

## T Cell Determinants from Autoantibodies to DNA Can Upregulate Autoimmunity in Murine Systemic Lupus Erythematosus

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### Summary

(NZB × NZW) F1 (BWF1) mice develop spontaneous T cell autoimmunity to V<sub>H</sub> region determinants of syngeneic anti-DNA before the onset of clinical disease. In this study, we characterized the immunogenicity, MHC binding, and lymphokine secretion patterns induced by T cell determinants from the V<sub>H</sub> region of one such anti-DNA mAb (A6.1) and examined their role in the regulation of autoimmunity. Determinants were identified by proliferation of syngeneic splenic T cells from young, unprimed BWF1 mice in response to overlapping 12-mer peptides representing the entire V<sub>H</sub> region sequence. Immunization of young BWF1 mice with any of three determinants (A6H 34-45 [p34], A6H 58-69 [p58], and A6H 84-95 [p84]) elicited proliferative responses upon in vitro recall. Upon immunization with the whole A6.1 molecule, however, proliferative responses could be recalled only to the p58 peptide, defining this as immunodominant. The other two peptides (p34 and p84) elicited minimal or no proliferation and could be termed cryptic. Proliferative responses elicited by the cryptic determinants were restricted by a single class II (I-E<sup>d</sup> for p34 and I-A<sup>u</sup> for p84), whereas the immunodominant p58 determinant was restricted by both I-E<sup>d</sup> and I-E<sup>u</sup>. The cryptic p34 and p84 bound strongly to I-E<sup>d</sup> and I-A<sup>u</sup>, respectively, whereas the immunodominant p58 peptide bound poorly to I-E<sup>d</sup>. A6H p84 elicited T cells that secreted lymphokines in a pattern consistent with a Th1-like phenotype, whereas p58 induced a Th2-like cytokine pattern. Immunization with p34 or p84, or adoptive transfer of a p84-reactive T cell line to young BWF1 mice significantly increased IgG anti-DNA levels, accelerated nephritis, and decreased survival. In conclusion, in BWF1 mice, autoreactive T cells recognizing both cryptic and dominant self-determinants on anti-DNA autoantibodies escape deletion or anergy induction. Furthermore, since these cells are spontaneously activated before the onset of clinical disease, they may be involved in the development of the autoimmune process.

**S**LE is characterized by loss of tolerance to self-antigens and persistent production of autoantibodies (autoAb)<sup>1</sup> (1, 2). Both B and T cells contribute to the production of pathogenic autoAbs and development of autoimmune disease (1-3). Th cells have been shown to upregulate production of anti-DNA Abs both in vitro and in vivo (4-7). How

are these Th cells activated, and to which determinants are they responsive? In this study, we have focused on the interaction between Ig-derived peptides and T cells and their potential involvement in disease pathogenesis in a murine SLE model, (NZB × NZW) F1 (BWF1) mice.

Unprimed young BWF1 mice, but not MHC-matched nonautoimmune mice, develop spontaneous T cell autoimmunity to V<sub>H</sub> region peptides of syngeneic IgG anti-DNA mAbs (reference 8 and Singh, R. R., F. Ebling, E. Sercarz, and B. H. Hahn, manuscript submitted for publication). According to our working model, in autoAb-mediated diseases, autoAb molecules may be processed and presented to autoreactive T cells, which would then stimulate B cells, finally resulting in increased production of pathogenic autoAbs and acceleration of clinical disease.

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<sup>1</sup> Abbreviations used in this paper: autoAb, autoantibody; BWF1, (NZB × NZW) F1; BUN, blood urea nitrogen; CWF1, (BALB/c × NZW) F1; HEL, hen egg lysozyme; PPD, purified protein derivative; SI, stimulation index.

Herein, we determine the regulatory role of T cell determinants from an mAb directed against DNA, termed A6.1, derived from a BWF1 mouse. In particular, we have studied the immunogenicity, MHC class II restriction, lymphokine secretion patterns, and pathogenic potential of three different T cell determinants (A6H 34-45 [p34], A6H 58-69 [p58], and A6H 84-95 [p84]). Whether dominant or cryptic, these determinants elicited T cell proliferation and cytokine release and accelerated autoAb production and clinical disease in BWF1 mice.

## Materials and Methods

**Animals.** Female BWF1 and other mice were either obtained from the Jackson Laboratories (Bar Harbor, ME) or bred in the UCLA Rheumatology Vivarium. To determine immunogenicity and class II restriction, the following strains were used: BALB/c, B10.GD, NZW, NZB, and (BALB/c × NZW) F1 (CWF1) mice. The MHC class II molecules of these strains are shown in Fig. 2. Animals were maintained in accordance with guidelines of the UCLA Animal Research Committee.

**L Cell Transfectants.** The L cell transfectants RT 2.3.3H-D6 (I-A<sup>d</sup>), RT 7.7H714.3 (I-E<sup>α</sup>Aβ<sup>d</sup>), and RT 10.3B-C1 (I-E<sup>d</sup>) were kindly provided by Dr. Ronald Germain (National Institutes of Health, Bethesda, MD) and were used as described (9). The L cell transfectant 6A2 (I-A<sup>u</sup>) was kindly provided by Dr. Patricia Jones (Stanford University, Stanford, CA).

**Peptides.** Peptides used in initial screening tests were synthesized on pins, as previously described (8). For these studies, 110 overlapping 12-mer peptides were made, each overlapping its neighbor by 11 amino acids (e.g., 1-12, 2-13, etc.), representing the entire amino acid sequence of the V<sub>H</sub>D<sub>H</sub> region of mAb A6.1. Each peptide, at concentrations of 1-10 μM, was incubated with 1-5 × 10<sup>5</sup> T cell-enriched splenic cells from 12-14-wk-old female BWF1 mice and 3-6 × 10<sup>5</sup> irradiated syngeneic splenic cells (APCs). After a 48-h incubation at 37°C in 7% CO<sub>2</sub>, cells were labeled with [<sup>3</sup>H]thymidine, and proliferation was measured 18 h later. Several peptides consistently stimulated splenic T cells to proliferate (mean stimulation indices [SI] 5.2-8.1 with <18% SEM) and were thus defined as T cell determinants (Singh, R. R., F. Ebling, E. Sercarz, and B. H. Hahn, manuscript submitted for publication).

Larger quantities of six peptides, three T cell determinants (see Fig. 1), and three control peptides were synthesized in the UCLA Peptide Synthesis Facility using Fmoc chemistry. Three peptides, none of which elicited spontaneous proliferation of naive BWF1 T cells, were used as controls: (a) A6H 93-107 (p93) (ARD-SPYYYGSSYGFA), also derived from A6.1 V<sub>H</sub>, binds to the class II molecule I-A<sup>u</sup> (see Table 1) and induces a T cell proliferative response upon immunization (data not shown). (b) A modified p58 (p58M) (FYNQAFKGAATL), in which lysines at positions 5 and 9 in the wild-type peptide were substituted with alanines, did not stimulate unprimed syngeneic T cells and was used as a negative control (8). (c) One I-E<sup>d</sup>-binding immunodominant determinant of hen egg lysozyme, HEL 106-116 (HEL p106) (NAWVAWRNRCK), was used as a positive control. Upon immunization, it elicits vigorous T cell proliferative responses in BWF1 mice. The synthetic peptides were analyzed for purity by HPLC and by mass spectrometry. Each peptide chromatographed essentially as a sharp single peak. All purified peptides were found to have the expected molecular mass.

**Immunization.** Mice were immunized at the base of their tails

and hind footpads with 100 μg of purified A6.1 or 50 μg of different Ig-derived peptides, or with 5-7 μg of HEL p106, emulsified 1:1 in CFA (Difco Laboratories, Inc., Detroit, MI). These immunizing doses gave optimal proliferation.

**Proliferation Assays.** 8-10 d after immunization, draining LNs and spleens were collected. Lymphocyte suspensions were prepared by dissociating LN and spleen tissues separately; cells were washed twice in HBSS medium. The LN or spleen cells (5 × 10<sup>5</sup> cells per well) were incubated separately in serum-free HL-1 medium (Ventrex Laboratories, Portland, ME) with 2 mM glutamine for 5 d in flat-bottomed, 96-well microtiter wells without peptide, with different concentrations of relevant or irrelevant peptides, or with purified protein derivative of the tubercle bacillus (PPD; 700 U/ml) (Evans Medical Ltd., Langhurst, UK). The culture wells were pulsed with 1 μCi of [<sup>3</sup>H]thymidine for the last 18 h of the assay. Cells in each well were harvested onto printed Filtermat A paper (Wallac, Turku, Finland) with a semiautomatic cell harvester (Skatron Instruments, Sterling, VA), and beta emissions were measured in a beta counter (Beckman Instruments, Inc., Fullerton, CA). Results are recorded as mean cpm in triplicate wells; deviations from the mean were <10% for all experiments. Positive control responses were those to PPD. Negative controls were wells without antigen and wells containing an irrelevant peptide. Results are shown as SI: the ratio of cpm obtained with cells cultured in vitro with the relevant peptides versus cpm of cells incubated with medium alone, or as the mean triplicate Δcpm, i.e., actual values of cpm obtained with cells stimulated in vitro with the relevant peptide minus the background (cells incubated with medium alone).

**Class II-Peptide-binding Assay.** Class II-peptide-binding assays were performed using purified I-E<sup>d</sup>, I-A<sup>d</sup>, and I-A<sup>u</sup> class II molecules (in vitro I-E<sup>u</sup>-specific binding assays are not available), as previously described (10). In brief, purified mouse class II molecules (5-500 nM) were incubated with 5 nM <sup>125</sup>I-labeled peptides for 48 h in PBS containing 5% DMSO in the presence of a protease inhibitor mixture. Class II-peptide complexes were separated from free peptide by gel filtration on Sephadex G-50 or TSK2000 columns (Tosoh, Montgomeryville, PA), and the fraction of peptide bound was calculated as described (10). In the inhibition assays, peptide inhibitors were typically tested at concentrations ranging from 1.2 ng/ml to 120 μg/ml. The data were then plotted, and the dose yielding 50% inhibition was measured. Each peptide was tested in two to four completely independent experiments.

**Inhibition of Peptide-induced T Cell Proliferation by mAbs to Different Murine MHC Class II Molecules and to CD4<sup>+</sup> T Cells.** Various dilutions of culture supernatants or purified mAbs to class II molecules were added to LN or spleen cell cultures or peptide-reactive T cell line cultures containing the relevant peptides. The following mAbs were used: MK-D6 (anti-I-A<sup>d</sup>) (11), 10.2.16 (anti-I-A<sup>k,u</sup>) (12, 13), 14-4-4S (anti-I-E<sup>α,β,d</sup>) (13, 14), 34.4.4S (anti-I-E<sup>d</sup>), and GK1.5 (anti-CD4). (Since I-A<sup>z</sup> and I-E<sup>z</sup>, the designations for NZW class II genes, are identical to I-A<sup>u</sup> and I-E<sup>u</sup> [15], we have used the latter throughout this report.)

**Establishment of Peptide-specific T Cell Lines and Hybridomas.** T cell lines reactive to A6H peptides were prepared from LN cells of adult BWF1 and one other strain (NZB for p34, BALB/c for p58, and NZW for p84), immunized s.c. with 50 μg of individual peptide in PBS, in 1:1 emulsion with CFA. After 10 d, the draining LNs were collected, and single-cell suspensions were cultured at a concentration of 5 × 10<sup>5</sup> cells/ml in DMEM supplemented with 0.05 mM 2-mercaptoethanol, 2 mM l-glutamine, 1 mM pyruvate, gentamicin, and 10% FCS (complete medium) and with either rIL-2 or rIL-4. After 7-10 d, the cells were stimulated with the relevant peptide and syngeneic irradiated splenic cells. The peptide specificity

of T cell lines was confirmed by incubating  $2 \times 10^4$ – $10^5$  cells per well with  $1$ – $5 \times 10^5$  irradiated syngeneic splenic cells in the absence or presence of different concentrations of peptides. Proliferation of T cell lines was measured by [ $^3$ H]thymidine incorporation for the last 18 h of a 3-d culture. Peptide-specific cell lines were maintained by periodic restimulation with the relevant peptide and rIL-2 or rIL-4 every 10–14 d.

T cell hybridomas were produced by fusion of peptide-reactive T cell lines from BWF1 mice with the  $\alpha$ - $\beta$  variant of BW5147 (11). Peptide-specific hybridomas were subcloned, and the most reactive ones were selected for study.

**Detection of Lymphokines in Culture Supernatants.** Mice were immunized with various doses of peptides in CFA. 9 d later, LN and spleen cells were collected and separately cultured with medium alone, with PPD, or with the relevant peptide. Culture supernatants were collected after 24–30 h for IL-2 detection and after 48–60 h for detection of IFN- $\gamma$ , IL-4, IL-5, and IL-10. IL-2 levels were detected by a standard bioassay using an IL-2 indicator cell line, a subclone of CTLL-2, which does not respond to rIL-4 (16). The proliferative responses of this cell line were blocked by the anti-murine IL-2 mAb, S4B6 (17). IL-2 standard curves were generated using rIL-2 (PharMingen, San Diego, CA). IL-4 levels were detected by a standard bioassay using an IL-4-dependent cell line, CT.4S (18) (kindly provided by Dr. William Paul, National Institutes of Health, Bethesda, MD). Proliferative responses of CT.4S were blocked by the anti-IL-4 mAb 11B.11 (19) (kindly provided by Dr. Craig Reynolds, Biological Response Modifiers Program, National Cancer Institute, Frederick, MD). IL-4 was also detected by a capture ELISA using mAb pairs purchased from PharMingen. Standard curves were generated using rIL-4, which was generously provided by Dr. Steven Gillis (Immunex Corp., Seattle, WA). IFN- $\gamma$ , IL-5, and IL-10 levels were determined by a capture ELISA using purified mAb pairs and rIFN- $\gamma$ , rIL-5, and rIL-10 as standards (PharMingen) (20).

**Testing Pathogenic Potential of A6H Peptides in BWF1 Mice.** Two strategies were used: immunization with peptides in adjuvants and adoptive transfer of peptide-reactive T cell lines. (a) Immunization of young BWF1 mice with p34 or p84: 12-wk-old BWF1 mice were immunized s.c. every 2 wk, at first with 100  $\mu$ g of individual peptide in 1:1 emulsion with CFA and subsequently with peptide in alum, until 20 wk of age. Control mice received PBS in CFA followed by PBS in alum. In another experiment, 11-wk-old BWF1 mice were immunized s.c. once either with all three determinants (p34, p58, and p84 separately in CFA on three consecutive days) or with a combination of two control peptides (p93 and HEL p106) in CFA. Both control peptides bind MHC class II molecules and are immunogenic in BWF1 mice, p93 binds I-A<sup>a</sup> as does p84, and HEL p106 binds I-E<sup>d</sup>, as does p34. (b) Adoptive transfer of peptide-specific T cell lines: a p84-reactive T cell line, which was maintained by periodic restimulation with peptide, irradiated syngeneic APCs, and IL-4, was activated by peptide + IL-2 + IL-4 + APCs 3 d before transfer. On the day of transfer, these cells were purified on Ficoll-Hypaque (Pharmacia, Piscataway, NJ), washed twice, and dissolved in HBSS.  $1$ – $4 \times 10^6$  cells per mouse were injected i.p. at times 0 and at 2 wk. Control mice received HBSS alone.

**Assessment of Clinical Disease.** BWF1 mice described in the above-mentioned experiments were monitored for survival and proteinuria and were bled for blood urea nitrogen (BUN), plasma creatinine, and IgG anti-dsDNA Ab. Proteinuria was estimated by examination of fresh urine using Albustix (Ames, Elkhart, IN) on a scale of 0 to 4+, where 0/trace = negative, 1+ = 30, 2+ = 100, 3+ = 300, and 4+ = >2,000 mg/dl. BUN was estimated using Azostix (Ames) (21). Plasma creatinine was estimated by a

colorimetric method using kits and standards purchased from Stanbio Laboratories (San Antonio, TX). In normal (10–40-wk-old BALB/c) mice, plasma creatinine levels ranged from 0.3 to 0.6 mg/dl. In unmanipulated BWF1 females, creatinine levels in pooled plasma of groups of eight mice at 10, 20, 30, and 40 wk of age were 0.6, 0.6, 1.3, and 4.0 mg/dl, respectively.

**Measurement of Serum IgG Anti-double-stranded (ds) DNA Ab.** Antibodies to dsDNA were measured in serum samples by a standard ELISA assay using calf thymus DNA, as previously described (21). Mean triplicate OD 405 nm values were recorded at serial serum dilutions. Anti-DNA titers are expressed as units per milliliter, using a reference positive standard of pooled serum from 8-mo-old BWF1 mice. A 1:100 dilution of this standard serum was arbitrarily assigned a value of 100 U/ml.

**Statistical Analysis.** Survival analysis using the log rank (Mantel-Haenszel) test was done to compare the proportion of surviving mice and the proportion of mice with no severe proteinuria in control and test groups. Student's *t* test and Mann-Whitney U test were also done to compare disease parameters between control and peptide-manipulated groups.

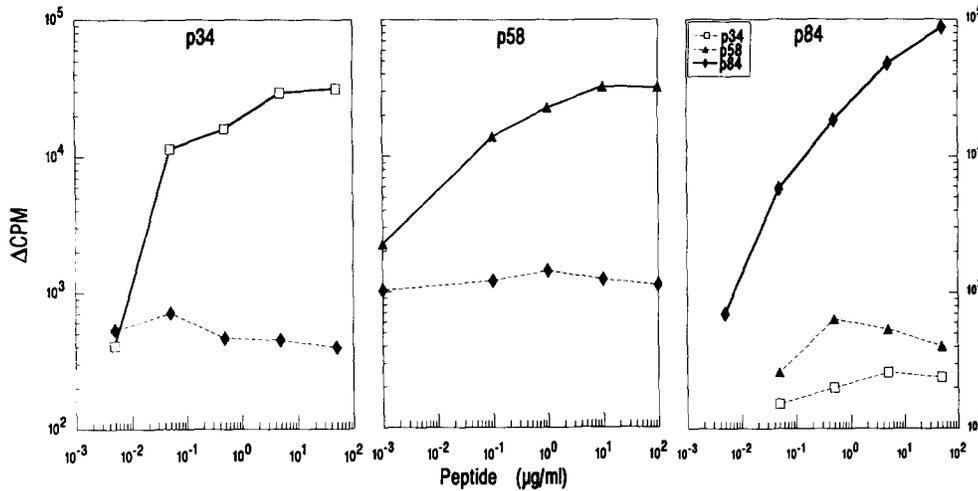
## Results

**Identification of T Cell Determinants in the V<sub>H</sub> of Anti-DNA mAb A6.1.** Using 12-mer overlapping peptides representing the entire amino acid sequence of the V<sub>H</sub>DJ<sub>H</sub> of mAb A6.1 (21), we found several clusters of overlapping peptides that consistently induced in vitro proliferative responses in splenic T cells from young nonimmunized BWF1 mice. The most stimulatory peptides in these clusters were p34 from the CDR1/FR2 region, p58 from the CDR2/FR3 region, and p84 from the FR3/CDR3 region (Fig. 1). In three additional BWF1 anti-DNA mAbs (two using the V<sub>H</sub> J558 gene family and one using 7183) (21), several peptides, including those similar to A6H determinants, were able to induce spontaneous proliferative responses (Singh, R. R., F. Ebling, E. Sercarz, and B. H. Hahn, manuscript submitted for publication).

**T Cell Immunogenicity of A6H Peptides in Young BWF1 Mice and MHC-identical Strains.** We compared the immunogenicity of these determinants in BWF1 females, in their parental strains, and in other MHC-identical strains. More specifically, the mouse strains bearing different combinations of H-2<sup>d</sup> and H-2<sup>u</sup> class II molecules (as in BWF1 mice) were immunized with the various peptides in CFA (Fig. 2), and 8–10 d later, LN cell proliferation assays were performed. A6H p34 elicited proliferative responses only in I-E<sup>d</sup>-bearing strains (i.e., BWF1, BALB/c, and NZB) and not in B10.GD, NZW, or B10.PL, whereas p84-induced responses were restricted to H-2<sup>u</sup> strains (BWF1, NZW, and B10.PL). A6H p58 elicited proliferative responses in either I-E<sup>d</sup>- or H-2<sup>u</sup>-bearing strains, but not in the strain lacking these class II molecules (B10.GD mice). In terms of the magnitude of the responses observed, p34 or p84 induced strong LN and spleen cell proliferative responses, whereas the response to p58 was weaker (Fig. 2).

To characterize further the responses against these determinants, peptide-specific T cell lines were generated from BWF1 mice immunized with individual peptides. Each T cell line reacted specifically to the immunizing peptide (Fig.





**Figure 3.** A6H peptide-derived T cell lines show no cross-reactivity with unrelated peptides. Proliferative responses of T cell lines derived from BWF1 mice immunized with the individual peptide ( $\square$  p34;  $\blacktriangle$  p58; and  $\blacklozenge$  p84) were tested for their specificity. A standard proliferation assay of these T cell lines was performed in the presence of relevant (—) or irrelevant (---) A6H peptides. Background cpm values ranged from 1,200 to 4,200. Results are expressed as  $\Delta$ cpm and represent one of at least three experiments.

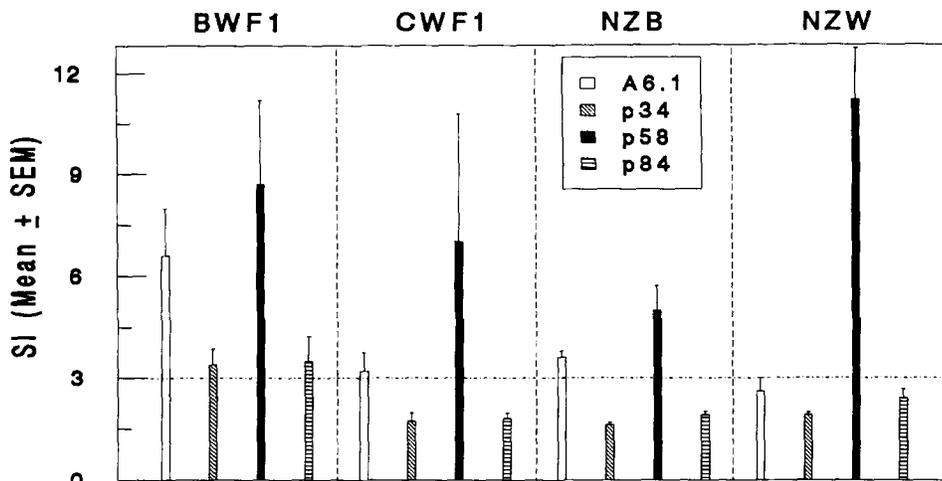
**Table 1.** Binding of A6H Peptides to Mouse Class II Molecules

Peptide	I-E <sup>d</sup>	I-A <sup>d</sup>	I-A <sup>u</sup>
	$\mu$ M	$\mu$ M	$\mu$ M
p34	0.044	>700	297
p58	18	>700	193
p84	>700	>700	0.087
p93	>700	>700	0.29

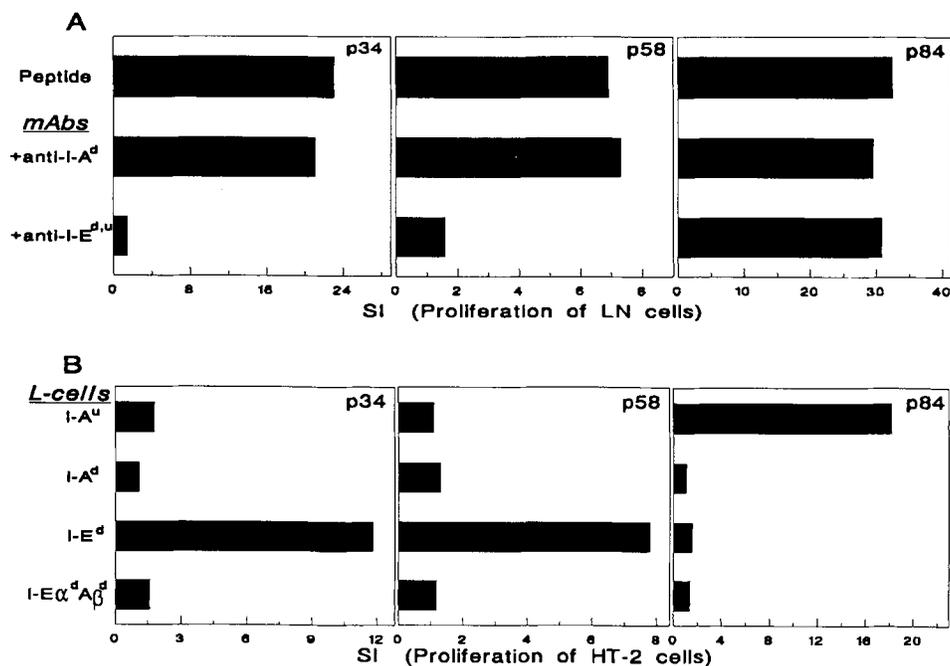
The amount of each peptide required to inhibit the binding by 50% of <sup>125</sup>I-labeled  $\lambda$  repressor 12-26 to I-E<sup>d</sup> and <sup>125</sup>I-labeled RO IV to I-A<sup>d</sup> and I-A<sup>u</sup> was measured (10). For each assay, the 50% inhibitory concentrations of a control standard inhibitor was also measured. These values were 0.15  $\mu$ M for  $\lambda$  repressor 12-26 binding to I-E<sup>d</sup>, 0.11  $\mu$ M for binding of OVA 323-336 to I-A<sup>d</sup>, and 8.0 nM for binding of RO IV to I-A<sup>u</sup>. The values represent the average of two to four completely independent experiments. Determinants p34, p58, and p84 elicit proliferative responses in naive BWF1 T cells; p93 does not.

I-E<sup>d</sup> molecules, marginally to I-A<sup>u</sup>, and not at all to I-A<sup>d</sup>. In contrast, p84 bound well to I-A<sup>u</sup>, but to neither I-A<sup>d</sup> nor I-E<sup>d</sup>. Finally, p58 bound relatively weakly to I-E<sup>d</sup>, marginally to I-A<sup>u</sup>, and not at all to I-A<sup>d</sup>.

**Determination of Class II Restriction of A6H Peptides.** BWF1 mice could present peptides in each of four class II molecules and, possibly, in their hybrids (15, 23). To determine class II restriction further, LN cell cultures from peptide-immunized BWF1 mice were stimulated in vitro with the relevant peptide and incubated with various dilutions of mAbs to class II molecules. As shown in Fig. 5 A, p34-induced proliferation was inhibited by anti-I-E<sup>d</sup> but not anti-I-A<sup>d</sup>. Proliferation induced by p84 in BWF1 and in NZW mice was not inhibited by anti-I-A<sup>d</sup> or anti-I-E<sup>u,d</sup>, suggesting that this peptide, in agreement with the direct binding data, is presented by I-A<sup>u</sup>. Finally, proliferation elicited by p58 was inhibitable by mAb 14.4.4S (anti-I-E<sup>u,d</sup>) in BWF1 (Fig. 5 A), BALB/c, and NZW (data not shown) mice, suggesting that both I-E<sup>d</sup> and I-E<sup>u</sup> molecules can present the p58 peptide.



**Figure 4.** Peptide p58 is immunodominant, and p34 and p84 are cryptic in most animals tested. 6–8-wk old BWF1 mice ( $n = 8$ ) and four each of CWF1 (H-2-matched) and parental (NZB and NZW) strains were immunized with purified A6.1 in CFA. Recall proliferative responses to the whole molecule and to the individual peptides were assayed in LN cells. Results are expressed as mean  $\pm$  SEM SI at equimolar concentrations of A6.1 or peptides. Background and PPD-positive control cpm  $\times 10^3$  values ranged from 1.6 to 2.4 and 88 to 167, respectively. Results from one of four similar experiments are shown here.



**Figure 5.** MHC class II restriction of A6H peptide-induced responses. (A) T cell proliferation induced by A6H peptides is inhibited by mAbs to class II molecules. LN cells from BWF1 mice immunized with peptides were incubated with an optimal dose of relevant peptide and increasing dilutions of mAbs. Results are expressed as SI from one of three similar experiments. Background cpm values ranged from 900 to 1,800. A6H p34-induced proliferative responses were inhibited by a mAb to I-E<sup>d</sup> but not by anti-I-A<sup>d</sup>, whereas p84-induced proliferation was not inhibited by anti-I-E<sup>d,u</sup> or anti-I-A<sup>d</sup>. (Anti-I-A<sup>u</sup> mAb 10.2.16 was stimulatory in all our experiments on BWF1 and NZW mice.) A6H p58-induced proliferation was inhibited by anti-I-E<sup>d,u</sup> but not by anti-I-A<sup>d</sup>. (B) Presentation of A6H peptides to peptide-specific T cell hybridomas or lines by L cells transfected with class II molecules. Proliferation of HT-2 (an IL-2/IL-4-sensitive cell line), cultured in the presence of supernatants obtained from T cell hybridomas or lines incubated with relevant peptides and various L cell transfectants, is shown as SI. Results are from one experiment representative of three. L cells transfected with I-E<sup>d</sup> but not those transfected with I-A<sup>d</sup>, I-E<sup>d</sup>Aβ<sup>d</sup>, or I-A<sup>u</sup> presented p34 to its specific T cell line or p58 to its specific T cell hybridoma, whereas the I-A<sup>u</sup> transfectant presented p84 to a p84-reactive hybridoma.

vant peptides and various L cell transfectants, is shown as SI. Results are from one experiment representative of three. L cells transfected with I-E<sup>d</sup> but not those transfected with I-A<sup>d</sup>, I-E<sup>d</sup>Aβ<sup>d</sup>, or I-A<sup>u</sup> presented p34 to its specific T cell line or p58 to its specific T cell hybridoma, whereas the I-A<sup>u</sup> transfectant presented p84 to a p84-reactive hybridoma.

Similarly, proliferation of the peptide-reactive T cell lines was inhibited by appropriate anti-class II mAbs (data not shown), consistent with observations in primary LN cell cultures.

Previous MHC assignments were confirmed by using murine class II-transfected L cells as APCs incubated with peptide-specific hybridomas or T cell lines (9). As shown in Fig. 5 B, p34 and p58 were presented by I-E<sup>d</sup>-transfected L cells, whereas p84 was presented by I-A<sup>u</sup>-transfected (6A2), but not by L cells transfected with I-A<sup>d</sup>, I-E<sup>d</sup>Aβ<sup>d</sup>, or I-E<sup>d</sup>.

Thus, several lines of evidence suggest that p34 binds I-E<sup>d</sup> and p84 binds I-A<sup>u</sup>. A6H p58 appears to bind both I-E<sup>d</sup> and I-E<sup>u</sup>.

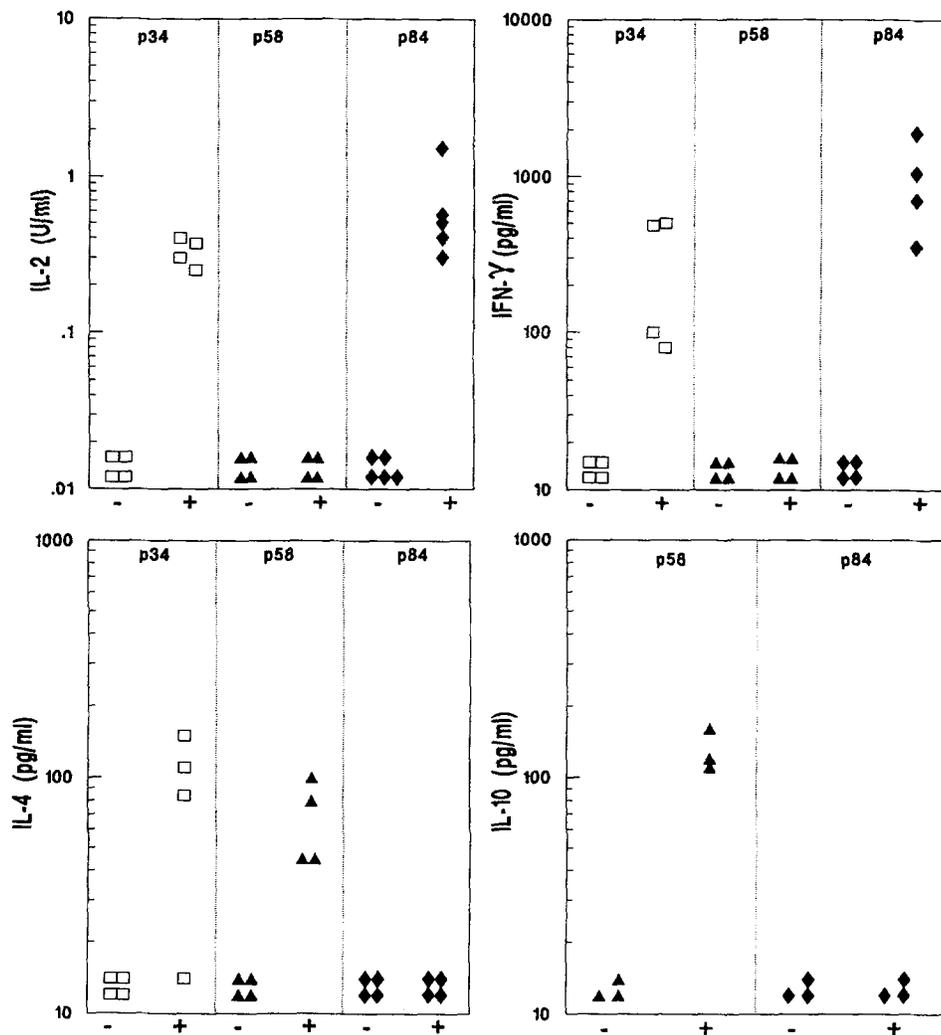
**Lymphokine Secretion Induced by A6H Determinants.** Primary LN cell culture supernatants from BWF1 mice immunized with various peptides were analyzed for cytokine content; results are shown in Fig. 6. Determinant p34 elicited release of IL-2 and IFN-γ, as well as IL-4, indicative of a Th0 (or Th1 + Th2) pattern of cytokine production. Culture supernatants from p58-immunized BWF1 mice contained IL-4/IL-10 and no IFN-γ/IL-2 (Th2 pattern), whereas supernatants from p84-immunized mice contained IFN-γ and IL-2 but not IL-4, IL-10 (Fig. 6), or IL-5 (data not shown), which is a Th1 pattern. The control peptide, HEL p106, also elicited a Th1 pattern (data not shown). In conclusion, each of the three peptides elicited a distinct cytokine profile, suggesting activation of distinct Th cell subsets (24).

**Immunization of Young BWF1 Mice with A6H Determinants**

**Increases IgG Anti-DNA Production, Accelerates Clinical Disease, and Reduces Survival.** Young BWF1 mice immunized with p34 or p84 had a significant increase in serum IgG anti-DNA Ab compared with PBS/CFA/alum-immunized mice ( $p < 0.05$ ) (Fig. 7 A). This increase in IgG autoAb was associated with an increase in the proportion of mice developing severe nephritis at an earlier age ( $p < 0.01$ ) (Fig. 7 B) and an increase in plasma creatinine levels ( $p < 0.05$ ) (Fig. 7 C). Mice were considered to have severe nephritis when they had  $\geq 300$  mg/dl proteinuria on at least two consecutive examinations 1 wk apart and BUN levels  $\geq 30$  mg/dl and/or plasma creatinine levels  $\geq 1$  mg/dl. 75% of mice developed severe nephritis 8–10 wk earlier in the p34- or p84-immunized groups than in PBS/CFA-immunized group. The increased IgG autoAb levels and impairment of renal function presumably led to decreased survival in peptide-immunized mice ( $p \leq 0.01$ ) (Fig. 7 D).

In another experiment, shown in Fig. 7 E, BWF1 mice immunized with a combination of all three determinants (p34 + p58 + p84) in CFA developed severe nephritis much earlier than mice immunized with a mixture of two control peptides, p93 + HEL p106, in CFA. A6H determinant-immunized mice also had increased serum anti-DNA Ab (mean  $\pm$  SEM,  $107 \pm 16$  vs  $63 \pm 15$  U/ml,  $p = 0.05$ – $0.1$ , Student's *t* test) and increased plasma creatinine (mean  $\pm$  SEM,  $0.99 \pm 0.1$  vs  $0.73 \pm 0.06$  mg/dl,  $p < 0.05$ , Student's *t* test).

**A p84-specific T Cell Line Hastens Onset of Severe Disease in Young BWF1 Mice.** Adoptive transfer of a p84-reactive



**Figure 6.** Different lymphokine secretion patterns induced in LN cell culture by A6H peptides. Culture supernatants from the LN cells of three to six immunized BWF1 mice were incubated in the absence (-) or presence (+) of relevant peptides and assayed for IL-2, IFN- $\gamma$ , IL-4, and IL-10. None of these cytokines were detected in the absence of antigen. The PPD-positive control levels of cytokines were similar in all three groups of mice immunized with individual peptides (data not shown). Results are shown as individual mouse values by a symbol. Note that p84 (◆) induced high levels of IL-2 and IFN- $\gamma$ , but not IL-4 and IL-10, whereas p58 (▲) induced the reverse cytokine pattern. A6H p34 (□) induced secretion of minimal levels of both types of cytokine (IL-2, IFN- $\gamma$ , and IL-4).

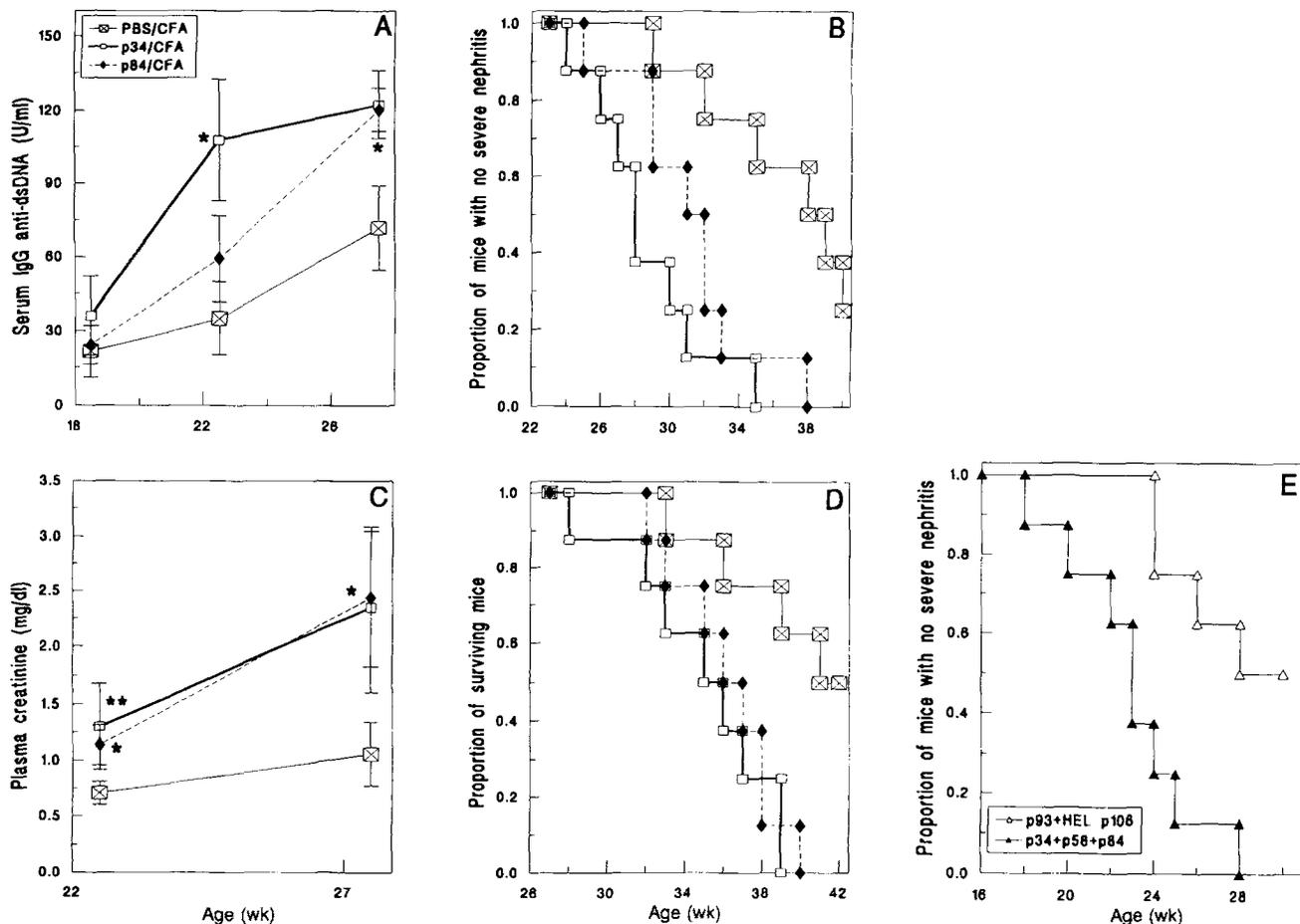
line resulted in an increase in IgG anti-DNA Ab, an earlier onset of severe nephritis, increased plasma creatinine levels, and decreased survival compared with mice injected with HBSS (Fig. 8, A-D).

## Discussion

There is ample evidence that B cells process endogenously synthesized Ig, and some of the resulting peptides are presented by class II molecules to T cells (25-27). Recent analyses of peptides eluted from class II molecules have provided further confirmation of very efficient natural B cell processing and presentation of endogenous Ig by class II molecules (28-30). It has been suggested that processing and presentation of self-Ig V region peptides leading to induction of Th or T regulatory cells may be important in regulation of immune responses and repertoire (25, 31, 32) and may also play a role in the induction and maintenance of autoimmune processes. To explore this possibility, we searched for autoreactive T cells capable of recognizing peptides derived from autoAb to DNA in autoimmune BWF1 mice. Multiple T cell determinants

were identified: three major determinants derived from four different anti-DNA mAbs share very similar motifs (Singh, R. R., F. Ebling, E. Sercarz, and B. H. Hahn, manuscript submitted for publication). Recently, peptides corresponding to CDR/FR regions similar to our A6H determinants (p58 and p84) have been eluted from class II molecules (28, 30). IgGV<sub>H</sub> 59-74 (XNADF~~K~~TPATLTV~~D~~KP), spanning CDR2/FR3, eluted from I-A<sup>b</sup> (28) is somewhat similar to p58 (A6.1 58-69 in the CDR2/FR3). Similarly, peptide (XSAV~~V~~YXAKRRQ), corresponding to the FR3/CDR3 region of Ig V<sub>H</sub>, eluted from HLA DR2/DR5 (30) is quite similar to p84 (A6.1 V<sub>H</sub> 84-95 in the FR3/CDR3) (Fig. 1).

Analysis of the hierarchy in determinant dominance in A6.1 revealed that p58, which showed the weakest binding to the class II molecule I-E<sup>d</sup>, proved to be the dominant determinant. Apparently, immunodominance in this instance depends on preferential processing of the determinant, perhaps owing to its easier availability, high affinity TCR binding, or promiscuity for class II molecules (I-E<sup>d</sup> and I-E<sup>n</sup>), rather than affinity for MHC molecules (20, 33, 34). It is interesting that some cryptic determinants bind strongly to class II mole-



**Figure 7.** Upregulation of systemic autoimmunity by autoAb-derived T cell determinants. (A-D) Groups of eight 12-wk-old BWF1 mice were immunized once with p34 (□), p84 (◆), or PBS (◻), in a 1:1 emulsion with CFA and subsequently in alum emulsion every 2 wk until 20 wk of age. Results are shown as mean  $\pm$  SEM (for serum IgG anti-dsDNA Ab and for plasma creatinine levels) and as the proportion of mice surviving and the proportion of mice with no severe nephritis. (A) Mean  $\pm$  SEM IgG anti-DNA levels were increased in BWF1 mice immunized with p34 or p84 compared with levels in PBS/CFA-immunized mice (\* $p < 0.05$ , Student's *t* test). (B) The log rank (Mantel-Haenszel) test was done to compare the proportion of mice not developing severe nephritis, which was higher in the control group than in mice immunized with p34 ( $p = 0.001$ ) or p84 ( $p = 0.009$ ). (C) Mean  $\pm$  SEM plasma creatinine levels were elevated in peptide-immunized mice (\* $p < 0.05$ , \*\* $p = 0.05-0.1$ , Mann-Whitney U test). (D) The increase in anti-DNA Abs and renal abnormalities was associated with decreased survival in peptide-immunized mice. Survival analysis was done using a log rank (Mantel-Haenszel) test ( $p = 0.008$ , p34;  $p = 0.01$ , p84). Results of another experiment are shown in (E). Eight mice immunized with one injection each of p34, p58, and p84 in separate emulsions in CFA had a statistically significant increase in the proportion of mice developing severe nephritis compared with mice immunized with two control peptides ( $p = 0.004$ , log rank test).

cules, again stressing the fact that crypticity is not synonymous with weak MHC binding. Weaker proliferative responses to p58 after immunization with the peptide itself, compared with cryptic p34 or p84, may be caused by its poor MHC binding capacity. Normally, dominant self determinants induce tolerance (22). However, because of its low MHC-binding affinity, some p58-reactive T cells might escape negative selection and thus be responsible for the presence of autoreactive T cells specific for this determinant. Self-tolerance may have eliminated other immunodominant T cell reactivities against better MHC-binding peptides.

Correlation between peptide and MHC affinity and cytokine pattern has recently been shown in various systems (35, 36). High affinity interactions tend to result in the activation of

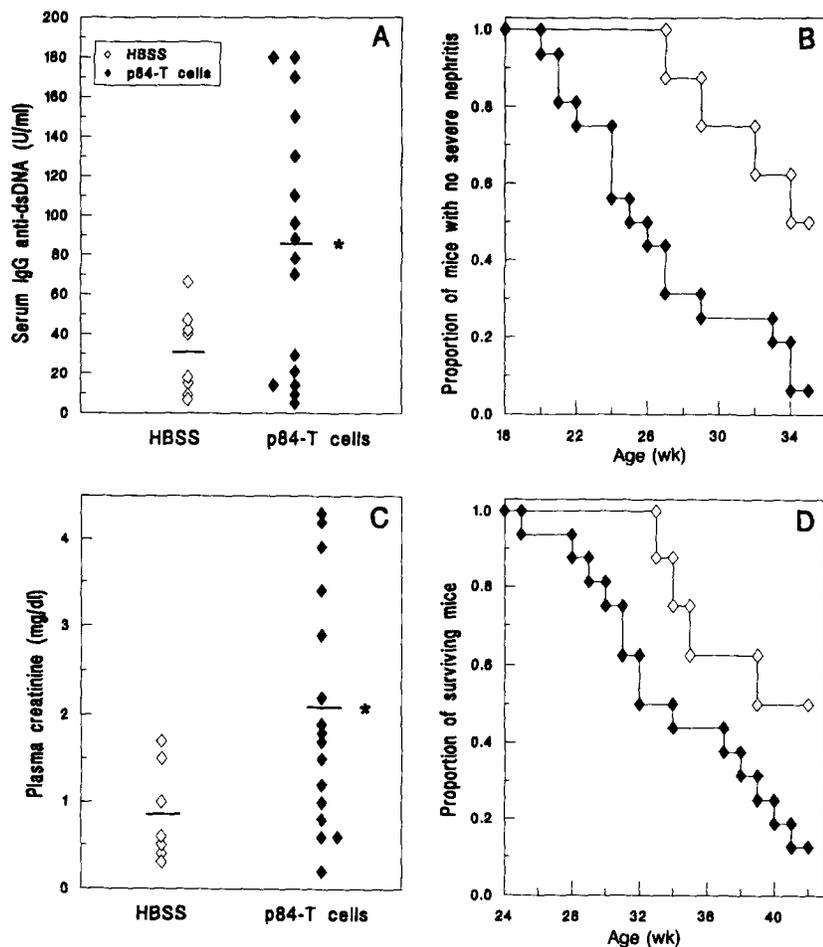
Th1 cells, as exemplified by p84 and HEL p106. By contrast, the Th2-like cytokine pattern induced by p58 may be due to its low MHC affinity. Autoantigen-specific Th2 responses may be of particular interest in the pathogenesis of autoAb-mediated systemic autoimmunity. Frequent injections of antibodies to IL-10, a Th2 cytokine, have been shown to delay substantially the onset of autoimmunity in BWF1 mice (37). In contrast, diversion of autoantigen-specific responses toward Th2 or inhibition of Th1 responses has been shown to be protective in several models of T cell-mediated, organ-specific autoimmune diseases (24, 38, 39). However, the strict requirement of Th2-like cells in the pathogenesis of SLE may be debated, since autoreactive Th clones of all subsets (Th0, Th1, and Th2) have been shown to induce IgG anti-DNA

production in vitro (7). Additionally, we have shown that anti-DNA Abs of the IgG2a isotype (usually Th1 dependent [40]) are capable of inducing lupus nephritis in normal mice (21). The Th2 cytokine-inducing p58 as well as p34 and p84, which elicit Th0 and Th1 cytokine patterns, respectively, increases in vitro IgG anti-DNA formation in ELISPOT assays (Ebling, F. M., R. R. Singh, and B. Hahn, manuscript in preparation). Since there may be cyclic evolution of Th1 and Th2 predominance, it would be interesting to follow their relative contributions at different stages of SLE.

It is interesting to analyze our findings in terms of the pathogenesis of autoimmunity in BWF1 mice. Spontaneous T cell autoimmunity to multiple V<sub>H</sub> region determinants from autoAb (anti-DNA mAbs), but not to those from a foreign (anti-HEL) mAb, develops before the onset of clinical disease in BWF1 mice. These determinants are recognized by autoimmune BWF1 T cells but not by T cells from MHC-matched, nonautoimmune CWF1 or normal BALB/c or NZW mice (Singh, R. R., F. Ebling, E. Sercarz, and B. H. Hahn, manuscript submitted for publication). Also, significant T cell proliferative responses to the whole autoAb molecule (A6.1) are seen in autoimmune but not in normal mice (Fig. 4). This might be caused by the presence of T cells that recognize regulatory/suppressive determinants, which do not permit reactivity to the whole self protein in normal mice. Alterna-

tively, autoreactive T cells may escape tolerance only in autoimmune backgrounds, or the Ig may be processed differently in autoimmune and normal mice.

Immunizations with dominant as well as cryptic determinants or adoptive transfer of a cryptic determinant-specific T cell line results in acceleration of clinical nephritis and decreased survival in BWF1 mice (reference 8 and Figs. 7 and 8). Thus, at the age when all of the peptide-treated BWF1 mice had succumbed, >60% of the PBS-treated animals were still alive. These determinants, when incubated with unprimed T and B cells from young BWF1 mice, increase IgG anti-DNA production in vitro (Ebling, F. M., R. R. Singh, and B. Hahn, manuscript in preparation). On the other hand, tolerance induction by i.v. administration of these peptides delays the onset of nephritis and prolongs survival in BWF1 mice (Singh, R. R., F. Ebling, E. Sercarz, and B. H. Hahn, manuscript submitted for publication). These predictable effects of determinants potentially involved in regulation support the notion that a Th-involved circuitry and spontaneous autoimmunity to these Ig-derived determinants constitute an immunoregulatory pathway in the pathogenesis of lupus, using some of the following mechanisms. (a) Regulatory/perpetuating role: these determinants may play a role in the maintenance of the autoimmune process, which has been induced earlier by some initiating antigen. A perpetual supply of these



**Figure 8.** Adoptive transfer of a p84-reactive T cell line to young BWF1 mice leads to a significant increase in IgG anti-DNA (A), an earlier onset of severe nephritis (B), increased levels of plasma creatinine (C), and a decrease in survival (D). 11-wk-old BWF1 mice received two i.p. injections of  $1-4 \times 10^6$  activated p84-reactive T cells ( $n = 16$ ,  $\blacklozenge$ ) or of HBSS ( $n = 8$ ,  $\diamond$ ) at a 2-wk interval. In A and C, serum IgG anti-dsDNA and plasma creatinine levels, respectively, of individual mice at 6 mo of age and means of each group (horizontal lines) are shown (\* $p < 0.01$ , Student's *t* test). The proportion of surviving mice (D) and the proportion of mice with no severe nephritis (B) in the two groups was compared using the log rank (Mantel-Haenszel) test ( $p = 0.055$  and  $0.008$ , respectively).

determinants from autoAb may induce the activation of peptide-specific Th cells, which in turn can help anti-DNA-producing B cells make more autoAb. (b) Induction or perpetuation by molecular mimicry: an Ig peptide that mimics a determinant on a self-Ag can induce autoimmunization. Recent work has shown that strikingly different antigenic determinants can cross-react with individual T cell clones (41). For example, administration of an MRL-lpr/lpr-derived Id-bearing Ig light chain, which has amino acid sequence homology with an antigenic region of the 70-kD U1 RNP polypeptide antigen, elicits autoAbs against the 70-kD U1 RNP autoantigen and DNA in BALB/c mice (42). (c) Induction/primary autoantigen role: because spontaneous autoimmunity to these peptides precedes the onset of clinical disease, it raises the possibility that they may have a role in disease

induction. Natural autoAbs and CD5 cells could provide one possible early source of these peptides. Peptides derived from natural autoAbs may activate Th cells capable of providing help for IgG anti-DNA Abs in genetically susceptible individuals with appropriate T cell repertoires. It has been shown that immunization with a polypeptide from the V<sub>H</sub> region of a human anti-Sm Ab induces autoAbs in BALB/c mice (43).

In conclusion, this study emphasizes that in autoimmune lupus mice, there are T cells capable of reacting to autoAb-derived peptides that have escaped the tolerance process. These T cells may be linked to an immunoregulatory pathway, in which determinants from Ig V<sub>H</sub> enhance autoAb production and clinical disease. Downregulation of this pathway may be of significant therapeutic benefit in autoAb-mediated systemic diseases.

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