The Journal of Immunology

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J Immunol 2001; 166:2913-2916; ;
doi: 10.4049/jimmunol.166.5.2913
http://www.jimmunol.org/content/166/5/2913

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Cutting Edge: Reversal of Murine Lupus Nephritis with CTLA4Ig and Cyclophosphamide[1]

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Cyclophosphamide (CTX) prevents progression of nephritis and prolongs survival in (NZB × NZW)F_1 (B/W) mice and is used to treat humans with lupus nephritis. To compare the efficacy of CTLA4Ig with CTX and determine whether there is an incremental benefit to combining CTLA4Ig with CTX, we treated B/W mice with CTX, CTLA4Ig, or both agents. In mice with mild renal disease, treatment delayed the onset of proteinuria and prolonged survival in all groups. In mice with advanced renal disease, treatment with both agents reduced proteinuria in 71% of mice, whereas mice treated with either agent alone had no such improvement. Survival was also markedly improved among mice treated with both agents. Thus, combination treatment with CTX and CTLA4Ig is more effective than either agent alone in reducing renal disease and prolonging survival of B/W mice with advanced nephritis. This striking reversal of proteinuria is unprecedented in animal models of SLE. *The Journal of Immunology*, 2001, 166: 2913–2916.

Lupus nephritis is a significant cause of morbidity and mortality among patients with systemic lupus erythematosus (SLE)[3] and is the major cause of death in the (NZB × NZW)F_1 (B/W) lupus-prone mouse. Early studies in this murine model showed that administration of the alkylating agent cyclophosphamide (CTX) significantly retarded the progression of kidney disease (1–4). After demonstration of similar results in humans, CTX has become the primary drug used for diffuse proliferative glomerulonephritis among patients with renal lupus (5). Selective biologic agents have also been used successfully in murine lupus models and have resulted in a slowing of the progression of renal disease (6–10). More recently, the value of interrupting T cell costimulatory signals has been demonstrated by the finding that CTLA4Ig, a soluble recombinant protein that contains the B7-binding domain of CTLA4, prevents the progression of renal disease and prolongs survival in B/W mice (11). However, although both CTX and CTLA4Ig have efficacy in murine lupus, these agents may result in different degrees of immunosuppression and may have different side effects. As an alkylating agent, CTX is not only toxic to cells that are in active cell cycle, but is also a carcinogen and mutagen. At doses used for lupus nephritis in humans, CTX therapy results in a reduction in peripheral lymphocytes (12) and an increased risk of some infections (13, 14). In addition, prolonged therapy commonly results in ovarian failure (15), azoospermia (16), and an increased rate of various malignancies (17). In contrast, CTLA4Ig does not reduce lymphocyte counts in B/W mice (11). While CTLA4Ig suppresses humoral immune responses to specific Ags in vivo, it does not appear to cause broad, long-lasting immunosuppression (18). These observations have recently been extended to humans with the demonstration that CTLA4Ig resulted in only transient suppression of humoral responses to soluble Ags and did not affect circulating lymphocyte counts in humans with psoriasis (19). Furthermore, these two agents may exert their beneficial effects on lupus by different mechanisms. CTLA4Ig specifically inhibits T cell activation by blocking a specific costimulatory interaction between APC and T cells (20). CTX, in contrast, is cytotoxic to a range of immune cells, many of which may have a role in the pathogenesis of lupus (12). The current study was therefore undertaken to directly compare the effects of CTLA4Ig and CTX on the course of murine lupus in B/W mice and to determine whether any additional benefit might be obtained from the concurrent use of both of these agents.

Materials and Methods

Mice

B/W mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and were housed in the American Association for the Accreditation of Laboratory Animal Care-accredited animal care facility at the San Francisco Department of Veterans Affairs Medical Center.

Reagents

CTLA4Ig was provided by Robert Peach (Bristol-Myers Squibb); it was produced by genetic fusion of the extracellular domain of CTLA4 and the hinge, CH2 and CH3 regions of Ig Cγ1 as described previously (21). CTX (Mead Johnson, Princeton, NJ) was reconstituted in sterilized water.

Experimental design

At age 6 mo, 100 female B/W mice were divided into two groups based upon their degree of renal disease. Group 1 had advanced renal disease (proteinuria $>$ 100 mg/dl) and group 2 had mild renal disease (proteinuria $<$ 100 mg/dl). Mice in each group were treated for 16 wk with CTX (50 mg/kg i.p. every 10 days), CTLA4Ig (50 μg i.p. three times per week), or both CTX and CTLA4Ig (50 mg/kg i.p. and 50 μg i.p. three times per week, respectively). An additional group with mild disease received saline (three times per week i.p.) as a control. Groups consisted of 14 mice per

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[1] This work was supported in part by grants from the Department of Veterans Affairs, the National Institute of Allergy and Infectious Disease, and by the Rosalind Russell Medical Research Center for Arthritis at the University of California at San Francisco.

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; B/W (NZB × NZW)F_1; CTX, cyclophosphamide.

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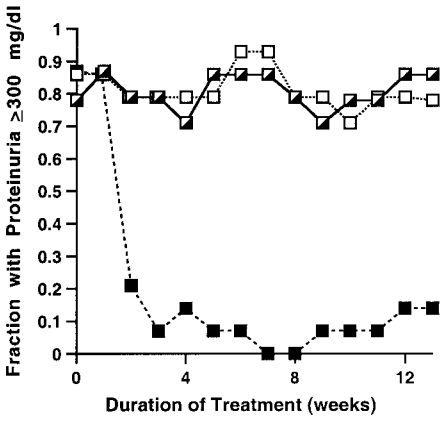

FIGURE 1. Proteinuria among mice with advanced renal disease. Proteinuria was measured during treatment with CTX (◪), CTLA4Ig (□), or combined CTX and CTLA4Ig (■). After 13 wk, the surviving mice in the CTX and CTLA4Ig groups had very advanced disease and did not produce adequate urine to assess them further.

treatment in the advanced disease group and 11 mice per treatment in the mild disease group. In a subsequent study, a cohort of B/W females with advanced renal disease was treated with these four regimens to evaluate the effects of treatment on total lymphocyte counts and lymphocyte subsets.

Fluorescence analysis of lymphocyte subpopulations

Absolute lymphocyte counts on heparinized whole blood were determined using a Technicon H1 automated cell counter (Technicon Instruments, Tarrytown, NY). Cells from individual mice were analyzed by flow cytometry as described previously (22). Cells were stained with the following mAb: anti-CD3 (hybridoma 145-2C11), anti-CD4 (hybridoma GK1.5), anti-CD8 (hybridoma 53-6), and anti-B220 (hybridoma RA3-6B2).

Measurement of Abs

IgG Abs to dsDNA in sera from individual mice were measured by an ELISA established in our laboratory previously (22).

Assessment of renal disease

Renal disease was assessed by colorimetric measurement of proteinuria using Uristix Ames Reagent Strips (Miles, Elkhart, IN).

Statistical analysis

Mean Ab titers were compared using the Student's *t* test. Proteinuria and survival rates were compared by χ-squared analysis.

Results

To examine the effects of CTLA4Ig and CTX on advanced lupus nephritis, we treated 6-mo-old B/W females with advanced renal disease (proteinuria >100 mg/dl) with CTX, CTLA4Ig, or both agents concomitantly. This represented an especially sick cohort of mice, as >80% of these mice had proteinuria ≥300 mg/dl (Fig. 1). Mice treated with either CTX or CTLA4Ig had no improvement in proteinuria. However, mice treated simultaneously with both CTX and CTLA4Ig exhibited a rapid and marked improvement in proteinuria. Within 3 wk the frequency of severe proteinuria (≥300 mg/dl) fell from 86% at baseline to 7% in mice receiving both CTX and CTLA4Ig (Fig. 1). In 10 of 14 mice, the level of proteinuria not only fell below 300 mg/dl (3+), but below 100 mg/dl (1+), reflecting at least a 67% reduction in proteinuria in individual mice (data not shown). This dramatic improvement was sustained throughout the course of treatment. Mice that received both agents also had significantly lower titers of circulating anti-dsDNA Abs compared with control mice and with mice that received either agent alone. Furthermore, anti-dsDNA Ab levels actually decreased in these mice after 12 wk of therapy (Table I). Mice treated with both agents had prolonged survival compared with the other treatment groups; 93% of mice receiving combined treatment were alive after 14 wk of treatment compared with only 36% in both the CTX alone and CTLA4Ig alone groups ($p < 0.05$; Fig. 2).

We also treated 6-mo-old B/W females with mild renal disease (proteinuria <100 mg/dl) with either CTX, CTLA4Ig, both agents concomitantly, or saline. Sixteen weeks after initiation of therapy, 80% of control mice had developed proteinuria ≥300 mg/dl, compared to 45% of CTLA4Ig-treated mice, 16% of mice treated with CTX alone, and 0% of mice treated with both CTX and CTLA4Ig (Fig. 3). Although absolute titers of anti-dsDNA Abs were lower in the CTX- and CTLA4Ig-alone groups, statistically significant reduction in anti-DNA Abs was only observed in mice treated with both CTLA4Ig and CTX (Table I). Interestingly, while there was no significant increase in dsDNA Ab titer among mice treated with CTLA4Ig, either alone or in combination with CTX, mice that received only CTX had an increase in dsDNA Ab during treatment. This difference was also seen in mice with advanced disease during treatment (Table I). Survival was maintained during treatment with either CTX or CTX plus CTLA4Ig (100%), and with CTLA4Ig alone (92%), compared to 40% in control mice (Fig. 4).

However, after treatment was stopped, there was progression of renal disease and mortality in all treatment groups (Figs. 3 and 4). Progression of disease in the CTX-alone group was especially marked and began soon after the last CTX dose (before week 16), such that by 20 wk, the percentage of CTX-treated mice with proteinuria ≥300 mg/dl was similar to that in the CTLA4Ig group (54 vs 64%). In contrast, the rate of progression of proteinuria in the combined treatment group was lower (18% at week 20). Nevertheless, this delay in progression of proteinuria among singly treated mice resulted in a prolongation in survival. Eighteen weeks after cessation of treatment, 27% of CTLA4Ig-treated mice, 36% of CTX-treated mice, and 54% of CTLA4Ig plus CTX-treated mice survived, compared with 8% of controls (Fig. 4).

Table I. *Development of anti-dsDNA Abs*[a]

	Advanced Disease			Mild Disease		
	Baseline	12 wk	*p*	Baseline	16 wk	*p*
Saline	2.6	4.3	NS	0.8	4.3	<.001
CTX	1.8	4.8	<.01	1.6	3.4	<.05
CTLA4Ig	2.0	3.0	NS	2.3	2.6	NS
CTX + CTLA4Ig	2.3	0.6*	<.001	2.3	1.7†	NS

[a] Expressed as geometric mean titer, among mice with either mild or advanced renal disease during treatment with saline, CTX, CTLA4Ig, or combined CTX and CTLA4Ig. Values of *p* are for comparisons between mean titers at the end of treatment and the beginning of treatment for each treatment. Anti-dsDNA Abs were not determined beyond 12 wk for mice with advanced disease because there were no surviving mice in the saline and single-treatment groups at 16 wk. *, $p < 0.001$ for CTX + CTLA4Ig vs saline at 12 wk; †, $p < 0.01$ for CTX + CTLA4Ig vs saline at 16 wk.

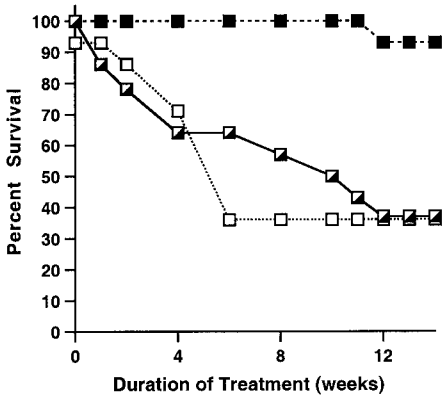

FIGURE 2. Survival among mice with advanced renal disease. Percent survival during treatment with CTX (◪), CTLA4Ig (□), or combined CTX and CTLA4Ig (■).

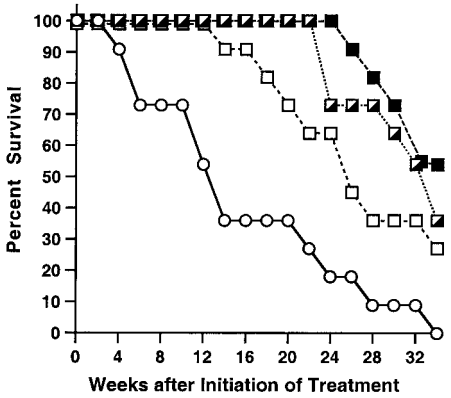

FIGURE 4. Survival among mice with mild renal disease. Percentage of mice surviving during and after receiving 16 wk of treatment with CTX (◪), CTLA4Ig (□), combined CTX and CTLA4Ig (■), or saline (○).

As a first step toward clarifying the mechanistic basis for the apparent synergism between CTX and CTLA4Ig, we evaluated the effects of therapy on circulating lymphocyte populations. In mice with mild or advanced disease, CTX appeared to effect primarily B cells and CTLA4Ig appeared to effect primarily $CD4^+$ T cells (Fig. 5). Specifically, CTX caused a significant (>50%) decline in the absolute number of B cells, whether it was administered alone or in combination with CTLA4Ig, but it did not alter T cells counts. In contrast, CTLA4Ig, either alone or in combination with CTX, caused a significant (>50%) rise in the absolute number of $CD4^+$ T cells, but did not alter B cell counts. Neither CTX nor CTLA4Ig altered the numbers of $CD8^+$ T cells (data not shown). The absolute lymphocyte counts were higher in mice with mild renal disease (Fig. 5, *A* and *B*), consistent with previous studies that have established that progressive lupus in B/W mice is accompanied by progressive lymphopenia.

Discussion

This study demonstrates that the combination of CTLA4Ig and CTX is an extremely effective treatment for active murine lupus nephritis. Previous studies in B/W mice demonstrated that CTX is very effective in treating murine lupus, especially lupus nephritis (1–4). Comparison studies in B/W mice (23) and humans (24) established it as the most effective of various immunosuppressive drugs commonly used in human SLE. When administered to B/W mice at the onset of renal disease, CTX stopped the progression of uremia and proteinuria and resulted in prolonged survival (1). However, the incidence of uremia and proteinuria, and the degree of kidney damage observed, remained unchanged from baseline levels, indicating that CTX did not reverse existing changes (1, 2). Our results are consistent with these earlier observations; CTX stabilized the level of disease at the level at which it was before the start of treatment. Similarly, CTLA4Ig retarded progression of renal disease, but did not reduce proteinuria. Thus, in this direct

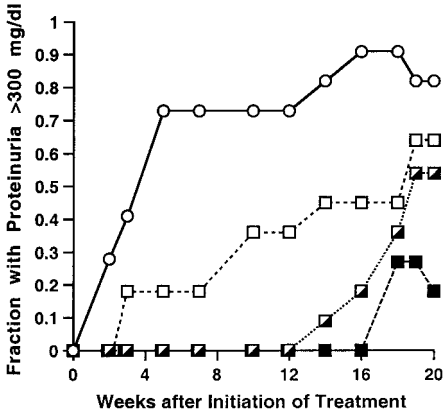

FIGURE 3. Development of proteinuria among mice with mild renal disease. Proteinuria was measured during 16 wk of treatment and 1 mo following cessation of treatment with CTX (◪), CTLA4Ig (□), combined CTX and CTLA4Ig (■), or saline (○).

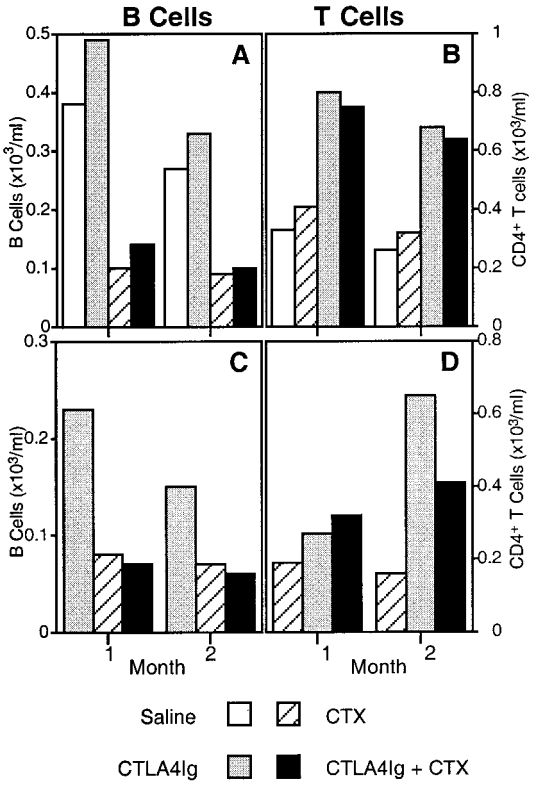

FIGURE 5. Lymphocyte subsets during treatment. Peripheral B cell counts (*A*) and peripheral $CD4^+$ cell counts (*B*) in mice with mild renal disease during the first 8 wk of treatment with CTX (▨), CTLA4Ig (▤), combined CTX and CTLA4Ig (■), or saline (□). Peripheral B cell counts (*C*) and peripheral $CD4^+$ cell counts (*D*) in mice with advanced renal disease during the first eight weeks of treatment CTX (▨), CTLA4Ig (▤), or combined CTX and CTLA4Ig (■).

comparison, both agents appear to have comparable efficacy in murine lupus nephritis and result in a parallel improvement in survival. However, despite similar efficacy as single agents, coadministration of CTX and CTLA4Ig to B/W mice with severe proteinuria was clearly more efficacious than either one alone, resulting in a rapid and sustained reduction in proteinuria in mice with advanced kidney disease. Recently, anti-CD40 ligand therapy in SNF_1 lupus-prone mice was shown to preserve kidney function in mice with established nephritis (25). In that study, mice with mild to moderate proteinuria at the start of treatment had some improvement in proteinuria after receiving anti-CD40 ligand. This suggests that other agents that interrupt T cell and APC costimulatory interactions might also have increased efficacy when combined with CTX.

The benefit of combining these two agents may be due to both similar and differing modes of action. CTX is an alkylating agent that damages DNA repair mechanisms and is toxic to both resting and dividing cells, although proliferating cells are generally more susceptible (26). CTX has multiple effects on the immune system with both immunosuppressive and apparent immunostimulatory effects (15, 27). In contrast to these broad and relatively nonspecific effects, CTLA4Ig specifically binds B7 molecules on APC with high avidity, interrupting CD28-mediated costimulation of T cells that have been activated via the TCR (21). In vitro, CTLA4Ig blocks T cell-dependent responses (21) and can result in Ag-specific unresponsiveness (28), suggesting that some of its in vivo immunosuppressive effects may result from the induction of Ag-specific tolerance. In vivo, CTLA4Ig suppresses cell-mediated immune responses (29) and T cell-dependent Ab responses (18).

In the current study, we found that these agents appear to have differing effects on the development of autoantibodies. The progressive increase in anti-dsDNA Abs observed in control mice was prevented by CTLA4Ig, but not by CTX. We also observed different effects on circulating lymphocyte subsets. CTX was more effective in reducing peripheral B cell counts, consistent with previous descriptions of the effect of CTX on peripheral B cells in mice and humans (30). CTLA4Ig, in contrast, was associated with an increase in $CD4^+$ T cell counts, particularly in mice with advanced disease. Although we did not observe a similar increase in $CD8^+$ T cells, the increase in $CD4^+$ T cells may reflect an improvement of lupus-induced lymphopenia, rather than a specific proliferative effect on $CD4^+$ T cells. In this model, we are also not able to assess whether autoreactive T cells are rendered anergic by this therapy. Taken together, however, these findings support the hypothesis that the benefit of combining these two agents is the result of different, complementary actions.

Overall, we found that CTLA4Ig is comparable in efficacy to CTX for the treatment of murine lupus nephritis in both early and advanced stages of disease. Thus, in the same model in which the effectiveness of CTX for lupus nephritis was first demonstrated, administration of an agent that blocks T cell costimulation has similar efficacy to CTX, the agent currently most widely used to treat human lupus nephritis. Furthermore, we found that the effects of these agents are complementary when used together. Combining CTLA4Ig with CTX resulted in a dramatic reduction in proteinuria, which is unprecedented in this model. The observed synergistic benefit of combining these agents, which was most apparent once renal disease was advanced, also suggests that these two immunosuppressants have differing mechanisms of action in lupus nephritis. These results therefore suggest the possibility for new treatment strategies for human SLE, which might serve to reduce some of the toxicities of CTX while maintaining, or even improving efficacy.

Acknowledgements

We thank Elizabeth Kaufman for valuable technical assistance and Robert Peach of Bristol-Myers Squibb for the generous provision of CTLA4Ig.

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