Nucleosome: A Major Immunogen for Pathogenic Autoantibody-inducing T Cells of Lupus

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Summary

Only a fraction (12%) of 268 "autoreactive" T cell clones derived from lupus-prone mice can selectively induce the production of pathogenic anti-DNA autoantibodies in vitro and accelerate the development of lupus nephritis when transferred in vivo. The CDR3 loops of T cell receptor β chains expressed by these pathogenic T helper (Th) clones contain a recurrent motif of anionic residues suggesting that they are selected by autoantigens with cationic residues. Herein, we found that ≈50% of these pathogenic Th clones were specific for nucleosomal antigens, but none of them responded to cationic idiopeptides shared by variable regions of pathogenic anti-DNA autoantibodies. Nucleosomes did not stimulate the T cells as a nonspecific mitogen or superantigen. Only the pathogenic Th cells of lupus responded to nucleosomal antigens that were processed and presented via the major histocompatibility class II pathway. Although the presentation of purified mononucleosomes to the Th clones could be blocked by inhibitors of endosomal proteases, neither of the two components of the nucleosomes—free DNA or histones by themselves—could stimulate the Th clones. Thus critical peptide epitopes for the Th cells were probably protected during uptake and processing of the nucleosome particle as a whole. The nucleosome-specific Th clones preferentially augmented the production of IgG autoantibodies to histone–DNA complex in vitro. In vivo, nucleosome-specific, $CD4^+$ T cells were not detectable in normal mice, but they were found in the spleens of lupus-prone mice as early as 1 mo of age, long before other autoimmune manifestations. Immunization of young, preautoimmune lupus mice with nucleosomes augmented the production of autoantibodies and markedly accelerated the development of severe glomerulonephritis. Previously, crude preparations containing nucleosomes were shown by others to have polyclonal mitogenic activity for B cells from normal as well as lupus mice. Identification here of pure mononucleosome as a lupus-specific immunogen for the Th cells that selectively help the pathogenic anti-DNA autoantibody producing B cells of lupus could lead to the design of specific therapy against this pathogenic autoimmune response.

The cause and cure of SLE remains elusive because the primary autoantigen(s) that drives the pathogenic autoimmune response in this disease is unknown. The pathogenic anti-DNA autoantibodies that are consistently found in the renal lesions of lupus (1–3) and cause immune deposit nephritis when administered in vivo (4, 5) appear to be the products of an antigen-driven immune response: i.e., they have undergone clonal expansion with IgG class switch and somatic mutations that generated cationic residues in their V_H regions (5–10). This pathogenic autoantibody response in both spontaneous and experimentally induced (graft-vs.-host) SLE is Th cell dependent and these Th cells are predominantly $CD4^+$ (11–19). We have cloned such special autoimmune Th cells from patients as well as (SWR × NZB)F_1 (SNF_1) mice with lupus nephritis. In marked contrast to the autoimmune NZB and the normal SWR parental strains, the SNF_1 progeny uniformly produce pathogenic anti-DNA autoantibodies under the influence of certain Th cells and develop severe lupus glomerulonephritis (2, 12, 20). Only 12% of 268 "autoreactive" T cell clones derived from 10 nephritic SNF_1 mice and, similarly, a mere 15% of ≈400 autoreactive T cell clones from five patients with lupus nephritis have the functional ability to selectively induce the production of pathogenic anti-DNA autoantibodies in vitro when cultured with syngeneic B cells (21–23). Moreover, representative pathogenic autoantibody-inducing Th clones derived from the nephritic SNF_1 mice rapidly induce immune deposit glomerulonephritis when transferred in vivo into

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young preautoimmune SNF_1 mice (24). A question of central importance in lupus is what autoantigen drives these pathogenic Th cells? Although DNA is a target antigen for the pathogenic anti-DNA autoantibodies, immunization with DNA does not lead to the development of SLE (25). We found that the V-D-J junctional regions (CDR3) of TCR β chains expressed by these pathogenic Th clones bear a recurrent motif of one or more anionic residues that are generated by N-nucleotide additions. This result suggested that the pathogenic Th clones of lupus may be selected by some autoantigen(s) with cationic residues (24). The importance of reciprocally charged residues in TCR-CDR3 and antigenic peptide interaction has been recently established in another system (26). Since the pathogenic Th clones of lupus preferentially help the select population of B cells that produce the pathogenic anti-DNA autoantibodies, it is likely that those autoimmune B cells in turn present the autoantigen(s) with cationic residues to these Th cells (see Fig. 1). Cationic proteins, such as, histones complexed with DNA could be taken up, processed, and presented by such anti-DNA B cells efficiently, as demonstrated in other antigenic systems (for a review see reference 27). Alternatively, peptides with cationic residues derived from the V regions of their endogenous, anti-DNA Ig (idiopeptides) could be presented by the anti-DNA B cells to the pathogenic Th cells (28). Herein, we tested the responsiveness of the pathogenic Th clones for some candidate autoantigens based on these hypotheses.

Materials and Methods

Mice. BALB/c, C57BL/6, NZB, SWR, and (NZB × NZW)F_1 (BWF_1) mice were from The Jackson Laboratory (Bar Harbor, ME). (SWR × NZB)F_1 (SNF_1) hybrids were bred at Tufts Animal Facility. Female mice were used for the studies.

Antibodies. The following mAbs: TIB120/M5 broadly reactive against I-$A^{b,d,q}$ and I-$E^{d,k}$; anti-I-A^d-HB3/MKD6, anti-J11d-TIB183, anti-Thy-1.2-TIB99, anti-CD4-RL-172.4, anti-CD8-TIB211, and anti-CD3-145-2C11, were obtained from the American Type Culture Collection ([ATCC] Rockville, MD. References in catalogue). The anti-I-A^b mAb HB38 (29), and the anti-TCR Vβ6 mAb 44-22-1 (30), were gifts from Brigitte Huber (Tufts University). The anti-IL-4 mAb 11B11 (31), and the anti-IL-2 mAb S4B6 (ATCC), were gifts from Dr. Miguel Stadecker (Tufts University). The anti-I-E^d mAb 14-4-4S (32), was from Dr. Laurie Glimcher (Harvard University, Cambridge, MA). The anti-I-A^q mAb MKQ7 (derived from BALB/c immunized with SWR B cells) was a gift from Dr. Phillipa Marrack (National Jewish Center for Immunology, Denver, CO). All mAbs were concentrated 10 times by 47% saturated ammonium sulfate (SAS) cut of hybridoma culture supernatants, dialyzed, and used at final dilutions, as specified.

Antigens. Pure mononucleosomes were prepared as described (32). Briefly, the nucleosomes were isolated by solubilizing chromatin from purified chicken erythrocyte nuclei with micrococcal nuclease (Worthington Biochem. Corp., Freehold, NJ). The solubilized chromatin was then stripped of H1, H5, and HMG proteins by gel filtration on CM sephadex C-25-120 (Sigma Chemical Co., St. Louis, MO). Stripped chromatin was then redigested with micrococcal nuclease and mononucleosomes were isolated by gel filtration on sephacryl S-400 (Pharmacia LKB, Piscataway, NJ), eluted with 5 mM Tris pH 8.0, 0.2 mM EDTA, and 20 mM NaCl. Column fractions were analyzed for monomers by PAGE of DNA and appropriate fractions were pooled (see Fig. 2 *A*). Histone-gel analysis showed the mononucleosome preparation to consist of core histones (see Fig. 2 *B*). "Nucleohistone" from calf thymus was purchased from Sigma Chemical Co. and was slowly dissolved in PBS by constant stirring for 1 wk (33, 34). Any insoluble material was removed by centrifugation at 2,000 *g* for 10 min. Gel electrophoresis of the above preparation showed the presence of oligo- and polynucleosomes. The DNA concentration of all nucleosome preparations was estimated by the diphenylamine reagent method (35). Calf thymus DNA (Sigma Chemical Co.) at various concentrations was used to develop a standard curve.

Histone octamers were prepared from core mononucleosome particles as described (36). Core particles were concentrated in a stirred cell (Amicon Corp., Danvers, MA) with an XM 50 membrane, and then further concentrated in a Centricon (Amicon Corp.) 30 to 1000 A_{260} Units per 1.8 ml. An equal volume of 4.4 M NaCl, 0.2 M KPO_4, pH 6.7 was added to the particles, followed by loading onto a hydroxyapatite column equilibrated with 2.2 M NaCl, 0.1 M KPO_4, pH 6.7. A_{230} monitoring indicated where peak fractions eluted, and the quality was further checked by running on a Laemmli 15% polyacrylamide-SDS gel. The nucleosome and histone octamer preparations were stored at 4°C. The other histone preparations and heparan sulphate were purchased from Sigma Chemical Co.

Synthetic peptides: CDR1 (TFTSYWMHWVKKRPGQGLE), CDR2 (NPSNGRTNYNEKFKRKAT), and a control peptide (WINTNTGEPTYAEEFKG) were synthesized by T-boc chemistry, purified by HPLC, sequenced, and verified to be >95% pure at the Tufts University peptide synthesis facility. The CDR1 and CDR2 peptides correspond to positions 28-46 and 52-69, respectively, of the CDR and flanking sequences of the V_H region that is shared by the major Id564 family of nephritogenic anti-DNA autoantibodies of SNF_1 mice (2, 4, 7). These CDR peptides also contain T cell epitope motifs (37). The control peptide corresponds to the CDR2 (position 50-65) of an irrelevant antimalarial antibody (38).

T Cell Clones. The seven cloned Th lines and eight Th hybridoma clones used in this study were derived from nephritic SNF_1 mice, subcloned, and maintained as previously described (21, 22, 24). Two of these previously characterized Th cell lines were growing poorly. They were therefore immortalized by fusion with the α/β TCR^- variant of BW5147 thymoma as described (24). In addition, 52H10.F11 is an insulin-specific T cell hybridoma derived from C57BL/6 (39), a gift from Brigitte Huber. For proliferation assays, T cells (lines) that had been rested for 7–10 d from their last feeding regimen were purified by centrifugation over Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) to remove dead cells/feeders. The enriched RPMI medium for growing and assaying the function of the Th clones has been described (21, 22) and will be referred to as final medium.

Isolation of Splenic T Cells. Whole spleen cells from which erythrocytes had been removed by hypotonic lysis were depleted of B cells by one cycle of panning (22). The nonadherent cells were further purified for $CD4^+$ T cells by two rounds of antibody/complement treatment as described (21, 40). Spleen cells were suspended at 20 × 10^6/ml in serum-free RPMI medium containing a cocktail of antibodies: anti-CD8 (1:4), anti-Ia (1:4), and anti-J11d (1:10) for 30 min on ice. The broadly reactive anti-Ia mAb, TIB120, was used for all the preparations. In addition, the anti-I-A^d mAb, MKD6, was used for preparing T cells from NZB, BALB/c, BWF_1, and SNF_1 strains, whereas the anti-I-A^b mAb, HB-38, was used for isolating T cells from C57BL/6. Antibody treatment was followed by incubation with a mixture of rabbit (1:20) and guinea

pig (1:10) complement (Pel Freeze Biologicals, Rogers, AR) at 37°C for 45 min. The isolated T cells were shown to be >95% pure by flow cytometry. For the isolation of $CD8^+$ T cells, the anti-CD4 mAb was substituted for the anti-CD8 mAb.

Preparation of Splenic APCs. Whole spleen cells depleted of erythrocytes were incubated at 20×10^6/ml in serum-free medium containing anti-Thy 1.2 (1:4) for 30 min on ice, followed by rabbit complement (1:5) at 37°C for 45 min. The APCs (1.5×10^6 in 200 μl) were either sham- or antigen-pulsed at 37°C for 2 h, and then washed three times, irradiated (3,000 rad), washed again, and cocultured at 5×10^5/well with T cells in 200 μl final medium in 96-well plates (Costar Corp., Cambridge, MA).

Other APCs. The L cell transfectants (gifts of Ron Germain, National Institute of Allergy and Infectious Diseases, Bethesda, MD): RT2.3.3HD6 ($A\alpha^dA\beta^d$), RT 10.3B-C1 (E^d), RT 7.7H714.3 ($E\alpha^dA\beta^d$), FT 7.1C6 ($A\alpha^bA\beta^b$), and the A20 B cell lymphoma (ATCC), were used as described (41, 42). These APCs were either sham- or antigen-pulsed for 18 h, washed three times, irradiated (18,000 rad), washed again, and then cocultured with the Th clones at a density of 5×10^4/well (L cells) or 2×10^5/well (A20).

Proliferation Assays. Each Th clone (10^5/well) or fresh splenic T cells (5×10^5/well) were cocultured in triplicate with irradiated, antigen- or sham-pulsed APCs in 200 μl final medium in flat-bottomed 96-well tissue culture plates (Costar Corp.) for 72 h. 6 h before harvesting, 1 μCi of [^{3}H]thymidine in 25 μl culture medium was added to each well. Incorporated radioactivity was measured in cpm as described (21, 22). As positive controls for stimulation, the anti-CD3 mAb was used. Stimulation index (SI)[1] for proliferation was calculated by dividing the mean cpm incorporated in cultures of T cells plus antigen-pulsed APC by the mean cpm in T cells plus sham-pulsed APC cocultures.

Blocking of Proliferation. All 10× concentrated mAb used for blocking T cell proliferative responses were added at 1:4 final dilution to the irradiated, antigen-pulsed APCs from 30 min before the addition of T cell clones and were left in the coculture throughout the 72-h period. Chloroquine (0.3 mM, Sigma Chemical Co.) was added to the APCs throughout the antigen pulse, after which the APCs were washed, irradiated, and used (43). The protease inhibitors, leupeptin and pepstatin A (Sigma Chemical Co.) were added to the APCs at 0.5 and 0.1 mM, respectively, before the commencement of antigen pulsing, and were maintained throughout the 72-h assay period, as described (44). For fixation, the APCs were exposed to 0.5% paraformaldehyde (Sigma Chemical Co.) for 30 s and promptly quenched with 0.2 mM glycine, washed twice in RPMI-10% FCS, incubated at 37° for 30 min, and rewashed two more times before antigen pulsing or coculture with T cells.

IL Production by T Hybridomas. 2×10^4 Th hybridoma cells growing in log phase were cocultured with irradiated, antigen- or sham-pulsed APC, with either 2×10^5 A20 B lymphoma cells per well or with 5×10^5 SNF_1 splenic APCs/well in 200 μl final medium in flat-bottomed 96-well plates (Costar Corp.). After 24 h, 100 μl of culture supernatant was harvested from each well, freeze-thawed and added to 2×10^4 HT-2 (IL-2 and/or IL-4 dependent cell line, gift from Brigitte Huber) cells per well. After an 18-h incubation, 1 μCi [^{3}H]thymidine was added to each well. 6 h later, radioactivity incorporated by the HT-2 cells was measured. IL production in response to anti-CD3 stimulation, served as a positive control. SI for interleukin production was calculated by dividing mean cpm in HT-2 cells incubated with culture supernatants from T hybridoma cells plus antigen-pulsed APC by that from T hybridoma plus sham-pulsed APC.

IL Secretion Profiles. Supernatants harvested from anti-CD3 stimulated Th clone cultures at 24 h were freeze-thawed and added in serial dilutions to 2×10^4 HT-2 cells/well in the presence or absence of 10 μl of anti-IL-2 (10× concentrated) and/or 2.5 μl of anti-IL-4 (10×) mAbs. [^{3}H]Thymidine incorporation during the last 6 h of a 24-h culture was determined, and compared with responses elicited by known amounts of murine rIL-4 (gift of Brigitte Huber) or rIL-2 (Cetus, Emeryville, CA) in the presence or absence of the anti-IL mAbs.

Help for Autoantibody Production In Vitro. SNF_1 splenic T cells or Th clones were cocultured with SNF_1 splenic B cells (plus macrophages) for 7 d according to previous procedures (12, 21). On day 7, culture supernatants were harvested, freeze-thawed, and assayed by ELISA for antibodies produced against ssDNA, dsDNA, histones, and histone–DNA complex. For these ELISAs, the supernatants were purified by 47% SAS cutting (12, 13, 21), dialyzed and used as described below.

ELISA. Total IgG and IgG class autoantibodies to ssDNA and dsDNA produced in helper assay coculture supernatants or in the sera of in vivo injected mice (see below) were measured by ELISA, as described (12, 13, 21), with one modification: for the anti-dsDNA assay, purified calf thymus DNA (Sigma Chemical Co.) was first sheared to 400–800 bp by sonication and digested with S1 nuclease before use. For the anti-ssDNA assay, this same preparation was boiled for 15 min and chilled on ice before use.

IgG class antibodies against histones and histone–DNA complex were measured as described (45–47). Briefly, 96-well plates were coated with 10 μg/ml total histones (Sigma Chemical Co.) overnight. For the antihistone–DNA complex assays, mBSA precoated plates were first coated with dsDNA (50 μg/ml) overnight, and then with total histones at 10 μg/ml overnight. This procedure gave more specific results than coating the wells directly with nucleosomes (46, 47). All remaining steps from the addition of test sample were identical to those of the anti-DNA ELISA (12, 13, 21). All samples were serially diluted and tested in triplicate. For subtraction of background binding, supernatants from Th clones cultured by themselves or serum from nonautoimmune AKR mice were used. Standard curves for each assay were obtained with known quantities of anti-DNA mAbs 564 and 205 as described (12, 21). For IgG autoantibodies to ssDNA, histones, and histone–DNA complex, 1 U/ml was considered to be equivalent to the activity of 1 μg/ml of mAb 564, which bound to all the three antigens (see below) and for IgG anti-dsDNA, 1 U/ml was equivalent to the binding of 0.6 μg/ml of the mAb 205.

In some experiments, the anti-DNA mAbs were digested with DNAse, dialyzed into high molarity NaCl (2 M), and then redialyzed into the ELISA sample buffer by Centricon (Amicon Corp.) before testing for binding to the above antigens or to histones that had been also digested with DNAse before coating onto ELISA plates, as described (48).

Pathogenicity of Nucleosomes In Vivo. 2-mo-old SNF_1 females were injected intraperitoneally three times, 2 wk apart, with either mononucleosomes (7 mice) or PBS (15 mice). For the first injection, nucleosomes (10 μg in 50 μl PBS/mouse) or PBS (50 μl/mouse) was mixed 1:1 with CFA (Gibco Laboratories, Grand Island, NY), whereas the remaining two boosters were in IFA. The mice were monitored weekly for proteinuria by albustix (20), and killed when they developed persistent proteinuria (defined as two consecutive weekly recordings of 300 mg/dl or greater). Sera was collected after killing for determination of blood urea nitrogen

[1] *Abbreviations used in this paper:* BUN, blood urea nitrogen; MHCII, MHC class II; SAS, saturated ammonium sulfate; SI, stimulation index.

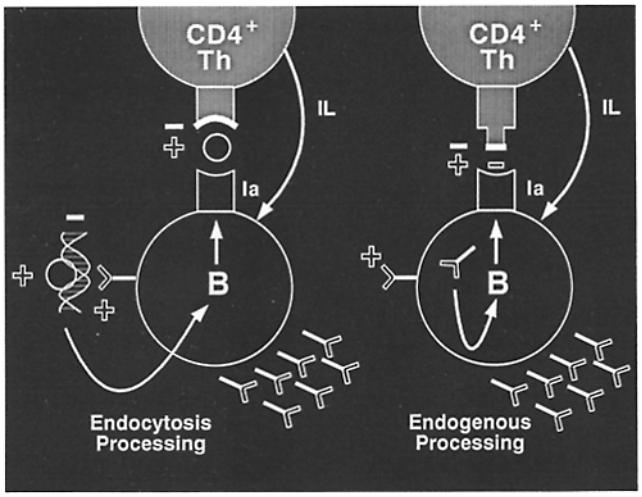

Figure 1. The pathogenic anti-DNA autoantibody-inducing Th clones have negatively charged residues (*solid white*) in the CDR3 of their TCR β chains. The Th clones could be specific for either a peptide with cationic residues (*black, inside white border*) derived from a DNA-binding protein that is endocytosed with anionic DNA, processed, and presented to the Th cells by the anti-DNA B cells (*left*), or a cationic idiopeptide processed from the V_H regions of the pathogenic anti-DNA autoantibodies produced by these B cells (*right*). This hypothetical scheme could also explain the preferential interaction between these Th cells and the cationic anti-DNA autoantibody-producing B cells. (*Ia*) MHC class II molecule.

(BUN) by azostix and measurement of IgG antibodies to ssDNA, dsDNA, total histones, and histone–DNA complex by ELISA. Sections from the kidneys were stained for detection and grading of glomerulonephritis by light microscopy, and immunofluorescence of frozen sections to detect the degree of IgG immune complex deposits was done as described (20, 24, 49).

Results

A Summary of the Properties of Pathogenic Autoantibody-inducing Th Clones. In previous work, we had cloned 268 T cell lines and hybridomas from the T cells that were spontaneously activated in vivo in the spleens of 10 SNF_1 mice with lupus nephritis. Only 12% of these T cell clones had the ability to preferentially augment the production of IgG anti-DNA autoantibodies when cultured with syngeneic B cells (22, 24). Most of these Th clones were $CD4^+$. Two of these cloned lines, L-1A and L-3A, had also been tested for pathogenicity in vivo, and they rapidly induced lupus nephritis upon transfer into preautoimmune SNF_1 mice (24). A recurrent motif of anionic residues that were generated mostly by N nucleotide additions, were found in the CDR3 junctions of the TCR β chains expressed by these Th clones (summarized in Fig. 3 from data in reference 24). 15 of these Th clones with the strongest autoantibody-inducing ability were studied here for their antigenic specificities, and their previously published features are summarized in Fig. 3. Herein, we also determined the interleukin production profile of the Th clones upon stimulation with anti-CD3 mAb. 11 of the 15 Th clones produced IL-2, but not IL-4 (Th1 type); three produced IL-4, not IL-2 (Th2 type), and one Th clone made both interleukins (Th0 type).

Pathogenic Th Clones Do Not Respond to Autoantibody V_H Region Peptides or to Heparan Sulphate Proteoglycan. The Th clones have anionic residues in their TCR Vβ-CDR3 suggesting that they may be specific for peptides with cationic residues and they preferentially help the B cells that produce an idiotypically connected set of cationic anti-DNA autoantibodies with homologous V_H but heterogeneous V_κ regions (2, 7, 21, 24). Hypothetically, those anti-DNA B cells could process and present to the Th clones their endogenous idiopeptides containing stretches of cationic residues in and adjacent to the CDR1 and CDR2 of the V_H region that they share (7). The two synthetic peptides corresponding to these regions also contained T cell epitope motifs (37). A control peptide corresponding to the CDR2 of an irrelevant antimalarial antibody (38) was also tested. Alternatively, since anti-DNA autoantibodies have been shown to cross-react with other polyanions besides DNA, such as heparan sulphate which is the major proteoglycan in the glomerular basement membranes (50), the anti-DNA B cells could process such an antigen for the Th clones with which they interact. Heparan sulphate, although predominantly anionic, does contain stretches of cationic residues (51). The synthetic CDR peptides ranging in concentration from 10^{-3} to 10^3 μM, or the intact pathogenic anti-DNA mAbs recurrently expressed by nephritic SNF_1 mice (2, 4, 7) at 1–10 μg/ml, or calf thymus DNA or heparan sulphate (0.1–10 μg/ml) were tested for stimulating the Th clones with appropriate APC. These Th clones are autoreactive, i.e., they spontaneously respond to splenic APC from older SNF_1 mice (21). However, we found that splenic APC from very young, 1-mo-old SNF_1 mice did not induce significant autoreactive proliferation of the Th clones and these APC were used for the assays. Among the Th clones, the lines were tested for proliferation and the Th hybridomas for IL production. None of the Th clones responded to any of these candidate autoantigens either when they were used to pulse the APC or when they were left in continuous culture with the APC (data not shown).

Pathogenic Autoantibody-inducing Th Clones Respond to Nucleosomes. 8 of the 15 Th clones shown in Fig. 3 responded to APC that were pulsed with either purified mononucleosomes or the "nucleohistone" preparation (Sigma Chemical Co.) containing nucleosome multimers (Tables 1 and 2). Results obtained with purified mononucleosomes are shown. The cloned Th lines are shown in Table 1. A nonautoantibody-inducing, autoreactive T cell line, L-5A (uses Vβ1/Jβ2.1 TCR genes), that is also derived from a nephritic SNF_1 mouse (21, 22, 24), is included. The nucleosome preparations did not stimulate any of the Th clones directly and the nucleosome-pulsed APC after washing and irradiation did not proliferate or produce ILs by themselves (Tables 1 and 2). Three of the pathogenic Th lines responded significantly. In the case of two of the cloned lines, L-3A and L-9.37, the proliferative responses to nucleosomes were as high as that to anti-CD3 mAb. The background SI for proliferation of these Th clones were calculated by averaging their responses to the synthetic V_H region peptides, intact autoantibodies, DNA or heparan

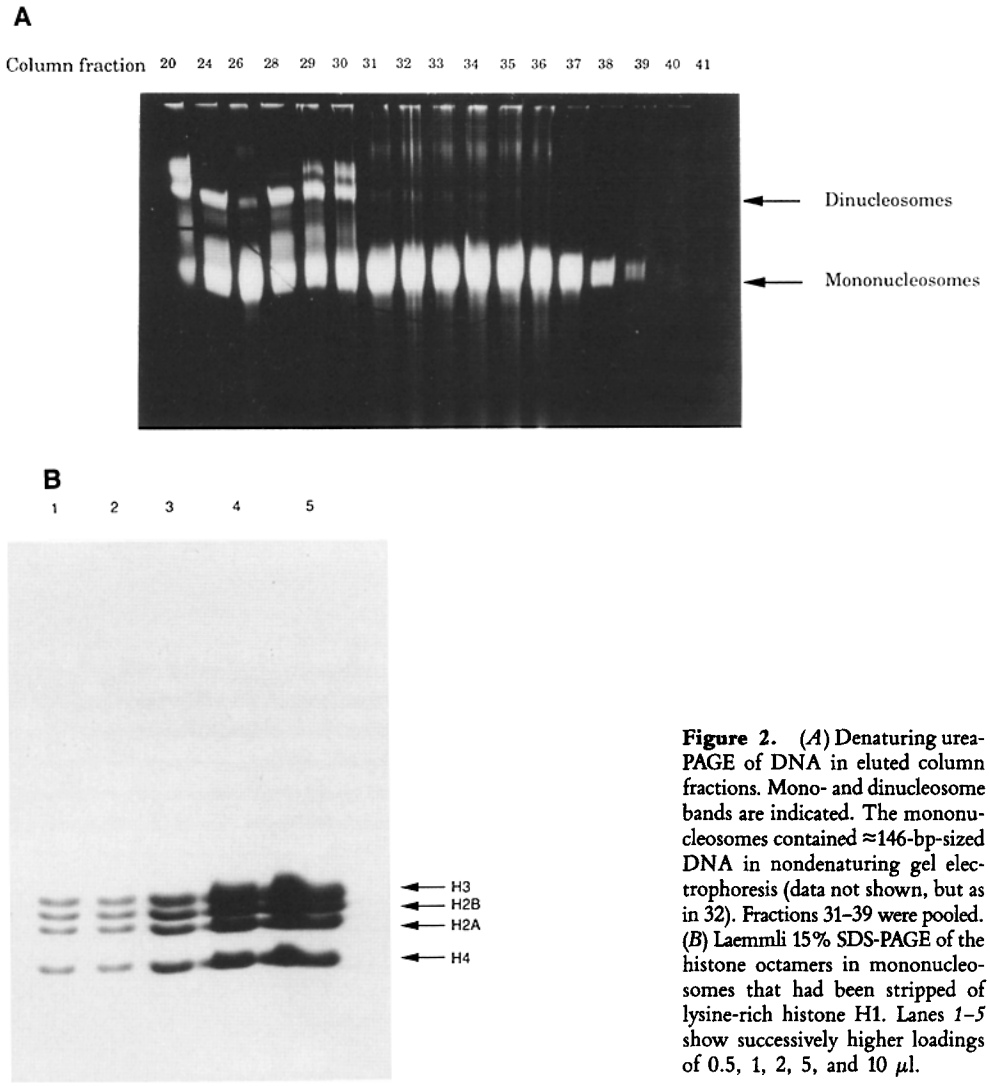

Figure 2. (*A*) Denaturing urea-PAGE of DNA in eluted column fractions. Mono- and dinucleosome bands are indicated. The mononucleosomes contained ≈146-bp-sized DNA in nondenaturing gel electrophoresis (data not shown, but as in 32). Fractions 31–39 were pooled. (*B*) Laemmli 15% SDS-PAGE of the histone octamers in mononucleosomes that had been stripped of lysine-rich histone H1. Lanes *1–5* show successively higher loadings of 0.5, 1, 2, 5, and 10 μl.

Th Cell Clone	Vβ	Junction	Jβ	Vβ/Jβ Usage	Interleukin Profile
L-1A	CASG	DAGAA	YNSPLYFAA	8.2/1.6	IL-4
H/L-9w.7	CASG	DRD	YAEQFFGP	8.2/2.1	IL-2
L-9.2	CASS	DGQ	YAEQFFGP	8.3/2.1	IL-2
H-16.B11	CASG	DAPGGG	AEQFFGP	8.2/2.1	IL-2
H-12.C4	CASS	DKNRG	LYFGA	8.3/2.4	IL-2
H-12.E4	CASS	DDRGGG	TEVFFGK	8.3/1.1	IL-2
L-3A	CASSQ	ATGG	EQYFGP	4/2.6	IL-4 + IL-2
L-9.10	CASSQ	DPGG	AEQFFGP	4/2.1	IL-2
L-9.37	CASSQ	GDWG	YEQYFGP	4/2.6	IL-4
H-15.G9	CAWS	PLPGQGD	ERLFFGH	14/1.4	IL-2
H-15.E3	CAWS	LEQLA	NTEVFFGK	14/1.1	IL-4
H/L-9.7	CTCS	AGLGEG	EQYFGP	2/2.6	IL-2
H-16.B6	CTCS	AGRDQAQH	FGE	2/1.5	IL-2
H-10.B5	CASSQ	DLGS	NTGQLYFGE	1/2.2	IL-2
H-16.G10	CASS	QGQGAV	EQYFGP	1/2.6	IL-2

Figure 3. A list of the pathogenic anti-DNA autoantibody-inducing Th clones derived from SNF_1 mice with lupus nephritis (24). (*L-*) Cloned lines and (*H-*) hybridomas directly derived from splenic T cells. (*H/L-*) Two of the established lines had to be rescued by fusion after initial characterization. Anionic residues in the CDR3 junctional region of TCR β chains of the Th clones are highlighted (24). IL profiles were determined in this study.

sulphate as described in the previous paragraph. These background values of SI + 3 SD for the three Th lines that responded to nucleosomes were 1.4 for L-3A, 1.7 for L-9.10, and 1.8 for L-9.37. In the case of the Th hybridoma clones, five responded significantly to nucleosomes, and IL production by four of them in response to nucleosomes was as high as that to anti-CD3 (Table 2). The background SI + 3 SD for IL responses of these Th hybridoma clones to the non-stimulating autoantigens described above (synthetic V_H peptides, autoantibodies, DNA, or heparan sulphate) ranged between 3.1 and 3.9. Splenic APC (B cells plus macrophages) from 1-mo-old SNF_1 mice ($H\text{-}2^{d/q}$), $I\text{-}A^d$ transfected L cells, or A.20 B cell lymphoma ($H\text{-}2^d$), were equally potent in presenting nucleosomal antigen(s) to these SNF_1-derived Th

Table 1. *Responses of Cloned T Cell Lines from Nephritic SNF_1 Mice to Nucleosomes*

T cell clone	T cell proliferative response (mean cpm) with: Nuc	APC	APC plus anti-CD3	APC plus Nuc	SI
L-1A	466*	1,016	18,631	1,028	1.0‡
L-9.2	772	1,452	5,676	1,467	1.0
L-3A	366	821	14,389	14,806	**18.0**
L-9.10	620	713	8,740	3,394	**4.8**
L-9.37	609	1,349	11,093	9,996	**7.4**
L-5A	369	573	15,442	553	0.9

* Representative of 5–12 experiments. Mean cpm of triplicate cultures is given; SD within each experiment were <10%. This experiment was done with pure mononucleosomes (Nuc) and splenic APC from 1-mo-old SNF_1 mice. In this and subsequent tables, APC plus Nuc means nucleosome-pulsed APC and APC alone means sham-pulsed APC. Nucleosome-pulsed, irradiated APC by themselves incorporated mean cpm of 269 ± 24.
‡ S.I., stimulation index (see Materials and Methods). In this and subsequent tables, significant SI (>3 SD above background) are shown in boldface.

clones (see below). Two T cell hybridomas that do not induce IgG anti-DNA autoantibodies are also shown in Table 2, because they express the same Vβ TCR as some of the pathogenic Th clones that respond to nucleosomes. H-14.E7 is derived from a nephritic SNF_1 mouse and uses Vβ14/Jβ2.1 genes (24, and Adams, S., and S. K. Datta, unpublished results). The other hybridoma, 52H10F11, is specific for insulin and it expresses Vβ4 TCR (39). For the latter, $I\text{-}A^b$ transfected L cells were used as APC (Table 2).

Nucleosomal Antigen Processing and MHC Restriction. As shown in Fig. 4, nucleosomal antigen presentation to the responder Th clones requires processing via classical MHC class II (MHCII) pathway. Results from the representative Th clone, L-3A that induces pathogenic autoantibodies in vitro and is

Table 2. *Responses of SNF_1-derived T Hybridomas to Nucleosomes*

Cloned T hybridoma	IL production (HT-2 cpm) by T hybridomas on culture with: Nuc	APC	APC plus anti-CD3	APC plus Nuc	SI
H/L-9w.7*	1,182‡	1,078	28,419	28,823	**26.7**
H/L-9.7	3,352	4,693	56,003	55,413	**11.8**
H-12.C4	1,233	1,337	71,326	45,455	**34.0**
H-16.B6	ND	565	64,020	56,093	**99.2**
H-15.G9	381	875	114,622	5,081	**5.8**
H-16.G10	ND	1,251	83,601	3,662	2.9
H-16.B11	ND	991	31,152	2,143	2.2
H-15.E3	ND	716	118,019	942	1.3
H-12.E4	ND	1,817	43,938	1,951	1.1
H-10.B5	496	1,338	198,140	1,995	1.5
H-14.E7	ND	2,088	71,703	2,037	1.0
52H10F11	ND	5,272	97,249	4,366	0.8

* All of the T hybridomas were derived from nephritic SNF_1 mice (Fig. 3), except for the insulin-specific 52H10F11 clone from C57BL/6 (see Materials and Methods) and $I\text{-}A^b$ transfected L cells were used as APC for the latter.
‡ Mean cpm of triplicate cultures from a representative experiment is given; the SD were <10% in each experiment. At least three experiments were done with each clone. This experiment was done with A.20 APC (except for hybridoma 52H10F11) and pure mononucleosomes. Nucleosome-pulsed, irradiated APC by themselves did not produce ILs: 534 ± 33 cpm on HT-2 cells. Similar results were obtained when splenic APC from 1-mo-old SNF_1 mice were used.

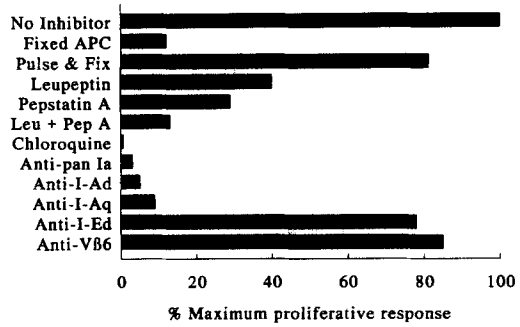

Figure 4. Effect of various inhibitors of antigen processing and presentation on the proliferative response of the nephritogenic Th clone L-3A to mononucleosomes presented by SNF_1 APC. Fixed APC were pulsed with antigen after fixation, whereas the order was reversed in Pulse and Fix APC. Anti-pan Ia mAb is TIB120 and its isotype-matched control is the anti-Vβ6 TCR mAb 44-22-1.

nephritogenic in vivo (24) are shown, but similar results were obtained with the other pathogenic Th clones that responded to nucleosomes. Antigen presentation was blocked (88% inhibition) by paraformaldehyde fixation of APC before pulsing with nucleosomes, but fixation after pulsing with the antigen did not cause significant inhibition. The lysosomotropic agent chloroquine, or the endosomal protease inhibitors, leupeptin or pepstatin A, also inhibited the Th cell response by 91, 60, and 71%, respectively. And leupeptin and pepstatin A together blocked stimulation by 87%. Treatment of APC with these agents did not have a toxic effect on the Th cells under the conditions of the experiments, as judged by their response to anti-CD3 mAb, which is consistent with previous work (43, 44). Presentation of nucleosomal antigen(s) by SNF_1 APC to the pathogenic Th clones was blocked by anti-I-A mAbs: anti-pan Ia TIB120 (anti-I-$A^{b,d,q}$, anti-I-$E^{d,k}$) inhibited by 97%, anti-I-A^d by 95%, and anti-I-A^q by 91%, but the anti-I-E^d mAb did not inhibit significantly (Fig. 4). Similar results were obtained when I-A^d transfected L cells or A.20 (H-2^d) lymphoma cells were used as APC for nucleosomal antigens, except that the anti-I-A^q mAb caused insignificant inhibition as expected, whereas the anti-I-A^d mAb caused 99.9% inhibition (data not shown).

In contrast to the parental strains' APC, the SNF_1 APC (H-$2^{d/q}$) were much more efficient in presenting nucleosomal antigen(s) to the SNF_1-derived Th clones (Table 3). APC from NZB (H-2^d) did present nucleosomes but the stimulation of the Th clones was much less than that with anti-CD3. The response of the Th clones to nucleosome-pulsed NZB APC could be blocked by anti-I-A^d mAb but not by the anti-I-A^q mAb (data not shown). The SWR (H-2^q) parental strain's APC presented nucleosomal antigens poorly and so did BALB/c, another H-2^d strain. APC from C57BL/6 (H-2^b) mice and I-A^b- or I-E^d-transfected L cells failed to present nucleosomal antigen(s) to these Th clones. However, I-A^d transfected L cells were very efficient in presenting nucleosomal antigen(s) (Table 3 and Fig. 5). Indeed, the I-A^d transfected L cells were as efficient as SNF_1 APC, although the SI with the latter were higher because the background cpm (Th clone plus APC without antigen) were usually lower than with the I-A^d L cell APC (Table 1 and Fig. 5). Results similar to those of the nephritogenic Th clone, 3A, were obtained with the other SNF_1-derived, pathogenic autoantibody-inducing Th clones that responded to nucleosomes (data not shown). All of the data shown in this paper were generated in experiments where the APC were first pulsed with nucleosomes, then washed extensively and irradiated before culture with the Th cells, to exclude any nonspecific mitogenic effect of the nucleosomes. However, we also did cultures of Th clones with irradiated APC, where the nucleo-

Table 3. *Nucleosomal Antigen Presentation to Pathogenic Th Clone by Different APC*

Type of APC	Proliferation (mean cpm) of L-3A with: APC	APC plus anti-CD3	APC plus Nuc	S.I.
SNF_1*	1,593‡	21,830	17,836	**11.2**
NZB	590	22,752	2,215	**3.8**
SWR	3,374	9,862	5,503	1.6
C57BL/6	529	5736	521	1.0
BALB/c	370	6,407	713	1.9
I-A^dL	1,066	13,931	10,066	**9.4**
$A\beta^d/E\alpha^d$ L	2,009	15,150	11,673	**5.8**
I-A^bL	1,533	14,516	1,448	0.9
I-E^d L	4,175	11,677	4,646	1.1

* MHC haplotypes of APC: SNF_1 (H-$2^{d/q}$), NZB (H-2^d), SWR (H-2^q), C57BL/6 (h-2^b), and BALB/c (H-2^d).
‡ Representative experiment with the nephritogenic Th clone L-3A is shown. Mean cpm of triplicate cultures is given; SD were <10%. Similar results were obtained with the other Th clones that responded to nucleosomes (data not shown).

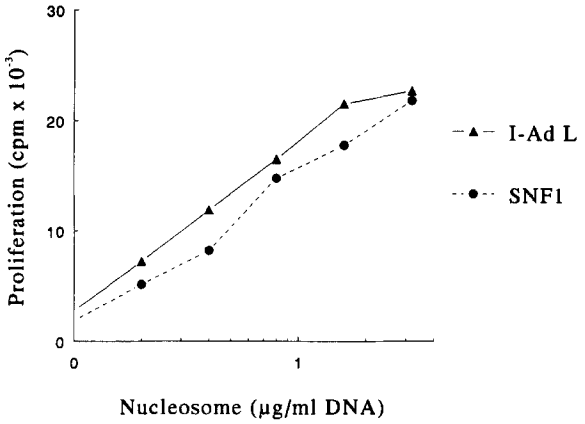

Figure 5. Dose-responses of the pathogenic Th clone L-3A to nucleosome presented by I-A^d L cells (▲) or SNF_1 APC (●). Mononucleosome concentrations used for pulsing the APC are given in μg/ml DNA equivalent.

somes were left continuously during the 72-h proliferation assay, instead of for just an initial pulse of the APC. The amount of nucleosomal antigens required for maximum stimulation of the Th clones in the former cases was about 10-fold less (data not shown).

Purified Histones Do Not Stimulate the Pathogenic Autoantibody-inducing Th Clones. The purified mononucleosomes that stimulated these Th clones consists of DNA (≈146 bp) and octamer of core histones (Fig. 2). Purified calf thymus DNA fed to the APC, either as high molecular weight DNA or sheared by sonication, did not stimulate any of the Th clones. All types of histones, such as core histone octamers purified from the mononucleosomes or a mixture of total histones or lysine-rich histones (H1, H2A, and H2B) or arginine-rich histones (H3 and H4) or H2A or H2B alone, all failed to stimulate any of the Th clones significantly (data not shown).

Nucleosome-reactive Th Clones Augment Production of Several IgG Autoantibodies. The pathogenic Th clone L-3A, when cultured with syngeneic B cells, not only augmented the production of IgG anti-DNA autoantibodies as described before (21) but also IgG autoantibodies to histones and histone–DNA complex (Fig. 6 *A*). Indeed, L-3A Th cells cocultured with SNF_1 B cells, augmented production of IgG antihistone–DNA complex antibodies by 152-fold ($P < 0.001$; Student's *t* test), but it augmented anti-ssDNA, anti-dsDNA, and antihistone antibody production by 18.8-, 13-, and 6-fold, respectively ($P < 0.01$). Similar results were obtained with other pathogenic autoantibody-inducing Th cells from SNF_1 mice (data not shown). Help by the autoimmune Th cells for the production of either cross-reacting anti-DNA antibodies or the production of different population of autoantibodies could explain this result. Therefore, prototypic anti-DNA mAbs that are pathogenic and recurrently expressed in nephritic SNF_1 mice (2, 4, 7) were also tested (Fig. 6 *B*). The pathogenic anti-DNA mAb 564 that cross-reacts with several nucleic acid antigens (52), also bound strongly to histones and histone–DNA complexes, and this binding could

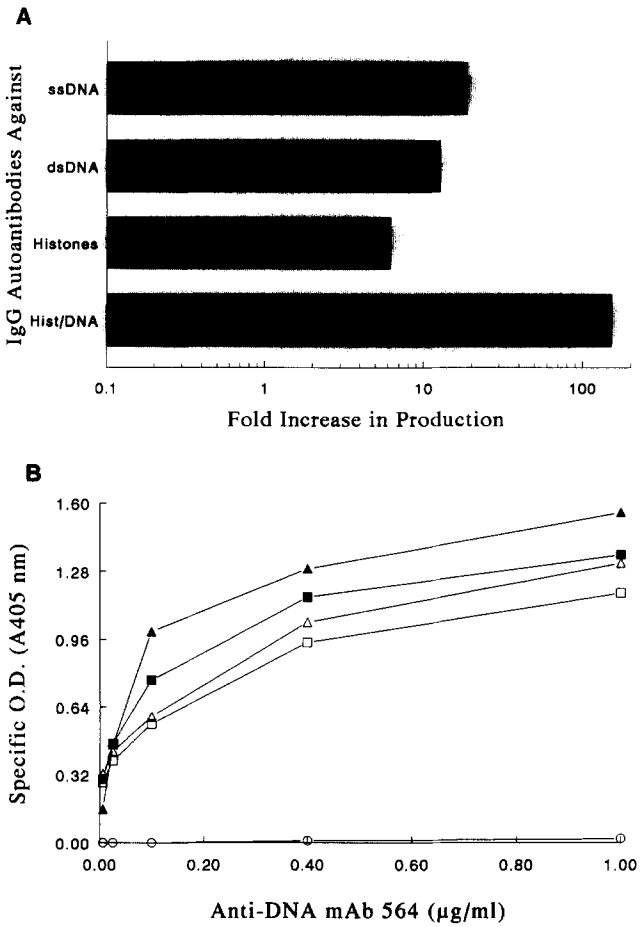

Figure 6. (*A*) Helper activity of the nephritogenic Th clone L-3A derived from SNF_1 mice with lupus nephritis. (*Bars*) Fold increases (log scale) in the production of IgG autoantibodies when the Th cells were cultured with syngeneic B cells as compared with B cells cultured alone. (*Hist/DNA*) Histone–DNA complex. (*B*) ELISA showing the binding of the prototypic, pathogenic anti-DNA mAb 564 of SNF_1 mice to ssDNA (▲), dsDNA (○), histones (△), and histone–DNA complex (■). The same mAb was digested with DNAse I and its binding to wells coated with histones that had also been digested with DNAse I is shown (□).

not be significantly reduced by DNAse digestion using described procedures (48). Similar results were obtained with other pathogenic anti-DNA autoantibodies of SNF_1 mice (2, 4, 7) that bind both ssDNA and dsDNA and belong to the major Id564 family (data not shown).

T Cells Reactive to Nucleosomal Antigens Are Present in the Spleens of Lupus Mice. Fresh $CD4^+$ T cells obtained from the spleens of SNF_1 and BWF_1 lupus-prone mice responded significantly to nucleosomes ex vivo, whereas splenic T cells from nonautoimmune strains or the NZB mice that rarely develop lupus nephritis failed to respond (Table 4). T cells from 3-mo-old NZB were tested before their spleens became abnormal from hemolytic anemia. The mean background SI + 3 SD of SNF_1 T cells to the nonstimulatory antigens described above (V_H region peptides, autoantibodies, etc.) was 1.6 and the corresponding value for T cells from the normal strains and NZB mice was 1.7. $CD8^+$ T cells did not re-

Table 4. *Responses of CD4⁺ T Cells from the Spleens of Different Mouse Strains to Nucleosomal Antigens*

Source of T cells	Proliferation in mean cpm and (SI) of CD4⁺ T cells cultured with:				
	Nuc	APC	APC plus αCD3	APC plus Nuc	APC plus Histones
SNF_1, 1 mo	1,378 ± 437*	3,064 ± 761‡	42,273 ± 6,156	**10,791** ± 1,493 (3.5)	4,328 ± 796§ (1.4)
SNF_1, 2 mo	1,921 ± 575	3,533 ± 896	97,327 ± 10,450	**19,263** ± 4,864 (5.5)	**17,645** ± 3,243 (5.0)
SNF_1, 4 mo	ND	2,541 ± 746	159,894 ± 16,147	**13,977** ± 556 (5.5)	ND
SNF_1, 6 mo	1,404 ± 108	2,421 ± 518	72,927 ± 6,861	**20,249** ± 4,385 (8.4)	**25,659** ± 3,979 (10.6)
BWF_1, 2 mo	1,147 ± 370	3,414 ± 143	42,555 ± 5,582	**14,375** ± 2,993 (4.2)	4,941 ± 638 (1.5)
NZB, 3 mo	1,280 ± 84	1,524 ± 139	47,926 ± 5,259	1,981 ± 181 (1.3)	1,518 ± 365 (1.0)
SWR, 6 mo	9,412 ± 762	10,670 ± 1,286	150,608 ± 16,532	10,340 ± 2,488 (1.0)	9,950 ± 1,019 (0.9)
BL/6, 5 mo	562 ± 89	917 ± 102	55,527 ± 6,842	975 ± 155 (1.1)	630 ± 178 (0.7)
BALB/c, 4 mo	680 ± 53	1,521 ± 155	48,962 ± 7,944	1,996 ± 442 (1.3)	1,171 ± 205 (0.8)

* Results from 3–5 experiments. Significant proliferation shown in boldface.
‡ Splenic APC from 1-mo-old mice of respective strains were used. Nucleosome-pulsed and irradiated SNF_1 APC by themselves incorporated 269 ± 24 cpm and BWF_1 APC, 236 ± 37 cpm.
§ Mixture of histones H1, H2A, and H2B. Similar results were obtained with total histones.

spond to the nucleosomal antigens (data not shown). In these experiments, T cell–depleted splenocytes from 1-mo-old mice of each strain were used as APC for their respective T cells, and they were pulsed with the nucleosomal antigens, washed extensively, and irradiated before culture with the T cells. Under such conditions, the antigen-pulsed APC did not stimulate syngeneic B cells from spleen (data not shown). Moreover, the nucleosomes did not stimulate the purified, splenic T cells directly (Table 4).

The SNF_1 mice had nucleosome-primed T cells as early as 1 mo of age and the SI increased significantly by 2 mo ($P < 0.001$; Student's t test). The SI to nucleosomes at 6 mo were even greater than at 2 or 4 mo of age ($P < 0.001$; Table 4). At 2 mo of age (but not at 1 mo), the SNF_1 T cells also had a population that responded significantly to histones, and the SI to histones increased markedly by 6 mo of age ($P < 0.001$). At 2 mo of age the BWF_1 lupus mice also had a significant T cell response to nucleosomes, but not to histones (Table 4). The response of the lupus T cells to nucleosomes presented by the APC could be blocked by anti-I-A^d mAb and similar results were obtained when I-A^d transfected L cells were used as APC (data not shown).

Nucleosomes Accelerate the Development of Severe Glomerulonephritis in SNF_1 Mice. 2-mo-old preautoimmune SNF_1 mice injected with purified mononucleosomes in adjuvant developed severe nephritis much earlier than age-matched SNF_1 mice injected with PBS in adjuvant (Fig. 7). The mice

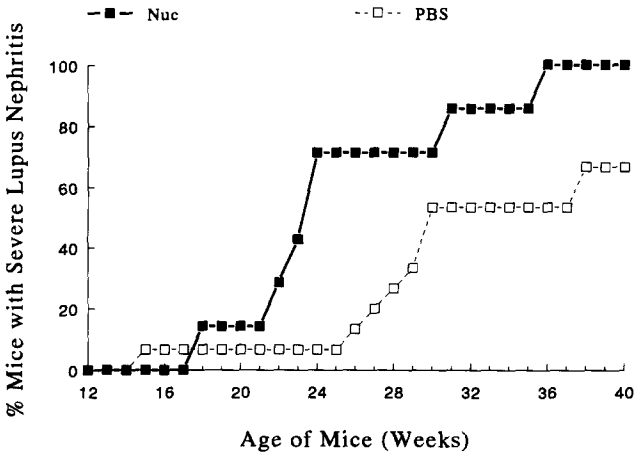

Figure 7. Incidence of severe lupus nephritis in SNF_1 mice immunized with mononucleosomes (*Nuc*) or saline (*PBS*). The mice received the last booster injection at 12 wk of age.

in Fig. 7 were considered to have severe glomerulonephritis when they had persistent proteinuria of 300 mg/dl or above for at least 2 wk and BUN levels at 30 mg/dl or above, and upon killing at this stage, their kidneys showed a 3^+–4^+ grade of nephritis by histopathology and a 3^+–4^+ degree of immune deposits by immunofluorescence (20). By 6 mo of age, ≈71.4% of the nucleosome-injected mice developed severe lupus nephritis, in contrast to ≈6.6% of the controls ($P < 0.001$), and by 9 mo, 100% of the former mice had severe nephritis as compared to 53% of the controls ($P < 0.05$). The incidence of lupus nephritis in the control SNF_1 group injected with PBS in adjuvant (Fig. 7), was similar to that of uninjected SNF_1 mice (20).

Levels of IgG class autoantibodies in serum were measured at about the midpoint of the study, 3 mo after immunization when the surviving mice were ~6 mo of age (Table 5). Serum samples obtained from killed mice that had developed severe nephritis earlier were also included. By this age, the nucleosome-injected mice had significantly higher levels of IgG anti-ssDNA and antihistone antibodies than the PBS-injected group overall ($P < 0.005$; Mann-Whitney U test). However, differences in anti-dsDNA levels in serum were not significant, as was the case with antibodies to histone–DNA complex ($P < 0.1$). The latter result was due to the marked variation in autoantibody levels in the nucleosome-injected mice, but their mean value was about 10-fold higher than that of the control group. This variation is expected since some of the nucleosome-injected mice had already developed severe nephritis by this age whereas others had not. Even those with severe nephritis did not always have high levels of serum

Table 5. *IgG Autoantibodies in the Serum of SNF_1 Mice 3 mo after Immunization*

Mice	Serum levels of IgG autoantibodies against:			
	ssDNA	dsDNA	Histones	Histone/DNA
		U/ml		
Nucleosome-injected mice				
N1*	2.3	2.22	30.78	1.53
N2*	1.9	2.22	37.04	11.76
N3	110.72	2.57	61.60	0.50
N4	58.9	39.74	27.62	50.20
N5*	141.98	2.49	216.96	15.20
N6*	6.43	2.00	2.36	0.30
N7*	1.92	3.36	13.43	2.95
Mean ± SEM	46.31 ± 20.67	7.80 ± 4.93	55.68 ± 25.72	11.78 ± 6.27
Saline (PBS)-injected mice				
S1	0.99	2.33	2.20	0.51
S2	0.77	2.38	3.19	0.94
S3	31.59	2.67	11.21	0.50
S4*	19.12	2.62	20.02	1.63
S5	38.90	2.74	6.06	3.20
S6	2.34	2.70	8.80	1.51
S7	0.85	2.58	9.35	1.97
S8	0.68	2.37	13.16	1.21
S9	0.93	2.71	4.12	1.33
S10	1.45	2.53	22.65	2.60
S11	0.19	0.13	0.61	0.17
S12	0.53	0.13	2.45	0.32
S13	0.93	2.09	25.81	1.34
S14	0.59	2.25	2.59	0.57
S15	1.92	1.47	3.19	0.41
Mean ± SEM	6.78 ± 3.03	2.11 ± 0.22	9.03 ± 2.09	1.21 ± 0.21

* Sera from these animals were obtained earlier when they developed severe nephritis (Fig. 7).

autoantibodies (Table 5), suggesting that the autoantibodies were probably deposited in tissues. Splenic $CD4^+$ T cells of SNF_1 mice from both groups responded strongly to nucleosomes since they were obtained from mice that were killed when they developed severe nephritis (data not shown).

Discussion

These studies show that the nucleosome is a major immunogen for the pathogenic autoantibody-inducing Th cells that arise spontaneously in lupus. The mechanism of autoimmunization by nucleosomes was so far unknown. Nucleosomes were suspected to be immunogens in lupus because they were detected in immune complexes with anti-DNA autoantibodies (53–55), but this phenomenon could occur secondarily because of indirect binding of the autoantibodies via DNA in the nucleosomes (50, 56). Even in normal individuals, most of the circulating DNA is found as part of oligonucleosomes that are released after apoptosis. Indeed, patients undergoing lifelong, chronic hemodialysis release large amounts of DNA in circulation as oligonucleosomes without developing lupus (57). Moreover, nucleosomes were found to stimulate B cells nonspecifically as a polyclonal mitogen, but this effect applied equally to B cells from lupus strains as well as normal mice, thus arguing against a specific role of nucleosomes in the pathogenesis of SLE (33, 34). Indeed, polyclonal mitogenic stimulation of B cells by nucleosomes would not explain the IgG class switch, somatic mutation, and clonal expansion of pathogenic anti-DNA B cells in lupus that are characteristic of an antigen-driven response (5–10). Our results for the first time show an antigen- and lupus-specific responsiveness of pathogenic autoantibody-inducing Th cells to nucleosomes. Not only pathogenic Th clones derived from nephritic lupus mice but also fresh splenic T cells from two different lupus strains were found to respond to nucleosomes. The $CD4^+$ T cells from lupus mice were already primed in vivo to this major autoantigen as early as 1 mo of age, long before autoantibodies and glomerulonephritis are detectable in these strains of mice. It is noteworthy that these types of T cells were not detectable in normal strains or in the autoimmune NZB parental strain that rarely develops nephritis. Thus, the presence of nucleosome-specific T cells predicts the development of lupus nephritis well in advance of other markers of the disease. Moreover, injection of nucleosomes into young preautoimmune SNF_1 mice led to increased autoantibody production and acceleration of severe lupus nephritis. This result could be in part due to polyclonal stimulation of B cells in vivo (33). However, injection of nucleosomes into normal mice whose B cells are also polyclonally stimulated by it or by injection of nucleosomes into rabbits does not result in the production of lupus-specific anti-DNA autoantibodies or nephritis. Mostly antihistone antibodies are produced in such normal animals (58–60). Therefore, the nucleosome-specific Th cells that are present only in the lupus-prone mice are necessary for the production of pathogenic autoantibodies and acceleration of nephritis.

About half of the pathogenic autoantibody-inducing Th clones from SNF_1 mice were specific for nucleosomal antigen(s). These special autoimmune Th clones are predominantly $CD4^+$. They arose spontaneously in the lupus mice with nephritis and they were a minor fraction of autoreactive T cells that were activated in vivo (24). These pathogenic Th cells were represented not only among cloned T cell lines but also among T cell hybridomas that rescued the in vivo–activated T cells directly from the nephritic lupus mice. These autoimmune Th clones not only had the functional ability to induce pathogenic anti-DNA autoantibodies in vitro like the freshly isolated T cells from the spleens of nephritic lupus mice, but they could also rapidly induce lupus nephritis when transferred into preautoimmune mice (12, 24). It is interesting that the majority of these autoimmune Th clones have the IL profile of Th1 cells. Th1 cells help in the production of IgG2a antibodies (61) and the major subclass of pathogenic anti-DNA autoantibodies found in the renal lesions of these lupus mice is IgG2a (2). However, some of the pathogenic Th clones were Th2 type, and lymphokines produced typically by Th2 cells are also known to be important in pathogenic autoantibody production (16, 62). Like our pathogenic Th clone L-3A, anti-DNA autoantibody-inducing Th clones with Th0 IL profile have also been isolated from other lupus mice (63).

The nucleosomes did not have any direct mitogenic effect on the T cells and since the nucleosome-specific Th clones expressed diverse TCR $V\beta$ genes, it is unlikely that nucleosomes provide a superantigen for the T cells. Moreover, other T cell clones expressing the same $V\beta$ TCR as the responders ($V\beta$ 4, 8.2, 8.3, or 14) failed to respond to nucleosomes. The presentation of nucleosomal antigens could be blocked by paraformaldehyde fixation or chloroquine treatment of the APC, indicating that they are processed by the classical endosome/lysosome MHCII pathway (43). The purified mononucleosomes that stimulated the Th cells consisted of only DNA (≈146 bp) and core histones. Yet, neither free DNA nor histones stimulated any of the Th clones. This result suggests that the nucleosomes as a whole are taken up for processing more efficiently either by special nucleosome receptors on the APC (64) or by the anti-DNA autoantibody receptors on the lupus B cells (Fig. 1). However, since $I\text{-}A^d$ L cells can present nucleosomal antigens efficiently, we have to postulate the ubiquitous presence of the nucleosomal receptors. An alternative possibility is that during antigen processing, critical Th cell epitopes in the histones are protected from degradation by being bound to DNA in the nucleosomes, as it occurs in other immunogens (65). The fact that antigen presentation could be blocked by leupeptin and pepstatin A, inhibitors of the endosomal proteases, cathepsin B and D, respectively (44), also suggests that a peptide(s) from the histone component of the nucleosomes contain the relevant epitope(s) for the pathogenic Th cells. The splenic T cells from older SNF_1 that responded to pure histones probably belong to a different subset than those responding to nucleosomes (Table 4), because splenic T cells from 1-mo-old SNF_1 or young B/WF_1 mice and the SNF_1-derived Th clones described above respond to nucleosomes only. The former subset of T cells may be specific for different epitopes on the histones than the nucleosome-specific Th cells, and they may

be involved in the production of those autoantibodies that are specific for histones, but do not cross-react with DNA (66, 67).

The APC from young SNF_1 mice could present nucleosomal antigens much more efficiently to the SNF_1-derived Th clones than either of the parental strain's (NZB or SWR) APC. More efficient antigen uptake due to the presence of anti-DNA B cells or cells with a higher density of nucleosome receptors in the SNF_1 APC could explain this result. However, the presentation of nucleosome by SNF_1 APC to the Th cells could be blocked equally by either anti-I-A^d or anti-I-A^q mAbs, suggesting that F_1-hybrid I-A molecules may be involved in presenting the T cell epitopes. This finding is consistent with previous results showing that the pathogenic Th clones would spontaneously proliferate more vigorously when cultured with APC from older nephritic SNF_1 mice than either of the parental strains' APC (21), and among the F_2 and backcross progeny of SWR × NZB crosses, the progeny mice with F_1 heterozygous MHC haplotype develop accelerated and severe nephritis (49). The pathogenic anti-DNA B cells that are expanded only in the older SNF_1 mice (but not in the parental strains) could present endogenous nucleosomal antigens much more efficiently to the Th (Fig. 1). However, nucleosome-specific T cells are present even in younger lupus mice, suggesting that the Th cells could be primed by other APC before their cognate interaction with the anti-DNA B cells. Indeed, I-A^d L cells and A.20 lymphoma cells could also present nucleosomal antigens efficiently to the Th clones, in contrast to splenic APC from BALB/c and NZB mice which are also H-2^d. The former continuous cell lines might be better in the uptake and/or processing of the complex nucleosomal antigens because of unknown reasons. The fact that I-A^d molecules are as good as I-$A^{d/q}$ (SNF_1-type) molecules in presenting nucleosomal epitopes to the SNF_1-derived Th clones suggests that these antigenic epitopes are promiscuous, i.e., the same epitope can be presented by different MHCII molecules even to the same T cell clone (68, 69). However, since I-A^d and I-A^q molecules are very homologous in structure in the regions that contact antigenic peptide and the TCR (70), they could both present the same T cell epitope to the same SNF_1-derived Th clone. Indeed, T cell clones specific for a peptide from cytochrome *c* can also respond equally to that peptide presented by I-E molecules of either k or b haplotype (71). However, in the SNF_1 mouse itself, the I-$A^{d/q}$ hybrid molecules are probably more effective in competing for nucleosomal antigen presentation than I-$A^{d/d}$ molecules (Fig. 4; 21, 49). These hypotheses can be tested further with I-A^q or I-$A^{d/q}$ transfected L cells which are not yet available.

The Th clones that responded to the nucleosomes expressed different TCR Vβ genes with different CDR3 junctional sequences. This heterogeneity would suggest that several peptide epitopes are generated from the complex nucleosomal antigen. However, the TCR repertoire for even a single peptide antigen can be highly diverse (72), and just a single residue change in an antigenic peptide would lead to usage of diverse TCR Vβ genes by the responding T cells demonstrating the plasticity of TCR repertoire (26). Thus, unlike experimentally induced autoimmune diseases, TCR Vβ usage by pathogenic T cells in spontaneous, systemic autoimmune diseases is usually heterogeneous.

About 50% of the SNF_1-derived Th clones did not respond to nucleosomes, indicating that other antigens are also involved in the pathogenic autoimmune response. This complexity is expected in a spontaneous and systemic autoimmune disease like lupus. We tested the second possibility that some of these Th clones are idiotype-specific (Fig. 1). Neither intact pathogenic anti-DNA autoantibodies (mAbs) that are expressed recurrently in the nephritic SNF_1 mice (2, 4, 7), nor synthetic peptides derived from the V_H-CDR regions that are shared by the pathogenic autoantibodies could stimulate the Th clones or fresh splenic T cells from either young or older nephritic SNF_1 mice. These synthetic idiopeptides contained cationic residues and T cell epitope motifs. However, self-idiotypes may be processed by the endogenous pathway in the autoimmune B cells (28), and thus may generate other idiopeptide epitopes. Even if such idiotype-specific Th cells are found in lupus, they would arise secondarily, since antigen-driven expansion and somatic mutation of the autoantibodies have to occur in the first place to generate such CDR idiopeptides. None of the Th clones responded to free DNA or polyanions like heparan sulphate proteoglycan that crossreact with anti-DNA autoantibodies.

The nucleosome-specific Th cells interact with and help the pathogenic anti-DNA autoantibody producing B cells preferentially, rather than any B cells producing IgG (22, 24). In spontaneous or drug-induced SLE, certain B cells produce autoantibodies that bind nucleosomes better than either free histones or DNA (45–47, 73). On the other hand, other B cells of lupus produce anti-DNA autoantibodies that bind to nucleosomes and histones via DNA (50, 56). The SNF_1-derived pathogenic autoantibodies of 564 family probably belong to the latter category. Their binding to histones could not be significantly diminished even after DNAse digestion, probably because some DNA bound by the autoantibodies in culture would remain protected from digestion (48). Nevertheless, both types of autoimmune B cells would present the nucleosomal autoantigen(s) to the pathogenic Th cells much more efficiently than other APC during the ongoing autoimmune response in lupus. Indeed, the pathogenic Th clones from nephritic SNF_1 mice not only augment the production of IgG anti-DNA autoantibodies but antihistone and antinucleosome (histone–DNA complex) antibodies as well.

Nucleosome is a naturally occurring and abundant particulate autoantigen generated by apoptosis of cells that happens daily. Particulate autoantigens from nucleus appear to be the target of autoantibodies (74). Now we have functionally characterized T cell clones of proven pathogenic role in lupus and the appropriate APC for the presentation of nucleosomal antigen to these T cells. We can therefore elute the naturally processed peptide, study the breakdown of tolerance to the autoantigen, and design ways to intervene against this pathogenic autoimmune response in lupus.

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