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Nucleosomes induce lymphocyte necrosis

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Nucleosomes are major autoantigens in systemic lupus erythematosus. These are found as circulating complexes in both lupus patients and lupus mice. The existence of a potential ubiquitous cell surface receptor specific for nucleosomes has been suggested. However, neither the nature nor the role of this receptor has been identified. Moreover, the consequence of receptor-nucleosome interaction on the target cell has not been studied in detail so far. We show here that purified nucleosomes induce cell death of normal and lupus lymphocytes *ex vivo* in a dose- and time-dependent manner whereas human dendritic cells were relatively resistant. Most importantly, nucleosome-induced cell death is primary necrosis. Moreover, necrosis could be abolished when nucleosomes were first treated with deoxyribonuclease I, proteinase K or with a specific monoclonal antibody. Finally, intravenous injections of purified nucleosomes result in a reduced spleen cell number compared to buffer-injected mice, indicating that circulating nucleosomes may behave similarly *in vivo*. Taken together, this is the first report indicating that nucleosomes are able to induce necrosis, which in turn could result in an amplification loop of the disease causing the inflammation observed in lupus patients.

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1 Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the production of autoantibodies (autoAb) directed against cell surface, nuclear and cytoplasmic proteins. Among these autoantigens (autoAg), nucleosome is of prime importance since autoreactive B and Th lymphocytes specific for nucleosomes have been identified in lupus patients and mice [1–4]. Moreover, anti-nucleosome autoAb are detected very early during the time course of the mouse disease and are potentially nephritogenic [1, 5] and the serum concentration of these autoAb is correlated with disease activity in human [5]. Nucleosome is a complex composed of approximately 180 base pairs (bp) of deoxyribonucleic acid (DNA) and two molecules of histones H2A, H2B, H3 and H4 as well as one molecule of histone H1. It is found as a circulating complex in the plasma of lupus patients and mice [6] and it has been shown that the level of circulating nucleosomes correlates with disease activity [7]. All these observations support the hypothesis that circulating nucleosomes are involved in lupus pathogenesis of SLE. Indeed, several studies have suggested the existence of a potential ubiquitous nucleosome-specific cell surface receptor [8, 9] and particularly on lymphocytes [10].

We looked for a potential effect of nucleosomes on living cells *in vitro* as well as *in vivo*. More particularly, we were interested in the consequence of triggering the receptor specific for nucleosomes. The results indicate a novel role of nucleosomes in autoimmune diseases and especially in SLE.

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Abbreviations: 7-AAD: 7-amino-actinomycin D **DNase I:** Deoxyribonuclease I **LDH:** Lactate dehydrogenase **Nuc:** Nucleosome **PI:** Propidium iodide **autoAg:** Autoantigen **autoAb:** Autoantibody

2 Results

2.1 Purified nucleosomes induce necrosis of mouse lymph node cells

Purified nucleosomes were incubated with fresh mouse lymph node cells (LNC) and cell proliferation was followed up by [^{3}H]thymidine incorporation (Fig. 1). Non-treated LNC, as well as ConA- and LPS-stimulated cells showed an impaired thymidine uptake when incubated with nucleosomes.

Culture supernatants were then analyzed for the presence of lactate dehydrogenase (LDH), which is a cyto-

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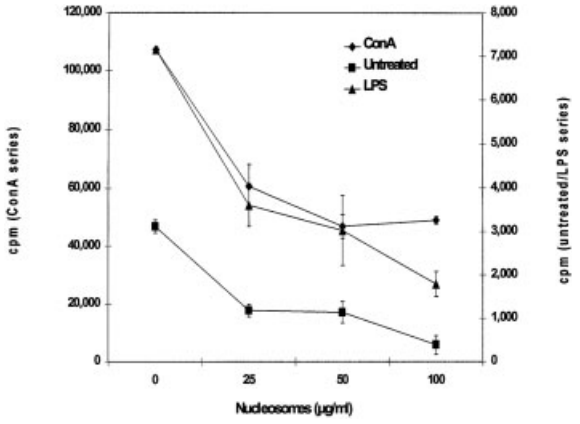
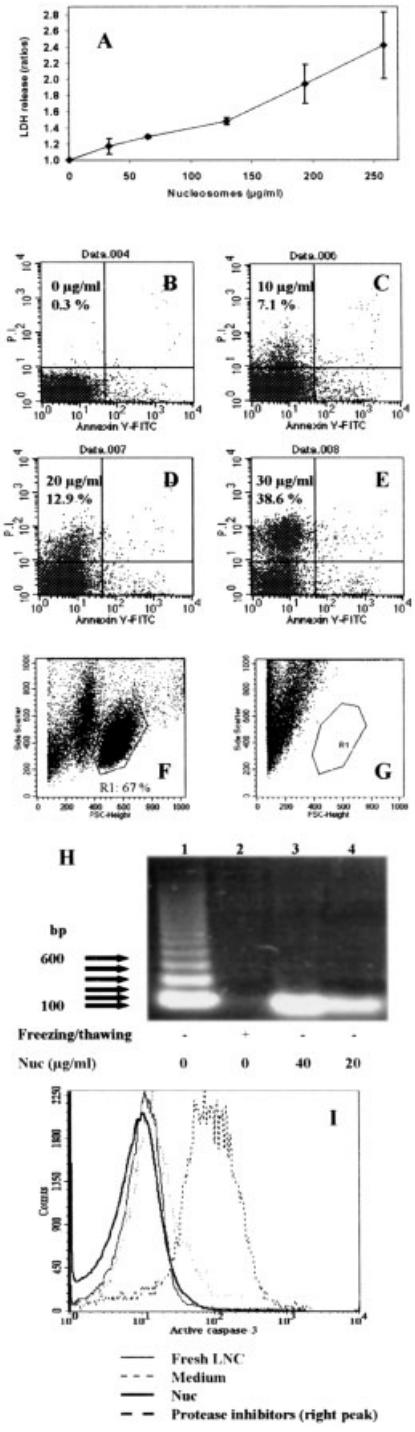


Fig. 1. Cell proliferation is impaired in the presence of purified nucleosomes. Non activated, ConA- and LPS-activated LNC were cultured for 24 h with nucleosomes. Cell proliferation was assessed by thymidine incorporation. As a comparison, nonactivated LNC cultured in the presence of a 1% Triton®X-100-containing lysis solution gave 257 cpm. The results are representative of three independent experiments.

solic enzyme released upon cell lysis. This phenomenon is associated with necrosis rather than with apoptosis. Whereas non-treated cells exhibit low LDH release after 24 h of culture, cell lysis is observed in nucleosome-treated cells in a dose-dependent manner, as shown in Fig. 2A**,** indicating that the cause for the nucleosome-induced inhibition of proliferation is cell death. To confirm the type of cell death, cells were analyzed by flow cytometry in the presence of annexin V-FITC and propidium iodide (PI). Thus, in the presence of 30 µg/ml of nucleosomes, about 40% of cells are annexin V-FITC-negative but PI-positive compared to only 0.3% in the absence of nucleosome (Fig. 2B–E). The gate used to select living cells is represented in Fig. 2F. LNC were cultured for 24 h without nucleosome and analyzed by flow cytometry. The cells in the gate represent 67% and a similar percentage is obtained with cells cultured in the presence of purification buffer. When nucleosomes alone were treated at the same conditions (Fig. 2G), no events were detected in the gate. The events detected represent nucleosome aggregates which are formed in culture medium. This does not represent contamination of the nucleosome preparation, *e.g.* with cell debris. Moreover, the extent of DNA fragmentation was examined. Untreated LNC showed the typical apoptotic DNA ladder on agarose gel after 24 h of culture (Fig. 2H, lane 1) corresponding to the background cell death (which was excluded in flow cytometry plots). This was used as positive control for apoptosis. On the other hand, fresh LNC were frozen/thawed several times; this was used as positive control for necrosis. In these conditions, it was not possible to detect any apoptotic DNA (Fig. 2H, lane 2). Like the frozen/thawed cells, nucleosome-treated LNC showed no apoptotic DNA. The unique 180-bp band observed in lanes 3 and 4 corresponds to DNA contained in purified mononucleosomes which can also be extracted by this method (not shown). This suggests that nucleosomes induce a switch in the mode of cell death from apoptosis to necrosis. Finally, cell death was analyzed by flow cytometry by intracellular staining using an anti-active caspase-3 Ab. As shown in Fig. 2I, a slight switch could be observed with cells cultured for 24 h compared to fresh cells. On the contrary, cells cultured in the presence of nucleosomes were not stained by this Ab. As a control, cells cultured with protease inhibitors entered apoptosis. We then confirmed the mode of cell death by morphological analysis by electron microscopy. Untreated LNC were characterized by a large nucleus/cytoplasm ratio and by a small rim of homogenous and electron-dense cytoplasm around the nucleus (Fig. 3A). Following nucleosome treatment, many cells became necrotic. The chromatin was clumped together (pyknosis), and the cytoplasm disintegrated resulting in a heavy decrease of electron density (Fig. 3B). There was no nuclear or cytoplasmic blebbing and no maintenance of the ultrastructure of the cytoplasm, suggesting the absence of apoptosis. In sharp contrast, during activation-induced cell death, a considerable number of cells presented signs of apoptosis such as the characteristic separation of chromatin, a condensed and marginated chromatin, nuclear blebbing, and maintenance of electron density of the cytoplasm (Fig. 3C). All these results demonstrate that nucleosome-treated LNC undergo necrosis and not apoptosis or secondary necrosis. It should be noted that morphological analysis of the cell by microscopy is the reference method to distinguish necrosis from apoptosis. It should also be noted that necrosis induction was dose dependent (Fig. 1, 2A–E) as well as time dependent (data not shown). Thus, it could be excluded that PI staining was due to the staining of the DNA part of nucleosomes on cell surface since the staining in the presence of a single nucleosome concentration was time dependent over a long period. Moreover, in binding experiments with FITC-nucleosomes, we never observed an increase in the number of PI-positive cells when increasing the amount of FITC-nucleosomes (see below). Moreover, we found that the number of living cells decreases linearly with the increase of PI-positive cells in the presence of increasing amounts of nucleosomes with a correlation factor R^2=0.90 (data not shown).



◀ *Fig. 2.* Nucleosome-induced cell death is necrosis. LNC were cultured with purified nucleosomes for 24 h and analyzed. (A) LDH released in culture supernatants. As a positive control, the ratio obtained with repeated freezing/thawing cycles of LNC is 5.9, whereas the ratios measured with cells incubated in the presence of purification buffer or 20 µg/ml Actinomycin D (an apoptosis-inducing agent) are 1.18 and 1.39, respectively. (B–E) Cells were analyzed by flow cytometry following Annexin V-FITC and PI staining. Nucleosome concentrations as well as percentage of Annexin V-FITC-negative and PI-positive cells observed are indicated. (F–G) Flow cytometry dot plots showing the gate used to select living cells and eliminate cell debris (F, R1) and showing purified nucleosomes without any cell (G) at 24 h. The percentage of cells contained in the gate is indicated. (H) DNA was extracted and analyzed for fragmentation on a 1% agarose gel. Lane 1, untreated cells; lane 2, necrotic cells following freezing/thawing treatment; lanes 3 and 4, nucleosome-treated cells. Lane 1 shows the typical apoptotic DNA ladder. DNA sizes are indicated. (I) Cells were analyzed by flow cytometry using a PE-conjugated anti-active caspase-3 Ab. In each case, the results are representative of three to ten independent experiments.

2.2 Mouse B cells are more sensitive to nucleosome-induced necrosis as compared to T cells

We investigated the nucleosome-sensitive LNC populations in mice. B and T cells were purified as described in Sect. 4. Following nucleosome treatment, both B and T cells underwent necrosis, as seen by flow cytometry analysis (Fig. 4A). However, B cells were more sensitive towards nucleosomes than T cells, especially at higher nucleosome concentrations. Nevertheless, high rates of cell death are observed with both cell types.

2.3 Human lymphocytes from normal individuals as well as from lupus patients are nucleosome sensitive

We first compared the sensitivity towards nucleosomes of mouse LNC and human PBMC by flow cytometry. In both cases, cells were gated on lymphocyte populations. As shown in Fig. 4B, both human and mouse lymphocytes are nucleosome sensitive and to about the same extent. On the contrary, human DC are less sensitive, but not fully resistant, under the same conditions (Fig. 4B). More importantly, human DC are activated by nucleosomes (Decker et al., manuscript in preparation).

Finally, PBMC from normal individuals as well as from lupus patients were analyzed by flow cytometry following nucleosome treatment. Three independent lupus samples were tested in three independent experiments. As shown in Fig. 4C, both types of cells were sensitive to nucleosome-induced cell death and to a similar extent. Similar results were obtained when comparing the percentages of PI-positive cells or with the LDH assay (data not shown).

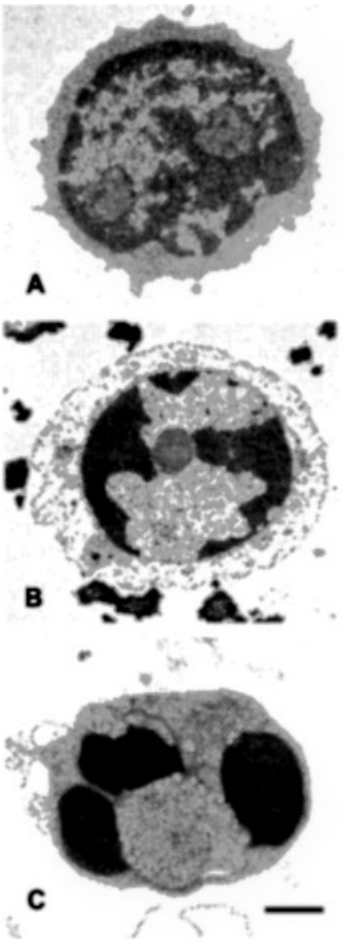


Fig. 3. Electron micrographs of lymphocytes. (A) Untreated lymphocytes. (B) Lymphocyte treated with nucleosomes. (C) Apoptotic lymphocyte. The bar in C is valid for all pictures and represents 1 μm.

2.4 Nucleosome-induced necrosis is dependent on nucleosome integrity

To be sure that the effect observed was due to nucleosomes and to see whether the effect depends on nucleosome integrity, we designed a series of control experiments. First, nucleosome preparations were submitted to either DNase I or proteinase K digestion before purification on sucrose gradient, resulting in partial digestion of the DNA and histone tails, respectively. This mainly yields nucleosome core particles of about 146 pb of DNA or nucleosome-like particles with impaired histone content, both with structures certainly different than the structure of native mononucleosomes. In both cases, pretreatment of nucleosomes abolished necrosis induction in LNC (Fig. 5A and B). Since the nucleosome structure is temperature sensitive, we tested necrosis induction by pre-heated nucleosomes. Whereas temperature

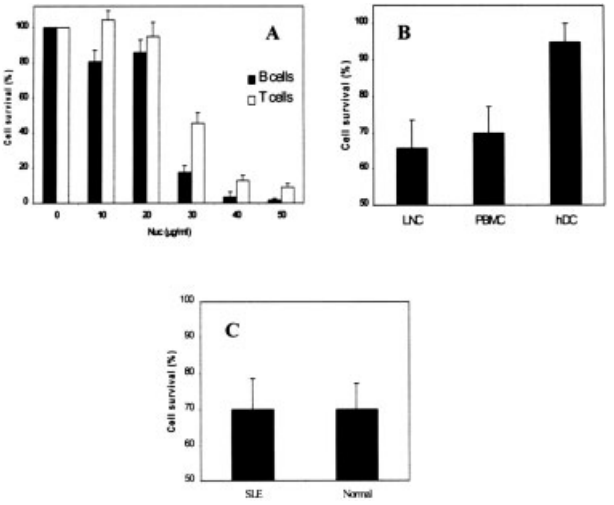


Fig. 4. Sensitivity of different cell types to nucleosome-induced necrosis. (A) Purified mouse B and T cells were cultured in the presence of nucleosomes for 24 h and analyzed by flow cytometry following staining with Annexin V-FITC and PI. The figure shows the number of surviving cells. Untreated cells represent 100% of cell survival. (B) Both mouse LNC and human PBMC are nucleosome sensitive. Cells were cultured in the presence of 40 μg/ml nucleosomes and analyzed as in (A). (C) Lupus PBMC (n=3) were also sensitive to nucleosome-induced necrosis. PBMC were cultured as in (B) and analyzed as previously. In all cases, the results are representative of three independent experiments.

under 40°C had little influence, heating to 60°C induced a 44% decrease in cell death induction and heating to 95°C nearly abolished cell death induction (Fig. 5C). Moreover, we compared purified individual nucleosome components, as well as nucleosomes "reconstituted" *in vitro* by pre-incubating purified DNA and histones, to purified mononucleosomes. A similar effect was observed with reconstituted nucleosomes and purified nucleosomes (Fig. 5D). On the contrary, purified DNA, histones or BSA, did not induce necrosis. Reconstitution of nucleosomes probably does not yield native nucleosomes. Nevertheless, this experiment shows that individual nucleosomal components do not present any activity whereas nucleosome-like particles as well as native nucleosomes induce cell death. Moreover, it should be noted that although DNA has been shown to bind to cell surface of mouse and human lymphocytes [8], it has no effect on cell survival. This also demonstrates that cell staining with PI is not due to the staining of the DNA part of nucleosomes bound to the cell surface. Finally, nucleosomes were pre-incubated with the nucleosome-specific mAb LG2–2 before addition of LNC. As shown in Fig. 5E, co-incubation of LG2–2 and nucleosomes resulted in inhibition of necrosis induction. No inhibition was observed when the same experiment

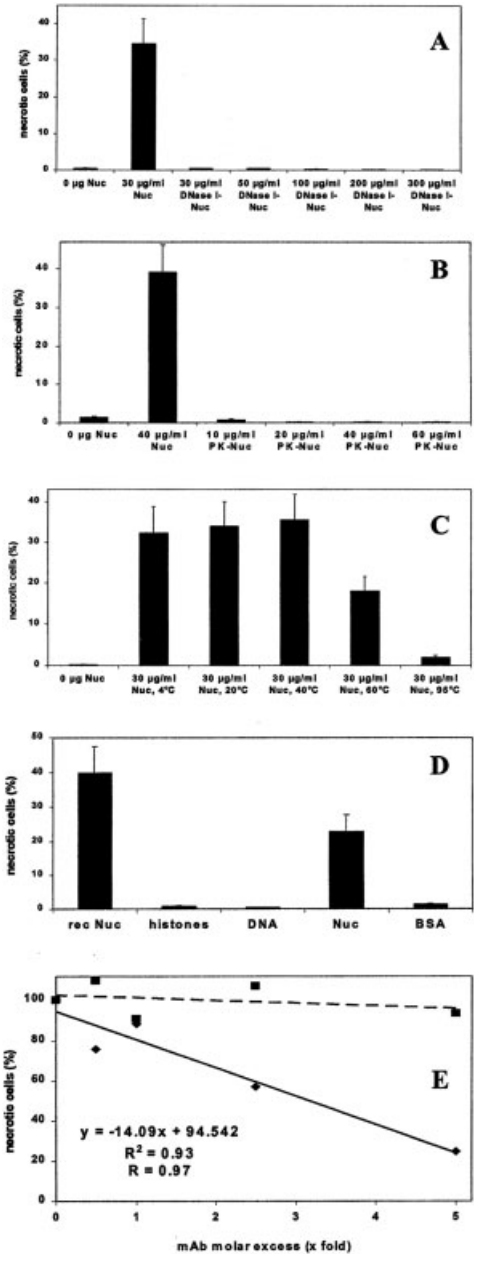


Fig. 5. Necrosis induction is specific for nucleosomes. LNC were cultured with purified nucleosomes for 24 h in various conditions and the percentage of necrotic cells was then determined by flow cytometry following Annexin V-FITC and PI staining. (A) DNase I digestion of nucleosomes. (B) Proteinase K digestion of nucleosomes. (C) Nucleosomes pre-incubated at different temperatures. (D) Necrosis induction by purified nucleosomes, purified histones, purified DNA, purified BSA and "reconstituted" nucleosomes (rec Nuc). (E) Nucleosomes were first incubated with LG2–2 mAb (or purified Ig; dotted line) before addition of LNC. Necrosis level was compared to the level observed in the case of LNC incubated with the same amount of nucleosomes but without any Ab (this value being taken as 100%). All the results are representative of three independent experiments.

was done in the presence of purified Ig of the same isotype. All these data demonstrate that necrosis observed in the presence of nucleosomes is not due to any contaminant and that necrosis is dependent on nucleosome integrity.

2.5 Cell-surface nucleosome binding

Since previous work has demonstrated that nucleosomes bind to the cell surface [8, 9], we checked our purified nucleosomes for the same activity. Fresh LNC were incubated with FITC-conjugated nucleosomes and analyzed by flow cytometry in the presence of PI. Living cells showed a dose-dependent staining and 24.16% of cells were positive with as few as 1 µg of FITC-nucleosomes (Fig. 6A, B). Only a weak staining (1.97%) was observed with the same amount of FITC-BSA

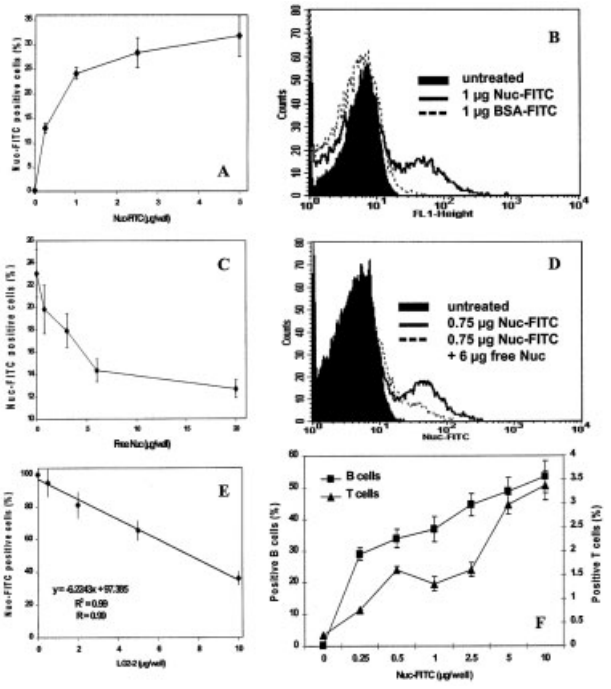


Fig. 6. Nucleosomes bind specifically to cell surface of lymphocytes. (A) Binding of FITC-nucleosomes was estimated by flow cytometry in the presence of PI. (B) No binding was observed with FITC-BSA in the same experimental conditions. The binding of 0.75 µg of Nuc-FITC could be inhibited in the presence of various amount of unlabeled nucleosomes (C, D) as well as in the presence of LG2–2 mAb (E, performed with 1 µg of Nuc-FITC incubated with cells treated with an FcR blocking Ab; 100% corresponds to cells incubated with FITC-Nuc in the absence of LG2–2). (F) The same binding assay than in (A) was performed with purified B and T cells. In all cases, values correspond to living cells. For each case, results are representative of three to five independent experiments.

(Fig. 6B). Under these conditions, pre-incubation of LNC with free nucleosomes before addition of FITC-nucleosomes impaired staining (Fig. 6C and D). For instance, a 45.3% inhibition of FITC-staining was observed with an eightfold excess of free nucleosomes. As a comparison, it was not possible to inhibit binding of FITC-nucleosomes with unlabeled free BSA (data not shown). We next estimated nonspecific binding of large molecular complexes using FITC-conjugated heat-aggregated IgG; no binding was detected under these conditions (data not shown). Moreover, when FITC-nucleosomes were pre-incubated with LG2–2 mAb before addition of fresh LNC pretreated with an FcR blocking mAb, a clear dose-dependent inhibition was observed (Fig. 6E). For instance, a reduction of 63.8% of the number of FITC-nucleosomes-positive cells was obtained with 10 μg/well of LG2–2. It should be noted that this mAb also inhibits nucleosome-induced necrosis (Fig. 5E). Finally, although both B and T cells are sensitive to nucleosome-induced cell death (Fig. 4A), we observed that nucleosome binding on B cells is higher (Fig. 6F). Actually, both B and T cells bind nucleosomes in a dose-dependent manner but with different efficiencies, which correlates with the different sensitivities to cell death observed with both cell types.

2.6 Nucleosomes induce *in vivo* elimination of spleen lymphocytes

We next looked for any *in vivo* effect of purified nucleosomes. However, it is rather difficult to demonstrate necrosis *in vivo* and particularly necrosis of circulating lymphocytes. We therefore tried to quantify lymphocytes in different organs following nucleosome injections into mice.

Because of the circulating property of both nucleosomes and lymphocytes, we decided to perform intravenous injections of purified nucleosomes. For this purpose, a total of 23 mice were i.v. injected with sucrose buffer or purified nucleosomes (two independent experiments performed with two different batches of purified nucleosomes). Thirty hours later, spleens cells were prepared and counted as described in Sect. 4. Using this protocol, B and T lymphocytes represent about 90% of the cells as evidenced by flow cytometry (data not shown). As shown in Table 1, spleens from nucleosome-injected mice contain less cells than spleens from buffer-injected mice (26.6% less cells) and this difference is statistically significant as evidenced using a two-tailed Student's *t*-test (p=0.003). There was no difference in the percentage of B and T lymphocytes in the spleen, as evidenced by flow cytometry following B220 and CD3 staining, respectively (data not shown). With the same procedure, we also checked the percentage and the total cell number of B and T lymphocytes in the blood and LN and we did not find any difference between both groups of injected mice, which indicates that the reduced number of spleen lymphocytes in nucleosome-injected mice is not due to nucleosome-induced cell migration or differential homing (not shown). Finally, we did not find any increase in the percentage of apoptotic cells in spleen, blood or LN strengthening the hypothesis that elimination of spleen lymphocytes results from necrosis. Likewise, no difference was observed for splenic DC and granulocytes between buffer- and nucleosome-injected mice, as measured by flow cytometry following staining for CD11c and Ly-6G, respectively, indicating that lymphocytes are the main target for nucleosome-induced cell death.

Table 1. *In vivo* nucleosome-induced elimination of lymphocytes[a)]

	Buffer	Nuc
Mice number	12	11
Mean cell number ($\times 10^{-6}$)	66.82	49.02
SD	13.69	11.94
p		0.003

[a)] Spleen cell numbers were determined 30 h after i.v. injection of purified nucleosomes or sucrose buffer. Mean cell numbers were compared using a two-tailed Student's *t*-test.

3 Discussion

We show for the first time that purified nucleosomes induce primary necrosis of lymphocytes. The cell death is not by apoptosis and not by secondary necrosis as shown by a large panel of techniques. It should be noted that we observed necrosis induction without any anti-nucleosome Ab. This result is particularly interesting since lupus is characterized by its inflammation state. On the other hand, SLE has been described as a disease with elevated rates of spontaneous apoptosis [11]. However, apoptosis is usually not associated with inflammation reactions and is even immunosupressive [12] since cell content is not released. On the contrary, necrosis induces inflammation and stimulates immune responses [13]. Thus, as a result of excessive apoptosis and deficient phagocytosis of apoptotic cells in lupus patients [14], secondary necrosis occurs [15] and nucleosomes are released into the circulation. Our results now indicate that nucleosomes induce primary necrosis of neighboring cells, and this phenomenon could be at the origin of the disease or could result in an amplification loop. There

are several reasons in favor for this consideration. First, necrosis induction could result in a local inflammation. As a consequence, reactive oxygen species released by macrophages could be responsible for the destruction of cells in the periphery. Secondly, necrosis has been shown to be associated with post-translational modifications of self Ag and generation of cryptic epitopes [16]. The release of such self Ag could be responsible for the break of peripheral tolerance and the spreading of the autoimmune response, which would be favored by the inflammation state induced by circulating nucleosomes. This hypothesis is supported by our observation that human DC are activated *ex vivo* by nucleosomes (Decker et al., manuscript in preparation). Thirdly, nucleosomes have been found in synovial fluid of rheumatoid arthritis patients and it has been suggested that the presence of nucleosomes characterizes inflammation [17]. Finally, lymphopenia is a well known manifestation of SLE and thus, according to our results, circulating nucleosomes could be involved in this process in patients. Altogether, the results described here are in agreement with clinical observations. It should be noted that it is difficult to judge whether the nucleosome concentrations used *in vitro* are comparable to physiological concentrations since few studies dealt with the quantification of circulating nucleosome concentrations in lupus patients or mice and the results are contradictory. For example, Amoura et al. [5] have shown that the mean concentration is about 100 ng/ml but can reach 1.2 μg/ml in some patients. On the opposite, using a different protocol, it was recently shown that this concentration is about 10 μg/ml and can reach 100–200 μg/ml in some patients ([7] and Ralph C. Williams Jr., Albuquerque, New Mexico, personal communication). Such a high concentration has also been described in synovial fluid from patients with inflammatory disorders where the mean nucleosome concentration was 14.1 μg/ml [17]. Since we could detect necrosis with 30 μg/ml nucleosomes, we are in the range of concentrations observed *in vivo* in the two latter cases. Moreover, the observed phenomenon might be induced following a local and transient cell death increase without any systemic dissemination. Thus, two different behaviors of nucleosomes seem to occur in patients depending on the local concentration. Whereas low nucleosome concentrations promote T cell activation, high concentrations induce cell death.

More importantly, both PBMC from normal individual and lupus patients were found nucleosome-sensitive, in line with the possibility that circulating nucleosomes could be involved in the proposed mechanism in patients. Altogether, our findings are consistent with the view that the level of circulating nucleosomes correlates with disease activity.

We also confirmed that our purified nucleosomes behave as previously described, as observed by the cell surface binding of FITC-nucleosomes. More importantly, we have clearly demonstrated that necrosis induction is dependent on nucleosomes with perfect integrity. This result is important and is relevant to the disease since it is believed that only nucleosomes are circulating, and not free histones or DNA [6]. We conclude that cell death is directly induced by nucleosomes and not by a soluble factor released following nucleosome treatment, since supernatants from nucleosome-treated cells had no effect on fresh cells (data not shown). However, it is still possible that cell death is due to cell-cell interactions following nucleosome-induced expression of a cell surface protein.

We have used anti-nucleosome Ab to prove that the phenomenon observed is due to nucleosomes. However, it should be noted that these Ab were either coated onto the culture plate or used with cells first treated with an FcR blocking Ab. As a consequence, we only observed inhibition of nucleosome activity when anti-nucleosome Ab were not able to interact with cells. We did not analyze the effect caused by the recognition of nucleosome/anti-nucleosome immune complexes.

Finally, we have shown that circulating nucleosomes may induce necrosis *in vivo* since i.v. injections of purified nucleosomes result in a clear diminution of spleen cell number in mice. This phenomenon was not observed in buffer-injected mice. Our data also suggest that this diminution in the number of spleen lymphocytes is not correlated to an increased cell number in other organs. Moreover, flow cytometry analysis revealed that both B and T lymphocytes were affected. Although we did not demonstrate necrosis *in vivo*, the reduction of cell number without any increase in the number of apoptotic cells is in agreement with cell lysis. Moreover, *in vivo* determination of lymphocyte necrosis is rather difficult to achieve. We used high amounts of nucleosomes to induce a massive effect in order to detect any difference between buffer- and nucleosome-injected mice. However, according to the blood volume in mice, we are still in the range of the highest nucleosome concentrations measured *in vivo* in the sera of lupus patients by Ralph C. Williams Jr., Albuquerque, New Mexico, (personal communication). Moreover, we have worked with normal mice. It is likely that in an autoimmune context the effect caused by circulating nucleosomes would be more severe since lupus patients have a decreased phagocytic activity, an impaired DNase I activity in serum as well as an improved sensitivity of lymphocytes towards cell death, this alterations being in favor of a higher half-life of nucleosomes in serum and a potential higher activity. In agreement, we have shown that DNase I-treated

nucleosomes do not induce necrosis. This could also explain why we did not clearly detect inflammation in nucleosome-injected mice. It also suggests that higher nucleosome concentrations are necessary to mimic the lupus conditions.

Recently, chromatin-containing immune complexes have been shown to activate B cells via Toll-like receptors [18]. However, no effect of chromatin alone has been described in that study. It should be noted also that B cell activation was observed with rheumatoid factor-positive B cells. As a consequence, the results described in that article can not been compared to those obtained in the present study since we have worked with purified nucleosomes, in the absence of Ig, and with non-autoimmune cells in most experiments.

In conclusion, circulating nucleosomes seem to be involved in two different mechanisms. First, at low concentrations, nucleosomes behave as an immunogen, like classical autoAg. They are engulfed by APC, processed and presented to Th cells. In these conditions they activate lupus T cells. Secondly, at local high concentrations, nucleosomes induce necrosis of neighboring cells, which could result in inflammation and the break of peripheral tolerance or Ag spreading. The signaling pathway(s) responsible for necrosis induction, the mechanism of activation of DC by nucleosomes, as well as *in vivo* inflammation studies in mice are currently under investigation.

4 Materials and methods

4.1 Animals and cell cultures

BALB/c and C57BL/6 mice were purchased from Janvier (Le Genest St Isle, France) and Charles River (Wilmington, MA). Spleens or LN were removed and cells were cultured in RPMI 1640 containing 10% heat inactivated FBS, 2 mM L-glutamin, 100 μg/ml penicillin/streptomycin, and 5×10^{-5} M β-mercaptoethanol. In some cases, LNC were treated with an FcR blocking mAb (PharMingen, San Diego, CA) for 30 min on ice according to manufacturer's recommendations. Some experiments were performed with $B220^+$ or $Thy1.2^+$ cells purified from LNC by negative selection using magnetic beads coated with either anti-Thy1.2 or anti-B220 mAb, respectively, according to manufacturer's recommendations (Dynal, Oslo, Norway). Purity was assessed by flow cytometry on a FACScalibur apparatus with CELLQuest software (Becton Dickinson, Mountain View, CA) using FITC-labeled anti-B220 and PE-labeled anti-Thy1.2 mAb (PharMingen). Purity was at least 95% of $B220^+Thy1.2^-$ cells and 97 % of $B220^-Thy1.2^+$ cells, respectively. Human PBMC were prepared from normal individuals and lupus patients accordingly to classical procedures and cultured in IMDM containing 10% human AB serum. Lupus samples were provided by Dr. Ina Kötter (Medizinische Klinik, Tübingen, Germany) and patients fulfilled the American College of Rheumatology criteria. Human DC were obtained from PBMC cultured in the presence of GM-CSF and IL-4 as described [19], except that the medium used was X-VIVO 15 containing 1% human serum. Samples of each cell batch were cultured in medium alone, with purified nucleosomes, with medium supplemented with the buffer used for nucleosome purification, and with medium supplemented with buffer and BSA, respectively.

4.2 Nucleosomes, histones, DNA, and mAb

Nucleosomes were prepared from calf thymus as described [20]. This method has been shown to produce native nucleosomes. Briefly, nuclei were purified using a Dounce homogenizer in the presence of a protease inhibitor cocktail (Sigma, Deisenhofen, Germany), digested at 37°C with micrococcal nuclease and lysed at 0°C for 30 min. Then, nucleosomes were purified on a 5–29% sucrose gradient in the absence of protease inhibitors and then filtered through a 0.2-μm filter. DNA and protein contents were checked on a 1.5% agarose gel and an 18% SDS-PAGE, respectively. Without any specification in the text, nucleosomes will refer to mononucleosomes. It should be noted here that histones are among the most conserved proteins. For instance, there is no difference in protein sequence between histone H4 from calf, human and mouse. In some cases, nucleosomes were digested with DNase I or proteinase K before purification on sucrose gradient. In other experiments, purified nucleosomes were incubated at different temperatures for 30 min before incubation with cells. For cell surface binding studies, nucleosomes were first dialyzed overnight against PBS at 4°C and then FITC-labeled as described [21]. Briefly, purified nucleosomes were allowed to react with FITC in sodium hydrogen carbonate buffer (0.05 M, pH 8) for 2 h at room temperature. Then the reaction was stopped with 8% (v/v) Tris buffer (1 M, pH 8) for 30 min at room temperature and free FITC molecules were removed by gel filtration through a Sephadex G-25 column.

Some experiments were done with purified DNA or histones. DNA was purified from mononucleosome fractions by phenol/chloroform extraction followed by ethanol precipitation. Purified histones were commercial preparations from Roche (Mannheim, Germany). In both cases, DNA and histone contents were checked as described above. Reconstituted nucleosomes were obtained by pre-mixing purified nucleosomal DNA and purified histones in equimolar ratio.

LG2–2 mAb was a kind gift of Dr. M. Monestier (Philadelphia, PA) and Dr. S. Muller (Strasbourg, France).

4.3 Cell death analysis

Cell survival was assessed by thymidine incorporation. Activated (with 2.5 μg/ml ConA or 2 μg/ml LPS) or nonactivated cells (2.5×10^5) were cultured in 96-well plates in the presence of purified nucleosomes for 24 h and then with tritiated thymidine (7.4×10^4–18.5×10^4 Bq/well, Amersham, Freiburg, Germany) for 18 h. Thymidine incorporation was measured using a scintillation counter (Microbeta, Wallac, Freiburg, Germany). LDH activity was measured with the Promega kit (Madison, WI) culturing 3×10^5 cells in 96-well plates for 24 h and according to manufacturer's recommendations. Results are expressed as ratios between nucleosome-treated cells and untreated cells. Positive controls for LDH release were obtained by incubation of cells with lysis buffer. Flow cytometry analyses were performed in the presence of annexin V-FITC and PI according to classical procedures. In some experiments, 7-amino-actinomycin D (7-AAD) was used in place of PI. Cells were gated to eliminate cell debris. Flow cytometry results are expressed either as cell survival compared to untreated cells (taken as reference or 100%) or as percentage of PI-positive/Annexin V-FITC-negative cells. The number of surviving cells, determined by the number of cells detected in the gate corresponding to living lymphocytes according to size and granularity parameters, was estimated by flow cytometry using the time option. DNA fragmentation was analyzed on a 1% agarose gel. Briefly, 4×10^6 cells were cultured in the presence of purified nucleosomes for 24 h in 24-well plates and DNA was extracted as described [22]. As a control, necrotic cells were obtained by freezing/thawing cells five times. Cell death was also analyzed by flow cytometry after cell fixation and permeabilization using a PE-conjugated anti-active caspase-3 Ab (PharMingen) after 24 h of culture. As a positive control, cells were cultured with 1 μl of a protease inhibitor cocktail (Sigma). For electron microscopy experiments, LNC were cultured for 8 h in medium alone or in the presence of nucleosomes or with both 10 μg/ml of ConA and 10 μg/ml of LPS to promote activation-induced cell death. A short incubation was chosen in order to distinguish primary necrosis from secondary necrosis. Ultrastructural analysis of cell death was assessed by transmission electron microscopy as described [23]. In all experiments, cells were treated as a negative control with the buffer used to purify nucleosomes. No effect could be observed on cells with this buffer in comparison to medium without buffer.

4.4 Cell surface nucleosome binding

Cells (10^5) were incubated with FITC-nucleosomes on ice in 10% FCS-containing PBS for 30 min. Cells were then washed and incubated with PI for flow cytometry analysis. FITC-BSA (Sigma) was used as negative control. In competition experiments, cells were first incubated with free nucleosomes for 15 min on ice and washed before addition of FITC-nucleosomes. In inhibition experiments with LG2–2 mAb, cells were first treated with an FcR blocking mAb (PharMingen), washed, and then incubated with LG2–2 and FITC-nucleosomes for 30 min on ice. FcR blocking treatment was done to avoid staining of cells due to internalization of LG2–2/FITC-nucleosomes immune complexes. Some experiments were performed with purified B or T cells. In this case, cells were triple-stained with Nuc-FITC, PE-conjugated anti-B220 or anti-Thy1.2 mAb and 7-AAD.

4.5 *In vivo* nucleosome-induced cell death

Mice were injected into the tail vein with 250 μg of purified nucleosomes per mouse i.v. or the same volume of sucrose buffer as a control. Thirty hours later, spleens were collected and independent cell suspensions were prepared by mechanical disruption through a 40-μm cell strainer and by lysis of red blood cells with 5 ml of hypotonic buffer per spleen, followed by two washes with PBS. Living cells were counted using a hemocytometer under a light microscope by the Trypan blue exclusion method. Differences in the number of spleen cells between nucleosome-injected and buffer-injected mice were tested using a two-tailed Student's *t*-test after having checked that both groups have a normal distribution and a similar variance.

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