of treatment, DEX-treated thymocytes had a 1.3-fold greater induction of total apoptosis. In contrast to DEX, cells cultured with various doses of DES were comparable to medium controls at all time points. After 48–50 h of incubation, all three thymocyte cell cultures (DEX, DES, and medium) had limited viability (<10% viable cells), and the majority of thymocytes were in the late apoptotic/necrotic stage, annexin$^+$/PI$^+$ (data not shown).

Dual Analysis of 7-AAD and Annexin V

The 7-AAD and annexin assays detected similar percentages of apoptosis in cultured thymocytes. Therefore, dual staining with 7-AAD and annexin was performed to ascertain whether both techniques identify the same populations. The 7-AAD^{intermediate} and 7-AAD^{bright} thymocyte subpopulations were also positive for FITC-annexin V (Fig. 4A) In contrast, the 7-AAD^{dull} subpopulation was negative for FITC-annexin V.

Backgating of FITC-annexin V$^+$ cells revealed that these cells were 7-AAD^{intermediate} and 7-AAD^{bright}, but not 7-AAD^{dull} (Fig. 4B).

Dexamethasone Induces Shifts in Cell Size

Changes in the size of thymocytes exposed to DEX or DES were determined directly by the CASY-1 Cell Counter and Analyzer System, which determines the diameter and volume of each cell, and indirectly by flow cytometric visualization of FS/SS plots. Freshly isolated murine thymocytes analyzed with CASY-1 revealed a pronounced peak between 4.9- and 6.2-µm cell diameter (which contains approximately 60% thymocytes). Before 12 h of exposure, there were no differences in any of the treatment groups. After 12 h of culture, the data from five experiments showed 51.4 ± 3.0% of the cells in medium within the pronounced peak for the control. However, this prominent peak was decreased in DEX-treated thymocytes (43.0 ± 3.4%, mean ± S.E. of five experiments; $P \leq 0.01$). In contrast, cells treated with DES were similar to thymocytes cultured in medium. The average percentages of cells in five separate experiments at this 4.9- to 6.2-µm gate were 50.7 ± 3.0%, 53.9 ± 3.0%, and 54.2 ± 3.0%, respectively, for the DES treatments of 10^{-5} M, 10^{-7} M, and 10^{-9} M. The trend of lower percentages of DEX-treated thymocytes within the 4.9- to 6.2-µm gate continued for the 24- to 26-h time point and the 48- to 50-h time point (data not shown).

By 12 h of DEX exposure, the FS/SS histograms revealed a subpopulation of thymocytes that were smaller and more granular (Fig. 5). This subpopulation was not as pronounced in the control thymocytes or in any of the DES treatments (FS^{low}/SS^{increased} population percentages: medium versus DEX, 19.3% versus 55.5%). This shift in the smaller subpopulation of thymocytes by DEX was not markedly evident before 12 h of exposure, whereas at the 24- to 26- and 48-h time points, there was a pronounced appearance of FS^{low}/SS^{increased} subpopulations in all cultures.

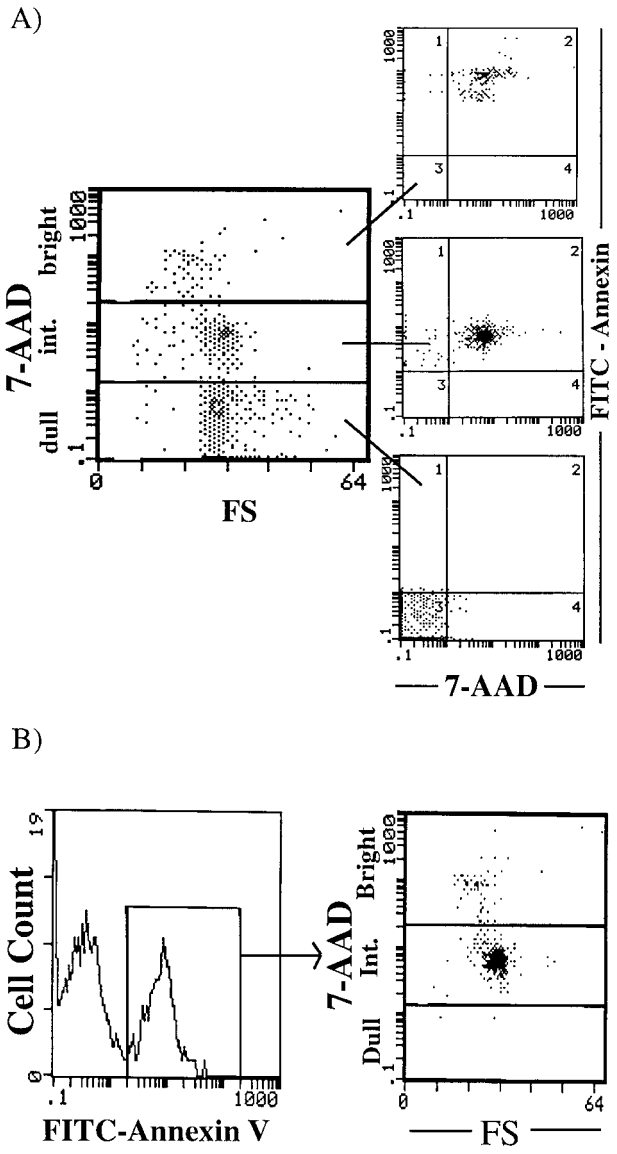

FIG. 4. Thymocytes from C57BL/6 mice were cultured in medium for 12 h. Cells were simultaneously stained with annexin V and 7-AAD, as indicated in Materials and Methods, and then analyzed flow cytometrically. Cells were assessed for 7-AAD fluorescence intensity as a function of FS and denoted subpopulations: 7-AAD^{dull}, 7-AAD^{intermediate}, and 7-AAD^{bright} (**A**). Each subpopulation was assessed for FITC-annexin V fluorescence as a function of 7-AAD. This figure is representative of three experiments. Conversely, the cells that were FITC-annexin V$^+$ were assessed for 7-AAD fluorescence as a function of FS (**B**).

Simultaneous Analysis of FS/SS With 7-AAD

At an initial kinetic time point (4 h of thymocyte culture), the FS/SS profiles of 7-AAD^{intermediate} (early apoptosis) and 7-AAD^{dull} (live) subsets were similar. A shift in FS (FS^{low}) was evident only in the 7-AAD^{bright} subset. This result suggests that FS/SS analysis is less sensitive in

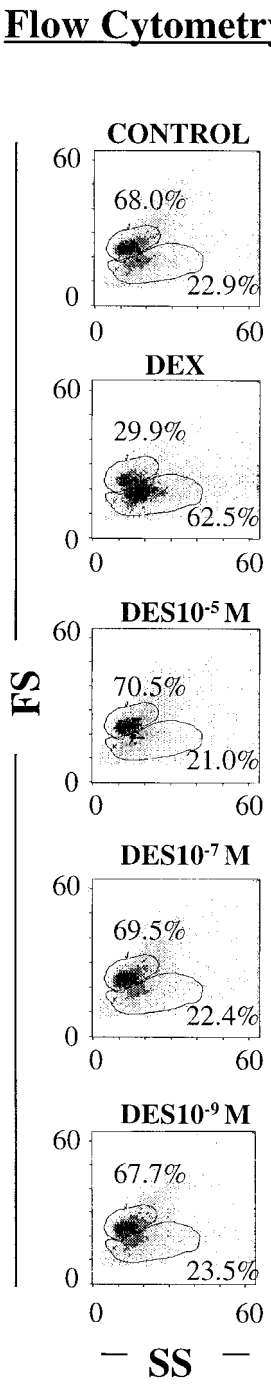

FIG. 5. Thymocytes from C57BL/6 mice were cultured in medium, DEX, or DES at 10^{-5} M, 10^{-7} M, and 10^{-9} M for 12 h and then analyzed by flow cytometry for forward and side scatter. Cells cultured in DEX show a shift to a lower FS and somewhat higher SS intensities. Data are representative of five experiments.

detecting early apoptosis during initial kinetic time points. At a later kinetic point (12 h of DEX exposure), the $FS^{low}/SS^{increased}$ subpopulation of thymocytes was evident. Gating on this subpopulation revealed that these cells were 7-AAD^{+} (7-$AAD^{intermediate}$ and 7-AAD^{bright}; Fig. 6B). Gating on the normal FS/SS subpopulation revealed that it contained only 7-$AAD^{intermediate}$ cells, not 7-AAD^{bright} cells.

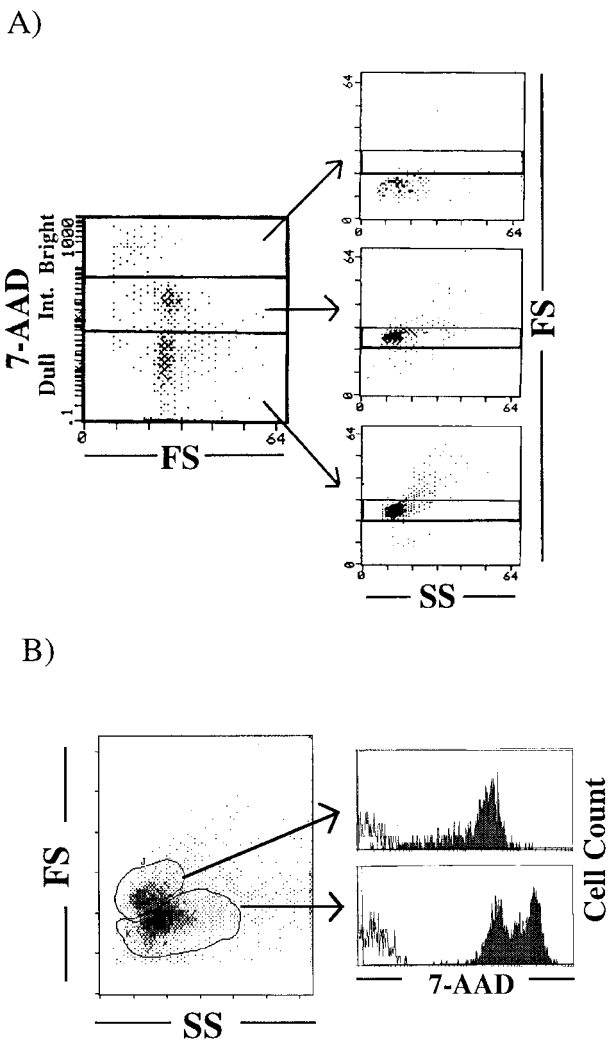

FIG. 6. Thymocytes from C57BL/6 mice were cultured in DEX for 4 and 12 h. Cells were stained as described in Materials and Methods and analyzed with flow cytometry for 7-AAD and forward and side scatter. After 4 h of incubation, subpopulations denoted as 7-AAD^{dull}, 7-$AAD^{intermediate}$, and 7-AAD^{bright} were assessed for FS/SS. Cells detected as 7-AAD^{bright} were the only subpopulation to reveal a $FS^{low}/SS^{increased}$ shift (**A**). After 12 h of incubation, gating of the $FS^{low}/SS^{increased}$ subpopulation of thymocytes revealed that these cells were 7-AAD^{+}. The unstained control cells are represented by the unshaded histogram (**B**).

This observation suggests that $FS^{low}/SS^{increased}$ subpopulation contains both early and late apoptotic/necrotic cells.

Cytopathological Identification of Apoptosis

An example of control cells compared with DEX-induced apoptotic cells is depicted in Figure 7. After 4 and 8 h of incubation with DEX, there was an increase in the number of apoptotic cells. The mean ± S.E. of apoptotic cells in four experiments was 2.3 ± 0.7% for the control thymocytes compared with 9.9 ± 1.9% for the DEX-treated thymocytes at the 4-h time point. Similarly, at the 8-h time point, the control and the DEX-treated thymocytes were 0.3 ± 0.17% and 14.3 ± 2.1% apoptotic, respectively. The 2- and 12-h time points also showed trends of higher percentages of apoptotic cells in the DEX-exposed cells

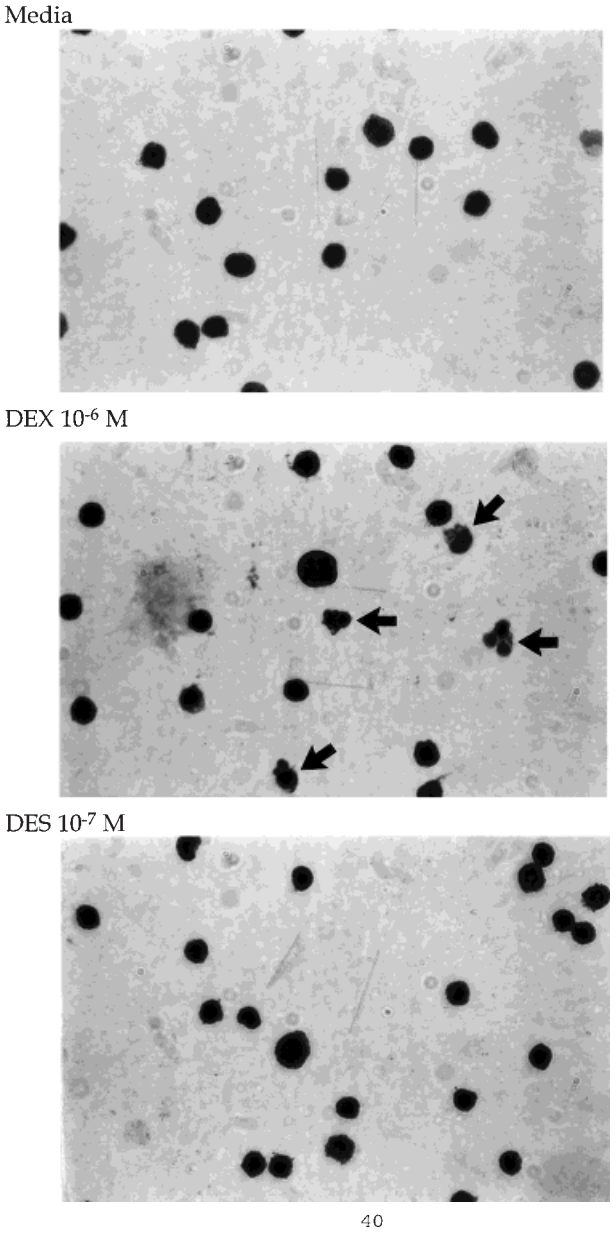

FIG. 7. Thymocytes from C57BL/6 mice were cultured in medium, DEX, or DES at 10^{-5} M, 10^{-7} M, and 10^{-9} M for 4 h and then subjected to Wright stain cytospin preparations. Representative photographs are shown of thymocytes from medium, DEX, and DES 10^{-7} M treatments. Apoptotic cells are denoted by arrows in the photographs of DEX-treated thymocytes. Magnification = ×250.

than in control. Overall, DEX-exposed thymocytes exhibited a significantly ($P \leq 0.01$) higher percentage of apoptosis than the control thymocytes. In contrast to DEX, thymocytes exposed to various doses of DES did not show an increase in apoptosis compared with the control at any time point studied. At the 24- to 26- and 48- to 50-h time points, very few intact cells were recovered by cytospin preparations, possibly because of increased fragility of the thymocytes at these time points in culture. The slides were therefore deemed unreadable.

DISCUSSION

In the present study, hormone-induced apoptosis of thymocytes was investigated by multiple assays. These included assays to assess the breakdown of membrane integrity as evaluated by staining with DNA-binding dyes (7-AAD for membrane changes and PI for analysis of cells with hypodiploid DNA content), translocation of phosphatidylserine to the external surface of the cell (annexin V/PI staining), and morphological changes (FS/SS, Cell Counter and Analyzer System, and cytopathological analysis). We found that after 4 h of culture, the ability of DEX to induce apoptosis was clearly evident by 7-AAD and annexin V, less markedly evident by PI staining, and not evident by FS/SS analysis. By 8 or 12 h of culture with DEX, induction of apoptosis was apparent by all four flow cytometric assays—7-AAD, annexin, FS/SS analysis, and PI. The PI assay detected a lower percentage of sub-G_0/G_1 populations in cultures of medium alone at 2- to 12-h time points. One limitation of PI staining, is that identification of early and late apoptotic/necrotic cells is not possible. Additionally, others have shown that PI was unable to detect apoptotic cells in freshly-isolated human thymocytes when the same cell population clearly showed fragmented DNA by PI analysis (24). Furthermore, the use of Vindelov's PI medium, which contains detergent, may have led to an inaccurate assessment of apoptosis: an underestimation, possibly due to disintegration of apoptotic cells, or an overestimatation, due to enumeration of apoptotic bodies as cells.

The 7-AAD assay is a simple assay that has reliably identified live (7-AAD^{dull}), early apoptotic (7-AAD^{intermediate}), and late apoptotic/necrotic cells (7-AAD^{bright}) (18 and the present study). This assay has been validated by simultaneous comparison with PI and cytopathological assays (18) and has been used in our laboratories (25; present study; and Hissong et al., unpublished observations) as well as others (26). Apoptotic populations can be identified by 7-AAD, even before shifts in FS/SS profiles can be visualized (Fig. 6). One caveat of using 7-AAD is that it interferes with Alamar Blue, a dye used for assessing lymphocyte viability and proliferation (15), and it may underestimate apoptotic cells in lymphocyte subpopulations contaminated with granulocytes (27).

FITC-annexin V staining presumably identifies early apoptotic cells (annexin$^+$/PI$^-$). This method of analysis has a few minor limitations. To accurately discriminate between apoptosis and necrosis, simultaneous measurement of annexin-V binding with PI should be performed. This simultaneous analysis becomes crucial when using noxious chemicals or infectious agents, which induce loss of membrane integrity through necrosis. Furthermore, in addition to strong binding affinity of annexin V to phosphatidylserine, it also shows minimal binding to other phospholipids such as phosphatidylcholine and sphingomyelin, which are found in the outer leaflet of plasma membranes (19). The percentages of apoptosis determined with 7-AAD and annexin V/PI staining were comparable at each time point. In dual staining with 7-AAD and annexin V, it became apparent that these two techniques detect similar

subsets of apoptotic cells. Given the similarities between these two methods of detection, 7-AAD is preferred because it is less costly.

With respect to detection of apoptosis by cytopathological identification, there was a significant increase in the percent of apoptosis detected at 4 and 8 h in the DEX-treated thymocytes. However, the need to subject cells to centrifugation is not appropriate for fragile apoptotic cells at later time points. A technique that is gentler on cells (i.e., that does not include a cytospin procedure), such as staining cell cultures with ethidium bromide and acridine orange, may be more effective at later time points when the cells are more fragile. We have used these dyes to microscopically detect and enumerate early and late apoptosis (15,25). One particular limitation of the ethidium bromide and acridine orange assay is that it tends to be tedious and relatively subjective. The cytospin method is nevertheless an efficient technique for detecting apoptosis at early time points.

We also used a recently developed CASY-1 cell counter and analyzer system in an attempt to quantify total apoptotic cells. Freshly isolated murine thymocytes, when analyzed by the CASY-1, have a distinct profile, forming a tight peak between 4.9–6.2 μm in diameter. The cells to the left of the peak are thought to be dead, and those to the right are thought to be larger cells (e.g., epithelial cells or macrophages) or aggregates of cells. We rationalized that cell shrinkage and apoptotic bodies that result from DEX-induced apoptosis would be identifiable by an increase in the number of smaller cells, visualized as an increase on the number of cells to the left of the peak. However, CASY-1 was unable to definitively identify small apoptotic bodies. Nevertheless, after 12 h of culture in DEX, there was a decrease in the percentage of cells within the 4.9- to 6.2-μm peak. In our experience, the CASY-1 was unable to definitively detect apoptosis in murine thymocytes using a 150-μm capillary tube but was able to demonstrate diminution of the pronounced lymphocyte peak. The sensitivity of a smaller capillary tube of 60 μm (which we do not possess) may be necessary to detect and quantify small murine apoptotic cells and/or bodies.

An additional objective was to evaluate whether DES, a synthetic estrogen, directly induced apoptosis of thymocytes, akin to DEX. Thymic atrophy is often noticed after in vivo DEX or DES treatment (16,28). Studies have shown that DES exhibits direct cytotoxic effects in prostate cancer cells via an apoptotic mechanism (29). We have shown that in vivo 17-β-estradiol treatment leads to decreased thymic weights (30) and cell numbers (31) in normal mice. Recently, we noted that freshly isolated thymocytes from 17-β-estradiol treated mice did not have marked apoptosis, despite pronounced thymic atrophy (Hissong et al. unpublished observations). By using multiple assays for apoptosis (PI, 7-AAD, annexin, FS/SS, and cytopathological assays), we found that thymocytes directly exposed to various doses of DES (synthetic estrogen) had levels of apoptosis similar to those of control cultures (Figs. 1–3, 5, 7). Although both DEX and DES diminished cellularity of the thymus in vivo, only DEX was able to directly induce apoptosis of thymocytes when exposed in vitro. Thus, our studies demonstrate that DES does not directly induce apoptosis of thymocytes. These results suggest that the two synthetic hormones, DES and DEX, induce hypocellularity of thymocytes, in vivo, by different mechanisms. Preliminary evidence suggests that exposure of thymocytes to 17-β-estradiol does not directly induce apoptosis, thus supporting our observations with DES. Further, in vivo studies using transgenic mice expressing anti-apoptotic oncogene, bcl-2, have shown that administration of 17-β-estradiol, but not DEX (32) can induce thymic atrophy, thereby suggesting that these two hormones have different modes of action.

It has been shown that thymocyte precursor cells migrate from the bone marrow throughout postnatal life (33). DES may act at the level of the bone marrow to affect the emigration of lymphocytes to the thymus. Previous studies from our laboratory (30,31) and from others (34) have shown that 17-β-estradiol markedly depletes the bone marrow via intrusion of the cavity by the growth of the bone (osteopetrosis). Alternatively, DES may act on thymic epithelial and/or stromal cells, which secrete cytokines that are critical in thymocyte development (35). Whether a DES metabolite(s) (or DES modified by binding to unknown molecules) induces direct apoptosis of thymocytes in vivo cannot be completely discounted. Future studies will focus on the direct affect of DES on bone marrow lymphocytes. We thus conclude that in vivo treatment with DES, unlike DEX, is not likely to directly induce apoptosis of thymocytes.

ACKNOWLEDGMENTS

The authors thank Mrs. Mary Nickle and Mrs. Audrey Reith for excellent care of animals, Ms. Joan Kalnitsky for flow cytometry work, Dr. M. Renee Prater, D.V.M., for help with photographs and microscopic identification of apoptosis, Dr. Francois Elvinger for suggestions about statistical analysis, Mr. Terry Lawrence for graphics work, and Dr. Bob Gogal for assistance with CASY-1. We also acknowledge Virginia-Maryland Regional College of Veterinary Medicine for a Graduate Research Fellowship awarded to Kelcey Becker.

LITERATURE CITED

1. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239–257.
2. Cohen JJ. Apoptosis. Immunol Today 1993;14:126–130.
3. Ogawa N, Dang H, Talal N. Apoptosis and autoimmunity. J Autoimmunity 1995;8:1–19.
4. Banda NK, Bernier J, Kurahara DK, Kurrle R, Haigwood N, Sekaly RP, Finkel TH. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J Exp Med 1992;176:1099–1106.
5. Duke RC, Chervenak R, Cohen JJ. Endogenous endonuclease-induced DNA fragmentation: an early event in cell-mediated cytolysis. Proc Natl Acad Sci USA 1983;80:6361–6365.
6. Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980;68:251–306.
7. Wyllie AH, Morris RG, Smith AL, Dunlop D. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol 1984;142:67–77.
8. Arends MJ, Morris RG, Wyllie AH. Apoptosis: The role of the endonuclease. Am J Pathol 1990;136:593–608.

9. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119:493–501.
10. Mainwaring PN, Ellis PA, Detre S, Smith IE, Dowsett M. Comparison of in situ methods to assess DNA cleavage in apoptotic cells in patients with breast cancer. J Clin Pathol 1998;51:34–37.
11. Cohen GM, Sun XM, Snowden RT, Dinsdale D, Skilleter DN. Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochem J 1992;286:331–334.
12. Lecoeur H, Ledru E, Prevost MC, Gougeon ML. Strategies for phenotyping apoptotic peripheral human lymphocytes comparing ISNT, annexin-V and 7-AAD cytofluorometric staining methods. J Immunol Methods 1997;209:111–123.
13. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 1986;83:2496–2500.
14. Hubert JF, Vincent A, Labrie F. Estrogenic activity of phenol red in rat anterior pituitary cells in culture. Biochem Biophys Res Commun 1986;141:885–891.
15. Zhi-Jun Y, Sriranganathan N, Vaught T, Arastu SK, Ansar Ahmed S. A dye-based lymphocyte proliferation assay that permits multiple immunological analyses: mRNA, cytogenetic, apoptosis, and immunophenotyping studies. J Immunol Methods 1997;210:25–39.
16. Ansar Ahmed S, Sriranganathan N. Differential effects of dexamethasone on the thymus and spleen: alterations in programmed cell death, lymphocyte subsets, and activation of T cells. Immunopharmacology 1994;28:55–66.
17. Robinson JP, Darzynkiewicz Z, Dean P, Dressler L, Tanke H, Wheeless L. Handbook of flow cytometry methods. New York: John Wiley and Sons, Inc.; 1993. p 8.
18. Schmid I, Uittenbogaart CH, Keld B, Giorgi JV. A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. J Immunol Methods 1994;170:145–157.
19. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 1995;184:39–51.
20. Carbonari M, Cibati M, Pesce AM, Sbarigia D, Grossi P, D'Offizi G, Luzi G, Fiorilli M. Frequency of provirus-bearing CD4+ cells in HIV type 1 infection correlates with extent of in vitro apoptosis of CD8+ but not of CD4+ cells. AIDS Res Hum Retroviruses 1995;11:789–794.
21. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139:271–279.
22. Petit PX, Lecoeur H, Zorn E, Dauguet C, Mignotte B, Gougeon ML. Alterations in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. J Cell Biol 1995;130: 157–167.
23. Swat W, Ignatowicz L, Kisielow P. Detection of apoptosis of immature CD4+8+ thymocytes by flow cytometry. J Immunol Methods 1991; 137:79–87.
24. Schmid I, Uittenbogaart CH, Giorgi JV. Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. Cytometry 1994;15:12–20.
25. Verthelyi DI. Effects of Estrogen on the B cell functions of normal mice. Blacksburg, VA: Virginia Tech; 1996.
26. Philpott NJ, Turner AJ, Scopes J, Westby M, Marsh JC, Gordon-Smith EC, Dalgleish AG, Gibson FM. The use of 7-amino actinomycin D in identifying apoptosis: Simplicity of use and broad spectrum of application compared with other techniques. Blood 1996;87:2244–2251.
27. Lecoeur H, Gougeon ML. Comparative analysis of flow cytometric methods for apoptosis quantitation in murine thymocytes and human peripheral lymphocytes from controls and HIV-infected persons. Evidence for interference by granulocytes and erythrocytes. J Immunol Methods 1996;198:87–99.
28. Greenman DL, Dooley K, Breeden CR. Strain differences in the response of the mouse to diethylstilbestrol. J Toxicol Environ Health 1977;3:589–597.
29. Robertson CN, Roberson KM, Padilla GM, O'Brien ET, Cook JM, Kim CS, Fine RL. Induction of apoptosis by diethylstilbestrol in hormone-insensitive prostate cancer cells. J Natl Cancer Inst 1996;88:908–917.
30. Ansar Ahmed S, Dauphinee MJ, Montoya AI, Talal N. Estrogen induces normal murine CD5+ B cells to produce autoantibodies. J Immunol 1989;142:2647–2653.
31. Ansar Ahmed S, Dauphinee MJ, Talal N. Effects of short-term administration of sex hormones on normal and autoimmune mice. J Immunol 1985;134:204–210.
32. Staples JE, Fiore NC, Frazier DE, Gasiewicz TA, Silverstone AE. Overexpression of the antiapoptotic oncogene, bcl-2, in the thymus does not prevent thymic atrophy induced by estradiol or 2,3,7,8,-Tetrachloridibenzo-*p*-Dioxin. Toxicol Appl Pharmacol 1998;151:200-210.
33. Donskoy E, Goldschneider I. Thymocytopoiesis is maintained by blood-borne precursors throughout postnatal life: a study in parabiotic mice. J Immunol 1992;148:1604–1612.
34. Seaman WE, Gindhart TD. Effect of estrogen on natural killer cells. Arthritis Rheum 1979;22:1234–1240.
35. Ansar Ahmed S, Talal N. Sex steroids, sex steroid receptors, and autoimmune diseases. In: Sheridan PJ, Blum K, Trachtenberg MC, editors. Steroid receptors and disease: Cancer, autoimmune, bone, and circulatory disorders. Marcel Dekker, Inc. New York (New York, USA) and Basel (Switzerland), 1988. p 289–316.