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Splenic T lymphocytes die preferentially during heat-induced apoptosis: NuMA reorganization as a marker

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SUMMARY

We are investigating nuclear events during apoptosis in mouse splenic lymphocytes cultured immediately after isolation (controls) or after heat treatment (42°C, 30 minutes), and have found that hyperthermia increased the level of apoptosis to double that of spontaneous apoptosis in controls within 6 hours. Immunolabelling for Nuclear Mitotic Apparatus Protein (NuMA) suggested that splenocytes were responding heterogeneously to the heat treatment. Whereas all nuclei in controls and about half of nuclei in heat-treated samples showed the usual diffuse nucleoplasmic labelling, 40-60% of nuclei in heated samples also contained numerous bright spots. We then examined whether the heterogeneity in NuMA organization might be an indication of a differential response of B and T lymphocytes to hyperthermia, and whether the presence of NuMA spots is related to the apoptotic process. NuMA labelling of heated fractionated splenocyte populations showed that 90% of nuclei in T-enriched cultures (≤4% IgG^+ cells), but only 25% of nuclei in B-enriched samples (≤80% IgG^+ cells), contained spots. As well, 2 hours after heat treatment of unfractionated cultures, ≥90% of nuclei that were accumulating DNA strand breaks, as detected by TUNEL, exhibited NuMA spots. These data indicate that cells with NuMA spots are targetted for, or have initiated, the death program. Since most T cells, but few or no B cells, were spotty after heating, we conclude further that hyperthermia induces apoptosis preferentially in splenic T lymphocytes. The observation that the proportion of T cells was, on average, threefold greater in control than in heated samples after 24 hours in culture reinforces this conclusion.

Key words: T lymphocytes, B lymphocytes, NuMA, Apoptosis, Hyperthermia, DNA fragmentation, Immunocytochemistry, Flow cytometry

INTRODUCTION

The Nuclear Mitotic Apparatus Protein (NuMA) was named for its localization in the nucleus during interphase and at spindle poles during mitosis (Lyderson and Pettijohn, 1980). The evidence indicates a function for NuMA in mitotic spindle organization and in post-mitotic nuclear reassembly (reviewed in He et al., 1995), but its function in the interphase nucleus is less clear. However, NuMA is an abundant nuclear protein that co-fractionates with the nuclear matrix (Kallajoki et al., 1991; Zeng et al., 1994a). Moreover, analysis of the cDNA predicted a coiled coil structure for NuMA (Yang et al., 1992; Compton et al., 1992, 1993) and NuMA has been localized to a subset of nuclear matrix core filaments by immunoelectron microscopy (Zeng et al., 1994b). Collectively, these data have led to the suggestion that NuMA may play a key role in nuclear matrix organization as a major component of nuclear matrix filaments (reviewed in Cleveland, 1995). In keeping with this view, we (Weaver et al., 1996) and others (Casiano et al., 1996; Hsu and Yeh, 1996; Zweyer et al., 1997) have shown that NuMA is an early target of apoptotic proteolytic cleavage. We have also found that the normally diffuse NuMA immunofluorescence labelling reorganized into a residual mass that was excluded from the condensed chromatin in fully collapsed apoptotic nuclei of dexamethasone-treated rat thymocytes (Weaver et al., 1996). On this basis, it has been proposed that NuMA fragmentation may play a signalling or regulatory role as the death program is initiated in the nucleus (Weaver et al., 1996).

In this context, the reorganization of NuMA labelling during apoptosis hyper-induced in mouse resting splenic lymphocytes by heat treatment (HT) may be important. We found that, immediately after heating, about half of the lymphocytes exhibited a unique pattern of NuMA labelling, consisting of many bright spots within a diffusely fluorescent nucleoplasm, and that this pattern was retained throughout subsequent stages of apoptotic nuclear collapse (Sodja et al., 1997). In the present study, we have examined more closely the significance of this novel NuMA pattern. The results indicate that it is the mouse splenic T lymphocyte subset that develops this pattern preferentially, and that cells with the spotty labelling are targetted for, or have initiated, the death program. Moreover,

the data show that heat treatment induced apoptosis preferentially in the T lymphocyte subset.

MATERIALS AND METHODS

Preparation of cells

Splenocytes were isolated from male Balb/c mice (Charles River) as previously described (Davis et al., 1993). Cells were resuspended at 2.5×10^6 cells/ml in complete Roswell Park Memorial Institute (RPMI) 1640 containing 10% CPSR-2 (Sigma), 2 mM glutamine (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml Fungizone, and divided into 1 ml portions in culture tubes.

Heat treatment

Cell portions were incubated for 30 minutes at 42.0±0.1°C in a Grant circulating water bath equipped with a Julabo PCH heater. At the end of the heat treatment, designated as 0 hours (t_0), some samples were fixed for flow cytometry and immunofluorescence labelling. The remaining samples were cultured at 37°C for up to 6 hours before processing.

Flow cytometry

Cells were pelleted in a benchtop centrifuge, resuspended in 1 ml phosphate-buffered saline (PBS), fixed by addition of 4 ml absolute ethanol (−20°C) and stored in PBS-ethanol at −20°C. Before analysis, samples were processed for propidium iodide fluorescence as in Walker et al. (1991). Red fluorescence was measured at 610 nm on a Coulter Elite ESP flow cytometer, with 2×10^4 cells counted for each sample. Data were then analyzed using Multicycle Cell Cycle software (Phoenix Flow Systems).

Immunofluorescence labelling

Total and fractionated splenocyte populations on poly-L-lysine-coated coverslips were fixed and permeabilized as previously described (Chaly et al., 1984, 1988). For single-labelling experiments, cells were labelled sequentially with anti-NuMA, a mouse monoclonal IgG1 (diluted 1:25, A-204, Matritech) followed by CY3-conjugated goat anti-mouse IgG (Fc fragment-specific) (diluted 1:200, Jackson Immunochemicals). All samples were counterstained with 0.2 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 5 minutes and mounted in Vectashield mounting medium (Vector Laboratories). Samples were viewed on a Zeiss Photomicroscope III with a Plan-Neofluar ×63, N.A. 1.25 oil immersion objective, and photographed using Ilford XP2-400 film. For confocal microscopy, optical sections 0.5 μm apart were collected with a Leica Confocal Scanning Laser Microscope equipped with a Krypton/Argon laser and a Zeiss ×63, N.A. 1.4 Planapo objective.

Labelling of DNA strand breaks

Samples fixed and permeabilized as above were assayed for DNA strand breaks using the method of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) (Gavrieli et al., 1992), as described in Weaver et al. (1996). After permeabilization, samples were incubated for 1 hour with 300 U/ml of terminal deoxynucleotidyl transferase in cacodylate buffer (Gibco BRL) with 10 μM biotin-16-dUTP (Boehringer-Mannheim) and the reaction was terminated by transferring samples to buffer containing 300 mM NaCl and 30 mM sodium citrate. The cells were then incubated sequentially in 2% bovine serum albumin in PBS for 10 minutes to block non-specific labelling, and in streptavidin-CY3 (diluted 1:100, Jackson) or avidin-FITC (diluted 1:100, Vector) in PBS. All steps were performed at room temperature. For samples double-labelled for immunofluorescence and TUNEL, antibody labelling was carried out first, as described above. To block non-specific binding, samples were incubated in 0.15% gelatin in PBS for 20 minutes. Samples were then washed in PBS and processed for TUNEL. After counterstaining with DAPI, samples were mounted and viewed as described above.

Enrichment of cell suspension for T or B cells

Total splenocyte populations were enriched for T or B cells at t_0. Enrichment for T cells was carried out as previously described (Wysocki and Sato, 1978). Briefly, 2.5×10^7 cells of each sample were pelleted in a benchtop centrifuge, resuspended in 3 ml of PBS with 5% fetal calf serum (Gibco), and incubated for 70 minutes at 4°C in bacteriological grade Petri dishes (Fisher Scientific) pre-coated with affinity-purified goat anti-mouse IgG (H+L) (Jackson Immunochemicals) at 5 μg/ml in 0.05 M Tris-HCl, pH 9.5. The non-adherent, T-enriched cell fraction was collected by aspiration.

B-cell enrichment was carried out by complement-mediated lysis as described in Roy (1984). For each sample, 5.0×10^7 cells were pooled by centrifugation, resuspended in 1 ml RPMI 1640 containing monoclonal mouse anti-Thy 1.2 (diluted 1:800, Sigma), and incubated on ice for 1 hour. Cells were washed with 1 ml RPMI medium, collected by centrifugation, resuspended in 1 ml RPMI with rabbit complement (diluted 1:20, Cedarlane), and incubated at 37°C for 1 hour. The non-lysed B-enriched cell fraction was washed twice with 3 ml of RPMI and collected by centrifugation.

To determine the proportion of B cells in total and fractionated splenocyte populations, samples were processed for immunofluorescence staining of cell surface IgG. Cells were incubated on ice for 20 minutes in RPMI containing CY3-conjugated goat anti-mouse IgG (Fc fragment-specific) (diluted 1:100, Jackson Immunochemicals), layered onto poly-L-lysine-coated coverslips, fixed for 1 minute in 3% paraformaldehyde in PBS, and permeabilized for 2 minutes in 0.2% Triton X-100 in PBS. Samples were then counterstained with DAPI, mounted and viewed by conventional fluorescence microscopy.

RESULTS

Kinetics of apoptosis

We have reported that heat treatment increases the rate of apoptosis with respect to controls within 6 hours (Sodja et al., 1997). To determine the effect of heat treatment on apoptosis over longer periods in culture, flow cytometry was carried out on samples cultured for up to 24 hours (Fig. 1). Fig. 1A shows data from a single representative experiment, and Fig. 1B shows the percentages of cells exhibiting reduced propidium iodide fluorescence as the means from three experiments. In control and heat-treated samples fixed at t_0, propidium iodide fluorescence produced a single peak corresponding to the DNA content of resting cells (Fig. 1A,B), as reported earlier (Daev et al., 1994). The proportion of the population with the sub-diploid DNA content characteristic of apoptotic cells increased over time in culture for both samples but, by t_6, was approximately double in heat-treated samples (36%) with respect to controls (15%). By 24 hours, greater than 50% of the population was apoptotic in both types of populations, but the proportion of apoptotic cells was still significantly higher in heated samples than in controls.

Behaviour of NuMA during spontaneous and heat-induced apoptosis

(1) NuMA distribution and chromatin morphology

As in earlier studies (Weaver et al., 1996), chromatin morphology was used to identify cells at different stages of

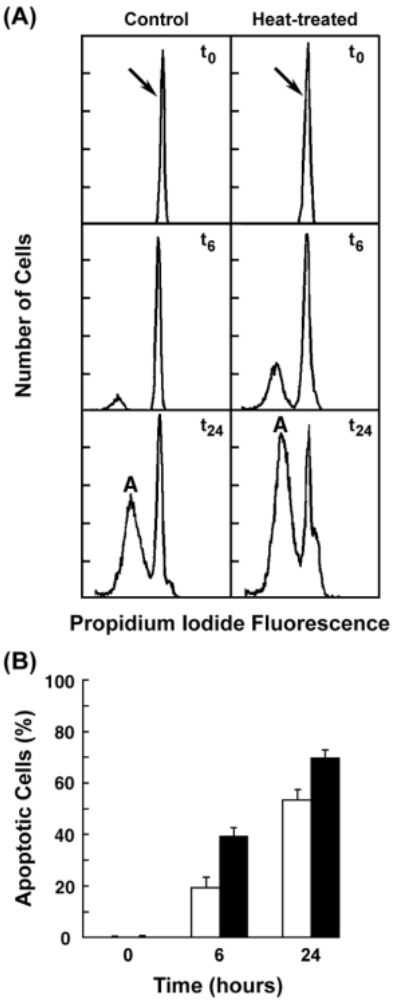

Fig. 1. Kinetics of spontaneous and heat-induced apoptosis. Splenocytes were cultured at 37°C (control) or heat-treated at 42°C for 30 minutes, then cultured at 37°C for 0, 6 or 24 hours. (A) Flow cytometric profiles of propidium iodide fluorescence from a single experiment for control and heat-treated splenocytes. 2×10^3 cells were counted for each sample. Arrows at the t_0 point indicate the peak containing diploid cells. The 'A's at t_{24} indicate the peak containing sub-diploid, apoptotic cells. (B) Histogram showing the percentage of apoptotic cells detected by flow cytometry in control (open bars) and heat-treated (solid bars) samples. Results are the means from 3 experiments; error bars show the standard deviation from the mean. By the Student's t-test, $P\leq0.001$ for the data at t_6 and t_{24}.

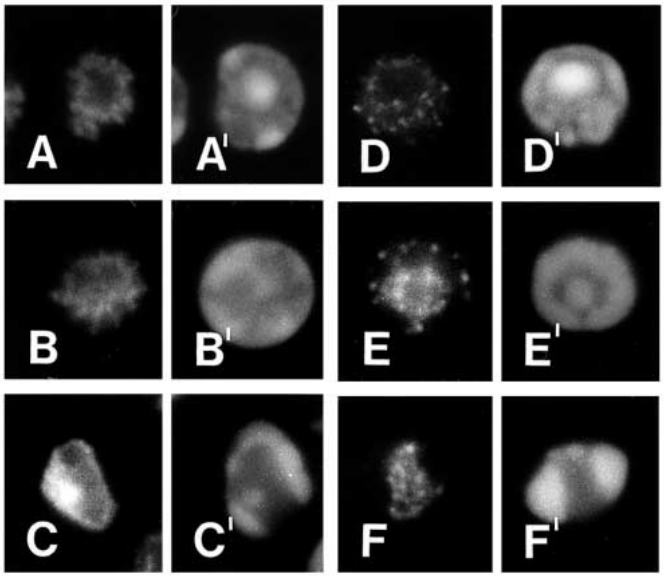

Fig. 2. Distribution of NuMA during control and heat-induced apoptosis. Control (A-C′) and heated (D-F′) samples were fixed at t_0 (A,A′, D,D′) or at t_2 (B-C′, E-F′), and were labelled with anti-NuMA (A-F) and DAPI (A′-F′). Control DN (DAPI normal) (A,A′), PC (partially collapsed) (B,B′) and FC (fully collapsed) (C,C′) nuclei exhibit diffuse NuMA labelling. DN (D,D′), PC (E,E′) and FC (F,F′) nuclei containing spots in addition to the diffuse labelling are shown for heat-treated samples. Magnification ×2600.

apoptosis. In cells with 'DAPI-normal' (DN) nuclei, the DAPI-labelling pattern was indistinguishable from that in freshly isolated splenocytes (e.g. Fig. 2A′,D′). Fully collapsed (FC) nuclei exhibited the chromatin morphology classically associated with apoptosis. That is, DAPI staining was untextured, and the chromatin was marginated (Fig. 2C′,F′) or formed a single ball (Fig. 4A′). In addition, some nuclei showed a morphology intermediate between DN and FC. That is, as in DN nuclei, the chromatin consisted of a broad irregular band at the nuclear periphery and, usually, a single central mass (cf. Fig. 2D′,E′). The DAPI staining, however, was very homogeneous, as in FC nuclei (cf. Fig. 2E′,F′). Such nuclei were characterized as partially collapsed (PC) (e.g. Fig. 2B′,E′), and have been described in greater detail elsewhere (Sodja et al., 1997). Both control and heated t_0 samples contained DN nuclei almost exclusively. FC nuclei were first detected at t_2 and became more prominent as the culture period was prolonged. PC nuclei were the least common in all samples, but were observed most frequently in heat-treated samples at t_2. The evidence indicates that PC nuclei represent an early transient stage of apoptotic nuclear collapse (Sodja et al., 1997).

In control samples, the organization of NuMA in DN nuclei and in the spontaneously apoptotic nuclei overall resembled that previously reported for other cell types (Weaver et al., 1996; reviewed in He et al., 1995). NuMA labelling was nucleoplasmic in DN and PC nuclei, surrounded by the thick band of peripheral heterochromatin, but faintly granular (Fig. 2A,A′,B,B′). Immunolabelling of similarly prepared HeLa cells and mouse 3T3 fibroblasts with the same anti-NuMA antibody produced the more usual diffuse nucleoplasmic staining (not shown). The slight granularity appears to be characteristic of these lymphocyte nuclei. Labelling remained nucleoplasmic in FC nuclei but was more variable in granularity and intensity, ranging from very bright (Fig. 2C,C′) to undetectable (not shown).

However, in heat-treated samples, an additional labelling pattern for NuMA was observed in some DN and apoptotic nuclei. In the uniformly DN t_0 samples, only about half of the nuclei exhibited the control NuMA labelling pattern (see Fig. 8). The other half contained many very bright spots in the nucleoplasm in addition to the faintly granular NuMA labelling (Fig. 2D,D′). These bright NuMA spots were also observed in the nucleoplasm of many DN nuclei at t_2, t_4 and t_6 (not shown).

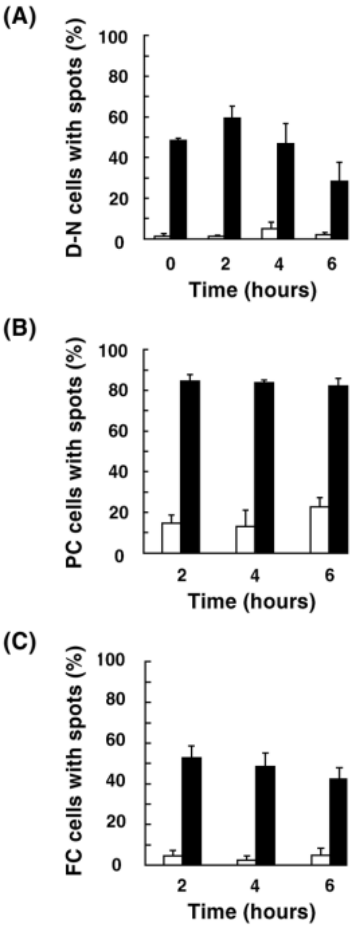

Fig. 3. Effect of culture on the frequency of NuMA spots. Control and heat-treated samples were fixed at intervals from t_0 to t_6 and labelled with anti-NuMA and DAPI. The chromatin morphology and NuMA distribution pattern were evaluated for 500 DN (A), 100 PC (B), and 250 FC nuclei (C) in control (open bars) and heat-treated (solid bars) samples. Histograms show the mean proportion of nuclei with NuMA spots from three separate experiments; error bars show the standard deviation from the mean.

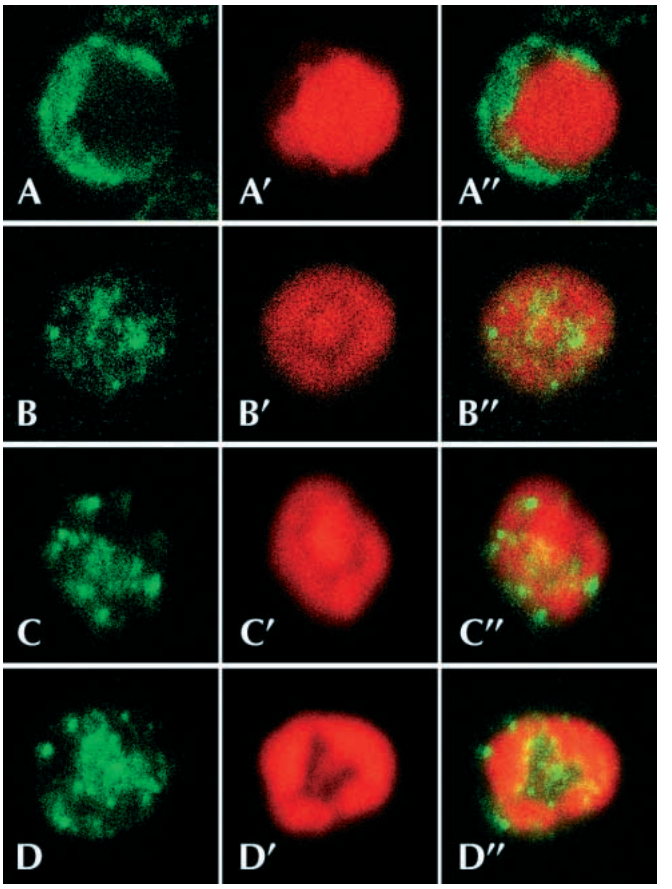

Fig. 4. Confocal fluorescence microscopy of NuMA distribution in relation to fragmented DNA. Control (A-A″) and heat-treated (B-D″) samples were fixed at t_2 and labelled with anti-NuMA (A-D), and with TUNEL (A′-D′); the corresponding merged images are shown in A″-D″. Labelling is shown for TUNEL-positive DN (B-B″), PC (C-C″), and FC (A-A″, D-D″) nuclei. Magnification ×3000.

Moreover, it seemed that most PC (Fig. 2E,E′) and many FC (Fig. 2F,F′) nuclei in these samples also exhibited the spotty NuMA labelling pattern.

To quantitate these observations, samples were scored to determine the proportion of DN, PC and FC nuclei with NuMA spots (Fig. 3). In controls, DN (Fig. 3A) and FC (Fig. 3C) nuclei with spots were rare (<5%) at all time points. PC nuclei with NuMA spots were more frequent, rising to 20% of the PC population by 6 hours (Fig. 3B). On the other hand, NuMA spots were observed in greater than 45% of the DN population fixed immediately after heat treatment, at t_0 (Fig. 3A). Furthermore, the proportion of nuclei with NuMA spots was much higher than in controls at all time points for PC and FC nuclei, as well as for DN nuclei. The data show that 30-60% of DN nuclei (Fig. 3A), about 80% of PC nuclei (Fig. 3B) and 40-50% of FC nuclei (Fig. 3C) were affected.

(2) NuMA spots and DNA fragmentation

The preponderance of apoptotic cells with NuMA spots in heated samples suggested that the spots might be an indication that cells have initiated the death program. We therefore examined the relationship between a known early apoptotic nuclear event, DNA fragmentation, and the organization of NuMA. Samples at t_2 were processed for TUNEL and then labelled with anti-NuMA. As anticipated, TUNEL labelled all PC and FC nuclei in control and heated samples. In addition, a small number of DN nuclei in both types of samples was also labelled. Such TUNEL-positive DN nuclei have been interpreted to represent nuclei in very early stages of DNA fragmentation (Weaver et al., 1996).

The distribution of NuMA relative to fragmented chromatin in these samples was examined by confocal microscopy (Fig. 4). In controls, NuMA staining was diffuse and excluded from the chromatin in FC nuclei (Fig. 4A-A″), as well as in DN and PC nuclei (data not shown). Similarly, in TUNEL-positive DN (Fig. 4B-B″), PC (Fig. 4C-C″) and FC (Fig. 4D-D″) nuclei of heat-treated samples, both the diffuse NuMA component and the NuMA spots were nucleoplasmic. However, the spots were distributed non-randomly, generally located adjacent to a chromatin mass.

The proportion of TUNEL-positive DN, PC and FC nuclei with NuMA spots in t_2 heat-treated samples was then determined by scoring three separate experiments (Table 1). The results show that DNA fragmentation was initiated preferentially in nuclei with NuMA spots. In each experiment, nearly all (>90%) TUNEL-positive DN nuclei, >80% of PC nuclei and ≥45% of FC nuclei had NuMA spots. The relatively low proportion of spotty FC nuclei is likely to be due to several factors. For instance, some FC nuclei showed no NuMA

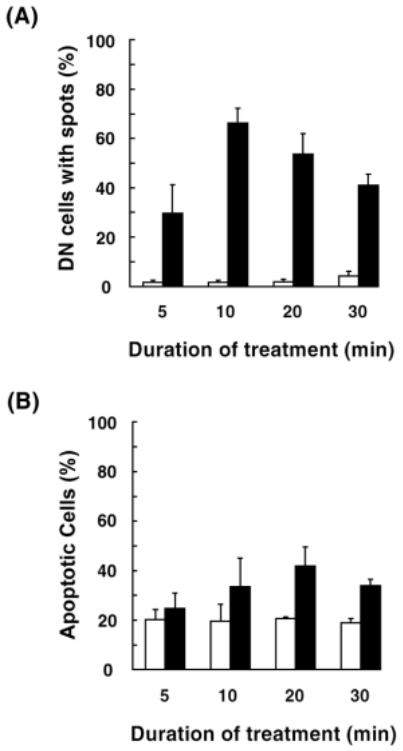

Fig. 5. Relationship between the duration of heat treatment and the appearance of NuMA spots (A) or the induction of apoptosis (B). Samples were heated for 5, 10, 20 or 30 minutes at 42°C. Heat-treated and parallel control samples were then fixed and labelled with anti-NuMA and DAPI (A), or were cultured for a further 6 hours and processed for flow cytometry (B). (A) Histogram showing the proportion of DN nuclei with NuMA spots in control (open bars) and heat-treated samples (solid bars) as determined by microscopy. Chromatin morphology and NuMA distribution were evaluated for 500 DN nuclei in each sample. The data are the means from three experiments; error bars show the standard deviation from the mean. The values for heat-treated samples were compared by the Student's *t*-test: P=0.01 for 5 and 10 minute samples; P=0.08 for 10 and 20 minute samples; P=0.05 for 20 and 30 minute samples.
(B) Histogram showing the percentage of apoptotic cells detected by flow cytometry in control (open bars) and heat-treated (solid bars) samples. Results are the means from three experiments; error bars show the standard deviation from the mean. The data for control and heat-treated samples were compared by the Student's *t*-test: P=0.4 for the 10 minute heat treatment; P=0.04 for the 20 minute heat treatment; P=0.01 for the 30 minute heat treatment.

labelling, as noted above, and in other FC nuclei, NuMA staining was so concentrated as to make spots unidentifiable.

(3) Effect of shorter heat treatment on NuMA spots and hyper-induction of apoptosis

To characterize NuMA spot formation further, we examined the length of heat treatment required for spots to appear. Splenocyte populations heated for 5-30 minutes were labelled with anti-NuMA and DAPI immediately after heat treatment, and the samples scored for the proportion of DN cells with NuMA spots (Fig. 5A). In all control samples, fewer than 5% of DN cells contained NuMA spots. In heated samples, however, the proportion of cells with NuMA spots varied with the duration of the treatment (Fig. 5A). A 5 minute treatment was sufficient to increase the proportion of cells with spots to 30%, and heating for 10 minutes increased the proportion further to 66% of the population. However, the proportion of cells with spots dropped somewhat as heat treatment was prolonged, to 53% and 41% for 20 and 30 minute treatments, respectively.

Table 1. Proportion of TUNEL-positive nuclei with NuMA spots in heat-treated samples[a]

Morphology of TUNEL-positive nuclei	% nuclei with NuMA spots[b]			Mean % nuclei with NuMA spots ± s.d.[c]
	Expt 1	Expt 2	Expt 3	
DN	95	100	91	95±5
PC	87	82	80	83±3
FC	45	56	59	53±7

[a]Splenocytes were heated at 42°C for 30 minutes, cultured at 37°C for 2 hours, fixed and labelled with anti-NuMA and TUNEL. Nuclei that fluoresced by TUNEL were evaluated as DN, PC or FC based on the morphology of chromatin by DAPI staining (see Fig. 2 legend).
[b]10-50 TUNEL-positive DN, 45-50 PC-like nuclei and 500 FC nuclei were evaluated for the presence or absence of NuMA spots. Values shown are from three separate experiments (expt).
[c]Values shown are the means from the three experiments ± s.d.

To examine the relationship between the appearance of NuMA spots and the induction of apoptosis in these samples, controls and cultures heated for various times were returned to the incubator for 6 hours and then processed for flow cytometry (Fig. 5B). The results indicate that the development of NuMA spots is not in itself a sufficient indicator of the hyper-induction of apoptosis. As anticipated, <20% of the population was apoptotic by t_6 in control samples. However, heating for 5 or 10 minutes did not significantly increase apoptosis above control levels (Fig. 5B), though a substantial proportion of the population contained NuMA spots (Fig. 5A). Hyperinduction of apoptosis to about 35-40% was observed only in samples heated for 20 or 30 minutes (Fig. 5B).

NuMA distribution in T and B cells

Splenocytes are a mixed population consisting of approximately equal proportions of T and B lymphocytes. Since NuMA spots were observed in approximately half of the heat-treated population, it seemed possible that NuMA spots were restricted to one of these lymphocyte subsets. To test this hypothesis, we prepared B-enriched and T-enriched lymphocyte populations for staining with anti-NuMA and DAPI, in addition to the usual total splenocyte samples.

First, the extent of enrichment was evaluated. The proportions of B cells in the populations were determined by labelling the cell surface with CY3-conjugated goat anti-mouse IgG, and nuclear morphology was monitored by DAPI staining (Fig. 6). As expected, surface IgG labelling showed 45-50% IgG^+ cells in the total splenocyte population (Figs 6A,A′, 7A,B). In samples enriched for B cells, about 80% of the population was labelled for cell-surface IgG (Figs 6B,B′, 7A). In samples enriched for T cells, on the other hand, less than 4% of the population showed labelling of the cell surface (Figs 6C,C′, 7B).

NuMA labelling of total and fractionated populations then demonstrated that the spots were heat-induced preferentially in T lymphocytes (Figs 7C,D, 8). NuMA spots were rare

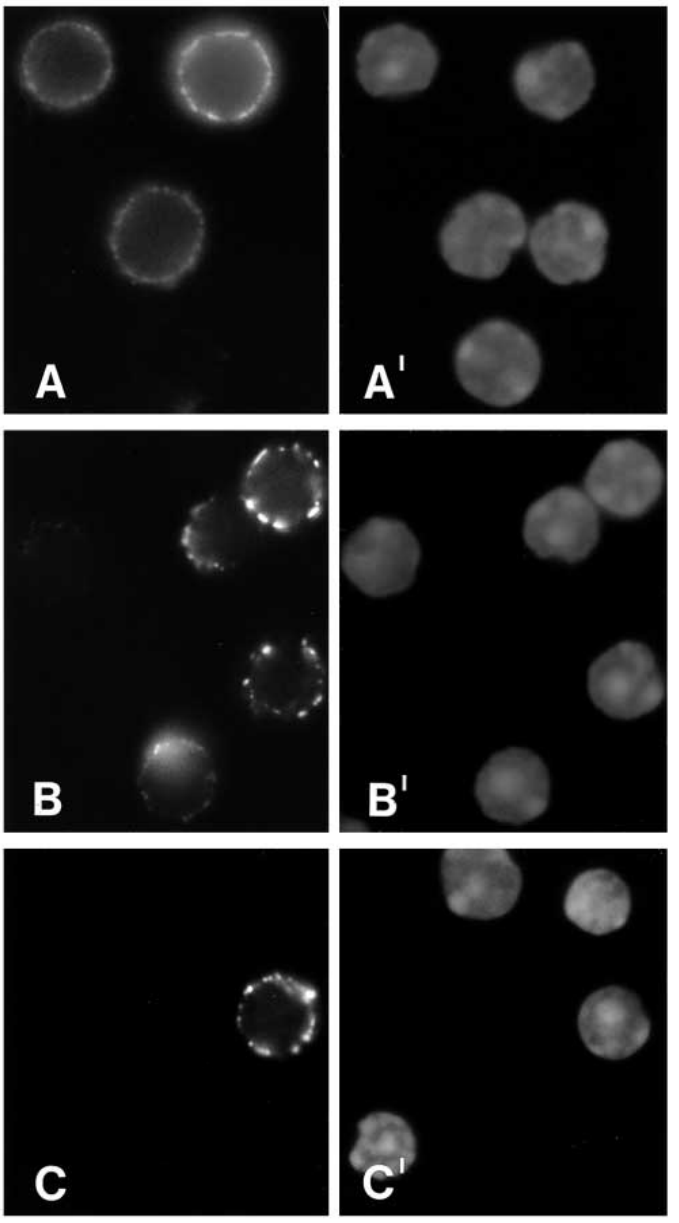

Fig. 6. Cell-surface IgG-labelling of total and fractionated splenocyte populations. Control and heat-treated samples were incubated with CY3-conjugated IgG (A-C), fixed and counterstained with DAPI (A′-C′). Surface IgG labelling is shown for control total (A,A′), B-enriched (B,B′) and T-enriched (C,C′) populations. Magnification ×1400.

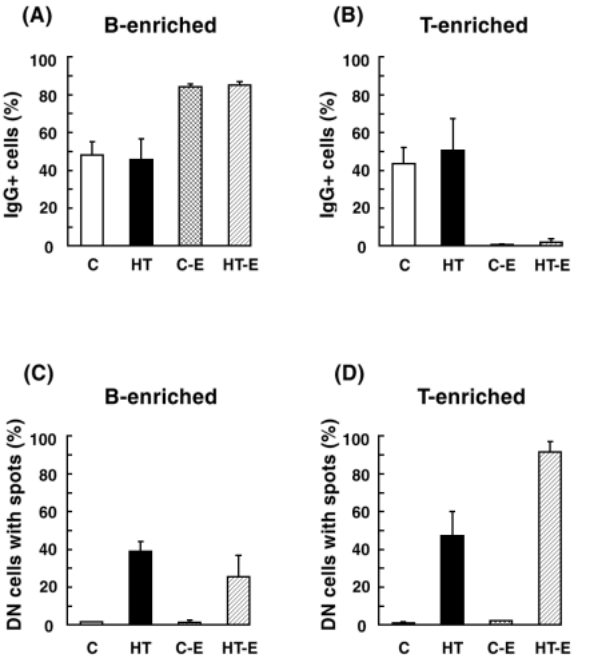

Fig. 7. Relationship between NuMA spots and lymphocyte subsets. (A-B) Histograms showing the proportion of IgG+ cells in total, B-enriched (A) and T-enriched (B) samples labelled with CY3-conjugated IgG and DAPI. Control total (open bars), heat-treated total (solid bars), control enriched (cross-hatched bars) and heat-treated enriched (hatched bars) samples were evaluated microscopically for the presence or absence of surface IgG labelling. For each sample, 500 DN nuclei were scored. Histograms show the mean values from three separate experiments; error bars show the standard deviation from the mean. (C-D) Histograms showing the proportion of DN nuclei with NuMA spots in total, B-enriched (C) and T-enriched (D) samples labelled with anti-NuMA and DAPI. Control total (open bars), heat-treated total (solid bars), control enriched (cross-hatched bars) and heat-treated enriched (hatched bars) samples were evaluated microscopically for the presence or absence of NuMA spots. For each sample, 500 DN nuclei were counted. Histograms show the mean values from three separate experiments; error bars show the standard deviation from the mean.

Table 2. Effect of culture on the ratio of IgG^-:IgG^+ lymphocytes

Experiment number	% IgG-labelled DN cells[a]		Ratio of IgG^-:IgG^+ cells	
	Control[b]	Heat-treated[b]	Control	Heat-treated
Expt 1	4.5	27	22:1	3.7:1
Expt 2	19	30	5.2:1	3.3:1
Expt 3	12	28	8:1	3.6:1

[a]Nuclei were evaluated as normal or apoptotic based on the morphology of chromatin by DAPI staining.

[b]Splenocytes were cultured for 24.5 hours at 37°C (control), or heated at 42°C for 30 minutes, then cultured at 37°C for 24 hours (heated). Samples were incubated with CY3-conjugated IgG for 20 minutes on ice, then fixed and counterstained with DAPI. In three separate experiments, 200 DN cells were evaluated for the presence or absence of cell surface IgG labelling.

(<5%) in all control samples (Figs 7C,D, 8A,A′). However, after heat treatment, 45-50% of the total population (Figs 7C,D, 8B,B′), but only 25% of the B-enriched population (Figs 7C, 8C,C′) had NuMA spots. Strikingly, moreover, more than 90% of the cells were spotty in the T-enriched samples (Figs 7D, 8D,D′).

Ratio of B:T cells at 24 hours

The data indicate that the T:B ratio is near 1:1 at t_0 (Fig. 7) and, according to flow cytometry, about 50-70% of the total splenocyte population dies within 24 hours (Fig. 1). If T and B cells die at the same rate, then the population of cells still alive at t_{24} should also consist of T and B lymphocytes in a 1:1 ratio. However, since essentially all TUNEL-positive DN nuclei in heated samples contained NuMA spots, the data from

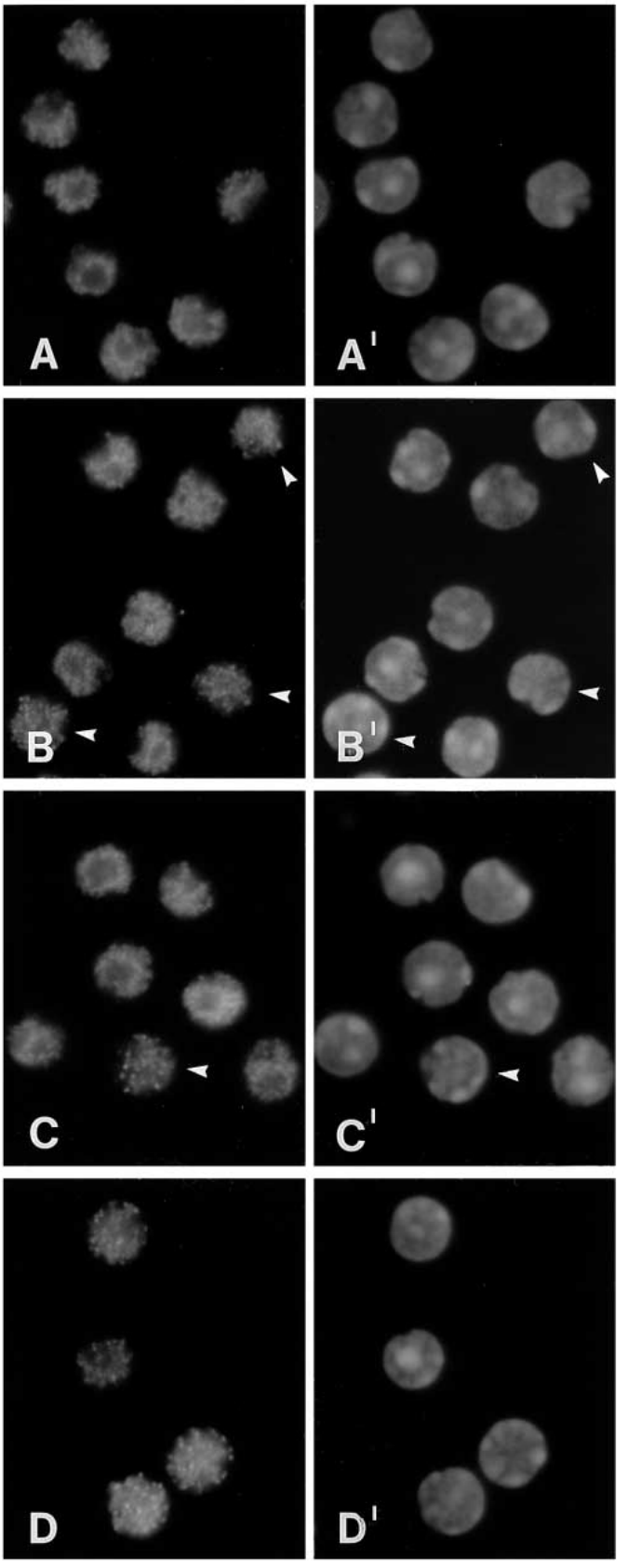

Fig. 8. Distribution of NuMA in total and fractionated splenocyte populations. Control and heat-treated samples were fixed and labelled with anti-NuMA (A-D) and DAPI (A′-D′). NuMA labelling is shown for control (A,A′) and heat-treated (B,B′) total cell populations, and heat-treated B-enriched (C,C′) and T-enriched (D,D′) populations. Arrowhead in B-B′, C-C′ identify nuclei with spots, and in C-C′ also identify a PC nucleus. No nuclei in A-A′ and all nuclei in D-D′ are spotty. Magnification ×1400.

NuMA labelling of fractionated populations imply that heat treatment hyper-induced apoptosis preferentially in T lymphocytes. If this is true, then the relative proportion of T cells in the heated cultures would be expected to decrease over time with respect to controls, reflecting the more rapid loss of these cells in heat-treated samples. To test this prediction, control and heated samples were cultured for 24 hours and labelled with CY3-conjugated IgG and DAPI. The proportion of B cells in the cultures was then determined by scoring for DN cells that were IgG^+.

The data show that the T and B subsets die at different rates in both control and heated samples, and are consistent with increased death of T lymphocytes after exposure to hyperthermia. Although the absolute percentages of IgG^+ cells varied between experiments, the percentage of B cells was consistently low (4-19%) in control samples. The preferential death of B cells in these samples was reflected in the high T:B ratios (Table 2). As predicted, however, the proportion of B cells in heated samples was consistently higher (27-30%). The decrease, with respect to controls, in the relative proportion of T cells in heated cultures was even clearer when the ratios of IgG^-:IgG^+ cells were compared. Whereas they ranged from 5.2:1 to 22:1 for control samples, they did not rise above 3.7:1 for heated samples.

DISCUSSION

Apoptosis is thought to play a central role in the immune system, functioning in the physiological control of T and B cell numbers, in the prevention of autoimmune responses, and in pathological immune responses (reviewed in Cohen, 1995; Abbas, 1996).

It is well known that lymphocytes die spontaneously in culture. Previous studies have indicated that spontaneous apoptosis affects 20-50% of the cell population within 16 hours for cultures of thymocytes (Migliorati et al., 1992; Clarke et al., 1993; Perandones et al., 1993; Rinner et al., 1996), isolated splenic B cells (Illera et al., 1993) and isolated splenic T cells (Perandones et al., 1993). In this study, surface IgG labelling was used to determine whether spontaneous or heat-induced apoptosis preferentially affected one of the lymphocyte subsets in a mixed population of T and B lymphocytes from the spleen. The results showed that, by 24 hours in culture, most of the remaining DN cells were IgG^-, suggesting that spontaneous apoptosis affected mainly the B cell subset. These results are in agreement with a recent flow cytometric study by Reap et al. (1995). Using a combination of cell surface antigen labelling and DNA staining of total mouse splenic lymphocyte populations, they showed that the B cells were more susceptible to spontaneous apoptosis than the T cells (Reap et al., 1995).

There have also been reports that lymphocyte subsets may respond differently to a given apoptotic stimulus. For example,

flow cytometric analysis of lymphocytes double-labelled for cell surface markers and DNA strand breaks has shown that $CD4^+$ cells are more resistant to irradiation *in vivo* than are $CD8^+$ or $CD4^-CD8^-$ cells (Zhang et al., 1995). As well, analysis of DNA content by flow cytometry has shown that mouse splenic T cells are more resistant to glucocorticoid-induced apoptosis than are thymocytes (Perandones et al., 1993).

The results presented here demonstrate a differential response of splenic B and T cells to heat treatment. Microscopic evaluation of surface IgG labelling showed that, by 24 hours, the ratio of DN T cells to B cells was consistently lower in heat-treated samples than in controls. This decrease in the relative frequency of T cells indicates that heat treatment increased the rate of apoptosis in total splenocyte cultures by inducing apoptosis in T cells. An increase in the rate of thymocyte cell death in response to heat has been previously reported (Sellins and Cohen, 1991; Migliorati et al., 1992). However, this study is the first to our knowledge to show that mature T cells are more susceptible to heat-induced apoptosis than mature B cells. It is also the first to demonstrate different morphological features in the same cell type, i.e. mature T cells, dying as a result of different signals, i.e. spontaneously and after heat treatment. Notably, the difference is in the organization of NuMA, a protein known to be an early target for apoptotic proteolytic cleavage (Hsu and Yeh, 1996; Weaver et al., 1996).

Splenic T and B cells also showed a differential response to heat treatment with respect to the NuMA labelling pattern. After a 30 minute heat treatment, essentially all cells in T-enriched samples exhibited NuMA spots. B-enriched cell populations, on the other hand, mainly exhibited the control diffuse NuMA pattern. Although the difference between total and enriched populations was less dramatic in B-enriched samples, this is probably because the B-enrichment protocol was somewhat less effective. Nonetheless, the proportion of cells with NuMA spots in the B-enriched samples was inversely correlated with the extent of removal of T cells. That is, in B-enriched cultures, only ≤80% of cells were IgG+, and about 25% of nuclei were spotty. This suggests that the cells with spots in B-enriched populations represented the remaining T cells.

The development of NuMA spots preferentially in T cells, which form about half of the total splenic lymphocyte population, explains our initial observation that about half of the population exhibited NuMA spots after heat treatment. Moreover, the lack of NuMA spots in B cells immediately after a 30 minute heat treatment indicates that B cells dying by apoptosis in heated samples do so without developing NuMA spots. This would account at least in part for the absence of NuMA spots in some of the PC and FC nuclei in heat-treated cultures.

The significance of NuMA spots in heat-treated samples, however, is unclear. On the one hand, observations with TUNEL showed that essentially all of the heat-treated cells in early stages of apoptotic DNA fragmentation contain NuMA spots. These data suggest that the spots may be a very early indicator of heat-induced splenic T cell apoptosis. Moreover, the very rapid appearance of NuMA spots in some cells after a 5 minute heat treatment suggests that this response does not require biosynthesis. On the other hand, apoptosis was hyper-induced only by heating for 20 minutes or longer, leading to the conclusion that the appearance of NuMA spots is not a sufficient sign that apoptosis has been initiated.

These two sets of data may not, however, be as contradictory as they first seem. We also found that, after a dramatic rise in the proportion of spotty nuclei in samples heated for 10 minutes, the proportion consistently dropped somewhat as heating was prolonged. This implies that some cells may recover from the induction of NuMA spots, i.e. return to the diffuse pattern, during the longer treatment times. When considered together with the TUNEL and other data above, these results suggest that cells which do not recover the control NuMA organization within 20 minutes become destined for apoptosis.

As well, these results further emphasize the important role that the NuMA protein plays in nuclear reorganization during apoptosis. Although the early events are still poorly understood, it appears that key structural proteins are redistributed and/or specifically degraded prior to, or coincident with, the onset of DNA degradation.

In vivo, T cell apoptosis has been implicated in immune deficiency associated with thermal injury (Teodorczyk-Injeyan et al., 1995). The results of this study show that mature splenic T and B lymphocytes also respond differentially to heat in vitro. This experimental system may, therefore, be a useful model in which to study the response of the immune system to fever or thermal injury.

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