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Simultaneous Analysis of Immunophenotype and Apoptosis of Murine Thymocytes by Single Laser Flow Cytometry[1]

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The study of the role of apoptosis in thymocyte development has been hampered by the lack of a means of directly immunophenotyping cells undergoing the early phase of apoptosis. This restriction has been overcome by single laser flow cytometry in which apoptosis is detected by Ethidium Bromide (EBr) staining and cell phenotype by binding of FITC-labelled antibody. The initial phase of apoptosis is observed as a cell population that stains faintly with EBr preceding the characteristically bright EBr-staining normally associated with cell death. Here we directly demonstrate using single laser flow cytometry that $CD4^+$ $CD8^+$ $CD3^{low}/CD3^{intermediate}$ thymocytes undergo apoptosis in vitro in response to glucocorticoid treatment.

Key terms: Programmed cell death, thymocyte deletion, apoptosis, immunophenotype, ethidium bromide

Apoptosis, or programmed cell death, is increasingly being recognised as the primary physiological mode of cell death. Unlike necrosis, which is cellular death and disintegration brought about by severe environmental alterations from the physiological norm (e.g., hyperthermia, anoxia), apoptosis occurs under physiological conditions and is an active process of cell suicide brought about by the cell itself in response to specific signalling or cytokine deprivation (reviewed in 12,19,21). Apoptosis has been demonstrated to occur in tissues undergoing processes as diverse as embryogenesis, metamorphosis, and immunological development, where selective deletion of certain cells without concomitant inflammation is advantageous (7,8,17).

Cells which had undergone apoptosis were originally identified microscopically by the characteristic formation of condensed bodies of nuclear material. Later it was recognised that a major hallmark of this process was the fragmentation of the genomic DNA into oligomers of ~200 base pair multiples, corresponding to the unit length of DNA coiled around a histone complex. This process was shown to be a result of the activation of a Ca^{++} dependent endonuclease (20,23) and to be ultimately responsible for the cell's death (3,11).

Apoptosis proceeds by fairly well defined steps. Initially there is a sustained moderate elevation in intracellular free Ca^{++} ions, followed by rapid cell shrinkage due to fluid loss, with a corresponding increase in cell density. Endonuclease mediated nuclear disintegration follows, with the appearance of crescentic nuclear morphology. The cell surface takes on a blebbed appearance, with fusion occurring between the endoplasmic reticulum and the plasma membrane. Finally, there is the formation of "apoptotic bodies," smaller membrane bound vesicles of nuclear material which are frequently engulfed by neighbouring phagocytes due to a specific receptor-mediated process (5).

Due to the increasing interest in the role of apoptosis in thymic development and immunological selection, we were interested in the development of a means of simultaneously analysing apoptosis and immunophenotype of cells. Previous studies have used subtractive procedures to immunophenotype cells which had undergone apoptosis by comparing viable cells with the starting population. This approach has inherent problems of antigenic modulation, e.g., when anti-CD3/TCR antibodies are used in vivo to induce apo-

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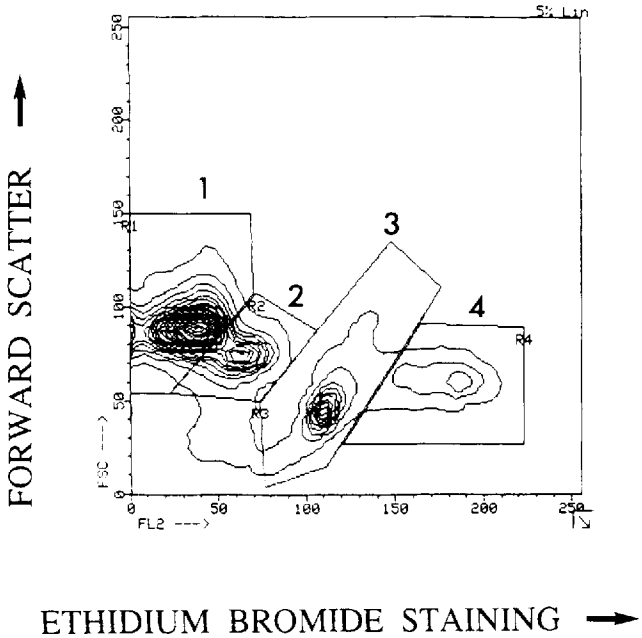

FIG. 1. The stages of apoptosis revealed by ethidium bromide staining. EBr staining resolved four distinct stages in a culture of thymocytes undergoing apoptosis. Contour plot of forward scatter against EBr fluorescence of murine thymocytes cultured for 24 h. Region 1—viable nonstaining cells. Region 2—FSC low/EBr dim cells. Region 3—EBr intermediate cells. Region 4—EBr bright cells.

ptosis (16). Here we report the development of a procedure for immunophenotypic analysis by single laser flow cytometry of cells undergoing the initial stages of apoptosis.

MATERIALS AND METHODS

Cell Preparation and Culture

Thymuses from 3- to 4-week-old CBA/Ca mice were dissected from animals sacrificed under ether anesthesia, and a single-cell suspension in sterile Ca^{++} free buffer (Buffer A, 133 mM NaCl, 4.5 mM KCl, 10 mM HEPES pH 7.4) was made by teasing apart lobes and passing through a loose-fitting glass homogeniser.

Cells were cultured at 37°C for up to 72 h in a humidified atmosphere of 5% CO_2 in RPMI 1640 (Gibco, Paisley, Scotland) supplemented with penicillin/streptomycin (Gibco) and 10% FCS (Flow, Rickmansworth, UK), at a density of 10^6 cells per ml. Additions to the cultures included methylprednisolone (Upjohn, Crawley, UK) (1×10^{-5} M) and phorbol ester (TPA) (Sigma, Poole, UK) (1.7×10^{-7} M).

Flow Cytometry

Cells from culture were placed immediately on ice, washed once with ice-cold murine-tonicity phosphate-buffered saline (134.3 mM NaCl, 2.7 mM KCl, 7.15 mM Na_2HPO_4, 2.85 mM KH_2HPO_4, pH 7.2) containing 0.1% w/v BSA and 0.1% w/v NaAzide (PBS/BSA/Az) and 5×10^5 cells incubated on ice for 45 min in 96-well, round bottomed microtitre trays (Nunc, Denmark) with appropriate concentrations of the following monoclonal antibodies: anti-CD3 (Clone 145-2C11, biotinylated, Pharmingen, San Diego CA); anti-CD4 (Clone YTS 191.1, phycoerythrin-conjugated, Caltag Laboratories, So. San Francisco, CA); anti-CD8 (Clone YTS 169.4, fluorescein conjugated, Caltag Laboratories, So. San Francisco, CA).

Where a second step avidin-FITC reagent was used, the cells were washed twice with PBS/BSA/Az and the appropriate dilution of avidin conjugate (Sigma) added. In addition, 50 μl of a 1 μg/ml solution of ethidium bromide (EBr) in Buffer A was added to appropriate wells at this stage. After 30 min on ice in the dark, plates were washed three times as before and resuspended in 200 μl PBS/BSA/Az prior to flow cytometric analysis. To assay cellular viability and metabolic activity (14), fluorescein diacetate (stock of 100 μg/ml in dimethylsulfoxide) was diluted 100-fold in RPMI 1640/2% FCS/0.1% NaAz and 50 μl added to wells, along with EBr as before, and trays incubated at room temperature for 15 min.

A total of 10,000 or 20,000 cells were analysed using a FACScan (Becton-Dickinson), with logarithmic amplification of fluorescence detection and side scatter, and linear amplification of forward scatter. Consort 30 software was used for acquisition and LYSIS software used for analysis.

Photography and Microscopic Scoring of Fluorescence

In addition to flow cytometry, some EBr stained cells were viewed microscopically and photographs taken using 400 ASA monochrome film (Ilford HP5). The same field was photographed with both brightfield and blue excitation of fluorescence. A minimum of 200 cells were counted to determine the proportion of cells exhibiting EBr fluorescence.

Extraction and Analysis of Thymocyte Genomic DNA

A modification of the method of Wyllie and Morris (22) was used to assess the fragmentation of thymocyte DNA. Control and cultured thymocytes were pelleted by centrifugation at 200 g at 4°C for 5 min, washed once with an ice cold Ca^{++} free buffer (Buffer A), and resuspended in 250 μl of the same buffer. An equal volume of lysis buffer (20 mM EDTA, 0.5% Triton X-100, 5 mM Tris-HCl pH 8) was added and cells incubated on ice for 15 min. Intact nuclei were pelleted by centrifugation at 500 g at 4°C for 10 min and the supernatant ultracentrifuged at 27,000 g at 4°C for 20 min. Soluble DNA in the supernatant was precipitated

FIG. 2. A timecourse of EBr staining characteristics of thymocytes cultured in RPMI 1640/FCS with and without methylprednisolone. Contour plots of forward scatter vs. ethidium bromide fluorescence. Populations arrowed correspond to the regions defined in Figure 1.

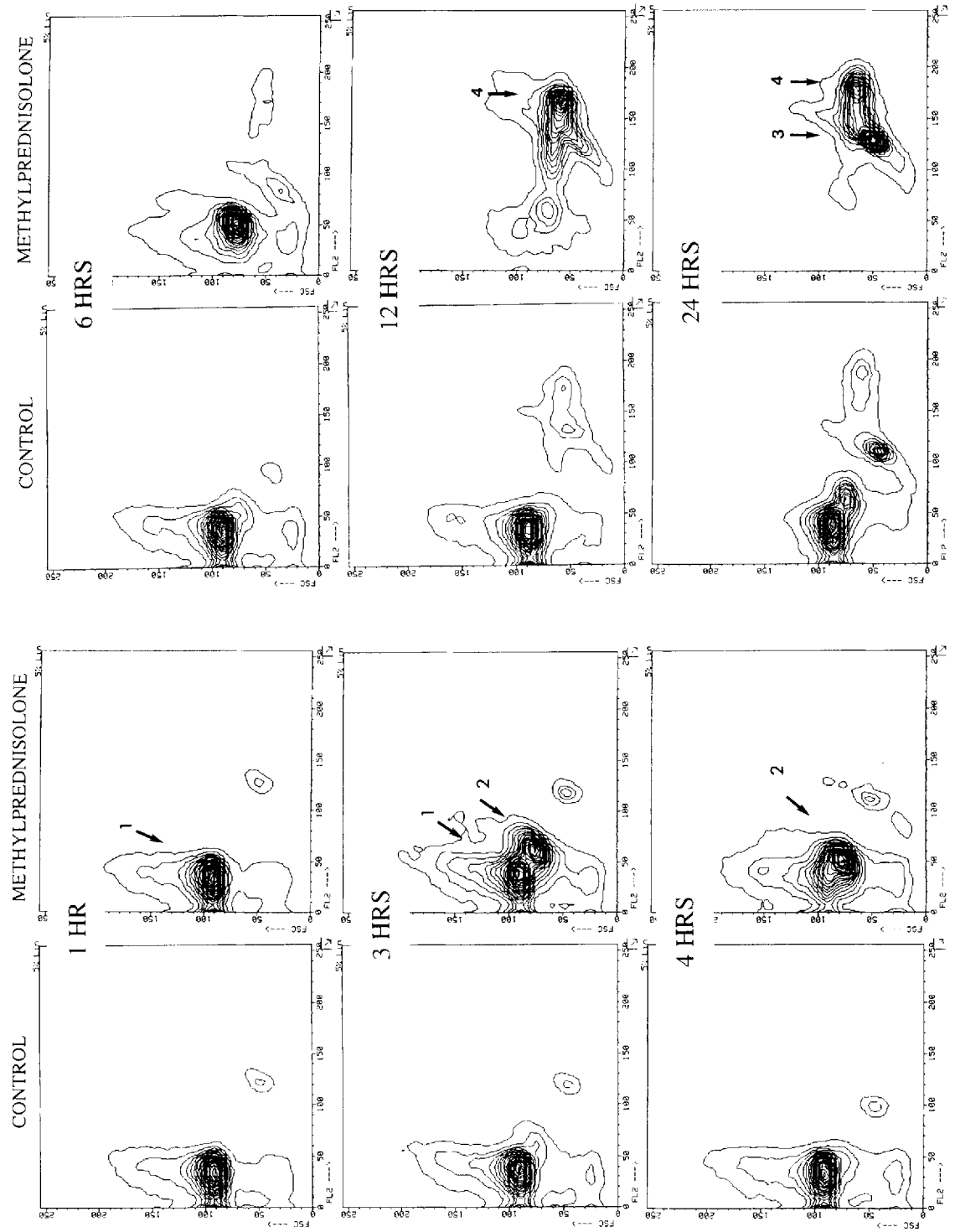
CONTROL
METHYLPREDNISOLONE
1 HR
3 HRS
4 HRS
6 HRS
12 HRS
24 HRS
FORWARD SCATTER
ETHIDIUM BROMIDE STAINING

FIG. 2.

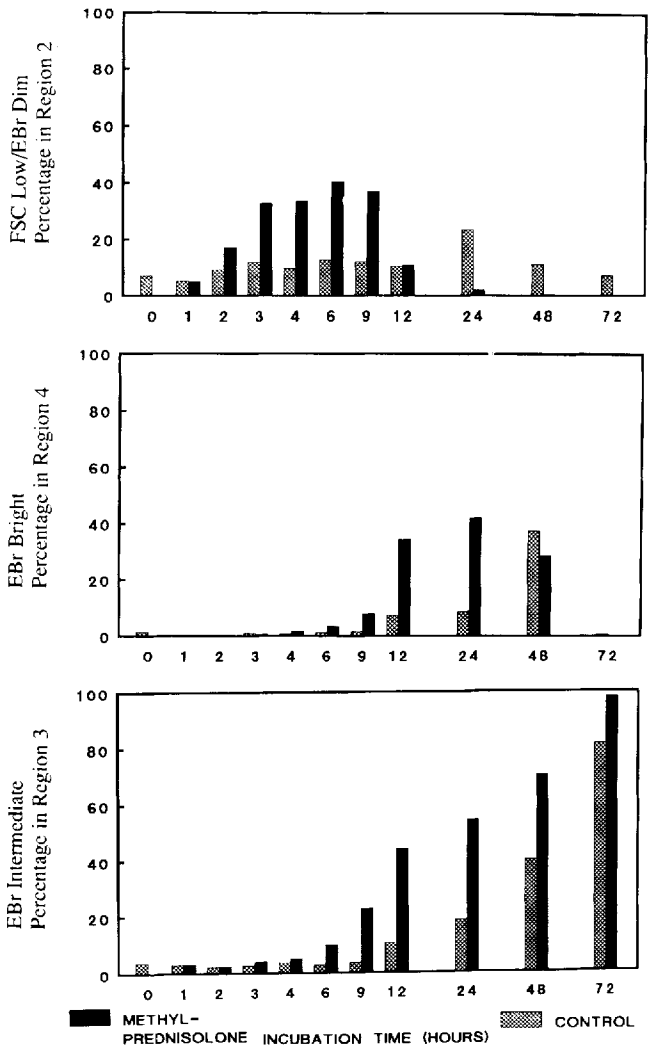

FIG. 3. A timecourse of the sequential movement of thymocytes through the stages defined by the regions 2, 3, and 4 in Figure 1, after culture in RPMI 1640/FCS with and without methylprednisolone. The results are expressed as the percentage of cells in the cultures within the defined region, calculated using LYSIS software.

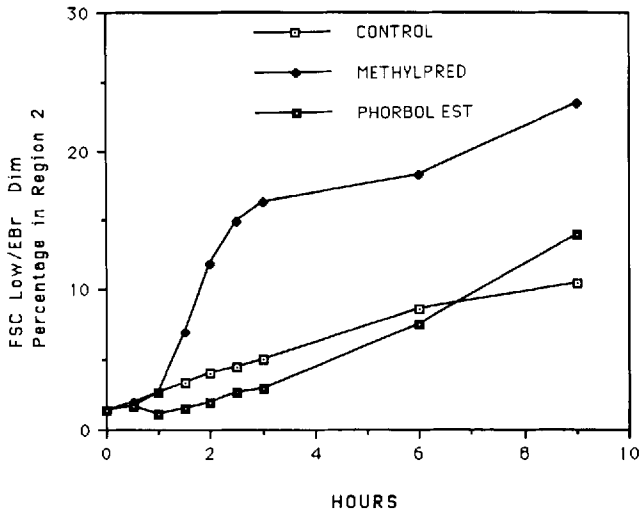

FIG. 4. A timecourse of the entry of thymocytes into the stage defined by Region 2 (FSC low/EBr dim). Thymocytes were cultured alone or in the presence of methylprednisolone or phorbol ester (TPA). In the early timepoints the entry into Region 2 by phorbol ester treated cells lags behind the control culture. However, by 9 h of culture, the total apoptotic proportion (Regions 2, 3, and 4 combined) were 18.1% in the control culture vs. 20.4% in the phorbol ester culture, compared with 45.9% in the methylprednisolone culture, demonstrating that phorbol ester delays thymocyte entry into apoptosis.

at −20°C for 2 h after the addition of 0.13 M NaCl/70% ethanol. DNA was pelleted by microcentrifugation in the cold for 15 min, dried, then resuspended in 10 mM TRIS-HCl/1 mM EDTA pH 7.4. The aqueous phase was extracted twice with chloroform and the DNA precipitated as before. Aliquots of DNA were loaded onto a 1.8% Agarose gel in TAE (0.04 M TRIS-acetate, 0.002 M EDTA, pH 8.0) buffer, and electrophoresed in a flatbed gel apparatus (BioRad, Watford, UK) at 30 V. Gels were stained using EBr at 0.5 μg/ml for 30 min, destained in 1 mM MgSO4 for 30 min, and photographed under transmitted UV light.

RESULTS

Flow Cytometry of Ethidium Bromide Stained Thymocytes

Thymocytes were cultured in medium alone or in the presence of methylprednisolone, a known inducer of thymocyte apoptosis, for up to 72 h. Cells were harvested at intervals after initiation of the cultures and stained with EBr as detailed in the methods. Figure 1 shows a contour plot of forward scatter (FSC) versus EBr fluorescence from an apoptotic culture of thymocytes. Four discrete populations with respect to EBr staining were visible, numbered 1 through 4. The sequential movement of cells through these populations in a limited timecourse of thymocytes cultured alone or in the presence of methylprednisolone was demonstrated in Figure 2. A more extensive timecourse represented in histogram form in Figure 3 shows percentages of cells in the four populations, calculated using the regions defined in Figure 1. Thymocytes from the viable state (Region 1) progressed to a lowered forward scatter/EBr dim stage (Region 2), which was acceler-

FIG. 5. Fluorescein diacetate staining of thymocytes undergoing apoptosis. Cultures of thymocytes in RPMI 1640 treated with methylprednisolone for 0, 3, and 6 h were stained simultaneously with fluorescein diacetate and EBr. Apoptotic stages based on the regions defined in Figure 1 were assessed for green fluorescein fluorescence, and data presented in histogram form. EBr negative = Region 1, EBr dim = Region 2, EBr bright and intermediate = Regions 3+4. The dotted lines indicate the fluorescence intensity of fully permeable "dead" cells, solid lines indicate maximum retention of fluorescein by "live" cells.

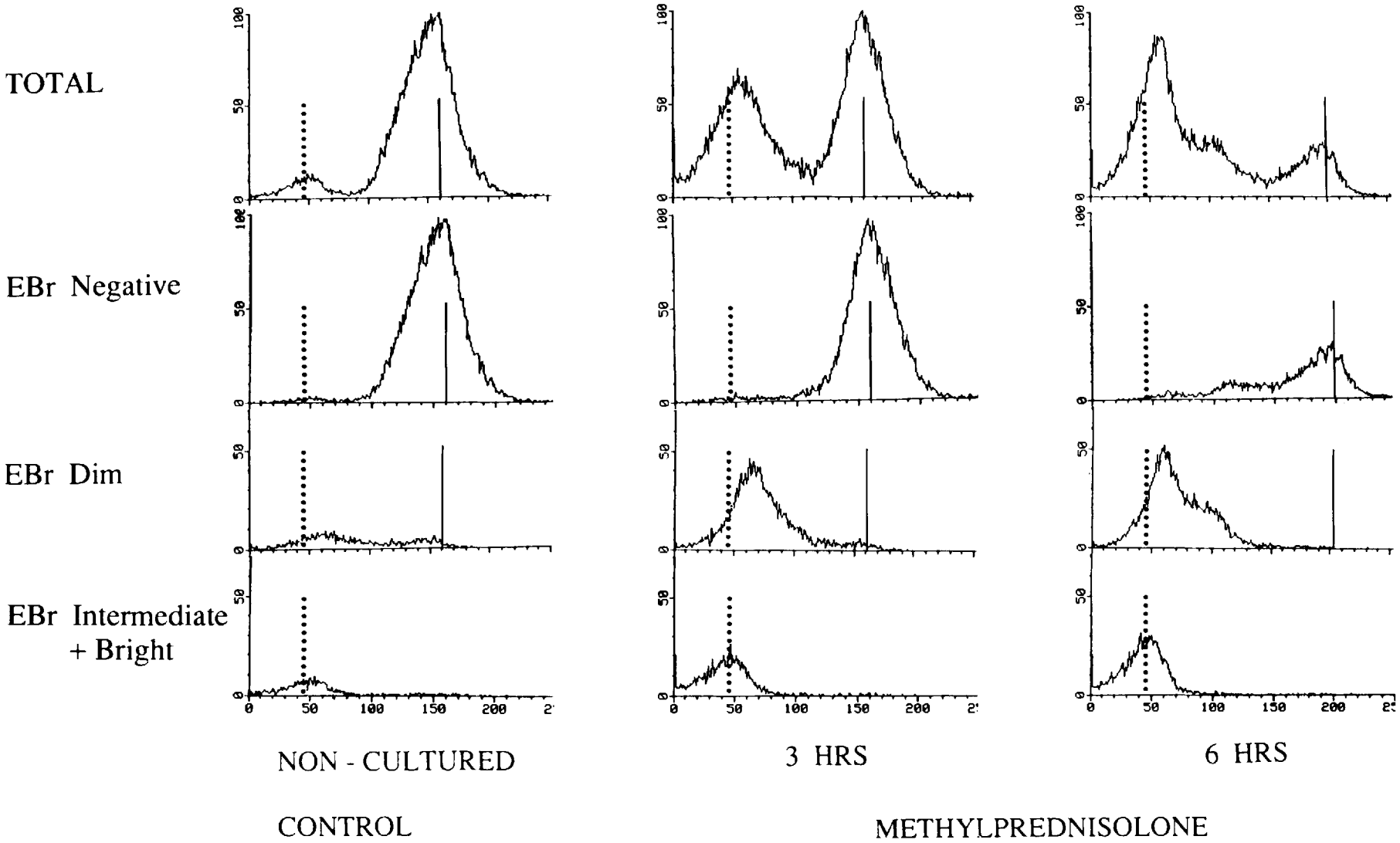
TOTAL
EBr Negative
EBr Dim
EBr Intermediate + Bright
NON - CULTURED
3 HRS
6 HRS
CONTROL
METHYLPREDNISOLONE

FIG. 5.

Table 1
Fluorescence Microscopic and Flow Cytometric Scoring of EBr Staining of Control and Methylprednisolone Treated Thymocytes[a]

	Microscopy[b]		Flow Cytometry[c]			
	% EBr(+)		% R3+R4	EBr (Intermediate + Bright)	% R2	EBr (dim)
Noncultured thymocytes	8		10		3	
Culture time (h)	C[d]	MePr[e]	C	MePr	C	MePr
4	1	2	6	10	10	35
18	12	57	13	50	19	40
24	42	89	39	95	30	3
42	51	97	47	98	20	0.5

[a]Thymocytes were cultured with and without 1×10^{-5} M methylprednisolone, stained with EBr and aliquots of the same sample analysed manually or by flow cytometry. Entry into Regions R3+4 correlates with manual scoring of EBr positivity. From these results, cells in Region R2 are not scored as positive by manual fluorescence microscopy.
[b]A minimum of 200 cells were scored for each point.
[c]Regions equivalent to those used in Figure 1 enabled calculation of percentages.
[d]Control cultures.
[e]Methylprednisolone treated cultures.

ated markedly in cultures supplemented with methylprednisolone. The cells subsequently exhibited bright EBr fluorescence (Region 4). Finally, the cells appeared less fluorescent, with a larger range of forward scatter values (Region 3). The effect of methylprednisolone was to synchronously initiate apoptosis, with the entry of nearly half of the thymocytes into the FSC low/EBr dim Region 2 after 6 h culture. This occurred without producing significantly increased numbers of brightly EBr-staining cells relative to the control culture. In contrast, Figure 4 shows that the effect of phorbol ester was to delay the entry of thymocytes into the FCS low/EBr dim Region 2.

Fluorescein Diacetate Staining of Thymocytes Undergoing Apoptosis

To assess the metabolic activity and viability of thymocytes in culture, fluorescein diacetate was used in conjunction with EBr. This nonfluorescent ester of fluorescein requires enzymatic cleavage to exhibit fluorescence, and the product is retained by cells with intact plasma membranes: thus it gives an indication of cell viability and metabolic activity. Figure 5 shows data from thymocytes incubated in the presence of methylprednisolone for up to 6 h. Regions, such as those used in Figure 1, based on FSC/EBr fluorescence enabled the green fluorescence intensity of the populations to be assessed. Cells in the FSC low/EBr dim (Region 2) exhibited an intermediate level of green fluorescence, suggesting either decreased esterase cleavage of fluorescein diacetate, or impaired ability to retain the fluorescent form of the dye.

Fluorescence Microscopy and Photomicrography of Ethidium Bromide Stained Thymocytes

To compare flow cytometric analysis of EBr stained thymocytes with assessment by fluorescence microscopy, thymocytes cultured with and without methylprednisolone were simultaneously assayed by both methods. The scoring of the flow cytometric data used regions equivalent to those in Figure 1, with cells scored as EBr positive (Regions 3+4), or EBr dim (Region 2). These data are presented in Table 1. The data again show that the effect of methylprenisolone was to synchronously cause a large number of thymocytes to enter Region 2. Comparing the two methods demonstrated that cells in Region 2 (exhibiting a low level of EBr fluorescence) are not detected by fluorescence microscopy. The fluorescence microscopy results closely paralleled the appearance of EBr positive (Regions 3+4) cells in the flow cytometric analysis.

Photomicrographs of thymocytes (Figure 6) illustrate the data in Table 1. Photographs were of identical fields taken with brightfield and fluorescence. They also demonstrate the lag between entry into Region 2 and the appearance of overtly EBr-staining cells. For example, at the 4-hr time point, both control and methylprednisilone cultures exhibited similarly low numbers of EBr staining cells. However, flow cytometry revealed that 35% of thymocytes were in the FSC low/EBr dim Region 2 (Table 1). High power microscopy revealed the formation of apoptotic bodies in the EBr bright cells (not shown).

Analysis of Thymocyte Genomic DNA

The degree of DNA fragmentation in thymocytes cultured alone or with methylprednisolone or phorbol es-

FIG. 6. Fluorescence microscopy of thymocytes undergoing apoptosis. Thymocytes were cultured in RPMI 1640 for 4, 18, 24, and 42 h, with and without methylprednisolone. After staining with EBr, identical fields were viewed by brightfield and fluorescence and photographed using a 40× objective. Aliquots of the same stained thymocytes were also analysed by flow cytometry and compared with microscopic counts of EBr fluorescence in Table 1.

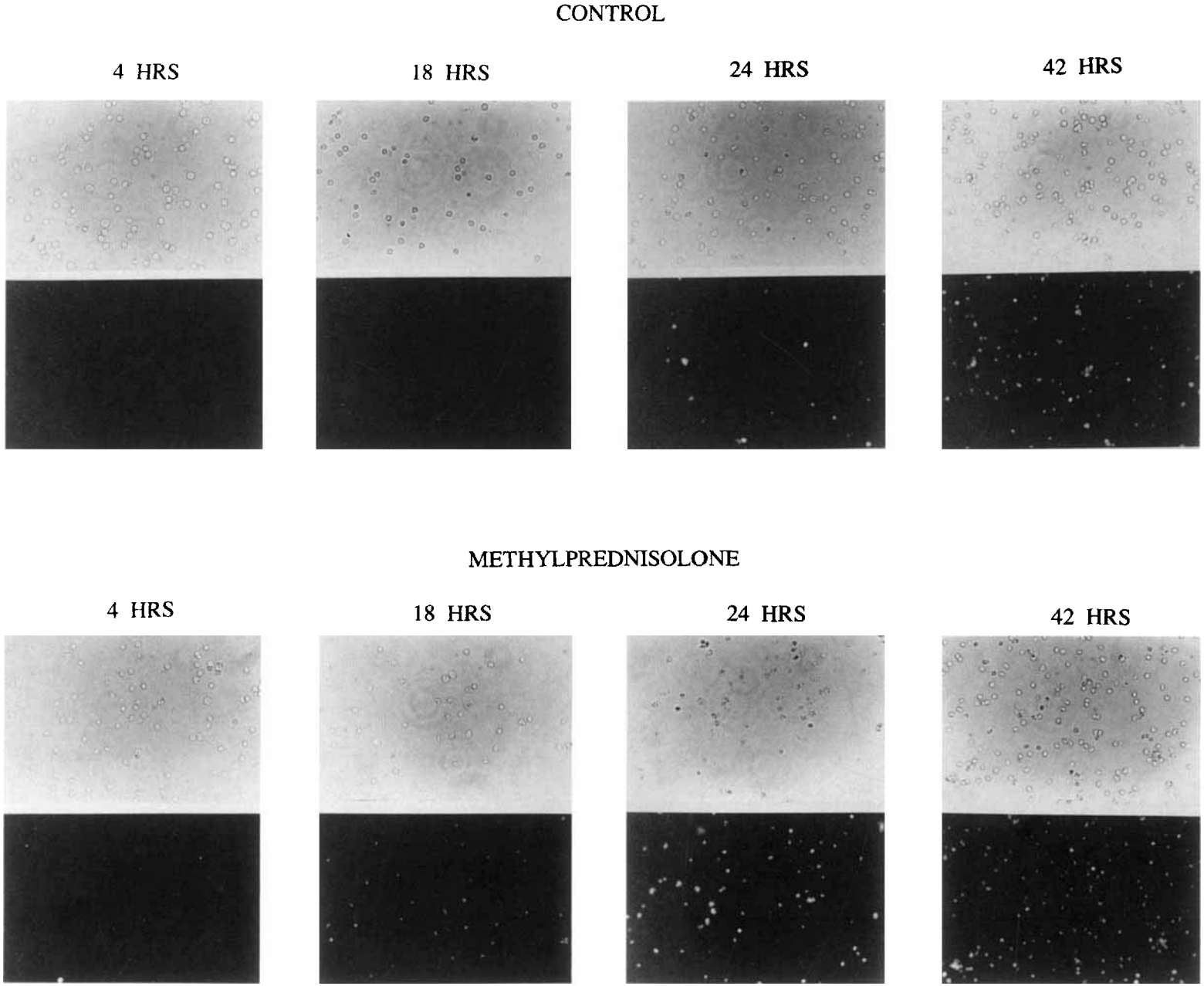
CONTROL
4 HRS
18 HRS
24 HRS
42 HRS
METHYLPREDNISOLONE
4 HRS
18 HRS
24 HRS
42 HRS

FIG. 6.

ter (TPA) was assessed by the agarose gel electrophoresis of soluble DNA fragments released after detergent treatment. Figure 7 shows a timecourse of DNA fragmentation in thymocytes cultured for up to 9 h. This figure demonstrated that the formation of 200 b.p. multiples was markedly accelerated by methylprednisolone and delayed by phorbol ester treatment, results consistent with the data illustrated in Figure 4.

Immunophenotype of Thymocytes in Apoptosis

Cultures of thymocytes incubated with or without methylprednisolone were stained with CD3 antibody and EBr. Contour plots of FITC fluorescence (X axis) vs. EBr fluorescence (Y axis) are presented in Figure 8. Anti-CD3 binding by thymocytes resolved four subpopulations, exhibiting negative, low, intermediate, and high expression of CD3, respectively. This figure demonstrates that the phenotype of thymocytes becoming dimly EBr staining (corresponding to the FSC low/EBr dim Region 2 stage) were of $CD3^{low}$ and $CD3^{intermediate}$ phenotypes. These $CD3^{low}$ and $CD3^{intermediate}$ cells in the control cultures also spontaneously started to enter the EBr dim population, but at a slower rate. Thymocytes which are CD3 negative or bright, which correspond to blasts and mature T cells, respectively, did not enter this population.

To ascertain the immunophenotype of thymocytes in apoptosis with respect to CD4 and CD8 expression, control and methylprednisolone treated cultures were stained either with EBr, or with a combination of anti-CD4 and anti-CD8. Figure 9 shows flow cytometric data from thymocytes which had undergone culture in the presence of methylprednisolone for zero and 6 h. The left half of the figure shows forward scatter vs. side scatter, with three gates based on forward scatter dividing the control cells into three similar size populations. Methylprednisolone resulted in a lowering of forward scatter, due to the entry of thymocytes into the FSC low/EBr dim stage (corresponding to Region 2 of Figure 1). This can be clearly seen in the right half of Figure 9. Table 2 shows the proportions of thymocytes from the control and methylprednisolone cultures which were stained by anti-CD4 and anti-CD8. The predominant phenotype of cells falling in forward scatter in response to methylprednisolone was $CD4^+CD8^+$. Although the numbers involved were small, $CD4^+CD8^-$ thymocytes also appeared to show some degree of sensitivity to methylprednisolone in three independent experiments, including the one presented here.

DISCUSSION

The technique described here originated from a search for a fluorescent DNA stain that would reveal apoptosis and allow the process to be followed in a single laser flow cytometer. When cells from a culture in apoptosis were stained with a low concentration of EBr, excess of which was carefully removed by washing, cells in apoptosis could be resolved as a weakly staining population. This stage preceded the brightly fluorescent state usually associated with the EBr staining of "dead" cells and could not be visualized with fluorescence microscopy. The conclusion that the weakly staining cells were entering the apoptotic state was established by their other properties, which were typical of apoptosis—a decrease in size, DNA nucleosome formation, and stimulation or inhibition by agents such as methylprednisolone or phorbol ester.

Surface property changes of thymocytes in the early stage of apoptosis may be responsible for the low level of EBr binding of the Region 2 cells. It would appear unlikely that intercalation of EBr into DNA could be responsible for such a low, but discrete, level of fluorescence. Populations of cells which may be analogous to the FSC low/EBr dim cells resolved in this study have been reported by other groups. Bohmer, (1,2) using a combination of acridine orange and ethidium bromide to stain leukemic cell lines which had either been treated with antineoplastic drugs or heat-shocked, demonstrated a dual-staining population. Similarly, Ellwart and Dormer (6) used a combination of Hoechst 33342 dye and propidium iodide, which resolved a propidium negative/wavelength-shifted Hoechst fluorescent population they called "early damaged cells" from Interleukin 3-deprived factor-dependent cells. This staining procedure was subsequently developed to demonstrate apoptosis per se by Dive et al. (4). The brightest EBr-staining population (Region 4) may be due to permeability of both cell and nuclear membranes. Fluorescence microscopy of EBr-stained apoptotic thymocytes suggests this to be the case. The thymocytes finally enter Region 3, which is characterised by a lower intensity of EBr staining and a larger spread of forward scatter. At this stage cells have extensively condensed nuclei and contain apoptotic bodies. On completion of this work we became aware of a similar method using ethidium bromide (18). Our method is distinguished by the careful removal of excess ethidium bromide, which permits a more detailed analysis of the stages of apoptosis and provides a method independent of surface antigen expression.

Data from three experiments (one of which is presented in Fig. 7) revealed that the classic hallmark of apoptosis, the formation of 200 b.p. multiples of fragmented genomic DNA, coincided with, or immediately preceded entry into, Region 2. Methylprednisolone ac-

FIG. 7. Nucleosome ladder of genomic DNA from thymocytes incubated for up to 9 h in RPMI 1640/FCS alone, or supplemented with methylprednisolone or phorbol ester. For each of the three culture conditions, lane 1 = 0.5 h, lane 2 = 1 h, lane 3 = 1.5 h, lane 4 = 2 h, lane 5 = 2.5 h, lane 6 = 3 h, lane 7 = 6 h, and lane 8 = 9 h. Lambda phage DNA restricted with the enzymes EcoRI and HindIII was used as a size marker.

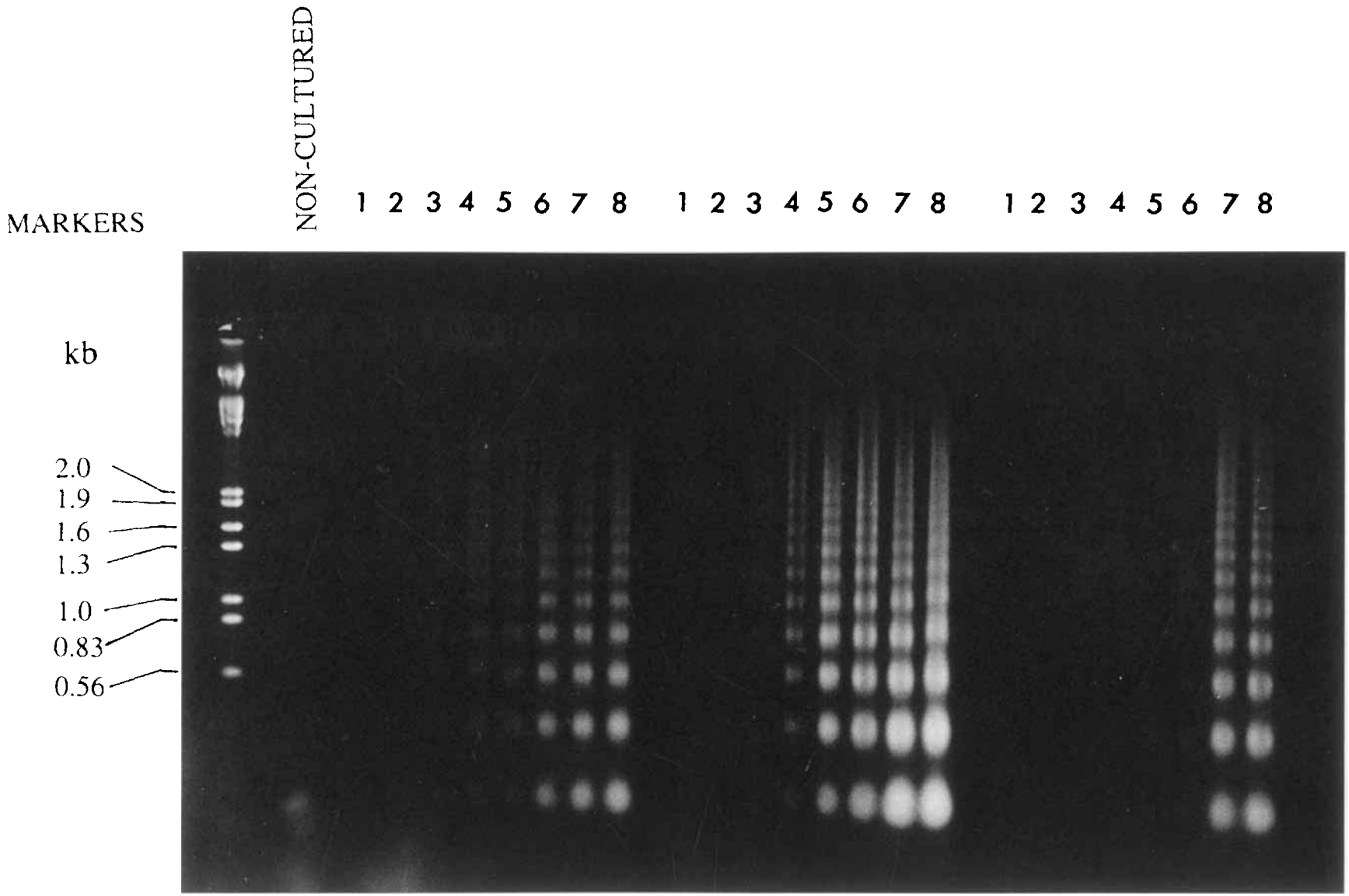
NON-CULTURED
MARKERS
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
kb
2.0
1.9
1.6
1.3
1.0
0.83
0.56
CONTROL
METHYLPREDNISOLONE
PHORBOL ESTER

FIG. 7

Table 2
CD4/CD8 Phenotype of Thymocytes Undergoing Apoptosis[a]

	Forward scatter gate							
	A (low)		B (interm.)		C (high)		Total = A+B+C	
Phenotype	C[b]	MePr[c]	C	MePr	C	MePr	C	MePr
$CD4^+CD8^-$	0.4[d]	1.4	2.1	2.6	4.3	4.7	7.8	8.7
$CD4^-CD8^+$	0.2	0.4	0.6	0.9	2.7	3.0	3.5	4.3
$CD4^+CD8^+$	28.7	50.3	35.2	19.9	22.6	14.6	86.5	84.8
$CD4^-CD8^-$	—	—	0.3	0.3	1.8	1.9	2.1	2.2
Total	29.3	52.1	38.2	23.7	32.4	24.2		

[a]The gating based on forward scatter shown in Figure 9 was used to determine the CD4/CD8 expression of thymocytes cultured with methylprednisolone for 6 h, or noncultured. The predominant population undergoing apoptosis (therefore falling in forward scatter) was $CD4^+CD8^+$. $CD4^+CD8^-$ thymocytes also exhibited sensitivity to methylprednisolone.
[b]Control thymocytes.
[c]Methylprednisolone treated cultures.
[d]Results expressed as a percentage of total.

celerated the rate of DNA fragmentation compared with control cultures, whereas phorbol ester delayed fragmentation, as previously reported (10). Flow cytometry of EBr-stained cells showed that phorbol ester delayed the entry of thymocytes into the FSC low/EBr dim Region 2 (Fig. 4).

The simultaneous staining of thymocytes with anti-CD3 and EBr, and the staining of thymocytes with anti-CD4 and anti-CD8 based on forward scatter characteristics, showed that cells entering the FSC low/EBr dim Region 2 in response to methylprednisolone treatment were of $CD3^{low}$ or $CD3^{intermediate}$ and $CD4^+CD8^+$ immunophenotype, with $CD4^+CD8^-$ thymocytes exhibiting some sensitivity. This agrees with previous demonstrations of the sensitivity to apoptosis of different thymocyte subpopulations. For example, Shi et al. (16) administered anti-CD3 and anti-TCR antibodies in vivo to 4–6-week-old mice and demonstrated deletion by apoptosis of thymocytes expressing low and intermediate amounts of CD3. These cells were also predominantly of $CD4^+CD8^+$ phenotype; however, significant numbers of $CD4^+CD8^-$ thymocytes were also deleted. In an experiment designed to reflect thymic selection more closely, Murphy et al. (13) used TCR transgenic mice to show deletion of $CD4^+CD8^+TCR^{low}$ thymocytes in response to challenge in vivo with the TCR-specific peptide antigen.

It was found that thymocytes spontaneously enter apoptosis in culture, but at a reduced rate compared with methylprednisolone cultures. Work by McConkey et al. (9) showed that IL-1 could inhibit apoptosis in anti-CD3 stimulated thymocytes by upregulating protein kinase C, suggesting that spontaneous apoptosis of thymocytes in culture may be a manifestation of cytokine deprivation. In the report presented here, phorbol ester, another upregulator of protein kinase C, was able to delay apoptosis in thymocytes (Figs. 4, 7).

The flow cytometric method of Ellwart and Dormer (6) may be used simultaneously with antibody binding to look at immunophenotype of cells in apoptosis, but this would require a flow cytometer fitted with a UV laser. We are currently attempting to set up three color analyses using the new generation of red fluorochromes as the second antibody label.

We have successfully used the technique reported here to investigate apoptosis in human B-cell chronic lymphocytic leukemia (in preparation). Such a generally applicable means of investigating programmed cell death should be useful for determining the relationship between surface structures on tumour cells, predisposition to apoptosis, and sensitivity or resistance to antineoplastic agents which exert their effects by inducing apoptosis (15). The ability to determine directly the immunophenotype of cells in apoptosis will make events in immunological selection during ontogeny more readily accessible to investigation and permit the analysis of apoptosis in complex mixtures of cells of different phenotypes.

ACKNOWLEDGMENTS

We thank Dr. J.D. Ansell, Prof. H.S. Micklem, and Dr. A.H. Wyllie for helpful discussion, and Mr. F. Johnston for photographs.

FIG. 8. Simultaneous staining of freshly isolated thymocytes, or thymocytes cultured in RPMI 1640/FCS with or without methylprednisolone, with EBr and anti-CD3 antibody. Contour plots of orange EBr fluorescence vs. green CD3 fluorescence. Open arrows define the $CD3^{negative}$ and $CD3^{bright}$ populations, whereas solid arrows define the $CD3^{low}$ and $CD3^{intermediate}$ populations. Note the movement of $CD3^{low}$ and $CD3^{intermediate}$ cells into the EBr dim stage, whereas the $CD3^{negative}$ and $CD3^{bright}$ cells remain EBr nonstaining. In this experiment, differences between the control and methylprednisolone treated cultures were not apparent before 3 h had elapsed.

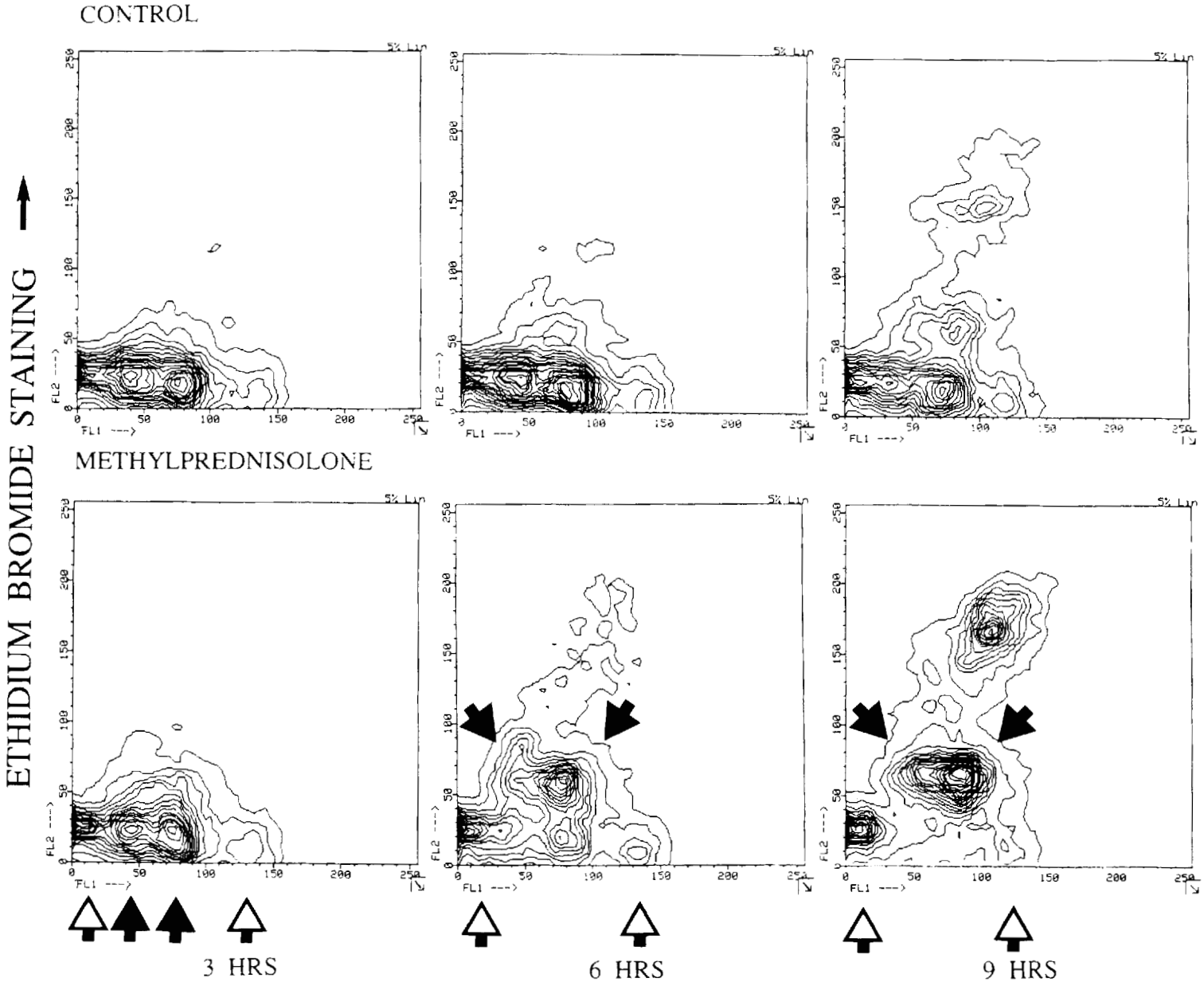
CONTROL
METHYLPREDNISOLONE
ETHIDIUM BROMIDE STAINING
ANTI - CD3 BINDING
3 HRS
6 HRS
9 HRS

FIG. 8.

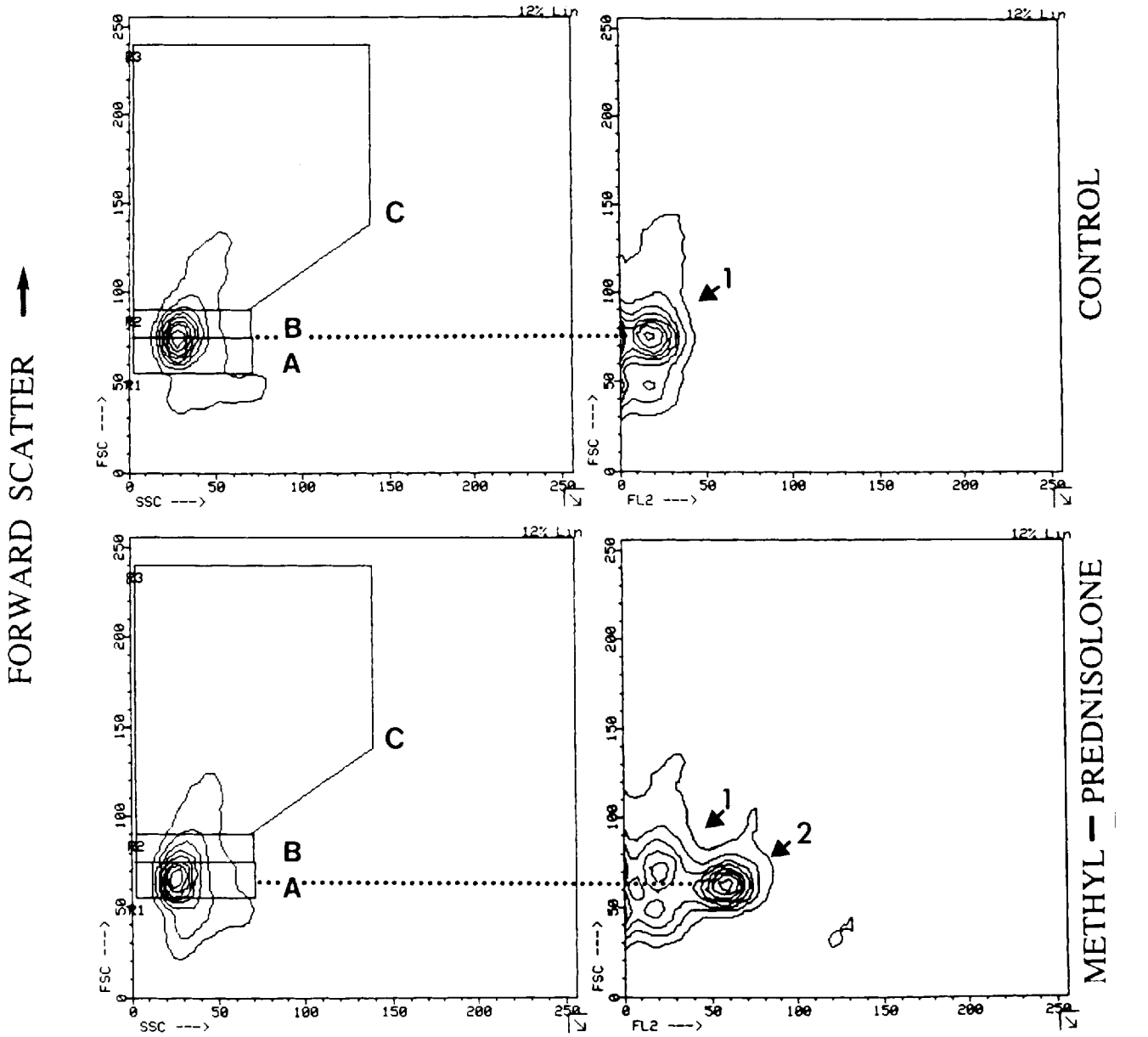

FIG. 9. Noncultured and 6-h methylprednisolone treated thymocytes were stained either with anti-CD4-phycoerythrin and anti-CD8-fluorescein, or EBr. The left half of the figure demonstrates the fall in forward scatter after methylprednisolone treatment, which is due to the movement of thymocytes into the FSC low/EBr dim stage, as shown in the right half. Arrowed are Regions 1 and 2. The three gates shown divide the cells according to their forward scatter. Percentages in the gates demonstrate the fall in forward scatter of the methylprednisolone treated thymocytes: control: A = 29%, B = 38%, C = 32%; methylprednisolone: A = 52%, B = 24%, C = 24%. The gates allow the immunophenotype with respect to CD4 and CD8 expression of thymocytes moving into the FSC low/EBr dim stage in response to methylprednisolone to be determined, as presented in Table 2.

LITERATURE CITED

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