Nucleosomal Peptide Epitopes for Nephritis-inducing T Helper Cells of Murine Lupus

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Summary

Nucleosome-specific T helper (Th) cells provide major histocompatibility complex class II–restricted, cognate help to nephritogenic antinuclear autoantibody-producing B cells in lupus. However, the lupus Th cells do not respond when components of the nucleosome, such as free DNA or histones, are individually presented by antigen-presenting cells. Thus critical peptide epitopes for the pathogenic Th cells are probably protected during uptake and processing of the native nucleosome particle as a whole. Therefore, herein we tested 145 overlapping peptides spanning all four core histones in the nucleosome. We localized three regions in core histones, one in H2B at amino acid position 10-33 ($H2B_{10-33}$), and two in H4, at position 16-39 ($H4_{16-39}$) and position 71-94 ($H4_{71-94}$), that contained the peptide epitopes recognized by the pathogenic autoantibody-inducing Th cells of lupus. The peptide autoepitopes also triggered the pathogenic Th cells of (SWR × NZB)F_1 lupus mice in vivo to induce the development of severe lupus nephritis. The nucleosomal autoepitopes stimulated the production of Th1-type cytokines, consistent with immunoglobulin IgG2a, IgG2b, and IgG3 being the isotypes of nephritogenic autoantibodies induced in the lupus mice. Interestingly, the Th cell epitopes overlapped with regions in histones that contain B cell epitopes targeted by autoantibodies, as well as the sites where histones contact with DNA in the nucleosome. Identification of the disease-relevant autoepitopes in nucleosomes will help in understanding how the pathogenic Th cells of spontaneous systemic lupus erythematosus emerge, and potentially lead to the development of peptide-based tolerogenic therapy for this major autoimmune disease.

The production of nephritogenic antinuclear autoantibodies in SLE is driven by cognate interactions between select populations of autoimmune Th and B cells (1–8). To further study this pathogenic mechanism, the special autoimmune Th cells of lupus were cloned from (SWR × NZB)F_1 (SNF_1)[1] mice with lupus nephritis, as well as from nephritic patients with SLE (3, 4, 7). In the SNF_1 model, representative pathogenic autoantibody-inducing Th clones rapidly induce immune-deposit glomerulonephritis when transferred into young preautoimmune mice (4). Although heterogeneous, the TCR-CDR3 junctional regions of the pathogenic Th clones recurrently contain anionic residues, suggesting selection by autoantigens with reciprocally charged residues that were presented to them by the anti-DNA B cells they preferentially helped (4, 8, 9). Indeed ~50% of such pathogenic Th clones that respond to nucleosomal antigens and nucleosome-specific T cells are detectable in SNF_1 mice long before they produce pathogenic autoantibodies (5). Moreover, immunization of preautoimmune SNF_1 but not normal mice with native nucleosome particles induces severe lupus nephritis (5). The pathogenic autoantibody-inducing Th cells of human lupus are also specific for nucleosomal antigens (8). Indeed, nucleosome-specific autoantibodies that are induced by such Th cells (4–8) play a central role in the development of lupus nephritis (5, 8, 10–12). Thus, nucleosomes are primary immunogens that initiate the cognate interaction between the pathogenic Th and B cells of lupus.

Identification of the critical Th cell autoepitopes in nucleosomes will help in understanding how the pathogenic Th cells of lupus emerge, and potentially lead to the development of peptide-based tolerogenic therapy. Mononucleosomes consist of double stranded DNA (dsDNA) wrapped around a core octamer of histones: two each of histones H2A, H2B, H3, and H4 (13). The fact that mononucleosome presentation could be blocked by leupeptin and pepstatin A that are inhibitors of endosomal proteases, sug-

[1] *Abbreviations used in this paper:* aa, amino acid sequence; dsDNA, double stranded DNA; SI, stimulation index; SNF_1, (SWR × NZB)F_1; ssDNA, single stranded DNA.

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gested that peptides from the histone component of the nucleosomes harbor the relevent epitope(s) for the pathogenic Th cells (5). However, the nucleosome-specific Th clones do not respond to free histones (5). This result suggested two possibilities: (*a*) the generation of critical histone epitope(s) may be dependent on the efficient uptake of the native nucleosome particle by specific receptors on APCs; or (*b*) the critical histone epitopes may be protected from degradation during processing by being bound to DNA in the intact nucleosomes (5). Therefore, to identify the critical epitopes, we adopted an alternative approach: a total of 145 overlapping 15-mer peptides, spanning all four core histones, were synthesized and the nucleosome-specific, pathogenic autoantibody-inducing Th clones were tested for response to these synthetic peptides. Herein, we report that the lupus Th cells do indeed recognize determinants from three different regions of the core histones, and these peptide epitopes can trigger pathogenic Th cells in vivo to precipitate severe lupus nephritis in SNF_1 mice.

Materials and Methods

Mice. BALB/c, NZB, SWR, and (BALB/c × SWR)F_1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). SNF_1 hybrids were bred at our animal facility. Periodic testing showed that the mice were free of specific pathogens, such as mouse hepatitis virus. Female mice were used for the experiments.

Cloned Th Cell Lines and Hybridomas. Cloned Th cell lines and hybridomas used here were previously derived from SNF_1 mice with lupus nephritis and maintained as described (3–5). Cloned Th lines were rested for 12 d before the assays. The B cell lymphoma APC, A20 ($I\text{-}A^d$), was grown as described (5).

Antibodies. The following mAbs were obtained from the American Type Culture Collection (Rockville, MD) and used as described (5, 6): anti-$I\text{-}A^d$ (HB3), anti-$I\text{-}A^{b,d,q}$ (TIB120), anti-HSA (TIB183), anti-Thy-1.2 (TIB99), anti-CD8 (TIB211), and anti-CD3 (145-2C11).

Cytokine ELISA. Anti-IL-2, anti-IFN-γ, anti-IL-4, and anti-IL-10, capture and biotinylated revealing antibody pairs and the respective standards were purchased from PharMingen (San Diego, CA). Streptavidin–horseradish peroxidase and the substrate 3,3′,5,5′-tetramethyl benzidine-dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). The cytokines were quantitated according to the manufacturer.

Synthesis of Peptides. All the peptides used for the results shown here, were either synthesized by pin method or Fmoc chemistry (Chiron Mimotopes, San Diego, CA). Overlapping 15-mer peptides (acetyl-peptide-diketopiperizine) were synthesized spanning the entire stretch of all four core histone (H2A, H2B, H3, and H4) sequences (GenBank). Each 15-mer overlapped the preceding amino acid by 12 residues. Purity of the peptides was checked by amino acid analysis. Longer, 24-mer histone peptides were synthesized as follows: $H4_{16\text{-}39}$, $H4_{71\text{-}94}$, and $H2B_{10\text{-}33}$. The "control" peptide was an $I\text{-}A^d$–binding 17-mer ovalbumin ($OVA_{323\text{-}339}$) peptide that is immunogenic in vivo (14). The 24- and the 17-mer were purified by HPLC and analyzed by mass spectrometry for purity. Relevant peptides of interest were also synthesized at Tufts University School of Medicine (Boston, MA), and results were similar to those obtained with the peptides from Chiron Mimotopes (data not shown).

Splenic $CD4^+$ T Cells and APCs. The splenic $CD4^+$ T cells were isolated as reported earlier (5, 6), from 3–4-mo-old SNF_1 mice by nylon wool column followed by the lysis of $CD8^+$ T cells and contaminating B cells. For peptide presentation, either mitomycin C–treated A20 B cell lymphoma or irradiated B+Mϕ APC were prepared as described (5).

	Help for IgG autoantibody production *		TCR usage		Nucleosome Response
Th clone	Anti-ssDNA	Anti-dsDNA	Vα/Jα	Vβ/Jβ	(SI)
H/L-3A	15.0	22.5	19.1/41	4/2.6	**24.0**
H/L9.37	6.7	4.2	8/14	4/2.6	**7.4**
H/L9.10	6.6	2.2	2.5/23	4/2.1	**4.8**
H16B6	22.4	6.6	2.5/37	2/1.5	**99.2**
H/L9.7	11.0	2.9	23.1/27	2/2.6	**11.8**
H12C4	41.8	2.6	13/47	8.3/2.4	**34.0**
H/L9w.7	26.2	7.0	2/15	8.2/2.1	**26.7**
H15G9	44.5	3.6	3/28	14/1.4	**5.8**
H15E3	16.3	2.4	16/13	14/1.1	1.3
L-1A	20.7	28.0	8/36	8.2/1.6	1.0
L-9.2	8.0	2.8	10/40	8.3/2.1	1.0
H16B11	202.1	7.1	10/14	8.2/2.1	2.2
H12E4	6.0	3.3	14/15	8.3/1.1	1.1
H10B5	12.5	2.0	2.5/42	1/2.2	1.5
H16G10	18.0	15.3	19.1/37	1/2.6	2.9

Figure 1. Summary of pathogenic autoantibody-inducing Th clones derived from SNF_1 mice with lupus nephritis (4, 5, 9). (*L*) Cloned Th lines; (*H*) Th hybridomas derived directly from splenic T cells. (*H/L*) Cloned Th lines that were fused with TCR-negative BW5147 thymoma to obtain Th hybridomas, after initial functional characterization (4, 5, 9). H/L hybridomas were retested to confirm that their nucleosome specificity and pathogenic autoantibody-inducing ability were still intact. SI for the nucleosome-reactive Th clones are shown in bold. (*) Help for IgG autoantibody production is expressed as fold increase in antibody production when Th clones were cocultured with syngeneic B cells, as compared with control cultures containing B cells alone. Optimal results from 10 to 15 experiments are given.

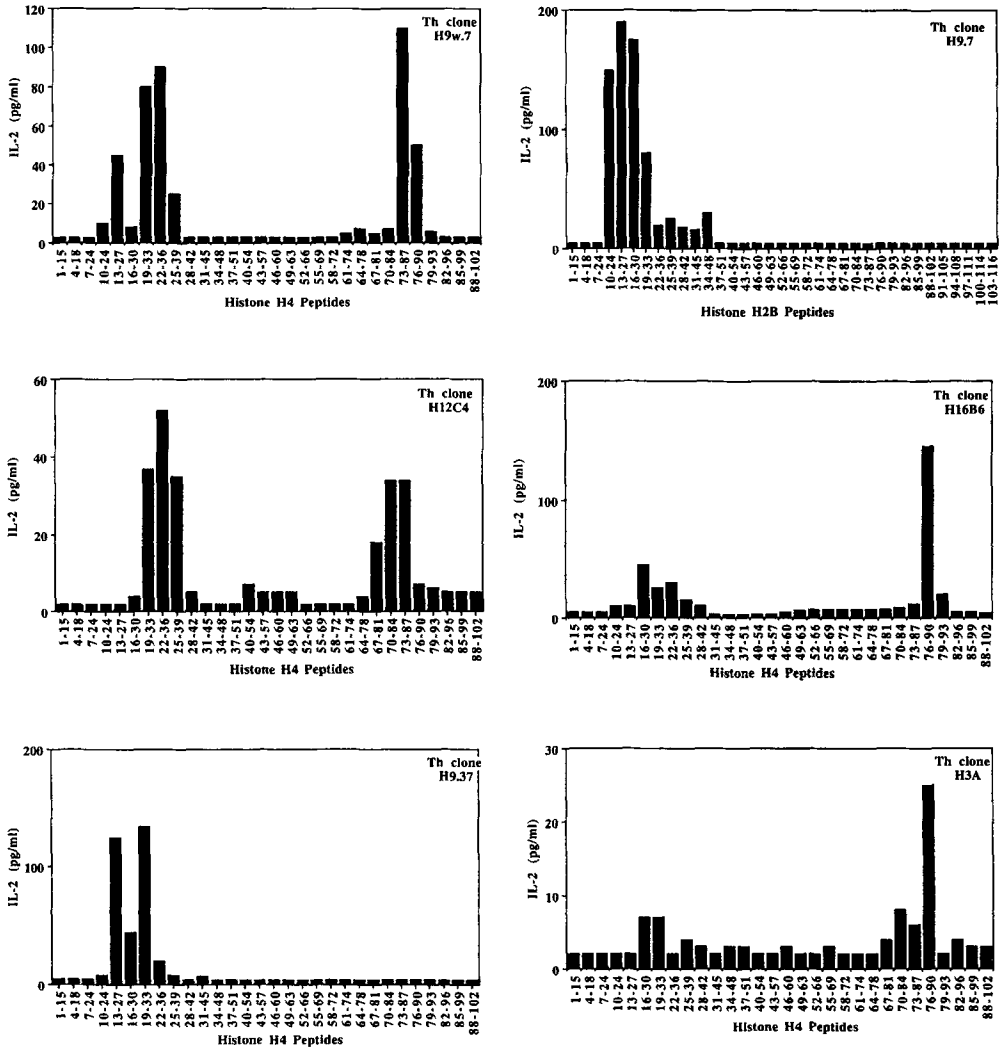

Figure 2. IL-2 production by nucleosome-specific, pathogenic autoantibody-inducing Th cell clones in response to histone H2B and H4 synthetic peptides using A20 as APCs. Mitomycin C–treated A20 were pulsed with 1 μM of the respective peptides for 18 h and were used as APCs. A representative of three to five experiments is shown. SD within each experiment was <10%. IL-2 production could not be detected in background cultures without peptide, whereas anti-CD3 antibody induced the Th clones to produce >2,000 pg/ml of IL-2 (not shown). Synthetic peptides (15-mer) spanning all four histones were tested with all 15 Th clones described in Fig. 1, and only the ones that responded are shown.

Proliferation and Cytokine Assays. Fresh splenic $CD4^+$ T cells (5×10^5/well) were cocultured in triplicate with irradiated B+Mϕ or mitomycin C–treated A20 lymphoma (10^6 cells) APCs, and different concentrations of control or test peptide in a 200-μl final volume in HL-1 serum-free medium (Hycor Biomedical Inc., Irvine, CA) for 96 h in flat-bottom 96-well plates (Costar Corp., Cambridge, MA). 18 h before harvesting, 1 μCi [^{3}H]thymidine/well was added. Stimulation index (SI) was calculated by dividing the mean counts per minute incorporated in cultures of T cells plus APCs and test peptide by the mean counts per minute in control peptide cocultures. For cytokine assays, culture supernatants were removed from duplicate coculture wells after 24–48 h.

Autoantibody Quantitation. IgG class autoantibodies to ssDNA, dsDNA, histones, and nucleosomes (histone–DNA complex) were estimated by ELISA (5, 6). Sera were diluted 1:100 and heat inactivated before use. Sera from normal BALB/c mice were used as negative control. Anti-DNA mAbs 564 and 205 were used to generate standard curves (5).

Pathogenicity of Histone-derived Peptides In Vivo. 18-wk-old prenephritic SNF_1 females (nine mice per group) were injected with either $H2B_{10-33}$, $H4_{16-39}$, $H4_{71-94}$, or control $OVA_{323-336}$ peptides (100 μg/mouse) emulsified in CFA. The animals received three more booster injections at 2-wk intervals with (50 μg/mouse) peptides adsorbed on alum (Pierce Chemical Co., Rockford, IL). The mice were monitored (4–6) and killed when they developed persistent proteinuria (two consecutive weekly readings of 300 mg/dl or greater). Sera were collected and blood urea nitrogen (BUN) was measured by Azostix (Miles Laboratories, Elkhart, IN). Kidney sections were stained with hematoxylin and eosin for grading of glomerulonephritis by a blinded observer as described (4–6).

Results

Summary of the Properties of Pathogenic Th Clones of Lupus. In earlier studies, we isolated 268 T cell clones from the spontaneously activated splenic T cells of 10 SNF_1 mice with lupus nephritis (4). About 15% of these T cell clones had the ability to preferentially augment the production of IgG anti-DNA autoantibodies, and representative pathogenic Th clones (H/L-3A, L-1A) rapidly induced immune-deposit glomerulonephritis when transfered into young pre-autoimmune mice (4, 6). A summary of the properties of 15 such pathogenic autoantibody-inducing Th clones is given in Fig. 1. The Th clones use a variety of TCR genes (Fig. 1), but their CDR3 junctional regions contain anionic

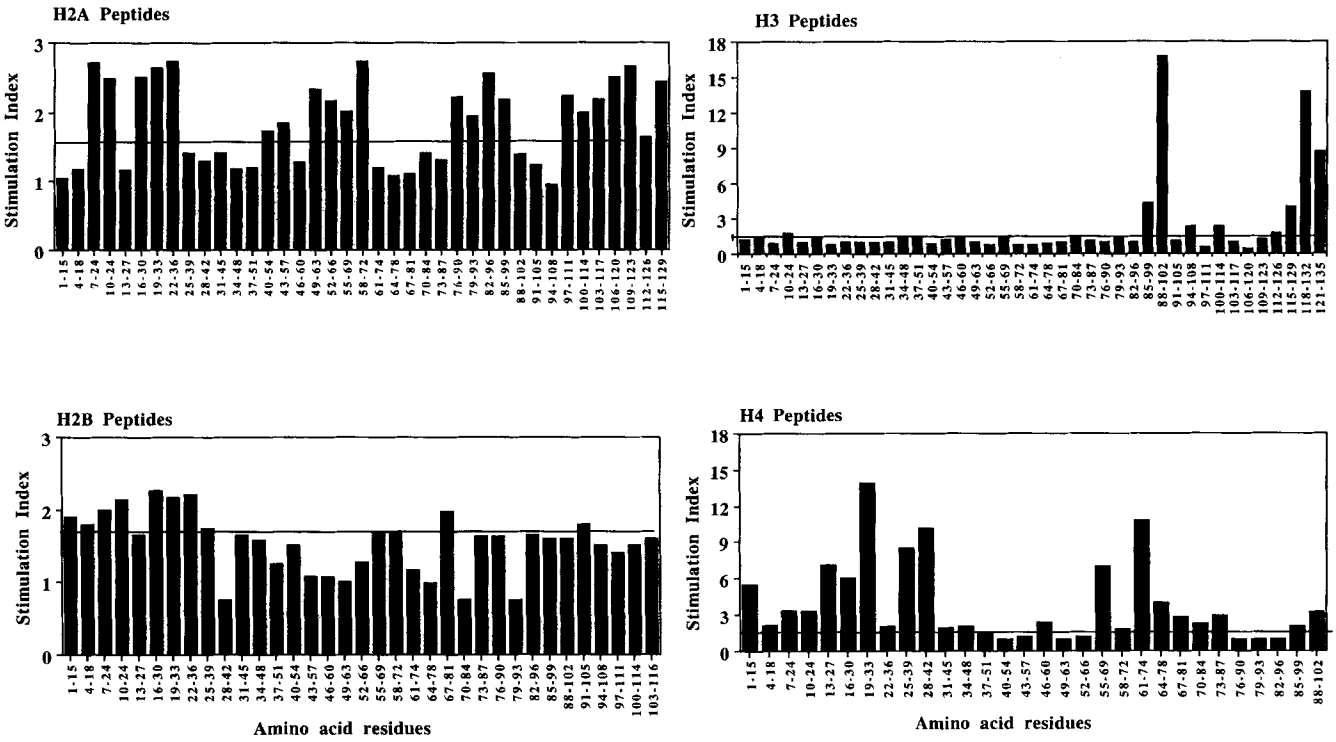

Figure 3. Proliferative responses of splenic CD4+ T cells from unmanipulated, 4-mo-old SNF_1 mice to overlapping 15-mer peptides spanning the entire sequences of histones H2A, H2B, H3, and H4. 1-mo-old SNF_1 splenic B+Mϕ were irradiated and pulsed with 1 μM of the respective peptides for 18 h and used as APCs. A representative of three experiments is shown. The results are expressed as stimulation indices. SD within each experiment was <10%. The background counts with control OVA peptide in these experiments were typically 350–900 cpm, and >3 SD above background cpm (*horizontal line*) was considered to be stimulatory.

residues suggesting that the antigenic determinants recognized by these TCRs might have reciprocally charged residues (4, 9). Indeed, half of these Th clones recognize nucleosomal antigens in the context of $I\text{-}A^d$ (5, and Fig. 1). All the Th clones augmented IgG anti-DNA autoantibody production by autologous B cells by 6–200-fold (Fig. 1, data from ref. 4), and their ability to induce IgG antinucleosome antibodies was even higher (5).

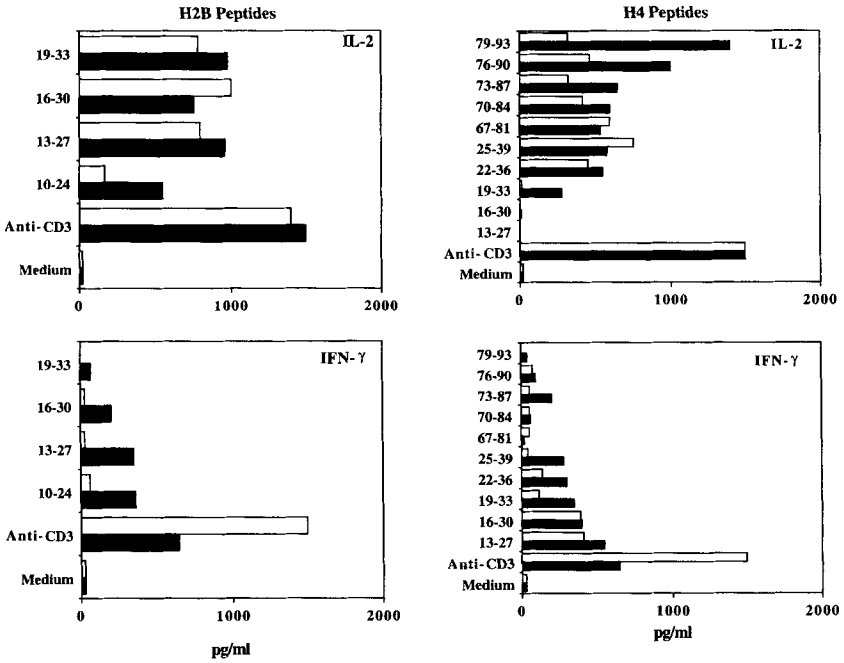

Figure 4. Cytokine production by splenic CD4+ T cells from unmanipulated, 3–4-mo-old SNF_1 mice to H2B and H4 synthetic peptides using either A20 (*filled bars*) or 1-mo-old SNF_1 splenic B+Mϕ (*unfilled bars*) as APCs. Representative results from three such experiments are shown.

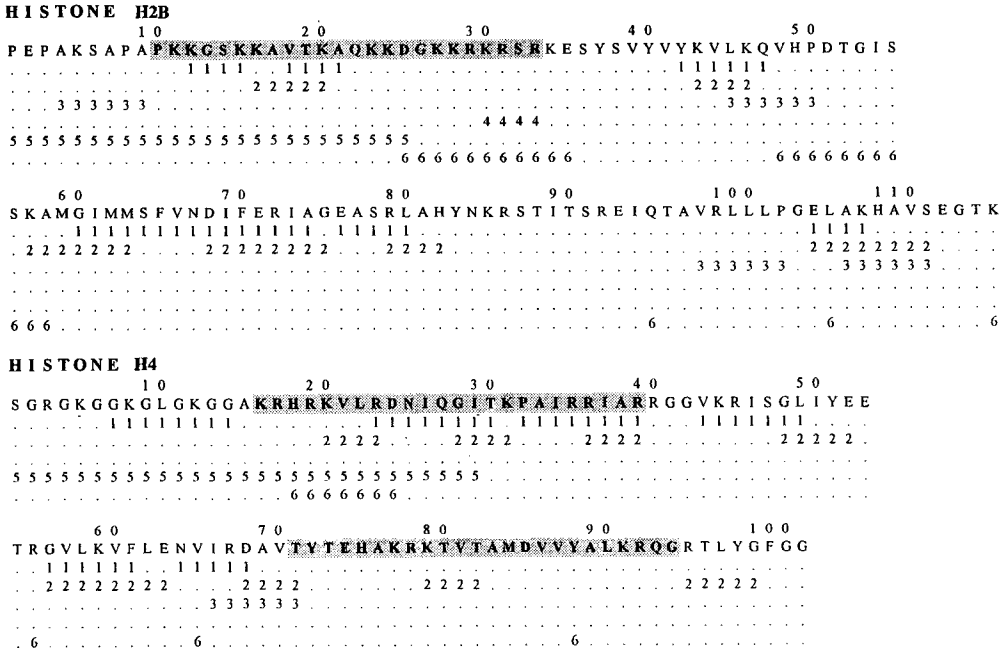

Figure 5. Analysis of amino acid sequences of histones H2B and H4. The highlighted regions contain the pathogenic Th cell epitopes and corresponding peptides (24-mer) were used for immunization. The regions that overlap with certain motifs of interest are designated by numbers: (*1*) Amphipathic regions (15); (*2*) Rothbard motif (16); (*3*) I-A^d binding motif (14); (*4*) I-E^d binding motif (17); (*5*) autoantibody binding regions (18, 19); and (*6*) Histone-DNA contact regions (13).

Identification of Pathogenic T_h Cell Determinants in Core Histones. To identify the critical Th cell epitopes, a panel of 15-mer overlapping peptides spanning the entire sequence of the core histones H2A, H2B, H3, and H4 were synthesized. The peptides were tested at 0.1, 1, and 10 μM, using A20 (I-A^d) APCs, for their ability to stimulate IL-2 production by the panel of 15 pathogenic autoantibody-inducing Th clones of SNF_1 mice. 1-μM concentration of the peptide was the optimum and was used for the assays. One cluster of overlapping peptides from histone H2B and two clusters from histone H4 peptides consistently induced IL-2 production by 6 of the 15 pathogenic Th clones (Fig. 2). All six of these pathogenic autoantibody-inducing Th clones had responded to native nucleosomes (5, and Fig. 1). The Th clone H9.7 responded to a cluster of overlapping peptides from H2B in the amino acid sequence (aa) region of 10-33 (Fig. 2). Clone H9.37 responded to a cluster of peptides from histone H4 in the aa 13-33 region (Fig. 2). The rest of the pathogenic Th clones responded to two clusters of peptides within histone H4 aa 13-39 and 67-93 regions (Fig. 2). The other histone peptides did not stimulate any of the Th clones.

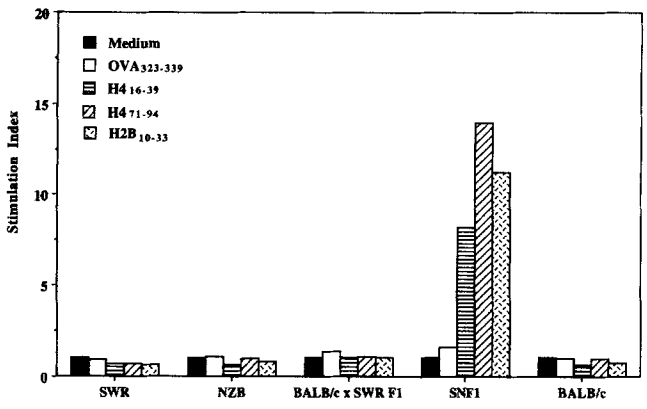

Figure 6. Proliferative response of 4-mo-old, splenic $CD4^+$ T cells to the longer (24-mer) histone peptides, $H2B_{10-33}$, $H4_{16-39}$, and $H4_{71-94}$ using 1-mo-old B+Mϕ from respective strains as APCs. Experiments were carried out as described in Fig. 3. A representative of three experiments is shown. The results are expressed as stimulation indices. SD within each experiment was <10%. Anti-CD3 stimulation elicited a SI of 33–44 in the strains tested.

Response to Core Histone Synthetic Peptides by T Cells from Unmanipulated SNF_1 Mice. To further test the in vivo relevance of these peptides, we screened them for their ability to stimulate proliferation of freshly isolated $CD4^+$ T cells from 3–4-mo-old, prenephritic SNF_1 mice (Fig. 3). SNF_1 lupus mice (but not normal mice) at this age spontaneously have nucleosome-specific T cells without any deliberate immunization (5). Peptides spanning certain regions of H3 and H4 stimulated greater proliferation (SI of 5–15) than peptides from H2A and H2B (SI of 2.5–2.8), but cytokine responses were equally high (see below).

Cytokine Induction by the Histone Peptides. The peptides in H2B and H4 that were thus localized for stimulating the pathogenic Th clones as well as the splenic T cells (Figs. 2 and 3), were further tested for their ability to induce cytokine production in the freshly isolated splenic $CD4^+$ T cells from unmanipulated, 3–4-mo-old SNF_1 mice. The peptides from histones H2B (region 10-33) and H4 (regions 13-39 and 67-93) induced T cells from the SNF_1 mice to produce Th1-type cytokines, IL-2, and IFN-γ (Fig. 4), but not IL-4, although anti-CD3 antibody did induce the production of >2,000 pg/ml of IL-4 by these T cells (data not shown). Although the H2B peptides had elicited relatively low proliferative response (Fig. 3), they stimulated high amounts of IL-2 (500–1,200 pg/ml) and IFN-γ (100–500 pg/ml) production, like the H4 peptides (Fig. 4).

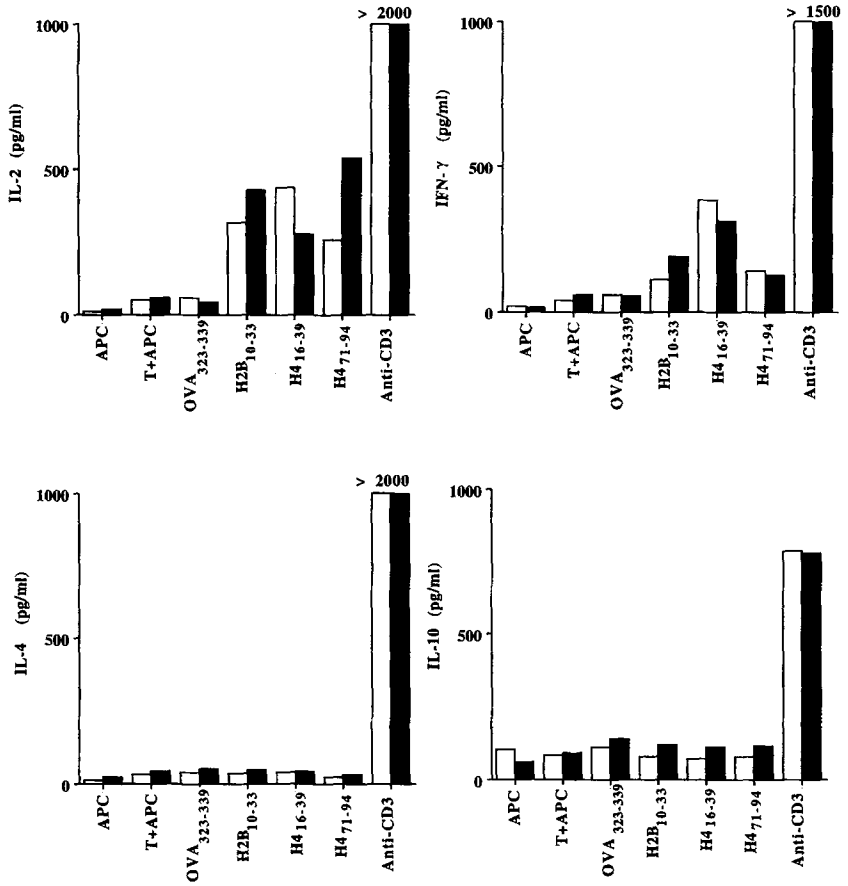

Figure 7. Cytokine production by $CD4^+$ T cells from unimmunized 3-mo-old SNF_1 mice in spontaneous response to the longer histone peptides using either A20 (*filled bars*), or 1-mo-old SNF_1 splenic B+Mϕ (*unfilled bars*) as APCs. Representative results from three such experiments are shown.

Selection of Histone Peptides for In Vivo Studies. We synthesized slightly longer 24-mer peptides, $H2B_{10-33}$, $H4_{16-39}$, and $H4_{71-94}$, to cover the amino acid sequences in the overlapping 15-mer peptides of H2B and H4 that stimulated the pathogenic autoantibody-inducing Th clones as well as the freshly isolated $CD4^+$ T cells from SNF_1 mice (Figs. 2–4). Even though some H3 and H2A peptides stimulated SNF_1 splenic T cells (Fig. 3), they were not selected because they did not stimulate any of the pathogenic Th clones. The sequences from histones H2B ($H2B_{10-33}$) and H4 ($H4_{16-39}$ and $H4_{71-94}$), which were selected for their stimulatory ability of SNF_1 Th cells, contained amphipathic and "Rothbard" T cell epitope motifs designated by numbers 1 and 2, respectively, in Fig. 5 (15, 16), but there were no $I\text{-}A^d$ (number *3* in Fig. 5; 14) or $I\text{-}E^d$ (number *4* in Fig. 5; 17) binding motifs. Nevertheless, the nucleosomal epitopes are presented by $I\text{-}A^d$ molecules on A20 APCs to the SNF_1 ($H\text{-}2^{d/q}$) T cells (5, and Fig. 4). The stimulatory peptides overlapped with histone regions known to contain B cell epitopes that are recognized by antihistone autoantibodies (number *5* in Fig. 5; 18, 19), as well as histone-DNA contact sties (designated by number *6* in Fig. 5; 13).

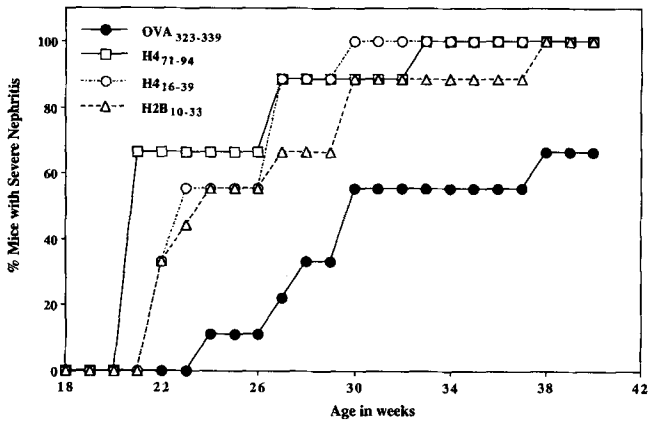

Figure 8. Incidence of severe glomerulonephritis in prenephritic SNF_1 mice immunized with histones $H2B_{10-33}$, or $H4_{16-39}$, or $H4_{71-94}$, or control $OVA_{323-339}$ synthetic peptides. Nine mice per group were immunized first at 18 wk age with 100 μg of the relevant peptides in CFA, followed by three more injections at 2-wk intervals with 50 μg of peptides adsorbed in alum. The mice received the last injection at 24 wk of age, except for those that had already developed severe nephritis.

In Vivo Relevance of Selected Longer Histone Peptides. The larger peptides $H2B_{10-33}$, $H4_{16-39}$, and $H4_{71-94}$ were first tested for their ability to stimulate $CD4^+$ T cells from 4–5-mo-old unimmunized SNF_1 mice, the parental strains SWR and NZB ($I\text{-}A^d$ and $I\text{-}A^q$, respectively), and from MHC haplotype-matched, nonautoimmune BALB/c ($I\text{-}A^d$) and (SWR × BALB/c) F_1 mice. As expected, all three longer peptides also induced a proliferative response in T cells from SNF_1 mice (Fig. 6). Histone H4 peptide, $H4_{71-94}$,

Table 1. *Antinuclear Autoantibodies in the Serum (Mean U/ml ± SEM) of Histone Peptide–immunized SNF_1 Mice*

Immunogen	Serum levels of IgG autoantibodies against:			
	ssDNA	dsDNA	Histones	Nucleosome
$OVA_{323-339}$	0.83 ± 0.25	1.32 ± 0.50	8.24 ± 1.71	2.08 ± 0.46
$H2B_{10-33}$	72.66 ± 26.66	18.36 ± 8.66	9.96 ± 1.73	11.43 ± 4.61
	(<0.05)	(>0.1)	(>0.1)	(<0.01)
$H4_{16-39}$	25.53 ± 8.15	11.03 ± 4.14	38.85 ± 1.24	18.95 ± 7.45
	(<0.025)	(<0.1)	(<0.025)	(<0.01)
$H4_{71-94}$	50.82 ± 22.72	12.14 ± 7.60	45.88 ± 12.2	20.63 ± 9.37
	(<0.01)	(<0.05)	(<0.01)	(<0.1)

Augmentation of autoantibody production in 21-wk-old SNF_1 mice after immunization with histone peptides. Results are expressed as mean ± SEM. *P* values (Mann-Whitney U test) in comparison to corresponding results in control $OVA_{323-339}$ immunized mice are given in parentheses.

elicited the highest response (SI of 14), whereas $H4_{16-39}$ peptide induced a SI of 8.5. The 24-mer $H2B_{10-33}$ peptide evoked a higher SI of 12.5 (Fig. 6), as compared with the smaller 15-mer H2B peptides spanning the same region (Fig. 3). The control $OVA_{323-339}$, a known I-A^d binding peptide, did not elicit any proliferative response in these unimmunized mice. These peptides did not stimulate $CD4^+$ T cells from any of the other strains tested (Fig. 6), whereas anti-CD3 antibody stimulated T cells from all the strains (data not shown). The longer histone peptides, like their 15-mer counterparts (Fig. 4), induced the production of Th1-type cytokines by $CD4^+$ T cells of unmanipulated SNF_1 mice, but not Th2 cytokines (Fig. 7).

Histone Peptides Accelerate the Devolpment of Severe Glomerulonephritis in SNF_1 Mice. 18-wk-old prenephritic SNF_1 mice injected with synthetic peptides from histones H2B ($H2B_{10-33}$) or H4 ($H4_{16-39}$ or $H4_{71-94}$) in adjuvant developed severe nephritis much earlier than age-matched SNF_1 mice injected with control peptide $OVA_{323-339}$ in adjuvant (Fig. 8). Mice were considered to have severe glomerulonephritis when they had persistent proteinuria of ≥300 mg/dl for 2 wk and BUN levels at ≥30 mg/dl; after killing at this stage, their kidneys showed a 3+ to 4+ grade of nephritis by histopathology. After only 3 wk from initial immunization, at 21 wk of age, 66.6% of the mice immunized with $H4_{71-94}$ had severe glomerulonephritis, whereas none of the mice in the other three groups had developed severe nephritis ($P = 0.009$, Fisher's exact test). 5 wk after the initial injection (at 23 wk of age), 66.6% of the $H4_{71-94}$, 55.5% of the $H4_{16-39}$, and 44.4% of the $H2B_{10-33}$ peptide-immunized mice had developed severe glomerulonephritis, but none of the control $OVA_{323-339}$-immunized mice ($P < 0.01$) had. 9 wk after the start of immunization (at 27 wk of age), 88.8% of histone $H4_{16-39}$ and $H4_{71-94}$ peptide-immunized mice, and

Table 2. *Proliferative Response (Mean cpm ± SEM) of T Cells from Peptide-immunized SNF_1 Mice*

Immunogen	Proliferative response of splenic $CD4^+$ T cells with APCs and				
	Medium	$OVA_{323-339}$	$H2B_{10-33}$	$H4_{16-39}$	$H4_{71-94}$
$OVA_{323-339}$	817 ± 98	35,650 ± 2,810	2,710 ± 890	1,658 ± 133	3,360 ± 759
		(43.6)	(3.3)	(2.0)	(4.1)
$H2B_{10-33}$	922 ± 109	477 ± 186	30,783 ± 3,714	4,384 ± 795	5,048 ± 715
		(0.5)	(33.4)	(4.8)	(5.5)
$H4_{16-39}$	742 ± 130	1,078 ± 186	1,300 ± 213	32,641 ± 3,524	3,418 ± 462
		(1.5)	(1.8)	(44.0)	(4.6)
$H4_{71-94}$	785 ± 165	1,191 ± 240	1,880 ± 339	2,707 ± 442	28,230 ± 2,483
		(1.5)	(2.4)	(3.5)	(36.0)

Recall responses to the immunizing histone and ovalbumin peptides. The proliferative responses from six mice of each group are shown as mean cpm ± SEM. SI are in parentheses.

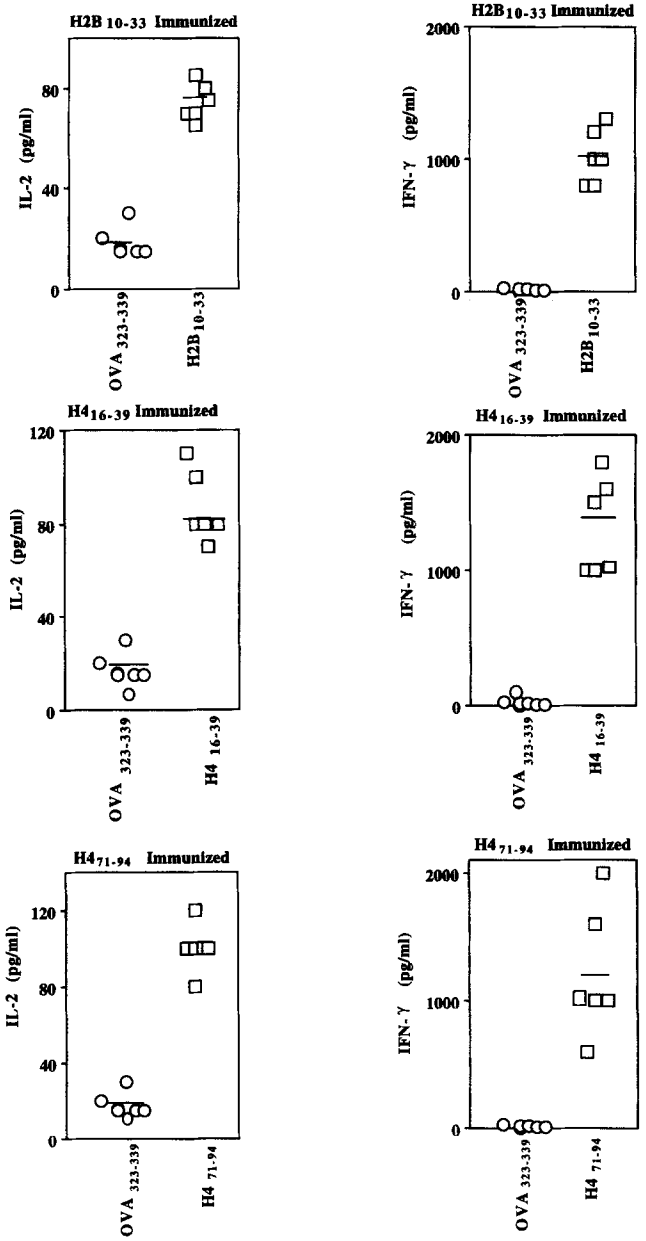

Figure 9. IL-2 and IFN-γ production by splenic CD4+ T cells of immunized SNF_1 mice in response to relevant histone peptides. Splenic CD4+ T cells of SNF_1 mice from each of the immunized groups, were cultured in presence of control $OVA_{323-339}$ (○) or relevant immunizing histone peptides (□) using SNF_1 B+Mϕ as APCs, and the supernatants were assayed for various cytokine production. Results shown are values from individual mice of each group and the mean value is indicated by the horizontal line.

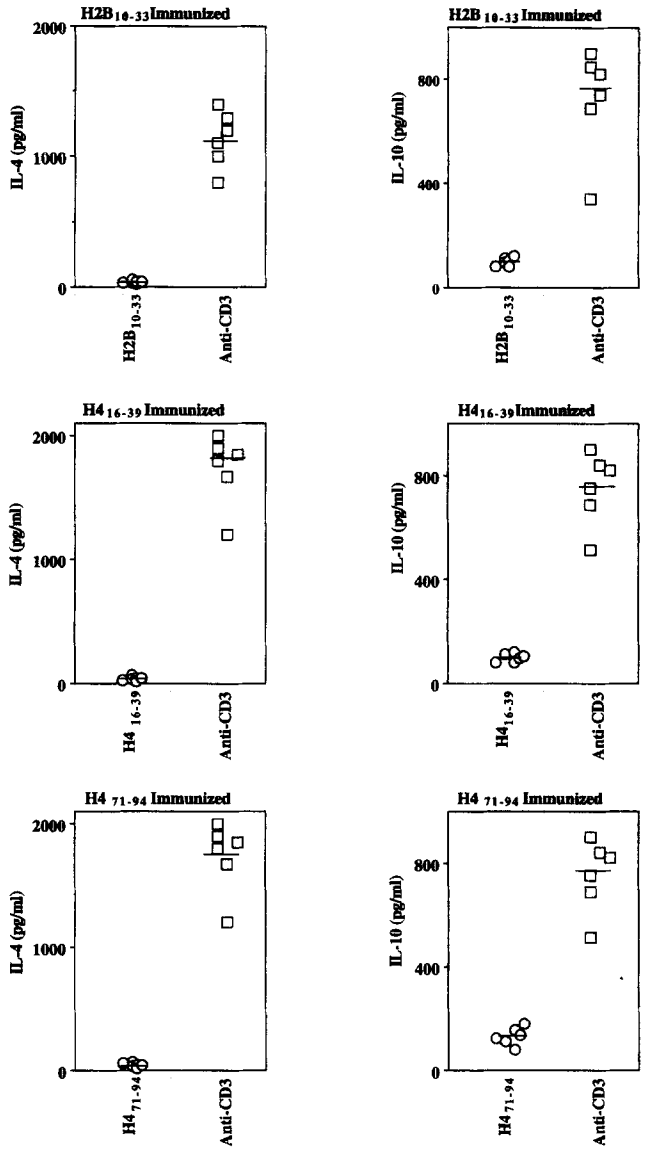

Figure 10. IL-4 and IL-10 cytokine response by splenic CD4+ T cells from the immunized SNF_1 mice shown in Fig. 9. The experiments were done as in Fig. 9. Results for the relevant immunizing histone peptide (○) or anti-CD3 stimulation (□) are shown after subtracting background values (T cells plus APCs with control OVA peptide) in each case. Values from individual mice from each group are shown with the mean value indicated by the horizontal line.

66.6% of $H2B_{10-33}$-immunized mice developed severe lupus nephritis, whereas the control group had a 22.2% disease incidence ($P = 0.015$). At 33 wk of age, $H4_{16-39}$- and $H4_{71-94}$-immunized mice had 100% and $H2B_{10-33}$ mice had 88.8%, whereas $OVA_{323-339}$-injected mice had only a 55.5% incidence of severe lupus nephritis ($P = 0.082$). By 40 wk of age, 100% of the histone peptide-immunized groups vs. only 66.6% of the control $OVA_{323-339}$ peptide-injected animals had developed the disease ($P = 0.206$; Fig. 8). Remarkably, the $H4_{71-94}$ peptide precipitated the disease more rapidly than the $H2B_{10-33}$ and $H4_{16-39}$ peptides during the period 3–8 wk after the initial immunization, i.e., between 21 and 26 wk age ($P < 0.01$–$P < 0.02$). By 27 wk of age, both $H4_{16-39}$ and $H4_{71-94}$ peptides were performing better in inducing lupus nephritis than $H2B_{10-33}$ (Fig. 8). The control $OVA_{323-339}$-immunized mice had the same rate of lupus nephritis as that of unimmunized or PBS/CFA-immunized SNF_1 mice (5).

Anti-nuclear Autoantibody Levels in Histone Peptide Immunized Mice. In unmanipulated SNF_1 lupus mice, the nephritogenic autoantibodies are specific for ssDNA, dsDNA, and nucleosomes (5). The levels of IgG autoantibodies with at least two of the pathogenic specificities were significantly elevated in the mice immunized with histone peptides when tested at 21 wk of age (Table 1). At later ages from 30 wk and beyond, there were no significant differences in serum autoantibody levels between the control and the histone peptide–immunized mice. This is expected, since elevated serum levels of autoantibodies precede the development of severe nephritis by several weeks, and marked fluctuations occur due to tissue deposition (5).

Recall Response to Immunized Histone Peptides by Splenic $CD4^+$ T Cells. In the histone peptide–immunized mice, splenic $CD4^+$ T cells had a significant proliferative response to the respective immunizing peptide at the time of killing (21–30 wk age; Table 2). The histone peptides also elicited a lower level of "background" or "spontaneous" T cell proliferative response in the other groups of SNF_1 mice that had not received the respective immunogen. The strongest spontaneous response was elicited by $H4_{71-94}$ in SNF_1 mice immunized with one of the other peptides. The background stimulation across the groups by these histone peptides is expected as the SNF_1 mice develop spontaneous lupus nephritis and possess nucleosomal peptide-specific T cells at the ages tested even without deliberate immunization (Figs. 6 and 7).

Cytokine Production Pattern by $CD4^+$ T Cells from Immunized SNF_1 Mice. The immunizing histone peptides also induced the production of Th1 cytokines IL-2 and IFN-γ by splenic $CD4^+$ T cells from SNF_1 mice immunized with the corresponding peptide (Fig. 9). However, IL-4 and IL-10 production in response to the peptides was not significantly above background levels (Figs. 7 and 10), although anti-CD3 antibody could stimulate production of the Th2 cytokines by the same T cell population (Fig. 10).

Discussion

Localization of the peptide autoepitopes in the core histones definitively establishes that nucleosomes are the primary immunogens for the spontaneously arising pathogenic Th cells of lupus, and excludes the possibility that nucleosomes stimulate indirectly by augmenting the display of other endogenous antigens or by increasing cytokine production by the APC (20). Herein, we have not only identified the peptide autoepitopes for the antinuclear autoantibody-inducing Th clones but have also established their disease relevance in vivo.

The nucleosome-specific, pathogenic autoantibody-inducing Th clones that responded to the peptides from H2B and H4 core histones, each have a single functional TCR α and β chain (4, 9). Yet, some of the Th clones responded to peptides from two different regions of H4 that did not have an apparent primary sequence homology except for the presence of charged residues (Figs. 2 and 5). Moreover, Th clones that responded to the same histone peptides (Fig. 2) have different TCRs (9, and Fig. 1). Such examples of degenerate recognition have been described for other T cells (21–23). This plasticity of TCR repertoire has implications for the selection of autoimmune Th cells in the thymus or in the periphery by environmental molecular mimics.

The overlapping peptides from the same regions of H2B (aa 10-33) and H4 (aa 16-39 and 71-94) core histones that were recognized by the pathogenic autoantibody-inducing Th clones also stimulated splenic T cells from unmanipulated SNF_1 mice, ex vivo. These peptides induced the production of high levels of Th1-type cytokines IL-2 and IFN-γ by the freshly isolated splenic T cells from the lupus mice. This finding is consistent with the observation that the pathogenic anti-DNA autoantibodies of SNF_1 mice are predominantly IgG2a, IgG2b, and IgG3 in isotype (5), which are induced by Th1 cells (24). Immune complexes consisting of these particular IgG isotypes mediate complement fixation, as well as activation of inflammatory cells, by binding to their Fcγ receptors, both of which are the hallmarks of lupus nephritis.

Interestingly, the lupus Th cell stimulatory regions of H2B and H4 did contain T cell epitope motifs that have been previously described (15, 16), as well as B cell motifs, i.e., the sites localized to be the targets of antihistone autoantibodies (18, 19). Thus, autoimmune B cells with specificity for the overlapping epitopes in the nucleosomal histones could present the autoepitopes to the Th cells efficiently after uptake and processing (4, 5, 7, 25, 26). Indeed, our earlier work indicated that a single pathogenic lupus Th clone could help a dsDNA-, a ssDNA-, a histone-, a high mobility group protein-, or a nucleosome-specific B cell in a polyclonal population, because each of those B cells by binding to its respective epitope on the whole chromatin, could take it up and process it, and then present the relevant peptide Th-epitope in the chromatin to the Th clone (5, 8). With the ongoing autoimmune response and intermolecular help, epitope spreading may occur across other histone components of the nucleosome particle (Fig. 3), as well as other relevant autoantigens associated with pathogenic autoantibody production (5, 8, 25, 27–30). In addition to the B cell epitope sites, the peptides of interest to the Th cells of lupus, contained the sites in histones that remain in contact with DNA in the nucleosome (13 and Fig. 4). This feature could protect the epitopes from degradation during processing by APCs, which could explain why the native particle is more immunogenic for the lupus Th cells.

The H2B and H4 peptides that were specifically recognized by the spontaneously arising, pathogenic autoantibody-inducing Th cells of lupus mice during the natural history of their disease were also pathogenic in vivo. Immunization of prenephritic SNF_1 mice with $H2B_{10-33}$, $H4_{16-39}$, or $H4_{71-94}$ peptide markedly accelerated the development of severe glomerulonephritis and augmented the production of pathogenic antinuclear autoantibodies. The $H4_{71-94}$ peptide precipitated lupus nephritis earliest, next the $H4_{16-39}$, followed by $H2B_{10-33}$ (Fig. 8). Interestingly, the nucleosomal core

histone H4 is also recognized by pathogenic autoantibody-inducing Th cells in human lupus (9), but the critical peptide epitopes have not yet been identified. Splenic T cells from the immunized SNF_1 mice that had developed severe nephritis, again produced Th1-type cytokines on rechallenge with the immunizing peptide. The skewing to a Th1-type response was probably not due to immunization with CFA because alum was also used. Moreover, T cells from unimmunized SNF_1 mice also produced Th1-type cytokines in spontaneous responses to these particular peptides (Figs. 4 and 7). This result is consistent with the class of nephritogenic autoantibodies produced by these mice as mentioned above.

How could autoimmunization to nucleosomes, the normal product of apoptosis (31), occur in lupus? The histone peptide epitopes that we have identified here could be cryptic, thus allowing nucleosome-specific T cells to escape to the periphery (27). However, normal mice do not develop lupus upon immunization with nucleosomes whereas, lupus mice do, and they have nucleosome-primed T cells early in life even without any deliberate immunization (5). Abnormal antigen processing due to the prevalence of nucleosome-binding B cells in lupus mice, and other intrinsic defects of the immune system of lupus in handling cross-reactive environmental antigens (5, 25, 26, 32–35) could contribute to the presentation of the nucleosomal autoepitopes. Indeed, MHC class I–bound histone peptides have been isolated, but so far, no histone peptides have been detected among the naturally processed peptides eluted from MHC class II molecules from nonlupus APCs (36). We can begin to address these issues as well as anticipate the development of peptide-based tolerogenic therapy in lupus with the identification of the critical autoepitopes.

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