Brief Definitive Report

Continuous Administration of Anti-Interleukin 10 Antibodies Delays Onset of Autoimmunity in NZB/W F_1 Mice

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Summary

We have previously shown that continuous administration of anti–interleukin 10 (anti-IL-10) antibodies (Abs) to BALB/c mice modifies endogenous levels of autoantibodies, tumor necrosis factor α (TNF-α), and interferon γ, three immune mediators known to affect the development of autoimmunity in "lupus-prone" New Zealand black/white (NZB/W)F_1 mice. To explore the consequences of IL-10 neutralization in NZB/W F_1 mice, animals were injected two to three times per week from birth until 8–10 mo of age with anti-IL-10 Abs or with isotype control Abs. Anti-IL-10 treatment substantially delayed onset of autoimmunity in NZB/W F_1 mice as monitored either by overall survival, or by development of proteinuria, glomerulonephritis, or autoantibodies. Survival at 9 mo was increased from 10 to 80% in anti-IL-10–treated mice relative to Ig isotype-treated controls. This protection against autoimmunity appeared to be due to an anti-IL-10–induced upregulation of endogenous TNF-α, since anti-IL-10–protected NZB/W F_1 mice rapidly developed autoimmunity when neutralizing anti-TNF-α Abs were introduced at 30 wk along with the anti-IL-10 treatment. Consistent with the protective role of anti-IL-10 treatment in these experiments, continuous administration of IL-10 from 4 until 38 wk of age accelerated the onset of autoimmunity in NZB/W F_1 mice. The same period of continuous IL-10 administration did not appear to be toxic to, or cause development of lupus-like autoimmunity in normal BALB/c mice. These data suggest that IL-10 antagonists may be beneficial in the treatment of human systemic lupus erythematosus.

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The (New Zealand black × New Zealand white [NZB × NZW[1]]) F_1 hybrid mouse develops a severe autoimmune disease that closely resembles SLE in humans (1–3). NZB/W F_1 mice spontaneously develop a fatal immune complex–mediated glomerulonephritis around 6–9 mo in female animals, and 12–18 mo in male animals. Previous attempts to define the underlying cause of autoimmunity in NZB/W F_1 mice have focused on the potential role of MHC genes. Indeed, IFN-γ, a cytokine which upregulates expression of MHC class II antigens in a wide variety of cell types (4–6), accelerates the development of autoimmunity in NZB/W F_1 mice (7–10). More recently, several studies (11–14) have suggested that the TNF-α gene, which is located within the MHC complex (15–17), may be involved in the pathogenesis of lupus nephritis in NZB/W F_1 mice. These studies reveal that NZB/W F_1 mice produce exceptionally low levels of TNF-α, and that this correlates with a restriction fragment length polymorphism in the TNF-α gene (11), and a polymorphism in simple dinucleotide tandem repeats in the 5′ regulatory region of the TNF-α gene (13, 14). A causative role for these observations is implied by the fact that replacement therapy with recombinant TNF-α significantly delays development of nephritis in NZB/W F_1 mice (11, 12).

IL-10 is a cytokine produced by subsets of activated T cells, B cells, and macrophages which mediates a variety of both immunostimulatory and immunosuppressive properties in mouse and human in vitro assays (for a review see references 18 and 19). In a recent effort to evaluate the physiological role of IL-10, we have treated BALB/c mice continuously from birth until 8 wk of age with neutralizing anti-IL-10 Abs (20, 21). Consistent with the known in vitro properties of IL-10 (18, 19), anti-IL-10–treated mice are characterized by elevated levels of endogenous IFN-γ and TNF-α (20, 21). The elevated IFN-γ in turn leads to the depletion of a numerically small subset of B lymphocytes, termed Ly1, CD5, or B-1 B cells (20), a population from which most murine autoantibodies are derived (22). These studies in normal mice suggested that neutralization of IL-10 may produce some de-

[1] *Abbreviation used in this paper:* NZB/W, New Zealand black/white.

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sirable consequences in NZB/W F_1 mice, i.e., elevation of endogenous TNF-α and reduction of autoantibody production, and some undesirable consequences, i.e., elevation of endogenous IFN-γ. In this report, we directly address the overall effects of continuous anti-IL-10 treatment on development of autoimmunity in NZB/W F_1 female mice. Our data indicate that neutralization of IL-10 significantly delays onset of autoimmunity in these mice because of an upregulation of endogenous TNF-α.

Materials and Methods

Mice. NZB/W F_1 mice were bred in our colony at DNAX Research Institute using NZB females purchased from The Jackson Laboratory (Bar Harbor, ME) and NZW males purchased from Simonsen Laboratories (Gilroy, CA). Only female F_1 mice were used in this study because of their more rapid onset of autoimmunity.

Anti-IL-10 Treatment. Groups of 17–23 B/W F_1 female mice were treated with either a rat IgM (designated SXC.1) or a rat IgG1 (designated JES 2A5) mAb specific for murine IL-10. Details about the production and characteristics of these Abs are given elsewhere (23, 24). Similarly, groups of 21–23 age-matched B/W F_1 female mice were treated with either of two isotype-matched control mAbs, designated J5/D (IgM) or GL113 (IgG1). Anti-IL-10 and isotype control mAbs were harvested as ascites from athymic nude (nu/nu) mice, purified by two sequential ammonium sulfate precipitation steps, dialyzed against PBS, and quantitated by protein electrophoresis and measurement of optical density. Treatment consisted of 3 parts: during the first week after birth, 0.2 mg of mAb per mouse was injected intraperitoneally either four or two times per week for the IgM and IgG1 Abs, respectively. During the second week, 0.5 mg of mAb per mouse was injected intraperitoneally either three or two times per week for the IgM and IgG1 Abs respectively; and from 3 wk of age and onwards, 1 mg of mAb per mouse was injected intraperitoneally either three or two times per week for IgM and IgG1 Abs, respectively.

Assessment of Renal Disease. Renal disease was assessed either via the development of proteinuria or by histological evaluation of glomerulonephritis. Proteinuria was measured colorimetrically using dip sticks (Albustix; Miles Laboratories, Inc., Elkhart, IN), and graded according to the following code: trace, 10 mg/dl; 1+, 30 mg/dl; 2+, 100 mg/dl; 3+, 300 mg/dl; and 4+, 1,000 mg/dl. Severity of glomerulonephritis was evaluated histologically by tissue sectioning and staining, and was graded on a 0–4+ scale based on the intensity and extent of histopathologic changes, as described previously by Berden et al. (25). Briefly, the score system for evaluating severity of glomerulonephritis was as follows: a grade of 0 was given to kidneys without glomerular lesions; 1+ lesions corresponded to minimal thickening of the mesangium; 2+ lesions contained noticeable increases in both mesangial and glomerular cellularity; 3+ lesions were characterized by the preceding conditions with superimposed inflammatory exudates and capsular adhesions; and in 4+ lesions, the glomerular architecture was obliterated in >70% of glomeruli, and tubular cast formation was extensive.

Autoantibody ELISA. Serum antibodies specific for double- or single-stranded DNA (ds or ssDNA) were quantitated by ELISA as described elsewhere (26). Briefly, 5 μg/ml ds- or ss-DNA was used to coat ELISA plates (Flow Laboratories, McLean, VA) in an overnight incubation at 4°C. Antigen-coated plates were subsequently blocked for 1 h with PBS containing 0.05% Tween 20, 0.02% NaN_3, and 0.1% BSA, then incubated for 1 h at room temperature with test or standard sera diluted from 1:100 in twofold serial dilutions. Plates were then washed with PBS-0.05% Tween 20, and incubated for 1 h with 1 μg/ml horseradish peroxidase (HRP)–conjugated anti–mouse IgG or IgM (Zymed Laboratories, Inc., S. San Francisco, CA). Absorbance was measured using a microplate reader (Vmax; Molecular Devices, Menlo Park, CA) 30 min after the addition of 1 mg/ml 2,2'-Azinobis (3-ethylbenzthiazoline-6-sulfonic Acid); ABTS (Sigma Chemical Co., St. Louis, MO). Anti-DNA titers were expressed as units, using a reference standard of pooled serum from 4-mo-old MRL/MpJ-lpr/lpr mice provided by Dr. Shelby Umland (Schering-Plough Corp., Kenilworth, NJ). A 1:100 dilution of this standard serum was arbitrarily assigned a value of 100 U/ml.

Serum Concentration of TNF-α. Serum levels of TNF-α were evaluated using a cytokine-specific ELISA, commercially available from Endogen, Inc. (Boston, MA).

Results

Continuous Administration of Anti-IL-10 Antibodies Delays Onset of Autoimmunity in NZB/W F_1 Mice. In normal mice, continuous administration of anti-IL-10 Abs from birth until adulthood alters the animals' levels of IFN-γ, TNF-α, and autoantibodies (20, 21), three mediators known to affect the development of autoimmunity in lupus-prone NZB/W F_1 mice (3, 7–14). To determine whether IL-10 neutralization in NZB/W F_1 mice may indeed influence their development of autoimmunity, groups of 20–23 female NZB/W

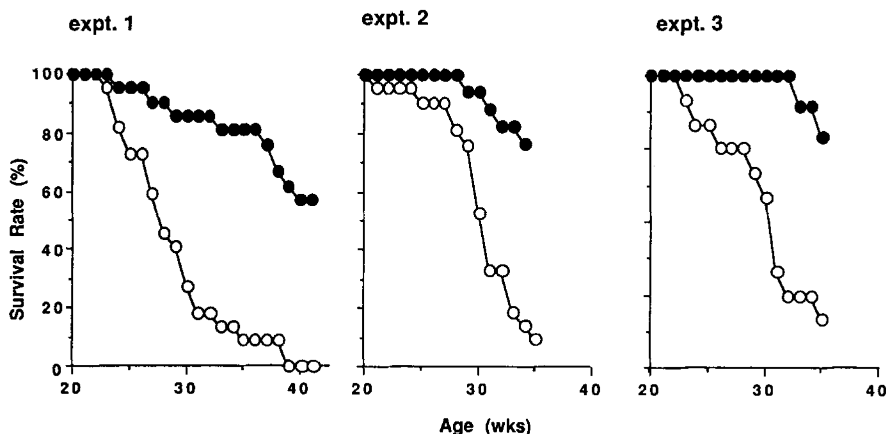

Figure 1. Anti-IL-10 Ab treatment delays onset of autoimmunity in NZB/W F_1 mice. (*Expt. 1*) Groups of 23 female mice were injected three times per week from birth and throughout the entire study with either SXC.1 rat IgM anti–mouse IL-10 Ab (●) or an isotype control Ab designated J5/D (○). Animal survival was monitored over the following 41 wk. (*Expts. 2* and *3*) Groups of 20 female mice were injected twice per week from birth and throughout the entire study with either JES 2A5 rat IgG1 anti–mouse IL-10 Ab (●) or an isotype control Ab designated GL113 (○). Animal survival was monitored over the following 35 wk.

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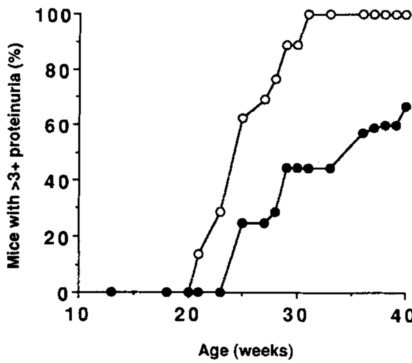

Figure 2. Development of proteinuria in NZB/W F_1 female mice treated with anti-IL-10 Abs. Groups of 22 female mice were injected three times per week from birth and throughout the entire study with either SXC.1 anti-IL-10 Ab (●) or an isotype control Ab (○). Development of renal disease was assessed by measuring proteinuria using Albustix dip sticks from weeks 12–42. Data show proportion of mice with more than 300 mg protein/dl in their urine. Similar results were obtained in two additional experiments.

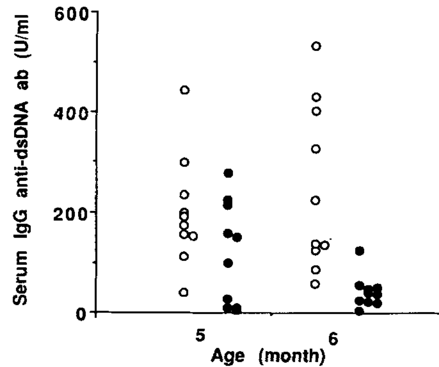

Figure 3. Autoantibody production in anti-IL-10–treated NZB/W F_1 mice. NZB/W F_1 female mice were injected with SXC.1 anti-IL-10 Ab (●) or an isotype control (○) three times per week from birth and throughout the entire study. Sera collected at 5 or 6 mo were evaluated for content of IgG Ab binding ds-DNA using an ELISA described in Materials and Methods. Each circle represents an individual mouse. Serum IgM anti-ds DNA Abs were also decreased in anti-IL-10–treated NZB/W F_1 mice (data not shown).

F_1 mice were injected three times per week from birth until 40 wk of age with either SXC.1 rat IgM anti–mouse IL-10 mAb, or equivalent amounts of an isotype control Ab designated J5/D. Specific details of the Ab administration throughout this time period are given in Materials and Methods. As shown in Fig. 1, expt. 1, isotype control Ab-injected mice died between 25 and 38 wk of age. Their survival curve was in fact indistinguishable from that of untreated control NZB/W F_1 mice maintained in the same DNAX animal facility (data not shown). In contrast, NZB/W F_1 mice injected continuously from birth with SXC.1 anti-IL-10 Abs showed a substantially enhanced survival curve over the 41-wk monitoring period (Fig. 1, *expt. 1*). Survival at 9 mo was increased from 10 to 80% in anti-IL-10–treated NZB/W F_1 mice relative to isotype control–treated NZB/W F_1 mice (Fig. 1, *expt. 1*). Similar results were obtained in two additional experiments of this nature, each using similar numbers of NZB/W F_1 mice per group, although monitoring the animals for slightly shorter time periods (Fig. 1, *expts. 2* and *3*). In experiments 2 and 3, animals were treated continuously from birth with either JES 2A5 rat IgG1 antimurine IL-10 Ab, or the isotype control GL113 Ab. Thus, this second anti-IL-10 Ab also prolonged the survival of NZB/W F_1 mice.

The survival curves shown in Fig. 1 presumably reflected death due to development of autoimmunity, suggesting that neutralization of IL-10 delayed development of autoimmunity in NZB/W F_1 mice. This assumption was validated by the fact that the protective effect of anti-IL-10 treatment of NZB/W F_1 mice was evident at the level of development of proteinuria (Fig. 2), serum autoantibody levels (Fig. 3), and glomerulonephritis (Figs. 4 and 5). By each of these criteria, continuous treatment of NZB/W F_1 mice from birth through 35–42 wk with anti-IL-10 Abs substantially delayed the onset of autoimmunity in these animals. The results shown in Figs. 2 –5 were reproduced in three individual experiments, two of which used a second antimurine IL-10 Ab, designated JES 2A5, and its isotype control (GL113).

IL-10 Neutralization Delays Onset of Autoimmunity in NZB/W F_1 Mice because of Elevation of TNF-α. Several studies have implicated atypically low endogenous TNF-α levels as a contributing factor in the pathogenesis of autoimmunity in NZB/W F_1 mice (11–14). Since IL-10 neutraliza-

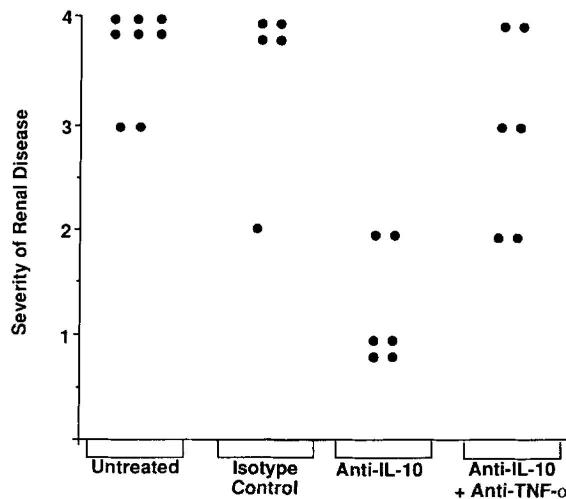

Figure 4. Histological evaluation of severity of renal disease in control and treated NZB/W F_1 mice. Kidneys were collected, sectioned, and stained by standard procedures. Severity of glomerulonephritis was graded according to the following score system: a grade of 0 was given to kidneys without glomerular lesions; 1+ lesions correspond to minimal thickening of the mesangium; 2+ lesions contained noticeable increases in both mesangial and glomerular cellularity; 3+ lesions were characterized by the preceding conditions with superimposed inflammatory exudates and capsular adhesions; and in 4+ lesions, the glomerular architecture was obliterated in >70% of glomeruli, and tubular cast formation was extensive. Results show number of animals in each group with a particular grading of renal disease, with each circle representing an individual mouse.

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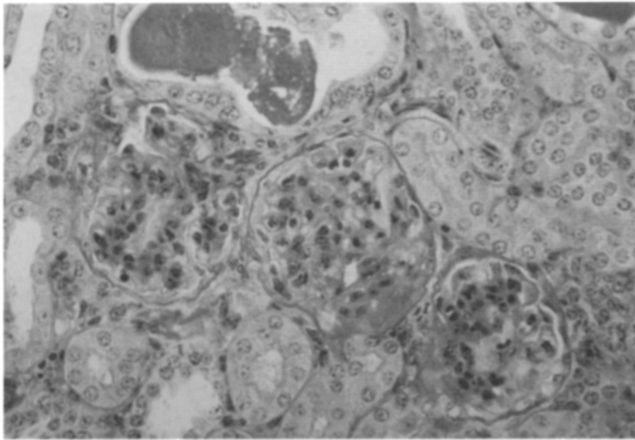

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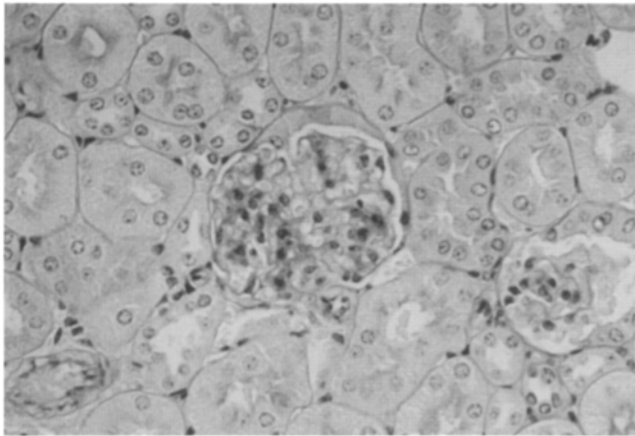

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Figure 5. Histological examination of kidneys from anti-IL-10–treated NZB/W F_1 mice (PAS staining; H250). (*A*) A typical grade 4 lesion observed in the isotype control–treated mice shown in Fig. 4. Note accumulation of amorphous PAS-positive materials in the mesangial matrix, and protein casts in renal tubules. (*B*) A grade 1 lesion in the anti-IL-10 Ab–treated group of mice. Note slight thickening of the mesangium.

tion in normal mice is accompanied by an increase in serum TNF-α levels (21), we considered whether the above protective effects of anti-IL-10 treatment of NZB/W F_1 mice could be explained by elevation of this cytokine. Indeed, treatment of NZB/W F_1 mice from birth until 8 mo of age caused a marked increase in serum TNF-α levels as compared with isotype-treated controls (Fig. 6). When NZB/W F_1 mice that had been treated from birth with anti-IL-10 Abs additionally received injections of neutralizing anti-TNF-α mAbs at weeks 30–35 of the experiment, the animals rapidly developed autoimmunity as indicated by a striking reduction in their survival (Fig. 7), as well as the development of both proteinuria (data not shown) and glomerulonephritis (Fig. 4). These data strongly suggest that the anti-IL-10–mediated delay in onset of autoimmunity in NZB/W F_1 mice is due to an elevation of endogenous TNF-α levels in these animals.

IL-10 Administration Accelerates the Onset of Autoimmunity in NZB/W F_1 Mice. The experiments described above suggest that IL-10 neutralization in NZB/W F_1 mice delays onset of autoimmunity in these animals by elevating their endogenous levels of TNF-α. This conclusion was supported by separate experiments which showed that continuous administration of IL-10 to NZB/W F_1 mice caused a small but significant acceleration of onset of autoimmunity. Groups of 20 female NZB/W F_1 mice were injected intraperitoneally three times per week from 4 to 35 wk of age with either 1 μg recombinant murine IL-10 or an equivalent volume of PBS. As shown in Fig. 8, expt. 1, PBS injected animals showed a typical survival curve, generally dying between 25 and 40 weeks. In IL-10 treated animals, the survival curve showed a more rapid onset of autoimmunity, with death occurring on average 5 to 6 weeks earlier (Fig. 8, *expt. 1*). Similar results were obtained in a second independent experiment involving identical numbers of animals monitored for a slightly shorter period of time (Fig. 8, *expt. 2*). The accelerated death of IL-10–treated NZB/W F_1 mice was accompanied by accelerated development of proteinuria and glomerulonephritis (data not shown), indicating that the survival curves reflected death

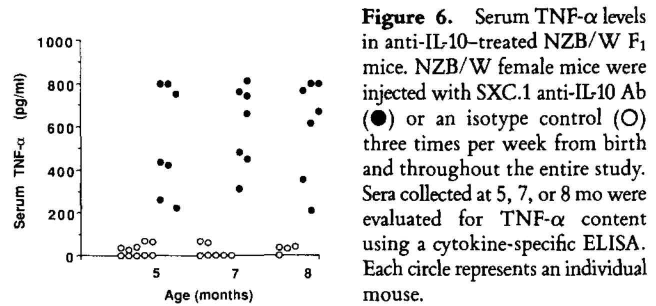

Figure 6. Serum TNF-α levels in anti-IL-10–treated NZB/W F_1 mice. NZB/W female mice were injected with SXC.1 anti-IL-10 Ab (●) or an isotype control (○) three times per week from birth and throughout the entire study. Sera collected at 5, 7, or 8 mo were evaluated for TNF-α content using a cytokine-specific ELISA. Each circle represents an individual mouse.

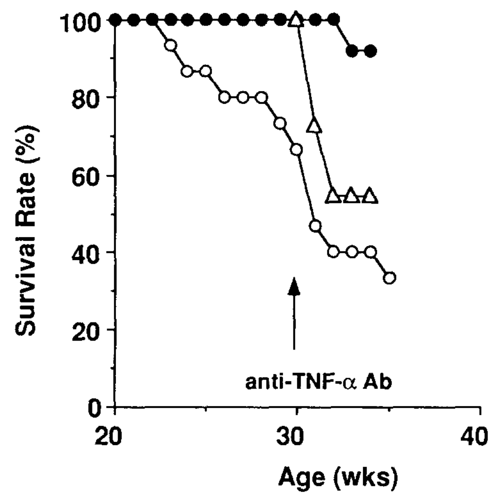

Figure 7. Anti-TNF-α Abs reverse anti-IL-10–mediated protection of NZB/W F_1 mice. Groups of 36 and 18 NZB/W female mice were injected twice a week with 2A5 anti-IL-10 Ab (●, △) or an isotype control Ab designated GL113 (○), respectively, from birth and throughout the entire experiment. Half of the animals injected with anti-IL-10 Ab additionally received XT22 anti-TNF Ab twice a week commencing at 30 wk after birth (△). Animal survival was monitored over a 34-wk period. Similar results were obtained in two additional experiments.

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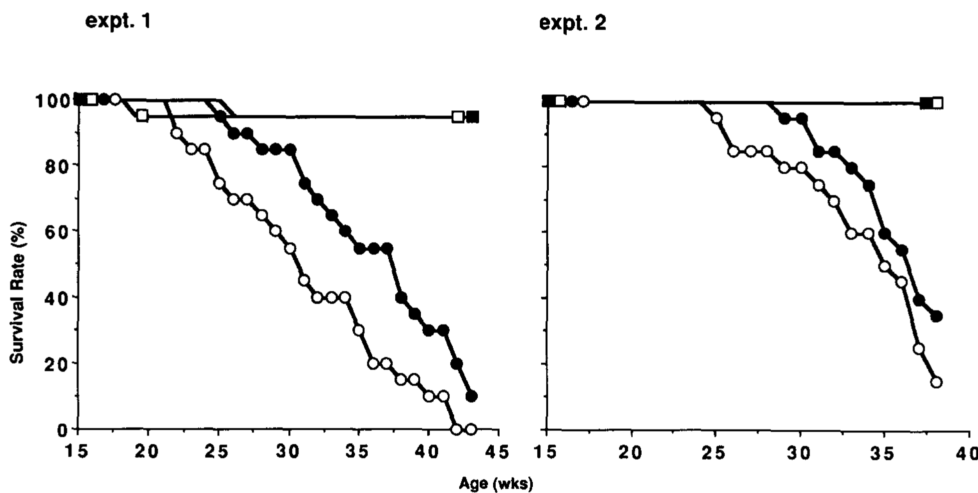

Figure 8. IL-10 administration accelerates onset of autoimmunity in NZB/W F_1 mice. Groups of 20 female NZB/W F_1 mice were injected three times per week from 4 wk of age and throughout the entire experiment with either 1 μg i.p. murine IL-10 (○) or an equivalent volume of PBS i.p. (●). The effect of identical treatment of groups of 20 BALB/c mice are shown for comparison (□,■).

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due to autoimmunity. Administration of equivalent amounts of IL-10 to normal BALB/c mice from 4 to 38–42 wk of age did not induce autoimmune disease in these animals, as evaluated by animal survival (Fig. 8) or development of proteinuria (data not shown).

Discussion

We show here that continuous treatment of lupus-prone NZB/W F_1 mice from birth until 38–42 wk of age with anti-IL-10 Abs substantially delays onset of autoimmunity in these animals. Conversely, continuous administration of IL-10 to NZB/W F_1 mice from 4 to 38 wk of age causes a significant acceleration in the development of autoimmunity in these animals, while having no overt consequence in normal BALB/c mice. The protective effect of anti-IL-10 Ab administration to NZB/W F_1 mice can be explained by the elevation of endogenous TNF-α levels that results from this treatment (21 and Fig. 6). This conclusion can be drawn from the fact that neutralizing anti-TNF-α Abs reversed the protective effect of anti-IL-10 treatment when introduced into the anti-IL-10–treated animals some 30 wk after experiment initiation (Fig. 7). These data predict a potential clinical use for IL-10 antagonists in treatment of human SLE. Such treatment may be particularly relevant to the subset of SLE patients expressing $DR2^+$ $DQw1^+$ histocompatibility antigens, since PBL from these patients produce atypically low levels of TNF-α (27). Conversely, the use of chronic long-term IL-10 administration may be contraindicated in SLE patients. On this point, it should be noted however that single-dose or short-term IL-10 administration had no significant effect on NZB/W F_1 mice (our unpublished observations), and long-term IL-10 administration had no significant effect on normal BALB/c mice (Fig. 8).

The mechanism of anti-IL-10–mediated protection of NZB/W F_1 mice from onset of autoimmunity suggests a protective role for TNF-α in this disease. Indeed this has already been demonstrated experimentally by direct cytokine administration (11, 12). While the basis of TNF-α–mediated protection has not yet been elucidated, several known properties of this cytokine suggest potential mechanisms of action in this disease model. One possible explanation relates to the fact that TNF-α significantly decreases IFN-γ–induced upregulation of MHC class II expression on different mature cells collected from NZB/W F_1 mice (14, 28). Aberrant upregulation of class II antigen expression is thought to be a causative mechanism in a variety of endocrine autoimmune diseases (29). Thus, suppression of this effect may be advantageous in NZB/W F_1 mice. Alternatively, whereas TNF-α does not suppress humoral immunity in normal or lupus mice (14, 30), there have been some reports that long-term administration of this cytokine causes suppression of cell-mediated immunity in these animals (30). The level of protection afforded by IL-10 neutralization in NZB/W F_1 mice is comparable to that previously obtained with direct administration of TNF-α (11, 12). The possibility of using either approach for treatment of SLE patients depends of course on translation of the current findings to the human disease situation, and the long-term toxicity of these reagents.

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