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LITHIUM CHLORIDE ENHANCES SURVIVAL OF NZB/W LUPUS MICE: INFLUENCE OF MELATONIN AND TIMING OF TREATMENT

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Abstract — Daily administration of 4 mg ^{6}LiCl to groups (15 mice/group) of female NZB/W mice starting at 8 weeks of age led to long-term survival (44 weeks of age) of 73% of the mice when injections were performed between 08:00 and 10:00 h and 67% of mice when injections were performed between 17:00 and 19:00 h. Untreated controls were dead by 34 weeks of age and the differences between untreated and treated groups was significant ($P \leq 10^{-4}$). In contrast, daily administration of melatonin (100 μg/mouse) did not significantly enhance survival when injections were performed between 17:00 and 19:00 h but did enhance survival when given between 08:00 and 10:00 h ($P \leq 10^{-3}$). Differences between the two melatonin groups was also significant ($P \leq 0.05$). Mice treated with Li plus melatonin exhibited survival curves identical to mice treated with Li alone. Therefore, the Li effect was dominant and survival was not altered by melatonin. Cessation of treatment in long-term survivors at 44 weeks of age led to the rapid death of 80% of the mice previously treated between 17:00 and 19:00 h (Li, Li + melatonin). In contrast, only 40% of the long-term survivors in the other groups had died by 66 weeks of age (22 weeks post-treatment). Thus the p.m. groups were less protected from disease reactivation than were the a.m. groups. Neither Li, melatonin, nor Li+melatonin influenced anti-gp70 or anti-ssDNA levels in serum, but Li treatment maintained renal function as determined by proteinuria scores. These findings indicate that the effectiveness of Li is probably not related to melatonin metabolism and immunomodulating influences, but the influence of other neuroendocrine variables cannot be eliminated.

Keywords: LiCl and SLE, NZB/W mice, LiCl and circadian rhythms, LiCl and melatonin, melatonin and murine SLE.

Lithium salts are an effective treatment modality in certain psychiatric conditions such as bipolar affective disorder (Johnson & Johnson, 1977; Johnson, 1984; Birch, 1988). In addition, Li salts can exhibit effects on immune regulation both *in vivo* and *in vitro* (reviewed in Hart, 1986, 1990, 1991a–c). The latter effects of Li salts on immune regulation have led to an investigation of the influence of Li in animal models of autoimmune disease, in particular many of the well-characterized murine models of diabetes (Zhao *et al.*, 1991; Lenz *et al.*, 1994) and systemic lupus erythematosus (SLE) (Krause *et al.*, 1992; Hart *et al.*, 1993, 1994; Hart & Lenz, 1993a, b).

In the NOD model of Type I diabetes, early treatment of female mice with low doses of LiCl (1–1.5 mg LiCl/day/mouse) were found to delay significantly pancreatic islet destruction and the development of disease parameters (Zhao *et al.*, 1991; Lenz *et al.*, 1994). In contrast, treatment of obese strain male mice, a model of Type II diabetes, with LiCl appeared to accelerate disease development (Zhao *et al.*, 1991). In murine models of SLE (Theofilopoulos & Dixon, 1987), initiation of Li treatment prior to the onset of overt disease activity in female NZB/W (Krause *et al.*, 1992; Hart *et al.*, 1994) or MRL-*lpr* (Hart *et al.*, 1993) mice led to enhanced survival. Initiation of treatment after disease activity was evident led to a dramatic decline in efficacy (Hart *et al.*, 1993, 1994). In contrast, early Li treatment of male MRL-*lpr* (Hart *et al.*, 1993) or male BXSB (Hart & Lenz, 1993a) mice was not effective in enhancing survival or inhibiting disease progression. Thus, there may be gender-dependent variables that are influencing the efficiency of Li. However, Li treatment of NZB/W females did not

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inhibit development of anti-nuclear or anti-DNA antibodies (Krause *et al.*, 1992; Hart *et al.*, 1994) and therefore the effectiveness of Li treatment does not appear to operate via estrogen-dependent steps in immune regulation. Interestingly, histological examination of kidneys from long-term survivors that were treated with Li revealed that treatment appeared to block the progression of tubulointerstitial renal disease without affecting glomerular disease (Krause *et al.*, 1992; Hart *et al.*, 1993; Done *et al.*, 1994). In addition, renal failure, the common cause of death in this model (Theofilopoulos & Dixon, 1987), was prevented.

The mechanism by which Li exerts this protective effect in female NZB/W mice has not been elucidated. It could directly affect specific targets in renal tissue or it could be an indirect effect. NZB/W mice have been reported to be deficient in tumor necrosis factor (TNF) (Jacob *et al.*, 1991; Gordon *et al.*, 1989), and Li can enhance TNF release and sensitivity (reviewed in Beyaert *et al.*, 1989). Thus, Li could enhance TNF function leading to enhanced survival. Li salts have also been shown to influence other circadian rhythms at the neuroendocrine level, specifically at the level of melatonin metabolism, which can in turn influence a number of other pluripotent neuroendocrine activities (Seggie *et al.*, 1987). As melatonin also has immunomodulating activities (Maestroni *et al.*, 1987; Pierpaoli & Maestroni, 1987), it is possible that Li is exerting its effect via this important mediator at multiple sites.

In previous studies (reviewed in Hart, 1986, 1990, 1991a–c), Li was administered as a single daily intraperitoneal injection in the morning between 08:00 and 10:00 h. Therefore, the present study was undertaken to determine whether the effectiveness of Li treatment was influenced by the time of day when the injections were made and whether the effectiveness was possibly related to melatonin metabolism.

EXPERIMENTAL PROCEDURES

Mice

Female NZB/W mice were obtained from Jackson Laboratory (Bar Harbor, MA, U.S.A.) at 6 weeks of age and maintained locally for 2 weeks prior to entrance into experiments. Mice were randomly allocated into groups of 15 mice and housed 5/cage in Thoren MaxiMiser Positive Individual Ventilation units with a HEPA filter system (Thoren Caging System Inc., Halseton, PA, U.S.A.). Deaths were recorded daily and, whenever possible, kidneys from dead mice were examined histologically to confirm that death was probably due to renal failure.

Reagents

^{6}LiCl (95.46% Li) was obtained from Oakridge National Laboratories (Oakridge, TN, U.S.A.), and was the same as that used previously (Hart *et al.*, 1993; Hart & Lenz, 1993b). Reagent grade melatonin was purchased from Sigma (St Louis, MO, U.S.A.). The dose of melatonin chosen for study was 100 μg/day/mouse, which is approximately 2–3.5 mg/kg, a concentration similar to those reported to have immunomodulating activity (Maestroni *et al.*, 1987; Pierpaoli & Maestroni, 1987). LiCl and melatonin solutions were prepared daily for injection in pyrogen-free water such that the desired dosage (4 mg ^{6}LiCl, 100 μg melatonin) could be given by 0.25 ml i.p. injection. Mice were injected daily with either LiCl, melatonin or an injection of both reagents at 08:00–10:00 h (a.m. groups), or 17:00–19:00 h (p.m. groups). Injections were performed 7 days/week at the intervals specified until death or sacrifice, or treatment was terminated at 44 weeks of age for survivors.

Proteinuria determination

Urinary protein was determined using the Ames Albustix method (Miles Inc., Etobicoke, ON, Canada). Urine was collected at regular intervals before 12:00 h and protein scores were assigned according to the supplier's scoring system (1 = 30 mg/dl; 2 = 100 mg/dl; 3 = 300 mg/dl; 4≥2000 mg/dl).

Analysis of mice at the time of sacrifice

Mice were bled for serum at sacrifice or when they appeared moribund. Kidneys were obtained after cervical dislocation, weighed and fixed in formalin.

Anti-single stranded DNA (ssDNA) antibody ELISA

Anti-ssDNA antibodies were determined by the ELISA method of Gunn (1986) utilized previously (Krause *et al.*, 1992; Hart *et al.*, 1993; Hart & Lenz, 1993a). Briefly, microtiter plates were coated with calf thymus ssDNA (Sigma), and blocked with bovine serum albumin. Serial dilutions (1/100–1/12,800) of serum were applied and incubated for 1 h at 37°C. Complexes which formed between the bound ssDNA and ssDNA-specific antibody were identified with an alkaline phosphatase system utilizing a goat anti-mouse IgG (Sigma). Optical density was determined with a Microplate Autoreader (Biotek Instruments) at 405 nm. The values are reported as individual values for a 1/100 dilution of serum.

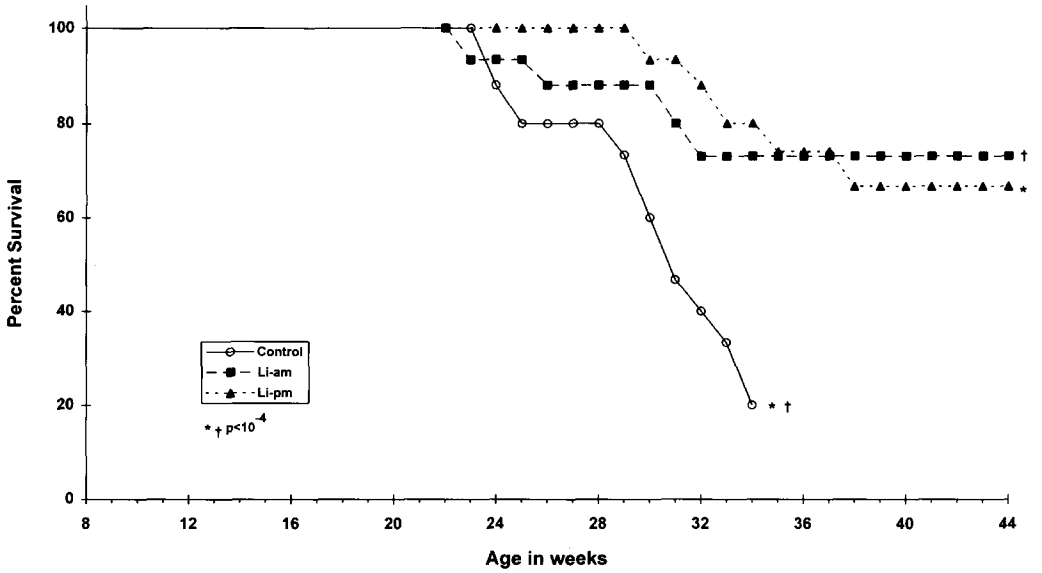

Fig. 1. Influence of the timing of exposure to 4 mg ^{6}LiCl on survival of NZB/W mice. Groups of 15 mice were untreated (○, control) or treated with 4 mg ^{6}LiCl between 08:00 and 10:00 h (■, Li-a.m.) or 17:00 and 19:00 h (▲, Li-p.m.) 7 days a week, and survival was monitored daily. At 34 weeks of age 12/15 untreated mice were dead and the remaining three mice were killed. Treatment was maintained until 44 weeks of age. At 44 weeks of age, the difference in survival between untreated and the treatment groups was significant ($P \leq 10^{-4}$).

Anti-gp70 IC assay

Serum levels of gp70-anti-gp70 immune complexes (gp70 IC) were determined by an ELISA combined with the precipitation of serum with polyethylene glycol (PEG; average mol. wt 6000; Siegtried, Zofingen, Switzerland) as described previously (Merino *et al.*, 1989). Briefly, 5 µl of serum samples were treated for 1 h at 4°C with 10% PEG. This treatment allowed only precipitation of antibody-bound gp70, but not free gp70. After washing the precipitates once, the quantities of gp70 in precipitates were determined by an ELISA. PEG precipitates were incubated in microtiter plate wells coated with affinity purified goat anti-serum gp70. After an overnight incubation at 4°C, the assay was developed with alkaline phosphatase-conjugated goat anti-serum gp70 antibodies and quantified by referring to a standard curve obtained from 2-month-old NZB sera with known amounts of gp70.

Histology

Kidneys were fixed in neutral buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin (H & E), periodic acid schiff (PAS) or periodic acid silver methenamine (PASM). The sections were assayed independently by two experienced observers blinded to the treatment protocol. The sections were scored on an arbitrary scale of 0 to +3, with 0 = normal, and 3 = most severe for degree of glomerular proliferation, crescent formation, capillary "spike" formation by silver stain, glomerulosclerosis, tubular atrophy, interstitial inflammation and vasculitis.

Statistical analysis

Significance of data was assessed by the utilization of one-way analysis of variance (ANOVA).

RESULTS

Influence of LiCl *and/or melatonin on NZB/W survival*

Groups of 15 mice/group were started on 4 mg ^{6}LiCl/day at 8 weeks of age and survival was monitored daily. As depicted in Fig. 1, injection of mice between 08:00 and 10:00 h or 17:00 and 19:00 h led to nearly identical enhancement of survival of NZB/W mice at 44 weeks of age. The difference between the Li-a.m. and Li-p.m. groups was not significant, as 11/15 mice survived in the a.m. group vs 10/15 mice in the p.m. group. In both groups survival was greatly enhanced compared to the untreated controls ($P \leq 10^{-4}$) (Fig. 1).

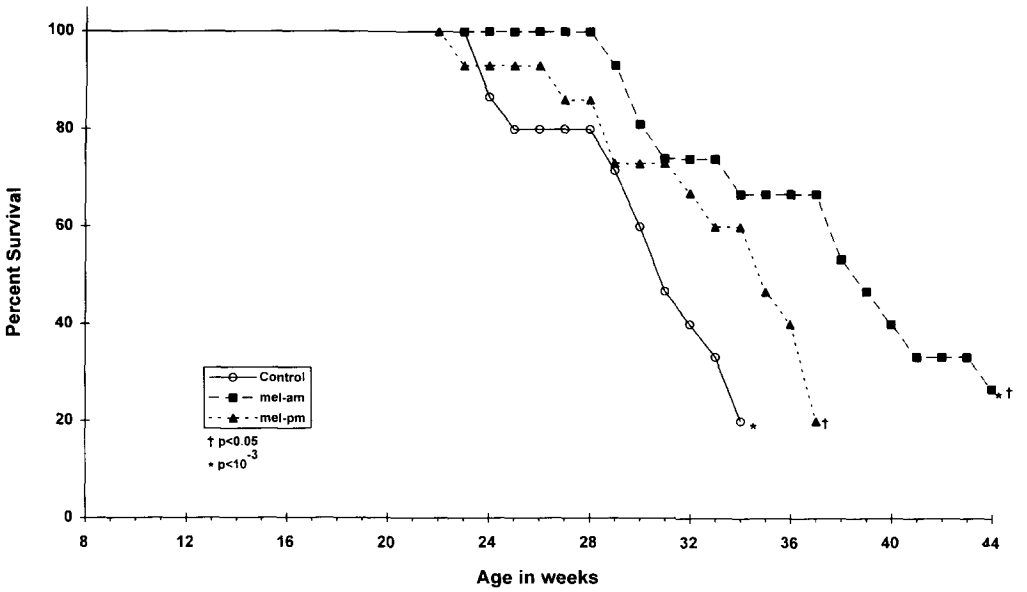

Fig. 2. Influence of the timing of exposure to melatonin on the survival of NZB/W mice. Groups of 15 mice were untreated (○, control) or treated with 100 μg melatonin between 08:00 and 10:00 h (■. mel-a.m.) or 17:00 and 19:00 h (▲, mel-p.m.) 7 days a week, and survival was monitored. Treatment was initiated at 8 weeks of age and maintained until 44 weeks of age. The difference between the untreated and melatonin-a.m. groups was significant ($P \leq 10^{-3}$), as was the difference between the melatonin-a.m. and melatonin-p.m. groups ($P<0.05$); however, the difference between the untreated and melatonin-p.m. groups was not significant.

When similar experiments were carried out with 8-week-old NZB/W mice receiving daily injections of 100 μg melatonin, a dose of melatonin (2–3.5 mg/kg body weight) comparable to those reported to alter immune responses in mice (Maestroni *et al.*, 1987; Pierpaoli & Maestroni, 1987), the results depicted in Fig. 2 were obtained. Survival of mice receiving melatonin between 17:00 and 19:00 h was only slightly enhanced compared to the controls and the difference was not significant (P = NS). In contrast, survival in the mice receiving melatonin between 08:00 and 10:00 h was significantly enhanced compared to the controls ($P \leq 10^{-3}$). Survival in the melatonin-a.m. group was also significantly enhanced compared to the melatonin-p.m. group ($P \leq 0.05$).

Finally, panels of mice were treated with LiCl plus melatonin to determine whether the influence of these two modalities on survival was complementary or antagonistic. As shown in Fig. 3, treatment of mice with 4 mg ^{6}LiCl plus 100 μg melatonin/day yielded survival curves identical to mice treated with 4 mg ^{6}LiCl/day alone, irrespective of whether the injections were performed in the morning or evening. Thus, the enhanced survival induced by these two modalities was not additive and the LiCl effect was dominant.

In the NZB/W model of SLE, the mice develop glomerulonephritis which proceeds to renal failure and death (Theofilopoulos & Dixon, 1987). Determination of proteinuria in the mice treated with the two modalities was performed to evaluate effect on renal function. As shown in Fig. 4, treatment with LiCl (a.m. or p.m.) led to retarded development of proteinuria, a finding consistent with enhanced survival. Interestingly, proteinuria in the melatonin-a.m. group progressed more slowly than proteinuria in the melatonin-p.m. group, a finding which again paralleled differences in survival curves in these two groups. The influence of Li + melatonin on proteinuria was not unlike that of Li alone.

Anti-ssDNA and gp70 immune complex profiles of untreated and treated mice

Mice were bled for serum when only 3 mice/group remained alive, or at 44 weeks of age 4–7 mice/group were bled and then killed. The serum was assessed for the presence of anti-ssDNA antibodies and gp70 immune complexes. This latter antibody, anti-gp70, has been reported to be nephritogenic (Izui *et al.*, 1981; Tokado *et al.*, 1991) and therefore may be important in the progression to renal failure. As depicted in Fig. 5, survivors in the various groups of treated animals all

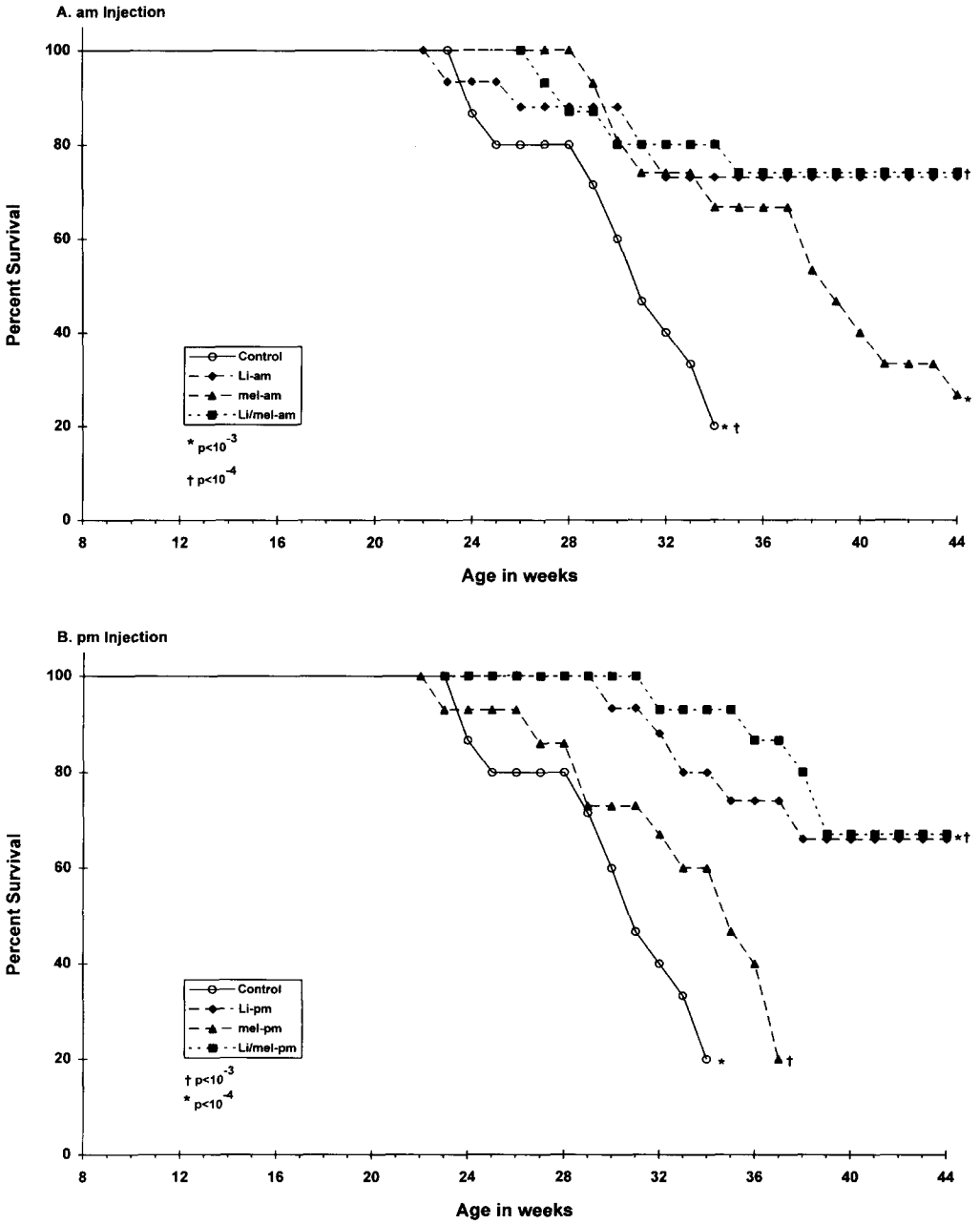

Fig. 3. Influence of melatonin on Li-mediated enhancement of NZB/W survival. Groups of 15 mice were untreated (○, control) or treated with 4 mg ^{6}LiCl (◆, Li), 100 μg melatonin (▲, mel) or Li+melatonin (■, Li/mel) between 08:00 and 10:00 h (A) or 17:00 and 19:00 h (B) 7 days a week. The significances of differences as determined by ANOVA are indicated in the figure.

had values for both anti-ssDNA or gp70 IC that were similar or elevated compared to the untreated mouse values. While some of these differences could be attributed to the fact that the sera were obtained from animals of different ages, considerable variation in antibody levels was noted within each group, again indicating there was no correlation with survival. However, it is clear that an absence of a nephritogenic antibody (anti-gp70) is not responsible for long-term survival in treated mice.

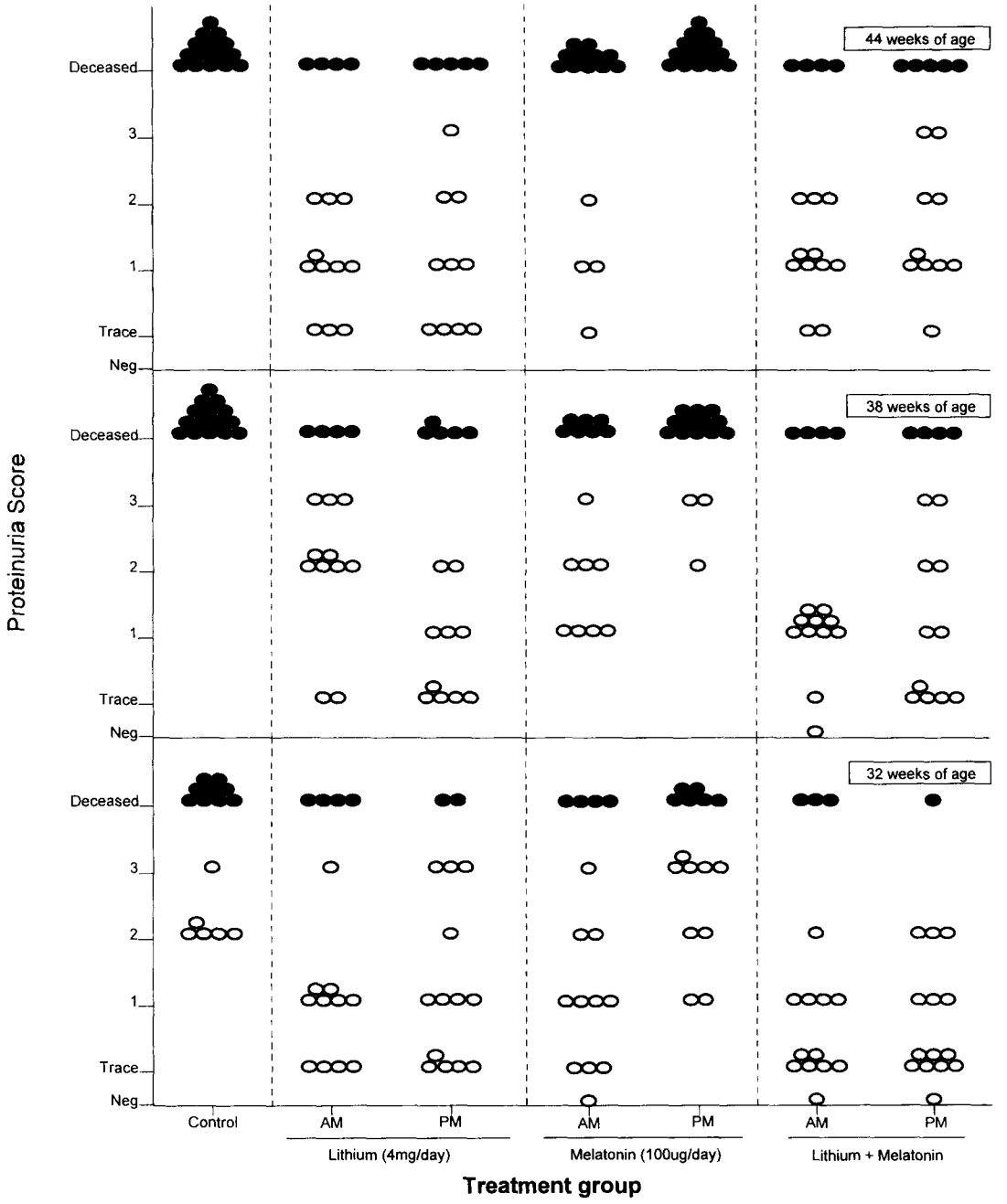

Fig. 4. Effect of treatment protocols on development and progression of proteinuria in NZB/W mice. Treatment was maintained from 8 to 44 weeks of age and proteinuria determined at the indicated time points as described in Experimental Procedures. There were 15 mice/group; live animals are indicated by the open circles while dead mice are indicated by the closed circles.

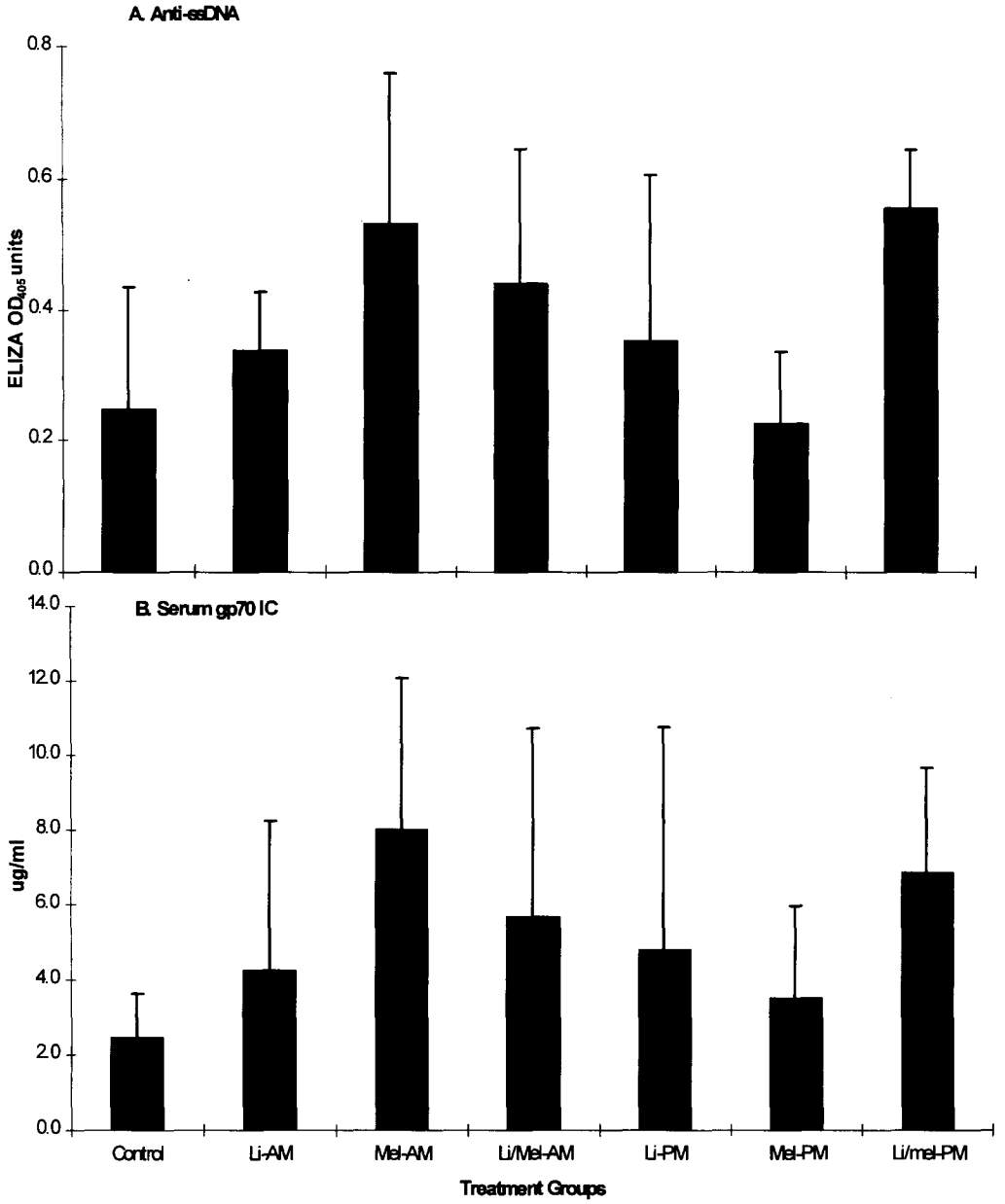

Fig. 5. Influence of treatment protocols on anti-ssDNA and anti-gp70 immune complex levels in the serum of NZB/W mice. Serum from untreated control mice ($n = 3$, 34 weeks of age), melatonin-p.m. mice ($n = 3$, 37 weeks of age) and the remaining groups of mice ($n = 4$–7, 44 weeks of age) were assessed for the presence of anti-ssDNA and gp70 IC as described in Experimental Procedures. Indicated values represent the mean ± S.D. for each group. Differences between groups were not significant ($P>0.05$, ANOVA).

Renal histology

At the time of sacrifice, samples of renal tissue were fixed in formalin and then processed for histologic examination. Similar to previous findings in Li-treated NZB/W mice (Hart *et al.*, 1994; Done *et al.*, 1994; Benediktsson *et al.*, in preparation), kidney sections from long-term survivors of Li-treated or Li+melatonin-treated mice showed less tubulointerstitial disease than tissue from untreated mice or mice with melatonin

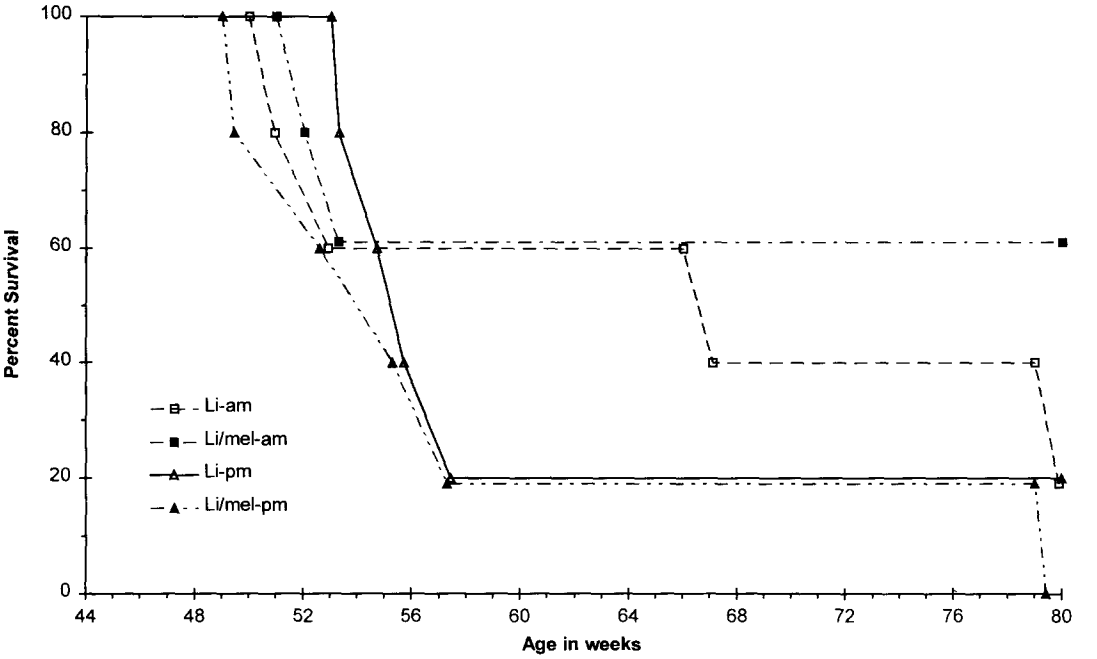

Fig. 6. Survival of treated NZB/W mice following cessation of treatment at 44 weeks of age. Randomly selected long-term survivors (5 mice/group) from the indicated treatment groups were maintained, and survival was monitored daily. Differences between groups were not significant, as determined by ANOVA.

alone. No statistically significant differences were noted when comparing the Li and Li+melatonin treatment groups. Interestingly, the Li and Li+melatonin-treated animals again showed evidence of glomerulonephritis (inflammatory cell infiltrates, structural changes), but these changes did not progress to tubular disease and renal failure.

Effect of cessation of LiCl *treatment on disease progression in* 44*-week-old survivors*

Randomly selected survivors to 44 weeks of age (5 mice/group) were maintained but LiCl treatment was stopped. Survival was monitored daily and proteinuria was assessed at intervals of 4–11 weeks post-cessation of treatment. By 13 weeks post-treatment cessation (57 weeks of age), 4/5 mice in the Li-p.m. and Li+melatonin-p.m. groups had died, while 2/5 mice in each of the a.m. groups were dead (Fig. 6). Furthermore, by 30 weeks post-treatment cessation (74 weeks of age), only one additional mouse (Li-a.m. group) had died (Fig. 6).

Therefore, similarly to previous results (Krause *et al.*, 1992; Hart *et al.*, 1994), LiCl treatment inhibited disease progression in this model of SLE, but did not completely stop the disease once treatment was terminated. This finding of enhanced survival was confirmed by the proteinuria data obtained on the mice following cessation of LiCl treatment. As shown in Fig. 7, with increasing time following cessation of LiCl and melatonin treatment, proteinuria scores gradually increased, with p.m.-group scores increasing somewhat faster than the scores of the a.m.-group. Thus, the trend in the proteinuria scores again paralleled the survival data.

DISCUSSION

The results presented in this report demonstrate that the effectiveness of LiCl to enhance the survival of NZB/W female mice is not dependent on the timing of the injections and that it is also not influenced by co-exposure to melatonin, a pluripotent component of a potential neuroendocrine target system. In contrast, the effectiveness of melatonin to influence survival was dependent on the timing of the injections, as might be expected for a mediator whose synthesis is regulated by circadian variables.

Administration of melatonin between 08:00 and

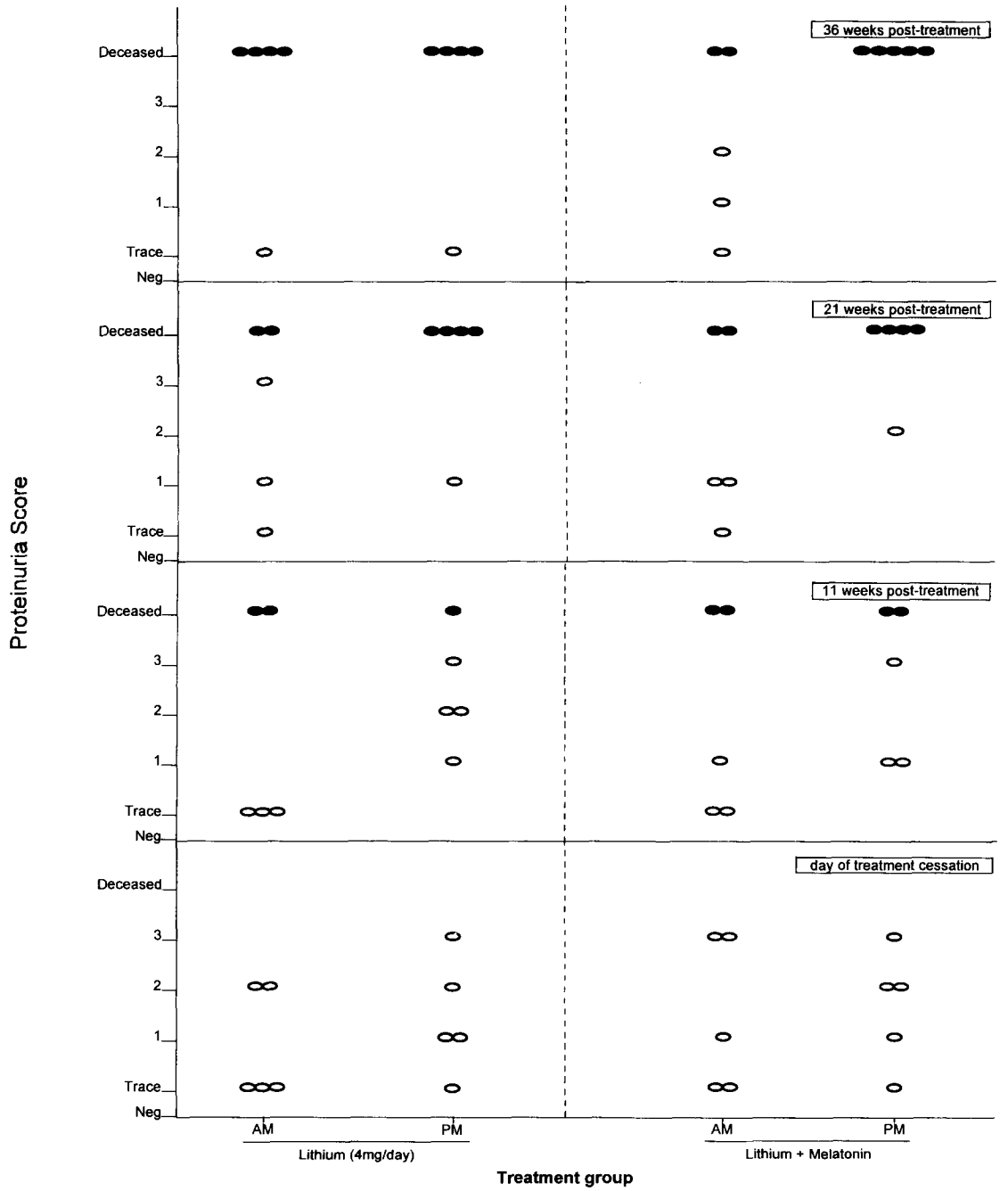

Fig. 7. Progression of proteinuria in long-term survivors following cessation of treatment. Proteinuria in the mice depicted in Fig. 6 was monitored at 11, 21 and 36 weeks post-cessation of treatment. Mice which died during this time period are illustrated by the closed circles.

10:00 h led to significant enhancement of the survival of female NZB/W mice when treatment was initiated at 8 weeks of age. Conversely, when the melatonin was administered between 17:00 and 19:00 h, no significant influence on survival was noted. As the normal peak of serum melatonin usually occurs between 00:00 and 02:00 h (Maestroni *et al.*, 1987; Pierpaoli &Maestroni, 1987), administration of exogenous melatonin prior to the onset of darkness had little effect, while administration in the morning during the light cycle led to alterations which enhanced survival. From the survival curves (Fig. 2), it is apparent that a.m. melatonin exposure extended survival compared to untreated controls, but only delayed disease progression rather than halting disease progression. The mechanism(s) responsible for this melatonin effect are not known. As melatonin has been reported to have immunomodulating effects (Maestroni *et al.*, 1987; Pierpaoli & Maestroni, 1987), one possible mechanism could be via an immunosuppressive effect. However, anti-ssDNA and gp70 immune complex levels were somewhat elevated in the melatonin a.m. group compared to the p.m. group (ssDNA, $P = 0.052$, NS; gp70, $P = 0.102$, NS). Thus, morning administration of melatonin is not effective as a general immunosuppressant. An alternative possibility is that melatonin is acting directly on the kidney. Receptors for melatonin have been reported in kidney tissue from a number of species (Pang *et al.*, 1993; Song *et al.*, 1993) and therefore melatonin could act directly on the target tissue. It is unclear how exposure of the kidney to a.m. melatonin induces a protective effect, but it is evident that such exposure prolongs survival and delays onset of severe proteinuria. Likewise, it is unclear why p.m. exposure of kidneys to melatonin is without effect. It may be possible that the agent is working in concert with other factors that are regulated in a circadian manner. However, the present results do indicate that further experiments with higher doses of a.m. administered melatonin are warranted, particularly since melatonin has been reported to up-regulate TNF production (Pioli *et al.*, 1993), a cytokine reported to also enhance survival of NZB/W mice (Jacob *et al.*, 1991; Gordon *et al.*, 1989).

It is apparent from the survival curves that administration of ^{6}LiCl is more effective than melatonin in enhancing survival, irrespective of the timing of the injections. Also in contrast to melatonin, ^{6}LiCl was equally effective when administered between 08:00 and 10:00 h or 17:00 and 19:00 h (11/15 vs 10/15 long-term survivors). Likewise, the influence of LiCl on the development and progression of proteinuria was independent of the timing of the injection. Also in contrast to melatonin, LiCl administration induced long-term survival of a subset of mice rather than just delaying the time to death. Based on these observations it is likely that LiCl and melatonin are exerting their influence on disease progression via different mechanisms. As LiCl administration also did not exert a detectable immunosuppressive effect on autoantibody expression, it may be possible that LiCl is influencing survival at the level of the kidney itself. If this is proven to be a correct speculation by future investigation, then the site of action is also likely different from that of melatonin.

Further support for the conclusion that LiCl and melatonin are influencing survival of NZB/W mice by different mechanisms comes from the experiments in which mice were treated with Li+melatonin, either in the morning or in the evening. In both cases, the long-term survivors in the Li+melatonin groups were identical to mice in the analogous groups treated with Li alone. Therefore, the LiCl effect is dominant and melatonin neither antagonizes the LiCl effect nor complements it.

As discussed earlier, LiCl or Li+melatonin administered between 08:00 and 10:00 h or 17:00 and 19:00 h was equally effective at inducing long-term survival as long as treatment was maintained. However, once treatment was stopped and a subset of such survivors maintained, it was apparent that the mice in the p.m. groups were less protected from disease reactivation (as measured by proteinuria progression and death) than were the mice in the a.m. groups. These differences were not significant, owing to the numbers of mice per group, but they may indicate a trend. If this trend is substantiated, then the effectiveness of Li may be influenced by other factors that are regulated in a circadian manner. Relevant to this point is the recent report that the biological effects of TNF, a cytokine reported to be influenced by Li (Beyaert *et al.*, 1989), can be influenced by circadian variables (Hrushesky *et al.*, 1994).

In summary, this report indicates that the effects of LiCl on the long-term survival of female NZB/W mice are probably not directly related to melatonin metabolism, one of the potential target systems. However, some aspects of LiCl effectiveness may be influenced by other components of circadian rhythm-modulated factors. Current investigations are focusing on the analysis of patterns of gene expression in kidneys from treated and untreated mice to understand better the mechanisms involved and how they are regulated.

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