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Apoptosis of γ/δ T Cells in Human Ehrlichiosis

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Expansion of activated T cells expressing the T-cell receptor (TCR) γ/δ, CD45RO, and HLA-DR antigens is a prominent feature of acute infection with *Ehrlichia chaffeensis* in humans. The fate of these activated cells and the resolution of the γ/δ T-cell response with return to the usual α/β T-cell populations in this disease are not clearly understood. At a morphologic level, apoptotic cells are present in the peripheral blood during the acute and resolution phases of the infection. Simple culture of density gradient-separated lymphocytes from the blood of patients with acute ehrlichiosis produced cell death rapidly in the media compared to α/β T cells. This loss of viability after incubation was apparently mediated by apoptosis, based on flow cytometric and morphologic analyses. The results suggest that most primed (CD45RO$^+$) and activated (HLA-DR$^+$) γ/δ T cells in acute ehrlichiosis might be subject to removal from the body by programmed or apoptotic cell death. (Key words: Ehrlichiosis; γ/δ T cells; Apoptosis) Am J Clin Pathol 1996;105:640–646.

Apoptosis is a form of programmed cell death that occurs during normal and pathologic cell turnover and is distinguished from necrosis by unique cellular events.[1–3] During apoptosis, nuclear chromatin is degraded into specific nucleosomal fragments via an energy-dependent process of gene-directed cellular destruction that is mediated by an endonuclease giving rise to the characteristic "ladder pattern" of 200 bp DNA fragments seen by electrophoresis.[3] On a morphologic level, apoptosis is characterized initially by water loss and cell shrinkage, followed by condensation of nuclear chromatin, nuclear disintegration, blebbing of the cell surface, and membrane-bound apoptotic bodies. This process has been studied by flow cytometric methods in which alterations of the light scatter pattern correlates with cell shrinkage, and a sub-G_0/G_1 (A_0) peak of DNA content is present based on leakage of nucleosomal fragments from cells fixed, permeable, and stained with a DNA intercalating dye such as propidium iodide (PI). Although detection of apoptosis by gel electrophoresis has clearly been a useful technique, flow cytometric methods have proven superior to investigate apoptosis of mixed cell populations, particularly when the cells of interest are limited in number and density-gradient separation may selectively loose apoptotic cells.[1,2]

Human ehrlichiosis is a recently described disease usually associated with fever, chills, headache, arthralgia, myalgia, nausea, and vomiting, similar to the acute phase of canine ehrlichiosis.[4–7] Laboratory abnormalities are mainly hematologic or manifested as an elevation of hepatic enzymes or both. The most prominent hematologic findings, occurring in 55% to 92% of patients at sometime in the course of their illness, include neutropenia, thrombocytopenia, lymphopenia, anemia, or any combination of these.[5–7] Elevations of hepatic enzymes occur in most patients at the time of presentation, and in at least 80% of patients sometime during the illness.[6,7] Characteristic intraleukocytic inclusions are detected in blood smears in the minority of patients with *E chaffeensis* infections. When seen by light microscopy, these appear round or ovoid, stain purple to dark blue using Leishman stain, or metachromatic with Giemsa stain, and are 2 μ to 10 μ in diameter. Electron microscopy shows each membrane-bound inclusion to contain up to 40 microorganisms with structural features consistent with Rickettsia of the genus *Ehrlichia*.[8] Human ehrlichiosis was initially thought to be secondary to infection with *E canis* or a closely related organism,[7,9] but more recent investigations have suggested that most disease is caused by a newly recognized species designated *E chaffeensis*.[4] However, a granulocytotrophic ehrlichia species closely related to *E phagocytophilia* and *E equi*

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appears to be a second species capable of producing human disease.[10] Although still under investigation, at least one report suggests that *Amblyomma americanum,* the Lone Star tick, may be a vector for *E chaffeensis.*[11]

A lymphocytopenia is almost always present early in the infection, but a lymphocytosis ensues during the treatment phase that is disproportionate to the remaining cell lines. We recently described this resurgent lymphoid hyperplasia as consisting mainly of T cells expressing the immunophenotype $CD3^+4^-8^-$ $V\gamma9^+/V\delta2^+$.[12] The present study suggests that resolution of this γ/δ T-cell hyperplasia involves the mechanism of apoptotic cell death.

MATERIALS AND METHODS

Specimens

Cases of human ehrlichiosis were identified by the Infectious Disease division on the basis of previously described symptomatology.[5]

Peripheral blood was obtained by venipuncture, in compliance with regulations of the Institutional Review Board of the University of Missouri. The diagnosis of ehrlichiosis was confirmed by clinical and laboratory data as previously reported.[5,12] All specimens from cases reported in this study were positive by polymerase chain reaction (PCR) performed as described.[13] The oligonucleotide primer sequences HE1 and HE3 were reported to be homologous to sequences within the 16S rRNA gene of *E chaffeensis* and amplified a 389 bp product from this species, but not from *E canis, E ewingii, E phagocytophilia, E sennetsu,* or *E risticii.*[13]

Immunophenotypic Analysis

For examination of unseparated cells, ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood specimens were immunostained and examined within 2 hours following lysis of erythrocytes according to the manufacturers recommendations (QPrep; Coulter Electronics, Hialeah, FL). Immunophenotypic analysis of lymphocyte subsets was performed as previously described to assist in immunologic evaluations (ref 12; data not shown).

The monoclonal antibodies (MoAbs) used to examine the issue of apoptosis in this study include: CD45 (HLe-1), which recognizes all leukocytes, and TCR α/β, which recognizes the α/β T-cell receptor (Becton Dickinson, Mountain View, CA); and TCRδ1, which recognizes all T cells expressing delta chains of the TCR (T Cell Diagnostics, Cambridge, MA).

For cell culture experiments, peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation (Ficol-paque, Pharmacia, Piscataway, NJ) and subjected to flow cytometric analysis within 4 hours of collection, as previously described.[12]

Identification of Apoptotic Cells

Morphology. Apoptotic cells were sought by light microscopic examination of peripheral blood smears and cytocentrifuged density-gradient-separated cell preparations stained with Wright-Giemsa. Cells that demonstrated a smaller size, shrinkage, and blebbing of cytoplasm and chromatin condensation, and nuclear fragmentation with intensely basophilic staining were deemed apoptotic. The percentage of apoptotic cells was determined from differential cell counts of 200 leukocytes.

Flow cytometric method. Examination of apoptosis was also performed using flow cytometric methods boased on measurements of light scatter, quantitative CD45 intensity, and PI fluorescence.[1,14] The usual method of examining apoptosis by DNA electrophoresis, whereas it does demonstrate the typical "laddering" effect of the regular fragmentation of genomic DNA into oligonucleosomal fragments of 180–200 bp, is insensitive to small numbers of apoptotic cells, is unable to determine the number of apoptotic *versus* nonapoptotic cells and is unable to discriminate subpopulations within complex mixtures of cells.

The method recently described by Carbonari and colleagues[14] uses the flow cytometric parameters of low forward angle light scatter (FALS) and decreased quantitative CD45 expression to determine the apoptotic population. This method circumvents several limitations and provides a simple, rapid means of determining small numbers of cells undergoing apoptosis. We used this method, but also examined DNA staining with PI.

Staining of the cells with PI (5 μg/mL in phosphate-buffered saline (PBS); Sigma Chemicals, St. Louis, MO) after fixation with cold ethanol results in a sub-G_0/G_1, or "A_0," dim peak of fluorescence that contains the apoptotic cells.[1,2] Three parts of cold 70% ethanol:distilled water (v/v) were slowly added to 1.0 mL of cell suspension in cold PBS containing 10% (v/v) fetal calf serum under constant agitation. Cells were fixed for 1 hour at 4 °C, followed by washing and incubation at 4 °C in PBS for 30 minutes. This method quantitatively correlates very well with the electrophoretic method.[1,2,14,15] In some experiments, fixation and PI staining were performed following immunostaining with monoclonal antibodies.

Observed alterations included cell populations with low FALS and similar 90° light scatter compared to nor-

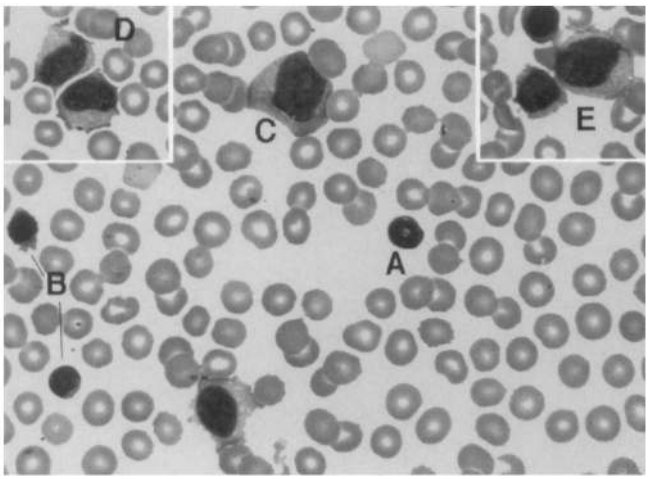

FIG. 1. Composite photomicrograph of peripheral blood lymphoid cells showing heterogeneity of morphology. A, A small apoptotic cell with classical "glassy" nuclear morphology and scant cytoplasm. B, Additional cells undergoing morphologic changes consistent with apoptosis. Cells C–E represent more typical cells found in the blood (Wright-Giemsa stain ×1,000).

mal lymphocytes, an A_0 peak determined by measurement of PI fluorescence intensity, and decreased quantitative CD45 expression.[14]

Cell Preparation and Culture

Peripheral blood mononuclear cells (PBMC) were isolated from sodium-heparinized whole blood by density gradient centrifugation, washed in sterile PBS, and resuspended in RPMI 1640 tissue culture medium (GIBCO Laboratories, Grand Island, NY), supplemented with 10% autologous human plasma, 25 mmol/L N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid, 5×10^{-5} mol/L 2-mercaptoethanol, 0.3 mg/mL L-glutamine, and 200 U/mL penicillin G.

Cell suspensions were cultured at a density of 5×10^4/well for 6 to 48 hours in 96-well flat-bottom microtiter plates (Corning Glass Works, Corning, NY) at 37 °C in 5% (v/v) CO_2, and 95% (v/v) air in a final volume of 200 μL of culture medium. At the time of termination of culture, cells were immunostained for CD45 and cell viability was assessed by PI dye exclusion (1 μg/mL PBS) of fresh, unfixed cells. The pattern of CD45 expression *versus* FALS was used to determine apoptotic cells with decreased CD45 expression and uptake of PI as described.[14]

RESULTS

Morphologic Examination of Unseparated Peripheral Blood Smears

Wright-Giemsa-stained peripheral blood smears were examined morphologically. A representative composite microphotograph from one case is shown in Figure 1. The lymphocytes were very heterogeneous and morphologically apoptotic cells (Figs. 1A and 1B) were smaller than non-apoptotic lymphocytes (Figs. 1C–1E), contained hyperchromatic nuclei without chromatin definition, and all had diminished, irregular cytoplasm. Additionally present were larger lymphocytes with more abundant basophilic cytoplasm and irregular to reniform nuclei with good chromatin definition. The percentage of morphologically apoptotic cells ranged from 1% to 4% at the time of initial examination to 5% to 8% during the resolution of lymphocytosis, which occurred over a period of 1 to 3 weeks (data not shown).

For comparison, a Wright-Giemsa-stained cytocentrifuge preparation of density-gradient separated PBMC is shown in Figure 2. Many of the lymphoid cells are large granular lymphocytes with reniform or cleaved nuclei and a prominent Golgi zone, moderate-to-large amounts of basophilic cytoplasm, and numerous small-to-prominent azurophilic granules. Occasional cells were large, without granules, and had more prominent chromatin clumping and prominent nucleoli. A single ehrlichial inclusion is present in one cell in this field. In some patients, significant numbers of plasmacytoid and/or plasma cells were also present (not shown).

Paired cytocentrifuge preparations and peripheral smears of patients obtained at the same time demonstrated a decreased proportion of apoptotic cells in the density gradient-separated PBMC preparations, consistent with observations of others.[14] In some cases, apoptotic cells were readily found in the smears, but required considerable searching in the cytocentrifuge preparation.

Flow Cytometric Analysis of Peripheral Blood Lymphocytes

As shown in Figure 3, the light scatter histogram of PBMC reveals an additional peak of lymphoid cells that

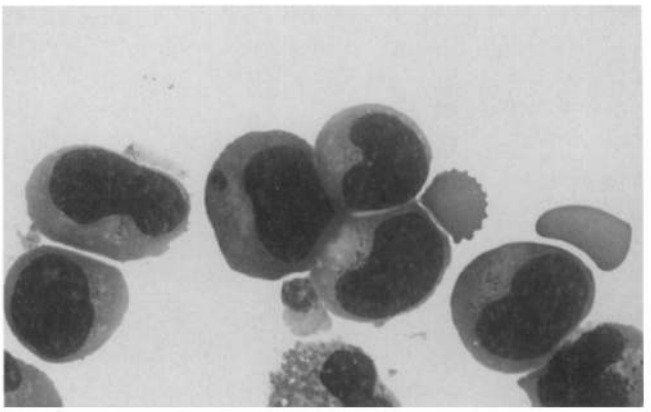

FIG. 2. Cytocentrifuged preparation of peripheral blood mononuclear cells following density gradient centrifugation and Wright-Giemsa staining (×2,000).

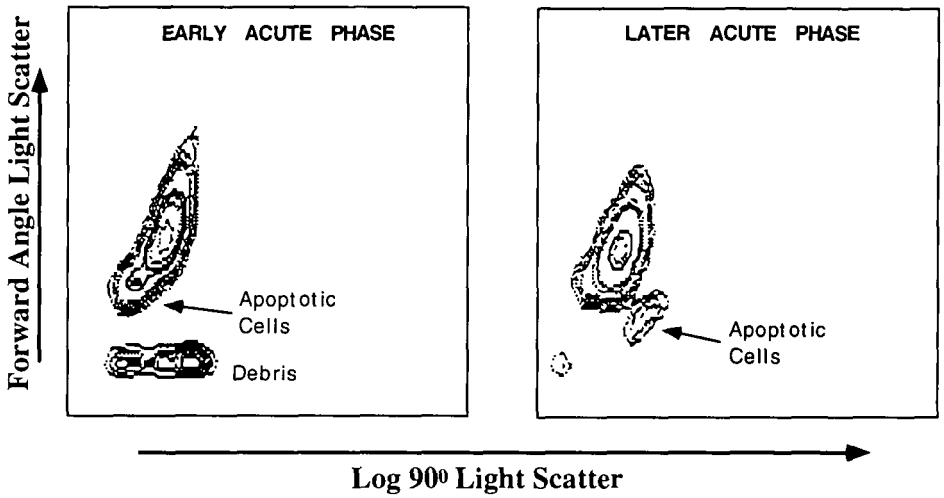

FIG. 3. Flow cytometric histograms of forward and 90° light scatter from typical preparations of density gradient-separated peripheral blood mononuclear cells taken from patients early and late in acute infection.

are somewhat smaller than most other lymphocytes, similar to the findings of others.[1,14,15] These cells have lower FALS, a correlate of cell size, and 90° light scatter that is variable, dependent on the timing of the specimen collected. Histograms from specimens collected early in the course of the acute illness (fig. 3, left panel) tended to have lower 90° light scatter than those collected during the later phase of the infection (Fig. 3, right panel). In the illustrated case, "early" was approximately 1 week from onset of symptoms, whereas "late" was 10 to 12 days and 2 days on antibiotic therapy.

Figure 4 illustrates a representative dual-parameter histogram of fluorescence intensity (FI) from CD45-stained cells *versus* FALS. In specimens collected during both the "early" and "late" phases of the infection, the smaller cells are associated with lower CD45 FI, which previously has been reported associated with apoptotic lymphocytes.[14] A similar histogram in Figure 5 shows that the CD45-dim cells are also predominantly expressing the γ/δ heterodimer (14%) of the T-cell receptor (TCR) rather than α/β TCR, although a small percentage of α/β T cells (4%) also have dim CD45.

Based on this dual population of CD45 FI, experiments were performed to determine the flow cytometric DNA content from each peak. Following immunostaining with CD45, the cell suspension was fixed in cold ethanol and stained with PI as described above. Figure 6 shows that the $CD45^{dim}$ population also has decreased PI-associated FI compared to the residual normal lymphocytes from the $CD45^{bright}$ peak. The percentage of apoptotic cells was thus calculated from the $CD45^{dim}$ peak.

Incubation-Induced Cell Death via Apoptosis From Acute Ehrlichiosis Patients

Based on earlier reports of spontaneous apoptosis of α/β T cells (on the basis of CD4 and CD8 expression) in patients infected with HIV or Epstein-Barr virus (EBV),[14,15] we investigated the same process associated with the γ/δ T cells in ehrlichiosis. The majority of γ/δ T cells from patients with acute ehrlichiosis rapidly underwent spontaneous apoptosis (as measured by flow cytometry) and cell death when placed into cell cultures, whereas the viability of α/β T cells from these same specimens remained high (Fig 7).

To determine what type of cells in ehrlichiosis patients underwent apoptosis, PBMC cultures from 3 individuals were incubated and evaluated for their surface phenotypes and viability by flow cytometry and the presence of

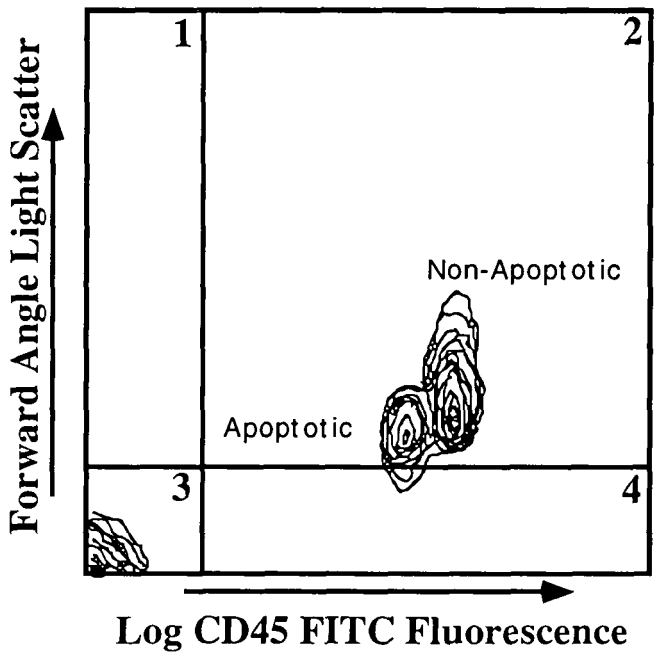

FIG. 4. Flow cytometric histogram of CD45 expression (x-axis) *versus* forward angle light scatter as a correlate of cell size.

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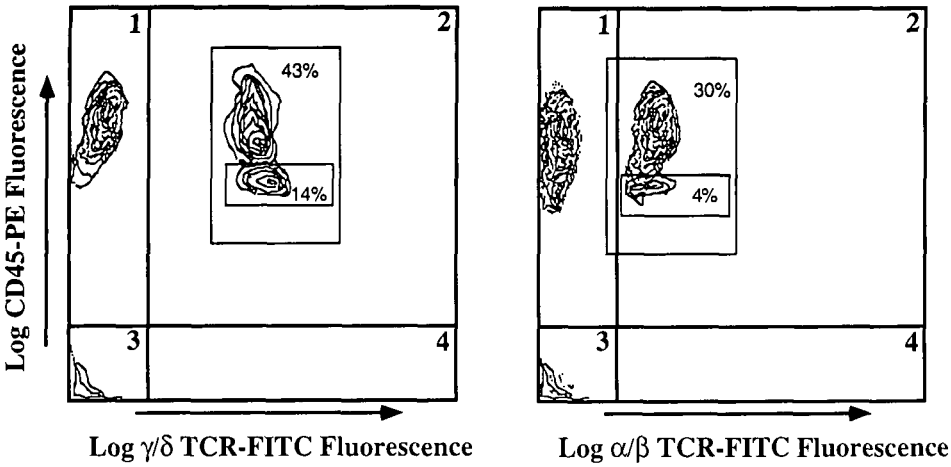

FIG. 5. Flow cytometric histograms of dual labeling of lymphocytes with CD45 (y-axis) and monoclonal antibodies reactive with γ/δ T cells (left panel) or α/β T cells (right panel).

morphologically apoptotic cells. Using PI uptake of live, unfixed cells as an indicator of cell viability, PI-negative cells were examined for surface expression of the α/β or γ/δ TCR.

Incubation in culture medium alone resulted in steadily reduced viability of γ/δ T cells as assessed by PI dye exclusion of unfixed cells. During the earlier phases of apoptosis (4–6 hours), the plasma membrane of cells is preserved and PI is excluded.[1] With more time, these cells begin to take up PI as they lose membrane integrity and subsequently die. In our experiments (Fig. 7), loss of cell viability as assessed by PI uptake was observed as early as 6 hours after incubation, increased with time, and further incubation led to about 70% cell death within 36 to 48 hours. In contrast, less than 10% of α/β T cells died in the same culture conditions during the observation period. The time course of apoptosis in ehrlichiosis disclosed that the appearance of apoptotic cells preceded the PI dye exclusion as a measure of the loss of cell viability, similar to observations of others.[1,2] These results suggested that γ/δ T cells in acute ehrlichiosis patients spontaneously died rapidly after incubation and that the residual population was comprised mainly of α/β TCR-expressing T cells, with loss of γ/δ T cells.

Apoptotic cells were easily identified on Wright-Giemsa-stained cytocentrifuged preparations by light microscopy (Fig. 8). A sizable proportion of γ/δ T cells that had been briefly incubated in the culture medium alone developed the condensed and fragmented nuclei

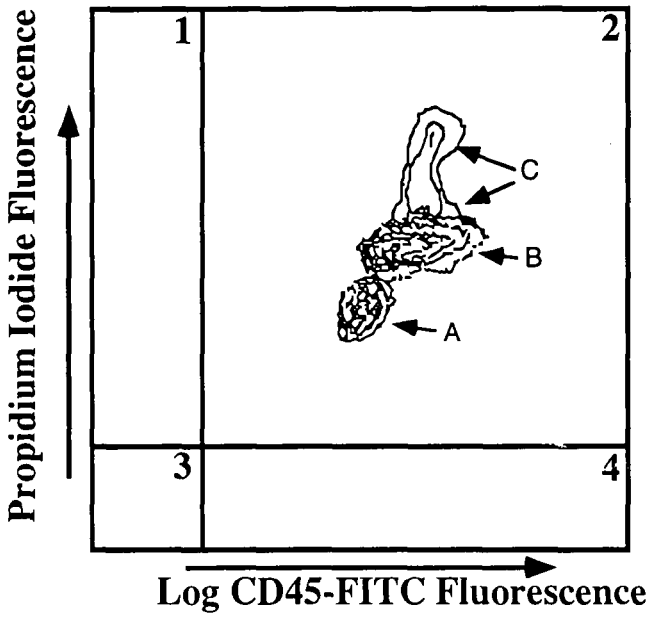

FIG. 6. Flow cytometric histogram of CD45 expression (x-axis) *versus* relative DNA content expressed as propidium iodide (PI) fluorescence (y-axis). The peak (A) contains apoptotic cells simultaneously expressing dim CD45 and dim PI fluorescence. Non-apoptotic cells expressing bright CD45, the G_0/G_1 peak (B), and the S/G_2+M peak (C).

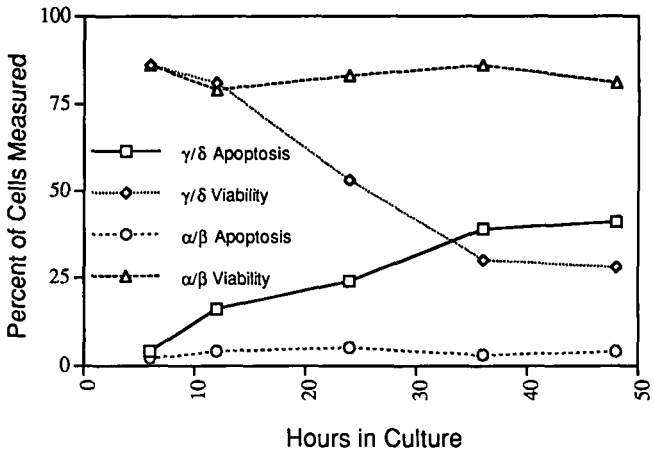

FIG. 7. Graph of cell viability and apoptosis of the α/β and γ/δ T cell populations over the 48-hour culture.

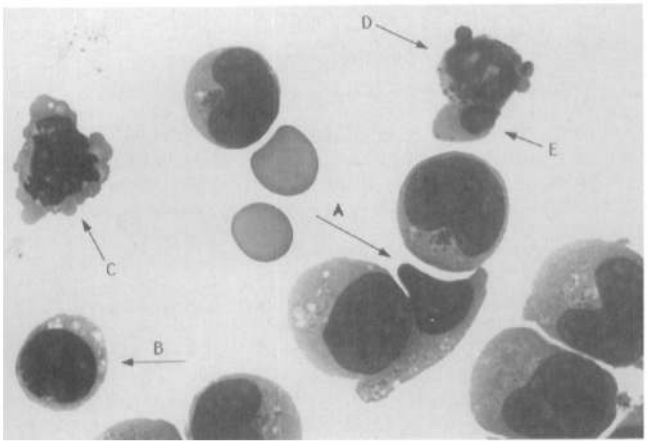

FIG. 8. Cytocentrifuged preparation of density gradient separated cells after 12 hours in culture. Note various stages of apoptotic changes (A–E) (Wright-Giemsa staining ×2,000).

with loss of cell volume, which is the characteristic hallmark of cells undergoing apoptosis.[1,3] In fact, in Figure 8 (8A–8E) illustrate what might be considered increasing morphologic stages of apoptosis.

Not only cultured PBMC spontaneously underwent apoptosis, but also whole blood specimens. When peripheral blood smears were made at various times, there was a morphologically detectable increase in apoptotic cells by 8 to 24 hours (data not shown).

DISCUSSION

Necrotic cell death is nonspecific and results from of an inability of cells to recover from injury, but programmed cell death, termed apoptosis, is an essential energy-requiring regulatory process in the immune system.[1,3] Antigen-induced apoptosis of mature human α/β T cells in the periphery has been described in patients with EBV mononucleosis and in HIV-positive individuals,[14,15] and now appears to also be involved in cell death of γ/δ T cells associated with ehrlichiosis.

The temporal change in light scatter involving the apoptotic peak compared to the normal cell peak is of interest. A hypothetical model might be proposed that lymphocytes lose water and therefore demonstrate decreased cell volume revealed as decreased FALS. As apoptosis continues, these cells lose CD45 FI and 90° light scatter with further shrinkage of the cytoplasm. As the cytoplasmic membrane becomes fixed and nuclear fragmentation ensues, there remains decreased FALS and acquisition of increased 90° light scatter. The finding of overlap between $CD45^{dim}$ and $CD45^{bright}$ cells within the decreased FALS region of the light scatter histogram supports the observations that water loss and cell shrinkage precede the downregulation or loss of surface molecules such as CD45.[14] This process is illustrated morphologically in Figure 8.

Human T cells are characterized by expression of a group of cell surface proteins designated the CD3/TCR complex. This complex consists of at least six proteins, including four invariant chains designated γ,δ,ϵ, and ζ, and two additional heterodimeric chains that may be composed of either α and β or γ and δ chains. Most peripheral blood T cells (>90%) express the α/β TCR, whereas only a minority express the γ/δ form.[16,17]

These γ/δ T cells are increased in certain infectious lesions, and modestly elevated in peripheral blood in certain disease states. Human ehrlichiosis is characterized by hematologic abnormalities including multi-lineage cytopenias. A lymphocytopenia has been present either at diagnosis, or at some time during the illness in most patients reported.[5] Early in the course of antibiotic treatment (48 to 72 hours), the lymphocytopenia corrects and is rapidly followed by a lymphocytosis of T cells that express CD3, but are negative for CD4 and CD8, as well as the major form of the TCR formed by the α/β heterodimer.[12] Instead, these hyperplastic $CD3^+4^-8^-$ T cells express the γ/δ heterodimer associated with $V\gamma9$ and $V\delta2$ chains,[12] a population of cells usually the distinctive minority of peripheral blood T cells, but constituting the major phenotype of peripheral γ/δ T cells.

In adults almost all $V\delta2^+$ T cells coexpress CD45RO, the low M_r isoform of the CD45 family of transmembrane protein tyrosine phosphatases (PTPase).[18] If indeed the CD45 program is similar in α/β and δ/δ T cells, then this would support the concept of a reactive lymphocytosis of memory γ/δ T cells in response to an undefined antigen(s). In patients with acute infectious mononucleosis, the majority of $CD4^+$ as well as $CD8^+$ T cells expressed CD45RO, which is considered a marker of memory T cells.[15] In the present study, incubation-induced apoptosis was demonstrated mainly within the γ/δ T-cell population, which we have previously shown to be $CD4^-CD8^-$[8], but expressed CD45RO.[12] The issue of memory in γ/δ T cells is not yet clear. Whereas CD45RO is thought to be associated with acquisition of memory by naive T cells following antigenic stimulation,[19] these studies used unfractionated T cells, so it is not clear if this same acquisition is expected with γ/δ T cells.

The γ/δ T cells in human ehrlichiosis also expressed HLA-DR,[12] which further supports the concept of T-cell activation. It appears that apoptosis occurs mostly in the population of $CD45RO^+$/HLA-DR^+ T cells, and thus the deletion of cells is of interest in the context of how (or if) memory with respect to human ehrlichiosis persists.

Apoptosis-mediated depletion of ehrlichia-activated γ/δ T cells may be important for the limitation of the exaggerated T-cell immune response during resolution of the infection. Apoptosis of activated T cells in acute ehrlichiosis should also provide some clues to help elucidate the pathogenesis of ehrlichial diseases. In addition, acute ehrlichiosis provides a useful model for study of the bifurcation of antigen-primed γ/δ T cells into the peripheral memory T-cell pool and programmed cell death.

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