Upregulation of Surface Markers on Dying Thymocytes

By Hidehiro Kishimoto, Charles D. Surh, and Jonathan Sprent

From the Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

Summary

Using terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) to detect cells undergoing early apoptosis, we have defined the surface markers expressed on $CD4^+CD8^+$ thymocytes undergoing spontaneous or steroid-induced apoptosis in tissue culture. Some surface markers, e.g., CD4, CD8, and heat stable antigen, are downregulated on apoptotic thymocytes. Surprisingly, however, other markers are upregulated; this applies to T cell receptor β/CD3, CD69, and CD25 expression. Upregulation of these markers is restricted to a discrete subset of apoptotic cells.

Immature $CD4^+8^+$ thymocytes have a rapid rate of turnover, and most of these cells are presumed to die in situ from "neglect", i.e., from a failure to receive the signals required for positive selection (1, 2). As in other tissues, T cell death in the thymus involves apoptosis and clearance by macrophages (2–4).

Consistent with their short life span, thymocytes die rapidly when dispersed and placed in tissue culture at 37°C; cell death is associated with DNA degradation, a cardinal sign of apoptosis, and it is potentiated by corticosteroids and ionizing irradiation (5–8). In this article, we examine the phenotype of dying thymocytes with the aid of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL). With this method, we show that apoptosis of immature $CD4^+8^+$ thymocytes in culture is associated with marked changes in surface phenotype.

Materials and Methods

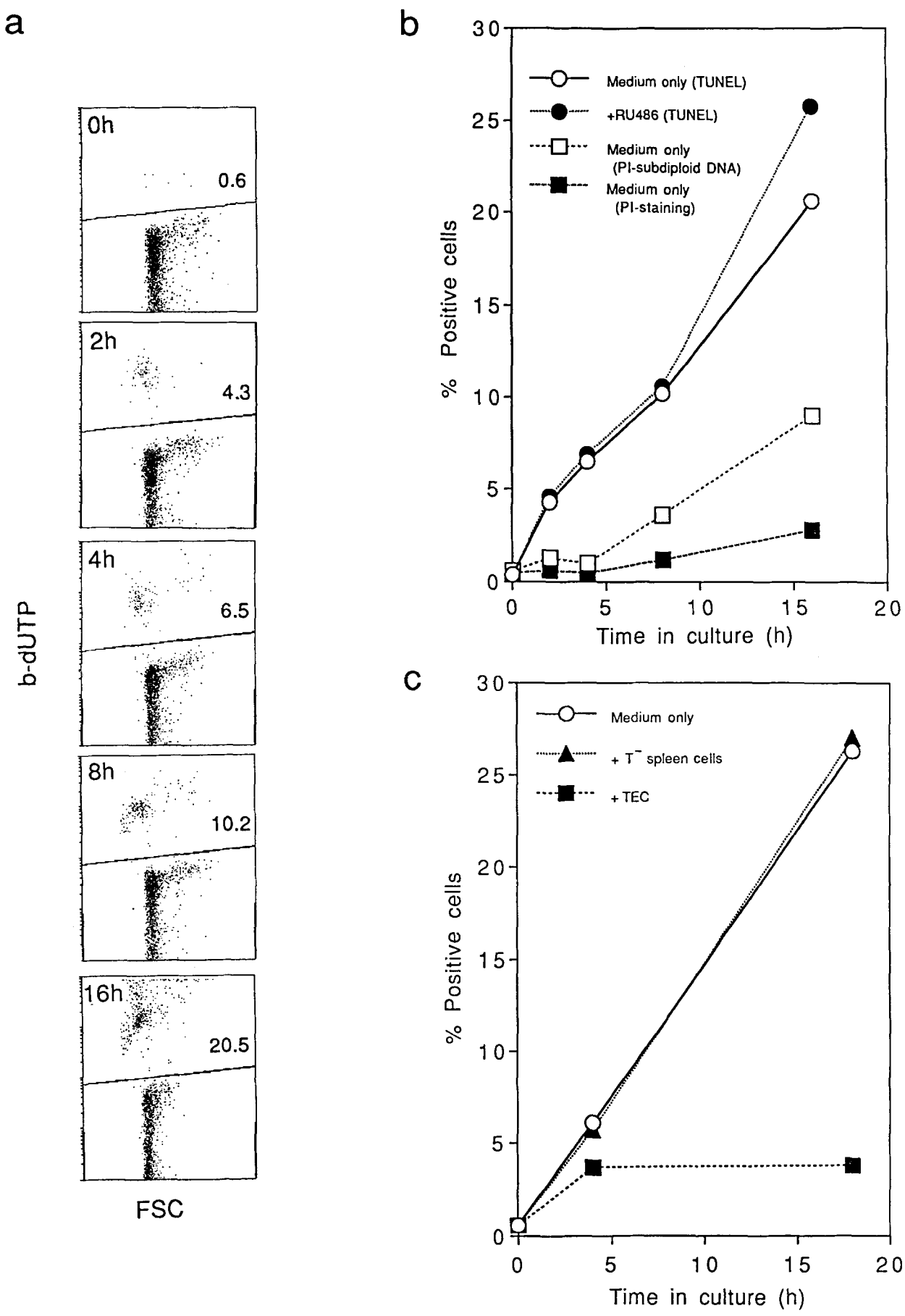
Antibodies. Antibodies specific for the following markers were previously described (9): Thy1.2 (Jlj, rat IgM), CD4 (RL172, rat IgM), CD8 (3.168.8, rat IgM), CD25 (7D4, rat IgM), CD44 (KM201, rat IgG), CD45RB (MB23G2, rat IgG), and HSA (J11D, rat IgM). Cytotoxic mAb specific for CD3 (C363.29B, rat IgG) (10) was used as ascites fluid. FITC-conjugated mAbs specific for TCR-β (H57-597, hamster IgG) (PharMingen, San Diego, CA), surface CD3-ϵ (2C11, hamster IgG) (PharMingen), intracytoplasmic CD3-ϵ (HMT3-1, hamster IgG) (11), CD4 (H129.19, rat IgG) (GIBCO BRL, Gaithersburg, MD), CD5 (53-7.3, rat IgG) (PharMingen) and CD69 (H1.2F3, hamster IgG) (PharMingen), and RED613-conjugated anti-CD8 (53-6.72, rat IgG) (GIBCO BRL) were used for direct staining.

Cell Purification. $TCR^{-/lo}$ $CD4^+$ $CD8^+$ thymocytes were purified from 6–8-wk-old C57BL/6 thymuses by treating cells with anti-CD3 mAb (C363.29B) and guinea pig C for 45 min at 37°C followed by positive panning on plates coated with anti-CD8 mAb. Thymic epithelial cells (TEC) were purified from deoxyguanosine-treated day 15 fetal thymic lobes (12) followed by removal of fibroblasts and $CD45^+$ cells by use of magnetic beads. T-depleted (T^-) spleen cells were prepared by mAb + C treatment (9).

Culture Conditions. Thymocytes (5×10^5) were cultured in 0.2 ml of RPMI medium supplemented with 5×10^{-5} M 2-ME, L-glutamine, and 10% FCS in 96-well tissue culture plates. Where indicated, cultures were supplemented with 10^{-7} M RU486 (Cold Spring Biochemicals, Wilmington, DE), 10^{-8} M dexamethasone (Sigma Chemical Co., St. Louis, MO), and/or 50 μg/ml cycloheximide (Sigma Chemical Co.). Freshly purified TEC (2×10^5) were cultured in 0.2 ml supplemented medium for 20 h in 96-well plates to form confluent monolayers; after removing 0.1 ml medium from the monolayers, thymocytes (5×10^5) were added in 0.1 ml fresh medium. In parallel, 5×10^5 thymocytes were cultured in 0.2 ml with 2×10^5 fresh T^- spleen cells or in medium alone.

Staining for Cell Surface Molecules and Flow Cytometry Analysis. Cells were either incubated with unconjugated mAbs followed by FITC-conjugated mouse $F(ab)_2$ anti-rat IgG (H+L) (Jackson ImmunoResearch Labs., Inc., West Grove, PA) or stained directly with FITC-conjugated mAbs and analyzed on a FACScan® (Becton Dickinson & Co., San Jose, CA) (9). In coculture studies, thymocytes were distinguished by staining with FITC-conjugated anti-Thy-1 mAb.

TUNEL Assay and DNA Staining. DNA breaks were detected by a modification of the end-labeling method (6, 13). Briefly, after surface staining, cells (10^6) were washed twice in PBS, fixed with cold 70% ethanol for 15 min, and refixed with 1% paraformaldehyde in PBS for 15 min on ice. The cells were then washed once in PBS, once in TdT buffer (100 mM cacodylic acid, pH 6.8, 0.2 mM cobalt chloride, 0.1 mM dithiothreitol and 100 μg/ml of BSA), and then incubated in TdT buffer supplemented with 0.1 U/μl TdT (Promega Corp., Madison, WI) and 5 μM biotin-21-dUTP (b-dUTP) (Clontech, Palo Alto, CA) at 37°C for 30 min. The cells were washed and incubated for 30 min with PE-conjugated streptavidin. To detect DNA content (14), cells were fixed in cold 70% ethanol, washed, and incubated in 0.5 ml PBS containing 50 μg/ml of propidium iodide (PI) (Sigma Chemical Co.) and 1 mg/ml of RNase A (Sigma Chemical Co.) for 15 min. Stained cells were analyzed on a FACScan®.



Results

The TUNEL method relies on the fact that nuclei of apoptotic cells contain DNA strand breaks and thus can incorporate nucleotides, for example, b-dUTP, in the presence of TdT. By staining cells before fixation for nucleotide incorporation, the surface phenotype of apoptotic cells can be examined. In initial experiments, we found that TdT-dependent b-dUTP incorporation was negligible when thymocytes were killed by treatments that do not induce apoptosis, for example, exposure to heat or mAb + C treatment (data not shown). In the experiments considered below, b-dUTP incorporation in the absence of TdT was extremely low (<1%) (see Fig. 2, *bottom*).

Death of Unseparated Thymocytes In Vitro. With the TUNEL method, apoptosis of thymocyte suspensions in tissue culture increased progressively and reached 20–30% by 16–18 h (Fig. 1, *a* and *b*). Apoptosis was not reduced by RU486, an inhibitor of corticosteroids (15) (Fig. 1 *b*). The detection of impending cell death with the TUNEL method proved to be appreciably more sensitive than with other methods, e.g., PI staining of unfixed cells or PI definition of subdiploid DNA (Fig. 1 *b*). Apoptosis of thymocytes was not prevented when thymocyte suspensions were cocultured with T^- spleen cells but was greatly reduced when cultured on monolayers of purified TEC (Fig. 1 *c*).

Surface Markers on Cultured TCR$^-$CD4$^+$8$^+$ Thymocytes. To study immature thymocytes, a population of TCR$^{-/lo}$ CD4$^+$8$^+$ (TCR$^-$ DP) cells was prepared from adult thymus by treatment with high concentrations of cytotoxic anti-CD3 mAb (10) + C; this treatment destroys >50% of thymocytes, and the surviving cells are depleted of TCR-α/β^{hi} and -α/β^{int} cells (see below). Before culture, the TCR$^-$ DP cells consisted almost entirely of CD4$^+$8$^+$ (CD4^{hi}8^{hi}) cells and were essentially devoid of typical CD4$^+$8$^-$ and CD4$^-$8$^+$ cells (Fig. 2, *top*). After 20 h culture in vitro in normal medium, CD4^{hi}8^{hi} cells were still prominent but were accompanied by a large population of CD4^{lo}8^{lo} cells merging into CD4^{lo}8$^-$ cells (Fig. 2, *bottom*). Gating on the apoptotic (TUNEL-stained) fraction of the cultured cells revealed that nearly all of these cells were CD4^{lo}8^{lo} and CD4^{lo}8$^-$. By contrast, the nonapoptotic cells displayed the same CD4^{hi}8^{hi} phenotype as the initial population before culture.

Surface Phenotype of Apoptotic Cultured TCR$^-$ DP Cells. Fig. 3 shows surface expression of a variety of markers on TCR$^-$ DP cells before (*bottom left*) and after culture for 20 h in vitro (*right*); staining of fresh whole thymocytes is included as a control. When the surface phenotype of the apoptotic (*R2*) versus the nonapoptotic (*R1*) populations of the 20-h cultured DP cells was compared, the expression of certain markers, e.g., CD5 (Ly-1) and CD44 (Pgp-1), was quite similar on the two populations; this also applied to NK 1.1 and Ly-6c expression (data not shown). Other markers, that

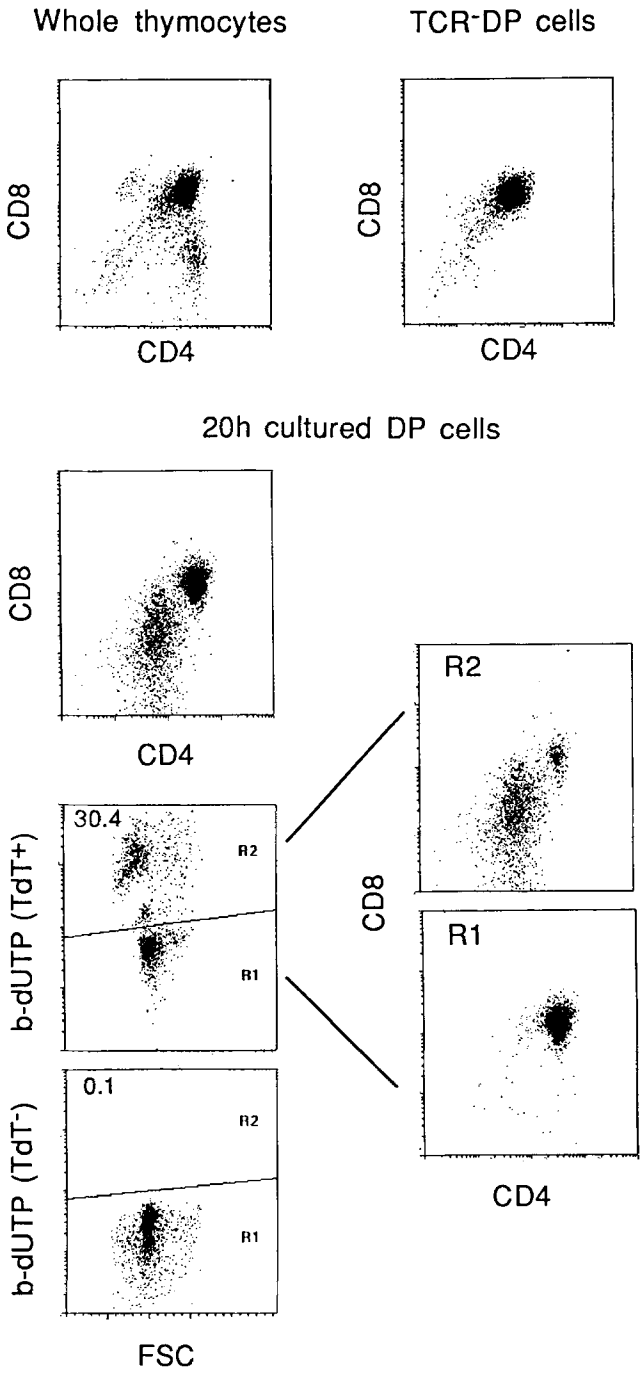


Figure 2. Downregulation of CD4 and CD8 expression on apoptotic thymocytes. The top of the figure shows CD4 versus CD8 expression on fresh whole thymocytes and fresh TCR$^-$ DP cells. The bottom panels show CD4 versus CD8 expression after culturing TCR$^-$ DP cells for 20 h in vitro. After culture, the cells were surface stained for CD4 and CD8 expression and then TUNEL stained (±*TdT*) after fixation. CD4 and CD8 expression on apoptotic (*R2*) versus nonapoptotic (*R1*) cells is shown on the right.

Figure 1. Apoptosis of thymocytes in tissue cultures detected with the TUNEL method. (*a*) TUNEL staining of thymocytes (see Materials and Methods) after culture in vitro for the intervals shown. (*b*) Rate of cell death of thymocytes in vitro as detected by three methods, that is, TUNEL, expression of subdiploid DNA, and PI staining of unfixed cells. (*c*) TUNEL staining Thy 1$^+$ thymocytes cultured in medium alone or with T^- spleen cells or TEC. Each point represents the mean of values from duplicate cultures.

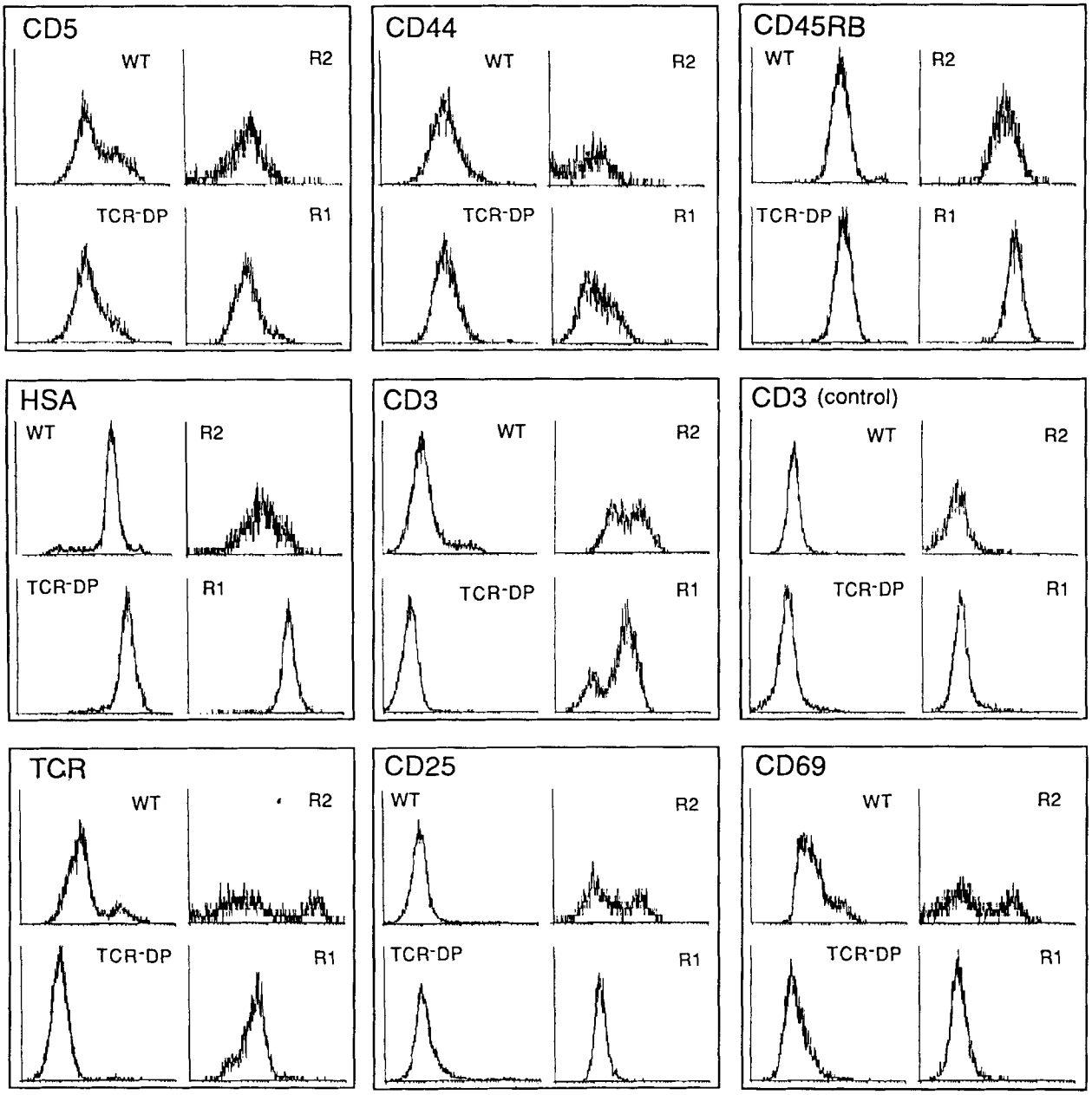


Figure 3. Surface marker expression on DP thymocytes cultured for 20 h in vitro. After culture, cells were surface stained for the markers shown and then TUNEL stained. The right portion of each panel shows staining of surface markers on apoptotic (*R2*) versus nonapoptotic (*R1*) cultured DP cells; control staining of whole thymocytes (*WT*) and fresh TCR$^-$ DP cells before culture is displayed on the left of the panels.

is, HSA and CD45RB, were expressed at a lower density on the apoptotic population than on live cells (Fig. 3) (twofold reduction for CD45RB and fivefold reduction for HSA). Surprisingly, the expression of four further markers, i.e., CD3 (CD3-ϵ), TCR-β, CD69, and CD25 (IL-2Rα), was biphasic on the apoptotic cells. For these markers, the bulk of the apoptotic cells showed the same level of expression as the viable (*R1*) cells. However, for 30–40% of the apoptotic cells, the four markers were expressed at much higher levels than on the viable cells; this was especially prominent for TCR-β expression.

The strong staining for CD3, TCR-β, CD69, and CD25 expression on a proportion of apoptotic cells contrasted with the low expression of these markers on the starting population of TCR$^-$ DP cells. With regard to artifact, it may be noted that the strong staining for surface CD3-ϵ and TCR-β (detected with hamster IgG mAbs) differed from the low staining with an isotype-matched control mAb specific for the intracytoplasmic domain of CD3-ϵ [Fig. 3, *CD3 (control)*]. Likewise, the strong surface staining for CD25 and CD69 (rat IgM) contrasted with the low staining for HSA (rat IgM).

As measured by forward scatter, the apoptotic cells ex-

pressing high levels of TCR-β and CD69 tended to be smaller than the cells expressing low levels of these markers (Fig. 4 *a* and data not shown). Double staining indicated that most of the TCR-β^{hi} cells were CD69^{hi}, and vice versa (data not shown). For TCR-β, surface staining for TCR-β expression on 20-h cultured double positive (DP) cells followed by fixation and PI staining (to detect DNA content) showed that ~50% of the TCR-β^{hi} cells had subdiploid DNA (Fig. 4 *b*). However, the majority of the cells with subdiploid DNA were TCR-β^{lo}, which rules against the possibility that the cells (or cell particles) exhibiting high TCR-β expression were simply "sticky."

Effects of Cycloheximide and Dexamethasone. As shown in Fig. 5 (*top*), culturing thymocytes for 20 h with cycloheximide (Cyh), an inhibitor of protein synthesis, prevented the nonapoptotic (*R1*) cells from upregulating TCR-β expression to the intermediate level. However, Cyh failed to prevent very high TCR-β expression on a subset of apoptotic (*R2*) cells; likewise, Cyh failed to impede upregulation of CD69 expression on the apoptotic cells. In addition, Cyh failed to prevent downregulation of CD4 and CD8. Culturing thymocytes for 20 h with dexamethasone (Dex) before staining (Fig. 5, *bottom*) markedly enhanced apoptosis, that is, from 34 to 98%. However, Dex-induced apoptosis caused little or no change in the proportion of TCR^{hi} and CD69^{hi} cells; CD5 expression on the apoptotic cells remained low. Confirming the findings of others (5), apoptosis induced by Dex was blocked by Cyh. However, Cyh failed to prevent the "background" apoptosis occurring in the absence of Dex.

Discussion

Information on the surface markers expressed on dying thymocytes (and other cells) is sparse and largely limited to the finding that negative selection of immature thymocytes in culture is preceded by partial downregulation of CD4 and CD8 (16, 17). The TUNEL method, with its capacity to detect cells in the earliest stages of apoptosis and allow surface staining before fixation, is ideal for defining the surface molecules expressed on dying cells. With this method, we show here that apoptosis of DP thymocytes in culture is associated with marked changes in surface phenotype. Some surface markers are expressed at the same level on apoptotic cells as on viable cells (CD5, CD44, NK 1.1, Ly-6c). However, other markers (CD4, CD8, HSA, CD45RB) are downregulated on apoptotic cells, while yet others (CD3/TCR, CD69, CD25) are upregulated.

Downregulation of surface markers on apoptotic cells might simply reflect progressive degradation of cell components as a by-product of apoptosis. Explaining surface marker upregulation is more difficult. Staining artifact seems unlikely since the intensity of surface staining on apoptotic cells did not correlate with Ig isotype or the species origin of the mAbs

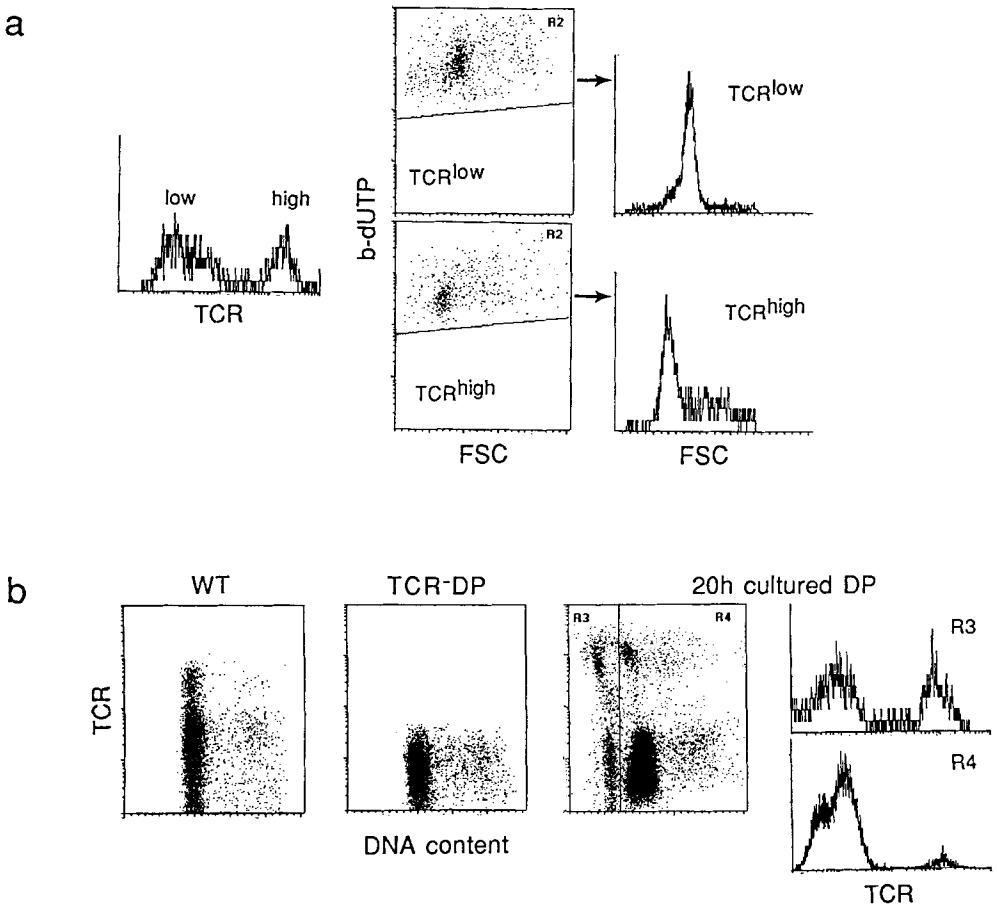


Figure 4. Features of TCR-β^{hi} apoptotic cells. DP thymocytes were cultured for 20 h in vitro and then stained as in Fig. 3. (*a*) FSC profiles of TCR-β^{lo} versus TCR-β^{hi} apoptotic (*R2*) cells. (*b*) Surface TCR-β expression versus DNA content (PI staining of nuclei) of WT, fresh TCR$^-$ DP cells, and 20-h cultured DP cells. To measure DNA, cells were surface stained for TCR-β and then fixed and stained with PI. For cultured DP cells, TCR-β expression on cells with subdiploid (*R3*) versus ⩾diploid (*R4*) DNA content is shown on the right.

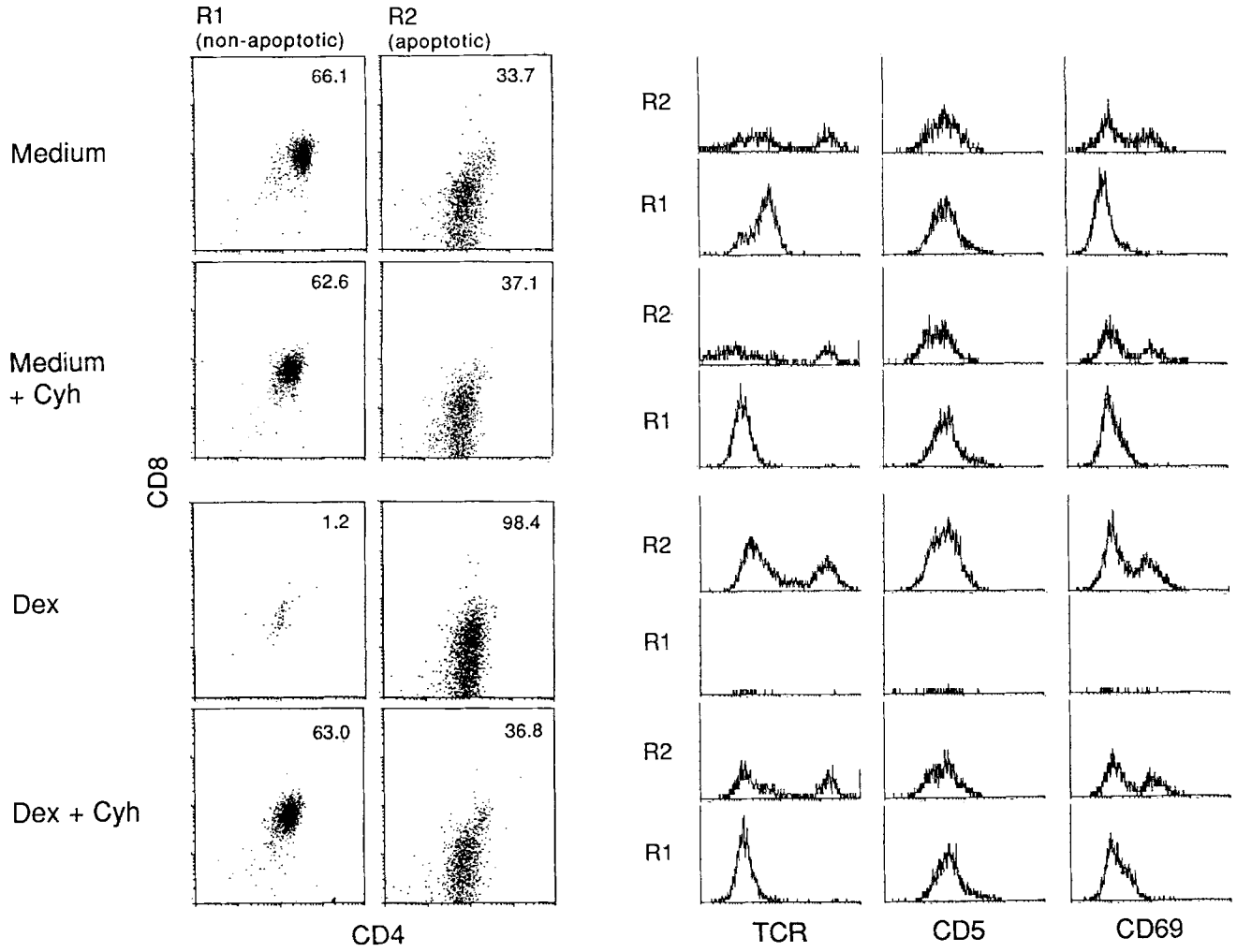


Figure 5. Surface markers on DP thymocytes cultured with cycloheximide (*Cyh*) and/or dexamethasone (*Dex*). TCR$^-$ DP thymocytes were cultured in medium alone or with Dex ± Cyh for 20 h and then stained as in Fig. 3. CD4 versus CD8 expression (*left*) and TCR-β, CD5, and CD69 expression (*right*) on apoptotic (*R2*) versus nonapoptotic (*R1*) cells are shown.

used. Protein synthesis was not involved because surface marker upregulation occurred in the presence of Cyh. Most (but not all) of the apoptotic cells showing surface marker upregulation were small in terms of forward scatter and contained subdiploid DNA. However, it is striking that, even for cells with subdiploid DNA, the upregulation of surface markers on apoptotic cells was restricted to a discrete subset of 30–40% of cells; this percentage applied not only to spontaneous apoptosis in culture but also to Dex-induced apoptosis.

More recently, double staining for TCR-β before and after fixation (using two colors) has shown that TCR-β expression on apoptotic DP cells is largely confined to the cell surface rather than the cytoplasm (our unpublished data). Indeed, intracytoplasmic TCR-β expression is lower in apoptotic cells than in viable cells. Upregulation of surface markers on apoptotic DP cells could thus be a reflection of a breakdown in intracellular trafficking leading to accumulation of markers on the cell surface. Why only a subset of apoptotic cells show surface marker upregulation, however, has yet to be resolved. It is also unclear why the markers upregulated on apoptotic cells include activation markers, that is, CD69 and CD25.

In conclusion, this article presents the first direct evidence that apoptosis causes a radical alteration in the expression of molecules on the cell surface. The factors controlling the redistribution of surface markers on apoptotic cells remain to be elucidated.

We thank Dr. P. Le for sharing his TUNEL protocol and Ms. Barbara Marchand for preparation of the manuscript.

This work was supported by grants CA38355, CA25803, and AI21487 from the United States Public Health Service. C.D. Surh is a Special Fellow of the Leukemia Society of America.

Address correspondence to Dr. Jonathan Sprent, Department of Immunology, IMM4, The Scripps Research Institute, 10666 N. Torrey Pines Road, La Jolla, CA 92037.

Received for publication 14 September 1994 and in revised form 3 November 1994.

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