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Dexamethasone Prevents Autoimmune Nephritis and Reduces Renal Expression of Ia But Not Costimulatory Signals

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Although glucocorticoids are a conventional treatment for lupus nephritis, the cellular and molecular mechanisms responsible for preventing renal injury are unknown. MRL-*lpr *mice develop an aggressive autoimmune nephritis. As these mice become nephritic, there is an increase in the renal expression of molecules that permit or facilitate immune interactions, including MHC class II (Ia) antigens, intercellular adhesion molecule-1 (ICAM-1), and proinflammatory cytokines. Because dexamethasone (Dex) alters Ia antigen expression and suppresses cytokine generation, the authors prophylactically treated MRL-*lpr *mice and investigated the relative importance of these molecules in inducing renal injury. MRL-*lpr *mice given Dex (0.4 mg/kg/d) from age 6 weeks were killed 4, 8, and 16 weeks after the initiation of therapy, and tissue was removed for histology and extraction of total RNA. Dex prevented lymphadenopathy and renal injury. Dex eliminated the marked Thy 1.2+ lymphocytic infiltrates within the kidney and preserved normal renal histology and urinary protein levels. Northern blot analysis of steady-state mRNA transcripts indicated Dex suppressed a four-fold increase in kidney major histocompatibility complex class II (Ia) molecule antigen mRNA seen by age 22 weeks (Ia/β-actin ratios = 0.64 ± 0.50* versus *2.32 ± 0.48,* P *< 0.01), but did not alter the costimulatory molecules ICAM-1 or tumor necrosis factor α (TNFα). Although all of these molecules are important mediators of inflammation, autoimmune nephritis was ameliorated without alteration of TNFα gene transcription or ICAM-1 transcription and surface expression. This study suggests that the benefit of steroids in nephritis stems from preventing lymphocyte infiltration into the kidney and decreasing immune interactions by limiting Ia expression. (Am J Pathol 1992, 141:743–751)

Glucocorticoid steroids have multiple and complex effects on immune responses and inflammatory injury. The therapeutic action of steroids in lupus nephritis may be related to the induction of lipocortin proteins and reduction of phospholipase A_2 activity, alterations in proinflammatory prostanoids, and redirecting mononuclear cell traffic.[1–3] In addition, glucocorticoids have been shown to reduce B cell immunoglobulin production,[4] major histocompatibility complex (MHC) class II (Ia) molecule transcription in macrophages[5] and renal tubular cells,[6] and alter the transcription, translation, and release of cytokines.[7–15] These actions have been exclusively based on *in vitro* analyses, and it is unknown if they are important in preventing autoimmune renal injury.

In our previous reports, an increase in the kidney expression of Ia molecules, ICAM-1, and the proinflammatory cytokine tumor necrosis factor α (TNFα) was associated with the progression of lupus nephritis in MRL-*lpr* mice.[16–18] In MRL-*lpr* mice, renal disease is aggressive and shares many pathologic features with human autoimmune lupus nephritis.[19] The *lpr* (lymphoproliferative) gene, which induces a massive proliferation of unique T cells before evidence of renal injury, accelerates renal disease in these mice. Glomerular injury and tubular interstitial mononuclear infiltrates are prominent by 4 months, and a 50% mortality rate is evident by age 5 to 6 months. Although steroids can retard the expres-

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sion of renal injury in lupus nephritis, a correlation between reduced disease activity and reduction in cytokine expression has not been reported in MRL-*lpr* mice.[20] Therefore, the current study was designed to establish which pro-inflammatory immune molecules expressed in the kidneys of MRL-*lpr* mice could be blocked by the chronic oral administration of Dex, a potent synthetic glucocorticoid. Our results indicate that the therapeutic action of Dex in autoimmune lupus nephritis correlates with reducing lymphocyte traffic to the kidney and preventing immune interaction by inhibition of Ia expression. In contrast, ICAM-1 and TNFα expression were not dampened by Dex, suggesting that although these molecules facilitate immune interactions, increased expression in the absence of Ia is insufficient to cause renal injury in the MRL-*lpr* mouse.

Materials and Methods

Mice and Materials

MRL/MpJ-*lpr*/*lpr* (MRL-*lpr*) (H-2^k) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in our animal facility on standard laboratory chow. Reagents were obtained from Gibco (Grand Island, NY), and all chemicals were obtained from Sigma (St. Louis, MO). Thy 1.2 monoclonal antibody (MAb) was obtained from Becton-Dickinson. The MAb 10-2.16 (anti I-A^k), and YN1/1.7.4 (anti-murine ICAM-1) were prepared as described and concentrated to approximately 0.5 mg/ml.[21] All antibodies were biotinylated using a standard protocol.

Dexamethasone Treatment

Dexamethasone sodium phosphate (Elkins-Sinn Inc., Cherry Hill, NJ) was added to the drinking water of female MRL-*lpr* mice starting at age 6 to 7 weeks, and once per week the concentration was adjusted to maintain a daily steroid intake of 0.4 mg/kg based on daily water consumption. Control groups received untreated water *ad libitum*. Mice were caged in groups of five, and water intake at the start of the study was not different between treated and untreated mice, nor between treated cages. One cohort of 20 mice (control and treated groups, n = 10) were followed for 16 weeks and then killed. One mouse from the control group and one from the steroid group died before study completion and were excluded from analysis. Mice in a second cohort of 20 mice (control and treated groups, n = 5 at each time point) were killed at 4 weeks or 8 weeks of treatment. Two mice in the untreated control group died before study completion. Sections of kidney tissue were snap frozen in OCT compound (Miles Inc., Elkhart, IN) for immunoperoxidase or fixed in 10% formalin for standard hematoxylin and eosin staining.

Immunoperoxidase Staining

Kidney sections were processed for immunoperoxidase staining as previously described.[16] Briefly, 4-μ croyostat sections were acetone fixed, blocked with 4% horse serum, incubated with saturating concentrations of primary antibody, followed by Vectastain ABC reagent (Vector Laboratories, Burlingame, CA). Peroxidase activity was detected by a 4- to 6-minute incubation with 3,3′ diaminobenzidine (0.5 mg/ml) containing 0.02% H_2O_2. Counterstaining was with methyl green and alcian blue. Irrelevant MAbs of the same isotype were used as negative controls.

Clinical and Histologic Scoring

Weights were obtained weekly, and mice also were assessed for dermatitis, and lymphadenopathy before being killed. Urinary protein was measured by dipstick (Albustix) and recorded as 0.5+ (< 30 mg/dl), 1+ (30 mg/dl), 2+ (100 mg/dl), or 3+ (300 mg/dl). Levels below 1.5+ are found in normal mice. In hematoxylin and eosin (H&E)-stained kidney sections, histopathologic glomerular damage was scored by a blinded observer as follows: 0 = no involvement, 1 to 3+ = glomerular changes in 0% to 25%, 25% to 50%, and 50% to 75% of total glomeruli respectively, and 4+ = greater than 90% involved with crescents or sclerotic glomeruli. In assessing infiltrative disease, arbitrary units of severity (0 to 4+) were graded by the same blinded observer, with 0 = no infiltrates visible, 1 = few infiltrates in some fields, 2 = moderate infiltrates easily found in most fields, 3 = moderate infiltrates in all fields, 4 = severe infiltrates with loss of normal surrounding histology.

RNA Preparation and Hybridization

Sections of spleen and kidney also were prepared for total RNA extraction. Total RNA was isolated from homogenized tissue with a single-step acid guanidium isothiocyanate-phenol-chloroform extraction method.[22] Total RNA (25 μg) was denatured with 1 mol/l glyoxal and 50% dimethylsulfoxide, electrophoresed through a 1.2% agarose gel, and transferred onto Gene Screen Plus nylon membranes (New England Nuclear, Boston, MA). The RNA was UV light crosslinked, and blots were prehybridized with 1 mmol/l (millimolar) ethylenediaminetetra-acetic acid (EDTA), 0.5 mol/l NaH_2PO_4 (pH 7.2),

and 7% sodium dodecyl sulfate (SDS) at 65°C. A specific 250-bp cDNA insert encoding murine TNFα was isolated by plasmid digestion with Rsa1.[23] A cDNA probe for murine ICAM-1[24] and a genomic probe for $A_{\alpha b}$[25] were as previously described. Probes were radiolabeled by primer extension using random hexanucleotides.[26] Blots were hybridized for 16 to 24 hours at 65°C and washed twice at 65°C with 1 mmol/l EDTA, 40 mmol/l NaH_2PO_4 (pH 7.2), and 5% SDS for 30 to 60 minutes, and twice with 1 mmol/l EDTA, 40 mmol/l NaH_2PO_4 (pH 7.2), and 1% SDS for 30 to 60 minutes at 65°C. After hybridization, the blots were exposed to Kodak X-AR film at −70°C for 4 to 10 days. Blots were reprobed with β-actin to ensure that approximately equal amounts of RNA were loaded in each lane. Steady-state mRNA transcript levels were quantified using scanning densitometry (Biosoft, Milltown, NJ). Autoradiographs were scanned using a high-resolution scanner, and a 256-level gray scale image was analyzed on a Mac II computer. Automatic integration of the density of the signal as well as area under the curve was performed with matched background subtraction. Accuracy of this method has been quoted to be within ±1% of other densitometry methods. The ratio of Ia, ICAM-1, or TNFα to β-actin was then calculated to standardize RNA levels and allow quantitative comparison of values between treated and control groups.

Statistics

Statistical analysis, where applicable, was performed using Statview SE+ (Abacus Concepts, Berkeley, CA). Differences between groups were compared by one-way analysis of variance and unpaired *t*-tests, and a P value of less than 0.05 was held to be significant. All results are expressed as mean ± SEM.

Results

Dexamethasone Attenuates Autoimmune Renal Injury in MRL-lpr Mice

Mice were assessed weekly for clinical signs of autoimmunity, including an aggressive nephritis that begins in untreated control mice by 4 months of age. Renal disease and proteinuria were abrogated by Dex (Table 1). Baseline urinary protein values of 0.75 to 0.8+ increased to greater than 2+ in control mice ($P < 0.05$), but remained unchanged in Dex-treated mice. We did not measure urine volumes in mice and because the dipstick method measures protein concentration, it may have underestimated proteinuria in polyuric mice. Results were consistent with histopathologic scoring of kidney sections (Table 2), suggesting differences in protein excretion accurately reflected disease. Glomerular injury in control mice ranged from increased cellularity and mesangial expansion to necrotizing vasculitis with crescents and complete sclerosis. The degree of renal injury predictably worsened with age in all control mice examined ($P < 0.01$). In contrast, normal glomerular histology was preserved in Dex-treated mice. Medullary, periglomerular, and perivascular interstitial infiltration by mononuclear cells was also limited in Dex-treated mice (Table 2). Immunohistochemical labeling of kidney sections from control mice indicated infiltrates in control mice were largely Thy 1.2+ T cells (not shown). All control mice had a generalized lymphadenopathy by 14 to 16 weeks of age, which worsened by 22 weeks of age, at the termination of the study. In contrast, none of the Dex-treated mice had palpable lymph nodes during the study. Although mice in each group had dermatitis by 22 weeks of age, it was more severe in the control group. Importantly, neither untreated or treated mice lost weight during the study, eliminating the possibility of immunosuppression by calorie reduction.[27] Dexamethasone reduces renal expression of Ia but not ICAM-1 or TNFα. Our previous studies have shown that renal disease in MRL-*lpr* mice is correlated with increased renal expression of Ia, ICAM-1 and the pro-inflammatory cytokine TNFα. Total RNA extracted from kidney sections from both control untreated mice and Dex-treated mice was analyzed for the level of steady-state gene transcripts of these molecules. A reduction in renal Ia expression was detected by as early as 4 weeks of Dex treatment (Ia/β actin ratio of 0.23 ± 0.05 *versus* 0.59 ± 0.05) (Figure 1). This reduction remained at 8 and 16 weeks of treatment (Figures 2, 3). Interestingly, expression of Ia in spleen was not reduced by Dex after 8 weeks (Figure 2) or 16 weeks of treatment (not shown). In contrast to Ia results, ICAM-1 expression was not different between treated and untreated mice either in kidney tissue at 8 and 16 weeks (Figures 2, 3) or in spleen at 8 weeks (Figure 2). Similarly, Dex did not reduce gene expression of TNFα in kidney at 4 and 16 weeks of treatment (Figures 1, 4B) or in kidney and spleen tissue at 8 weeks (Figure 4A).

To determine if steroids influenced tubular Ia and

Table 1. *Dex Reduces Urinary Protein in MRL*-lpr *mice.*

Treatment (weeks)	Urinary Protein†			
	Base	4	8	16
Control	0.8 ± .1	1.2 ± .2	2.4 ± .6*	2.0 ± .4*
Dex	0.8 ± .1	0.9 ± .1	1.0 ± 0	1.1 ± .3

* Indicates $P < 0.05$.
† Urinary protein measured by dipstick as in methods.
Mice were treated from age 6 weeks with Dex, and assessed for urinary protein by dipstick after 4, 8, and 16 weeks of treatment. Proteinuria in control mice was greater than treated mice after 8–16 weeks of Dex.

Table 2. *Dex Reduces Glomerular Injury and Mononuclear Cell Infiltrates in MRL-*lpr *Mice*

Treatment (wk)	Dex	n	Glomerular damage	Renal infiltrate location		
				Periglomerular	Perivascular	Medulla
4	–	4	0.5 ± .3	0.8 ± .5	1.5 ± .9	1.3 ± .8
	+	5	0.0 ± 0	0.2 ± .2	0.4 ± .4	0.0 ± 0
8	–	4	1.3 ± .6	1.8 ± .3	1.5 ± .6	2.5 ± .3
	+	5	0.0 ± 0	0.2 ± .2*	0.0 ± 0	0.2 ± .2*
16	–	9	2.1 ± .3	2.1 ± .4	3.2 ± .3	2.9 ± .2
	+	9	0.3 ± .2*	0.3 ± .2*	0.6 ± .4*	0.5 ± .3*

* Indicates significance of $P < 0.01$.
Kidney sections from mice treated for 4, 8, and 16 weeks of treatment were analyzed and compared with untreated control mice. Histopathologic damage or intensity of infiltrate were graded in hematoxylin and eosin-stained sections as in Methods, with 0 = none to 4 = severe. Results are given as means ± SEM.

ICAM-1 expression by post-transcriptional mechanisms, we analyzed kidney sections by immunohistochemistry using specific biotinylated antibodies to mouse ICAM-1 (YN1/1.7.4) and Ia (10-2.16). Consistent with the gene expression data from this study, there was enhanced expression of Ia within nephritic kidneys of control mice with localization primarily to cortical tubular cells, and mononuclear cell infiltrates (Figure 5A). Similarly enhanced levels of ICAM-1 were prominently displayed on tubular cells and infiltrates, and as well within the glomerular mesangium (Figure 5C). These patterns of expression were consistent with previous reports.[16,17] In Dex-treated mice, renal Ia was reduced because of both the absence of Ia-positive infiltrates and decreased tubular epithelial cell expression (Figure 5B). In contrast, tubular cell and mesangial ICAM-1 expression was not notably reduced with Dex treatment during the study period (Figure 5D).

Discussion

Tubular cell expression of Ia and ICAM-1, and kidney levels of IL-1 and TNFα, increase with the severity of autoimmune renal disease in MRL-*lpr* mice. Although Dex and other glucocorticoids have known anti-inflammatory and immunosuppressive activities, their cellular and molecular mechanisms of action *in vivo* in preventing autoimmune nephritis have not been defined.[20] Studies investigating the gene regulation of cytokines and cell surface molecules by glucocorticoids have largely used purified cell populations *in vitro.* Although providing valuable information, these *in vitro* studies can not duplicate the complex network of immune interactions occurring within the kidney during lupus nephritis. Using chronic oral administration of Dex, we assessed the relation of disease activity to the modulation of tubular epithelial cell Ia, and ICAM-1 as well as TNFα within the kidneys of MRL-*lpr* mice. Dex is a synthetic glucocorticoid several times more potent than prednisone and has been shown to be immunosuppressive in rodents.[28,29] Furthermore, addition of Dex to drinking water provided a well-tolerated and simple method for long-term administration.

The specificity of immune interactions between antigen-presenting cells and T cells is dependent on MHC molecule expression.[30–32] Tubular cell class II (Ia) mole-

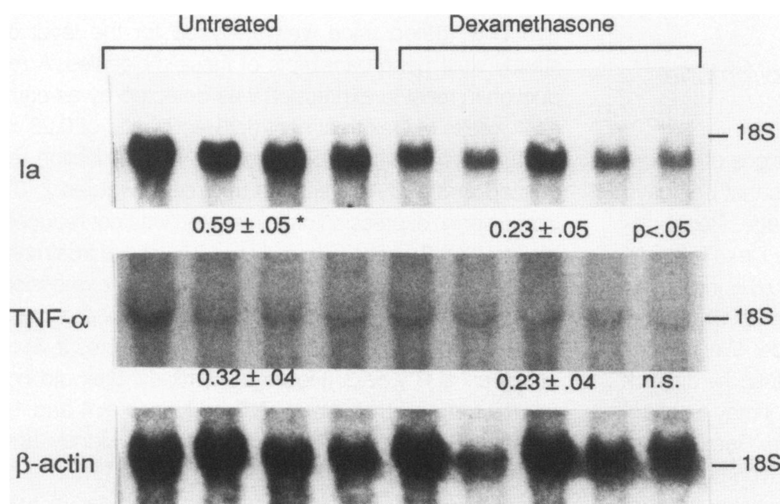

Figure 1. *Renal Ia (P < 0.05) but not TNFα mRNA was reduced by Dex. Samples were obtained from MRL-*lpr *mice treated for 4 weeks (n = 5). The blots were reprobed for β-actin. The mean ratio of Ia and TNFα to β-actin indicated below treatment groups were calculated by scanning densitometry, as in methods.*

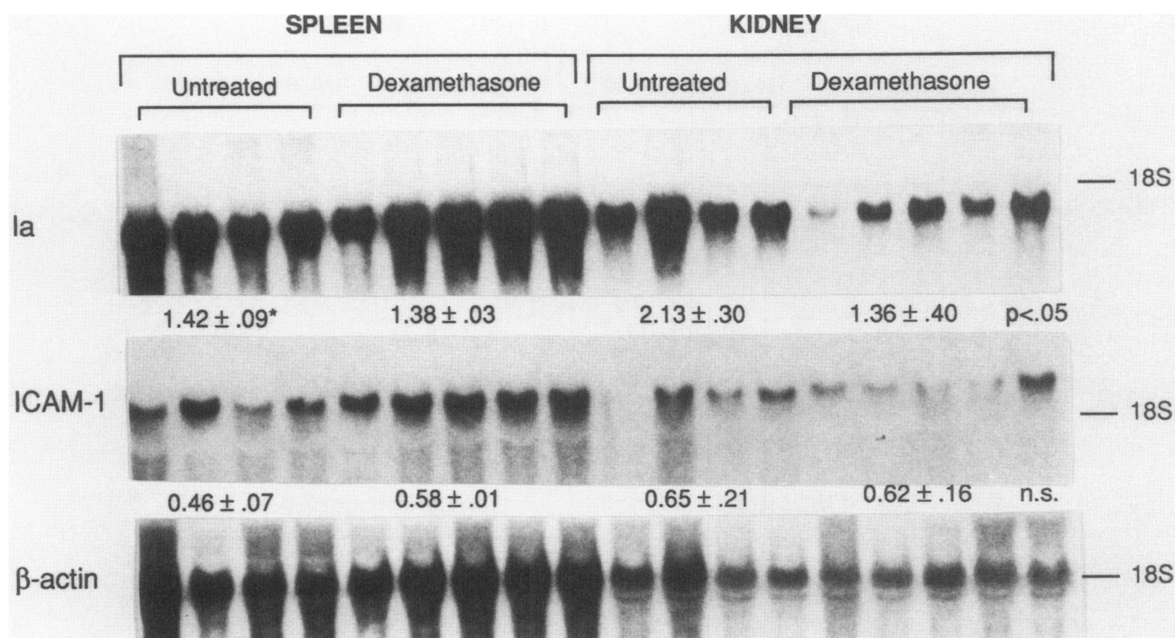

Figure 2. *Renal Ia mRNA obtained from MRL-*lpr *mice was reduced by 8 weeks of Dex compared to untreated mice (2.13 ± .3 vs. 1.36 ± .4, P < 0.05). No differences were found in spleen Ia/β-actin steady state mRNA transcript levels between groups (indicated by *) or in spleen and kidney ICAM-1/β-actin levels between treatment groups (P = n.s.). A slight increase in spleen ICAM-1 with Dex treatment was not significant (0.46 ± .07 vs. 0.58 ± .01, P = .08).*

cule expression was markedly reduced in Dex-treated mice as early as 4 weeks. It is therefore not surprising that immune injury was prevented in the absence of renal Ia expression. Previous reports of attenuation of disease in NZB/W mice using anti-Ia antibodies have also emphasized the primary importance of Ia expression in autoimmune nephritis.[33] Alternatively, tubular Ia expression in autoimmune nephritis may be an epiphenomenon of T cell infiltration and cytokine release, rather than a primary inciting event.[34] In fact, T-cell clones isolated from the renal cortex of MRL-*lpr* mice are capable of inducing Ia and ICAM-1 on tubular cells *in vitro* (manuscript in prep-

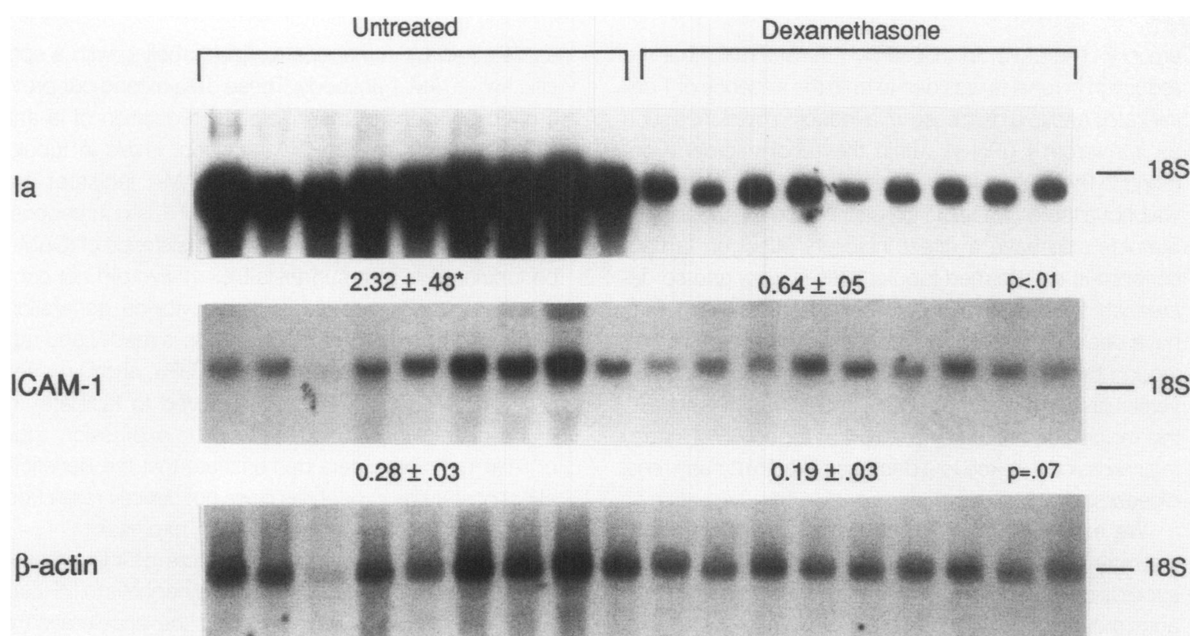

Figure 3. *Dex caused a marked decrease in renal Ia/β-actin mRNA levels after 16 weeks of treatment (n = 9) compared with untreated MRL-*lpr *controls (indicated by *, n = 9). In contrast, ICAM-1/β-actin mRNA levels were not altered (P = 0.07).*

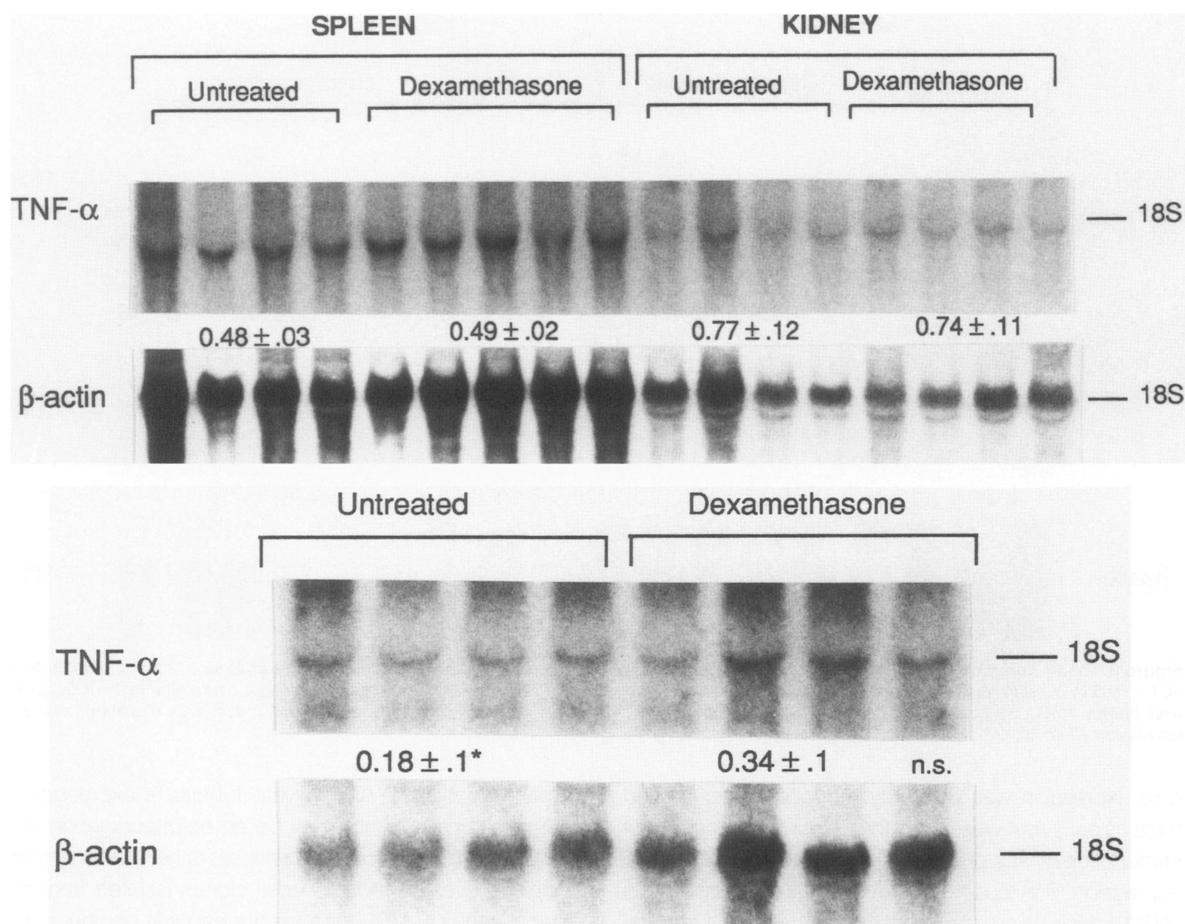

Figure 4. Top: *Dex did not alter TNFα/β-actin mRNA expression in kidney or spleen after 8 weeks of Dex.* **Bottom:** *A small increase in the mean kidney TNFα/β-actin mRNA level following 16 weeks of Dex treatment was not different from untreated controls indicated by * (0.18 ± 0.1 vs. 0.34 ± 0.1,* P = *0.3).*

aration). Therefore, in this study it is probable that the reduction in renal Ia was due to both the absence of T cell infiltrates and to a decrease in Ia-inducing cytokines such as interferon γ (IFN-γ) within the renal microenvironment.[35] The finding that splenic expression of Ia mRNA was not altered by Dex is consistent with this possibility. Steroids also have a direct inhibitory effect on Ia transcription in transformed tubular cells *in vitro,* and so decreased tubular Ia expression in this model also may have been mediated through a primary mechanism.[6] Although the role of class II molecules remains a controversial area in the pathogenesis of autoimmune disease, this report adds to the evidence that reduction of kidney Ia expression is linked to a decrease in autoimmune renal disease activity.

We examined the effect of Dex on other molecules that may facilitate T-cell interactions within the kidney. The expression of tubular cell ICAM-1 is also enhanced in autoimmune nephritis.[17] In contrast to Ia expression, Dex did not appreciably reduce total kidney ICAM-1 mRNA transcripts or tubular cell expression of surface ICAM-1 as detected by immunoperoxidase labeling with a specific anti–ICAM-1 antibody. These data extend our previous observations of the differential regulation of Ia and ICAM-1 steady-state mRNA transcript levels in tubular cells.[21] The presence of surface ICAM-1 indicates that post-transcriptional reduction of ICAM-1 is not responsible for preventing renal injury. The persistence of ICAM-1 on tubular cells also suggests Dex *in vivo* did not completely abrogate pro-inflammatory cytokine generation, as ICAM-1 expression on tubular cells is readily and rapidly induced by interleukin 1 (IL-1), TNFα, and low levels of IFN-γ. Although ICAM-1 is believed to facilitate immune interactions, particularly when Ia expression is low or limiting,[36] these data demonstrate that the beneficial effect of steroids in nephritis does not directly result from limiting tubular or mesangial ICAM-1 expression.

Cytokines are important mediators of inflammation and are associated with autoimmune nephritis in MRL-*lpr* mice.[18,37,38] Studies have reported the acceleration of lupus nephritis by administration of IFN-γ[39] or in the case of TNFα, either acceleration or amelioration of dis-

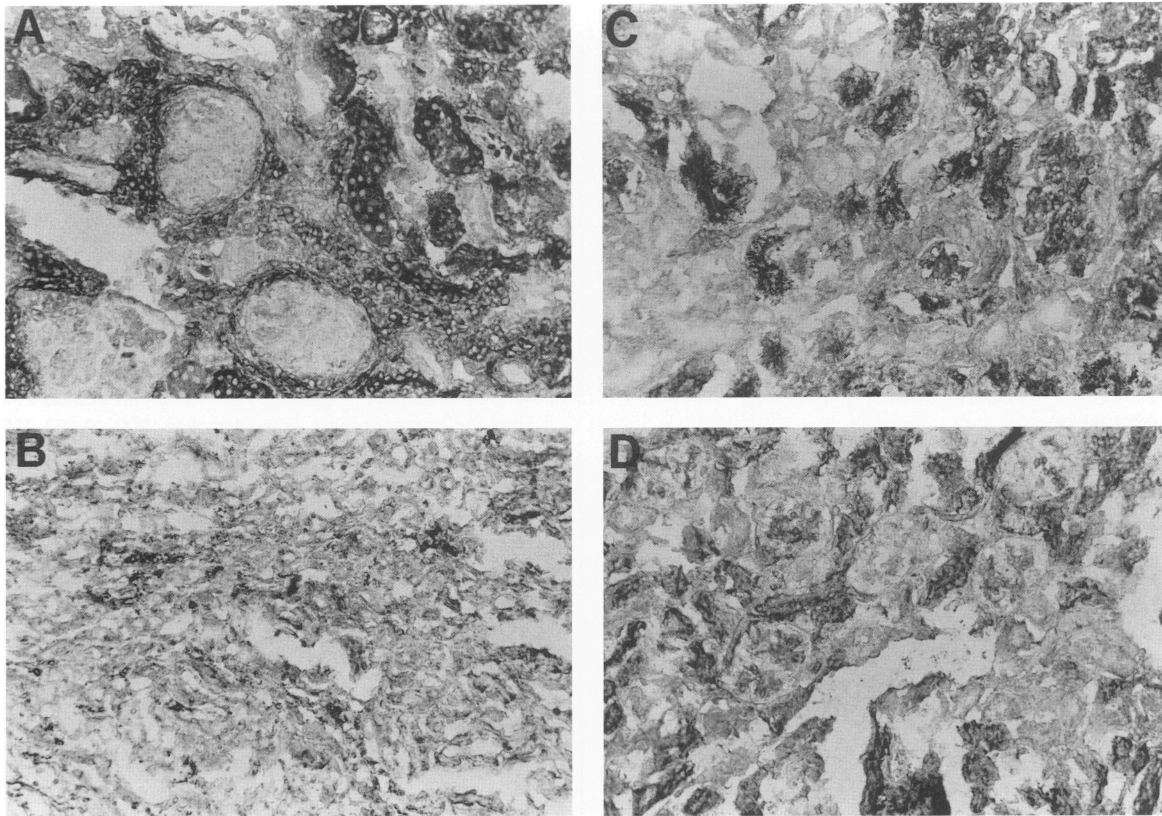

Figure 5. *Dex reduced renal tubular expression of Ia but not ICAM-1. Kidney sections from MRL-*lpr *mice 22 weeks of age were examined by immunoperoxidase as in Methods (approximate magnification ×450). Infilitrate and tubular expression of Ia as indicated by darkly stained immunoperoxidase product in untreated mouse* **(A)** *was markedly reduced following 16 weeks of Dex* **(B)**. *In contrast, moderate tubular and mesangial expression of ICAM-1 was not reduced by control levels* **(C)** *after Dex treatment* **(D)**.

ease.[40,41] Thus, steroid inhibition of endogenous cytokines might be expected to improve autoimmune nephritis. Glucocorticoid control of cytokine gene transcription may confer immunosuppressive activity by several mechanisms. Glucocorticoid-responsive elements (GREs) in the promoter/enhancer regions of cytokine genes may alter transcription rates.[8] Internalized glucocorticoid receptors also may complex with *c-jun* to prevent subsequent binding to AP-1 sites in promoter regions and thus alter gene transcription.[42] Alternatively, steroids may accelerate cytokine mRNA degradation by interaction with A-U–rich sequences in the 3′ untranslated regions of mRNA, which is a common motif in transiently expressed cytokine genes.[43]

Because pro-inflammatory cytokines may promote autoimmune disease, we examined total RNA from kidney and spleen tissue for TNFα mRNA. Interestingly, we did not detect reduced TNFα mRNA levels, despite *in vitro* studies reporting transcriptional inhibition of TNFα by steroids. These results are consistent with a similar inability to suppress TNFα mRNA transcription with Dex in response to endotoxin challenge *in vivo.*[15] Because the generation of TNFα *in vivo* is subject to complex controls by other cytokines, it is possible that incomplete suppression of IFN-γ antagonized the inhibitory effect of steroids on TNFα transcription.[5,44] The current study, however, clearly indicates that the therapeutic benefit of Dex in autoimmune nephritis is not related to a reduction in the gene expression of TNFα. Other reports have not correlated protection from immune injury by steroids *in vivo* and reduction of pro-inflammatory cytokine gene expression. Elevated kidney levels of IL-1β mRNA associated with renal allograft rejection were not reduced despite successful treatment and improvement with steroids.[45] Several mechanisms may be involved. Glucocorticoids may alter cytokine levels post-transcriptionally or prevent release.[13] Alternatively, as in the case of TNFα, toxicity may be reduced by protecting target tissues.[9] Therefore we can not exclude the possibility that Dex reduced TNFα protein or its toxicity to prevent renal injury. Glucocorticoids, however, have not consistently caused complete suppression of secreted TNFα, and it is unlikely that this alone can explain our results.[46,47] It may be that suppression of other molecules by Dex is required to ameliorate injury. For example, steroids inhibit nitric oxide synthase and thus may limit endothelial injury,[48] or may con-

fer immunosuppression by activating undefined genes that cause T cell apoptosis.[11]

In summary, therapeutic action of Dex in autoimmune nephritis is associated with a reduction in renal T cell infiltration and tubular cell expression of Ia. Because the effect of steroids are so complex, the relationship to Ia expression may be not cause and effect, but rather correlative. Although ICAM-1 and TNFα, which are enhanced in nephritis, may facilitate immune interactions and correlate with disease activity, our data support the concept that the clinical benefit of steroids in ameliorating nephritis stems from limiting lymphocyte traffic to the kidney and preventing immune interactions by inhibition of Ia expression.

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