Effect of Immunosuppressive Agents on the Immunogenicity and Efficacy of an Immunotoxin in Mice¹

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ABSTRACT
Immunotoxins (ITs) are potent cytotoxic agents used in the treatment of cancer, autoimmune disease, and graft-versus-host disease. Results from clinical trials demonstrate that many IT-treated patients, especially those with an intact immune system, develop anti-IT antibodies that may prohibit repeated IT dosing. We, therefore, evaluated a panel of novel immunosuppressive (IS) agents for their ability to inhibit the antitoxin immune response in mice receiving multiple courses of a ricin A chain (RTA)-containing IT and also assessed whether this suppression would result in an increase in IT-mediated antitumor activity. The results indicate that a 3-day pretreatment, plus one additional boost 2 weeks later, of a combination of hCTLA4Ig + anti-CD40L, virtually eliminated the anti-RTA response in normal mice receiving six weekly injections of an IT. When tested in BCL1 tumor-bearing mice, the concomitant use of a combination of hCTLA4Ig + anti-CD40L and six doses of the IT resulted in a 1.5-fold increase in tumor cell killing, as compared with treatment with IT alone. We conclude that a combination of IS + IT therapy should facilitate the administration of multiple courses of IT, as well as enhance its antitumor activity.

INTRODUCTION
ITs³ are antibody-toxin conjugates used in the treatment of cancer, autoimmune disease, and graft-versus-host disease. To date, several hundred cancer patients have been treated in Phase I trials. Approximately 30% of the patients treated with RTA ITs produce both HAMA and HARA. This is undesirable because the presence of these antibodies may alter the pharmacokinetics of the IT and, hence, its activity. To improve the efficacy of ITs and allow multiple courses to be administered, the immune response against the IT must be eliminated or at least markedly reduced. The utilization of chimeric (1) or humanized (2) MAb may solve the problem of the immunogenicity of the antibody but not the RTA.

The administration of different IS agents has been evaluated as a means of minimizing the host anti-IT antibody response. Classical immunosuppressive agents, such as cyclophosphamide (3) and cyclosporine A (4), were generally unsuccessful in significantly reducing HARA titers. The effectiveness of these agents was most likely limited by the dose which could be administered without hepatic or renal toxicities. A new generation of modified chemical IS agents, such as 15-deoxyspergualin (5), were also only moderately successful. Although the side effects associated with these new agents are substantially less, they still exert broad immunosuppression.

Recently, several cell-surface molecules that are crucial to the regulation of T-dependent B-cell activation have been identified. MAbs and fusion proteins targeting these molecules have been developed, and these reagents are highly immunosuppressive. Thus, anti-CD4 (6), anti-B7-2 (7), anti-CD40L (8), and hCTLA4Ig (which binds to mouse B7-1 and B7-2; Ref. 9) induce selective and prolonged immunosuppression in vivo to T cell-dependent antigens.

The goals of this study were to use the T or B cell-specific reagents to develop an IS regimen that would significantly reduce or eliminate the HARA response during multiple courses of IT therapy and also to determine whether suppressing this response would result in an increase in the therapeutic efficacy of a B cell-reactive IT in BCL1 tumor-bearing mice.

MATERIALS AND METHODS
Animals. Female BALB/c mice 6–10 weeks of age were purchased from Harlan/Sprague Dawley (Indianapolis, IN) and housed in the animal facility at the University of Texas–Southwestern Medical Center in Dallas.

JA12.5-dgA IT. Rat-anti-murine δ (JA12.5) was provided by Dr. J. M. Davie (Searle & Co., Skokie, IL) and purified by Damon Biotech (Needham Heights, MA). JA12.5 was conjugated to dgA using N-succinimidyl-3-(2-pyridyldithio)propionate (10, 11). The IT is referred to as JA12.5-RTA in the text.

Immunosuppressive Agents. hCTLA4Ig (12) was a gift from Dr. P. S. Linsley (Bristol-Myers Squibb, Seattle, WA). Hamster-anti-murine CD40L was a gift from Dr. R. J. Noelle (Dartmouth Medical School, Lebanon, NH) and was also purified from the MR1 hybridoma (13) obtained from American Type Culture Collection (Rockville, MD). Rat-anti-murine B7-2 was purified from the GL1 hybridoma (7) obtained from American Type Culture Collection. Rat-anti-murine L3T4 (anti-CD4) was purified from the GK1.5 hybridoma (14). Human,
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The resultant sera were transferred to micro Eppendorf tubes and stored at -20°C. Mouse anti-RTA titers were determined by an indirect ELISA using serial dilutions of the sera. Anti-RTA titers were determined from the linear portion of the standard curve. The values obtained for each mouse are the average of three separate ELISAs using at least three wells/dilution minus the absorbance obtained using the equivalent dilution of normal mouse sera. The standard curve for each ELISA is the average of at least six wells/concentration minus the absorbance obtained using secondary antibody alone. Reported titers are the average of five mice per treatment group ± SD.

**Determination of Tumor Burden.** Total viable spleen cell counts were determined by trypan blue exclusion. FACs analysis was performed using 96-well microtiter plates (Dynatech, Chantilly, VA). A single-cell suspension of splenocytes at 1 × 10^6 cells in 50 µl of PBS/azide was incubated with 5 µg of MAb 6A5 and rat IgG2b-anti-BCL1, idiotypes (18) in 5 µl of PBS/azide or 5 µg of normal rat IgG2b in 5 µl of PBS/azide for 20 min on ice. The plates were centrifuged at 1200 rpm for 7 min, wells were washed three times with 200 µl PBS/azide, and 5 µl of a 1:20 dilution of mouse-anti-rat kappa (MARK-I) were added to wells. Plates were incubated on ice for 20 min, and the cells were centrifuged at 1200 rpm for 7 min, washed three times with 200 µl PBS/azide per well, resuspended in 1 ml PBS, vortexed, and filtered through nylon cloth to remove aggregates. The percentage of BCL1 Ig+ cells was calculated as %6A5+ – % irrelevant rat immunoglobulin-.

**Treatment of BCL1-bearing Mice with Six Weekly Injections of IT plus a Combination of IS Agents.** Mice were injected i.p. with 5 × 10^6 splenocytes from BCL1-bearing mice (15), and groups of five mice were treated with the regimens described in Table 1. Mice were bled weekly for 6 weeks, and their abdomens were palpated weekly to assess tumor growth as manifested by the onset of splenomegaly (16). All mice were sacrificed by CO2 asphyxiation 48 h after the last IT injection (17), and the spleens were removed. The tumor burdens were determined by cell counts and FACS staining to enumerate BCL1 Ig+ cells.

**Murine-Anti-RTA Immunoglobulin ELISA.** Wells of 96-well flat-bottomed polystyrene plates (Corning, Rochester, NY) were coated with 50 µl/well of RTA at 10 µg/ml overnight at 4°C. Wells were blocked with 200 µl/well BSA Tween 20 for 30 min at room temperature and washed three times with dH2O. Fifty µl of primary antibody (serial dilutions of mouse sera from treated or normal animals) in blocking buffer were then added to each well. For the standard curve, 50 µl of purified murine-anti-RTA in blocking buffer were added and incubated at room temperature for 2 h. The wells were washed, and 50 µl of secondary goat-anti-murine IgG (H&L)-HRP antibody (Pierce) diluted 1:5000 in blocking buffer were added and incubated for 2 h at room temperature. Wells were washed, and 50 µl of HRP substrate (Bio-Rad Laboratories, Hercules, CA) were added. After 10 min at room temperature, the absorbance at 405 nm was read. Anti-RTA titers were determined from the linear portion of the standard curve. The values obtained for each mouse are the average of three separate ELISAs using at least three wells/dilution minus the absorbance obtained using the equivalent dilution of normal mouse sera. The standard curve for each ELISA is the average of at least six wells/concentration minus the absorbance obtained using secondary antibody alone. Reported titers are the average of five mice per treatment group ± SD.

**Statistical Analysis.** Statistical analysis was performed using a two-tailed Student’s t test. P < 0.05 was considered to be significant.
RESULTS

Suppression of the Anti-RTA Response in Normal Mice Injected with One Course of IT and IS Agents. The first goal of the study was to evaluate the panel of individual IS agents for their ability to decrease an anti-RTA response in normal mice injected with a single dose of the IT. Groups of mice were pretreated for 3 days with the each IS agent and then injected i.p. with 60 μg of JA12.5-RTA in PBS. In mice not receiving an IS, anti-RTA responses peaked on day 21. As shown in Fig. 1, hCTLA4Ig (P < 0.03) and anti-CD4OL (P < 0.004) were the most suppressive agents. Moreover, they did not induce the depletion of splenic CD4+ T cells. Anti-B7–2 and anti-CD4 were immunosuppressive but not at levels that were statistically different from those induced by the control antibodies (P > 0.05). Treatment with the control antibodies alone resulted in a modest suppression of the anti-RTA immune response. This effect may be similar to that observed during the i.v. administration of immunoglobulin (19).

Suppression of the Anti-RTA Response in Normal Mice Given Repeated IT Injections. We next examined the ability of the IS agents to suppress the anti-RTA immune response when four courses of 30 μg of IT were administered. The mice were pretreated with various IS agents or control antibodies as before and were then injected i.p. with four doses of IT administered at weekly intervals starting on day 0. The doses of all of the IS agents were 250 μg, and the antibody responses were followed for 6 weeks. Mice treated with the IT, together with the control antibody, had peak anti-RTA titers on day 35. As shown in Fig. 1, all of the IS agents significantly suppressed the murine-anti-RTA response; hCTLA4Ig and anti-CD4 suppressed by >99%, and anti-CD4OL and anti-B7–2 suppressed by 72 and 37%, respectively.

Fig. 1 The anti-RTA response in normal mice receiving various regimens of IT and IS agents. The percentage of control was calculated for the day corresponding to peak anti-RTA immunoglobulin titers. A, anti-B7–2; B, anti-CD4; C, anti-CD4OL; D, hCTLA4Ig. In A, mice were injected with 60 μg of JA12.5-RTA i.p. on day 0 + IS agent (50 μg of anti-B7–2, anti-CD4, or hCTLA4Ig or 500 μg of anti-CD4OL) i.p. on days −1, 0, and 1. Day 21 sera were used. In B, mice were injected with 30 μg of IT i.p. on days 0, 7, 14, and 21 + IS agent (250 μg of anti-B7–2, anti-CD4, anti-CD4OL, or hCTLA4Ig) i.p. on days −1, 0, and 1. Day 35 sera were used. The control represents treatment with an irrelevant species-matched antibody. Statistical analysis gave P values of <0.03, <0.001, <0.02, and <0.002, respectively, for the above groups. In C, mice were injected with IT + IS agents as in B, plus additional boosts with IS agents on days 7, 14, and 21. Statistical analysis gave P values of <0.007 for all IS agents tested.

To determine whether the anti-RTA responses could be completely prevented, three additional weekly boosts of the IS agents were administered. As shown in Fig. 1, the anti-RTA response was below detection in all of the IS-treated mice.

Suppression of the Anti-RTA Response in Normal Mice Treated with Multiple IT Injections by a Combination of IS Reagents. Because our long-term goal is to achieve complete suppression of the anti-RTA response so that multiple IT injections can be administered, we next evaluated a combination of two of the IS agents. Mice were injected with a combination of hCTLA4Ig, together with anti-CD4OL, followed by six weekly IT injections of 30 μg. The kinetics of the anti-RTA responses in mice pretreated with the IS regimen plus one boost on day 14 are shown in Fig. 2. Peak titers were reached on day 42, and all IS treatment protocols resulted in varying degrees of suppression of the anti-RTA responses (Fig. 3). Hence, as compared with the control antibodies, pretreatment alone with the IS decreased the anti-RTA titer by 71%. Pretreatment, followed by one or two additional weekly boosts of the IS agents, resulted in 89–95 and 97% decreases in the titers, respectively. Finally, pretreatment plus three additional weekly boosts of IS agents inhibited the anti-RTA response by 99%.

Evaluation of RTA-specific Tolerance Induced by IS Agents. To determine whether the concomitant administration of IS agents and IT induces tolerance against RTA, mice were initially injected with three weekly courses of IT ± IS agents or control antibodies. The anti-RTA antibody responses were decreased by 80, 92, 83, or 99% in mice treated with IT plus anti-CD4, anti-B7–2, anti-CD4OL, or hCTLA4Ig, respectively (data not shown). As shown in Fig. 4, IT plus a combination of hCTLA4Ig and anti-CD4OL suppressed the anti-RTA antibody response by >99%, as compared with mice treated with either IT or IT + antibody. Six weeks were then allowed to elapse, and the mice were challenged on day 56 with IT or KLH. The resultant anti-RTA titers in mice initially treated with IT + IS...
were equivalent to the primary antibody response of those treated with IT + control antibodies. In addition, the anti-KLH titers in the IT + IS-treated mice were the same as those in the mice treated with IT + control antibodies. Six weeks later (day 98), the mice were rechallenged with IT. Fig. 4 shows that mice initially treated with IT + IS generated a normal secondary anti-RTA immune response. These results demonstrate that immunosuppression is transient and that repeated administration of the IS agents are necessary. Because the anti-RTA titers were followed for an extensive period of time, it is likely that IS agents were inhibiting rather than delaying an immune response against RTA.
**Antitumor Response of the IT ± IS.** To evaluate the effect of suppressing the anti-RTA response on the ability of the IT to kill tumor cells *in vivo*, mice were injected with BCL1 tumor cells and then treated with the most effective IS regimen consisting of a combination of hCTLA4Ig + anti-CD40L. As shown in Fig. 5, treatment with the IS agents also abrogated the anti-RTA response in the tumor-bearing mice. With regard to the antitumor effect of the regimens, treatment with the IT + IS decreased the number of BCL1 tumor cells in the spleens by 88%, whereas treatment with the IT alone or with the IT and the control antibodies decreased the BCL1 tumor burdens by 63–64% (Fig. 6). In contrast, treatment of the BCL1 tumor-bearing mice with either the IS agents or the control antibodies did not result in a statistically significant change in the splenic tumor burden (Fig. 6), demonstrating that these IS agents *per se* do not affect the growth of the BCL1 tumor. Thus, optimal antitumor activity was achieved when little or no anti-RTA was generated.

**DISCUSSION**

The goal of this study was to develop an immunosuppressive regimen that would significantly reduce or eliminate the anti-RTA response generated after the administration of a RTA-containing IT and to determine whether suppressing this response would increase its therapeutic efficacy. The agents evaluated were anti-CD4, anti-B7–2, anti-CD40L, and hCTLA4Ig. Our studies demonstrate that although all of the IS agents were effective in suppressing or inhibiting the anti-RTA response in mice receiving one or four courses of IT, based on the doses which we used, hCTLA4Ig was the most potent. Because hCTLA4Ig is known to act synergistically with other IS agents, we combined it with our second most effective IS agent, anti-CD40L. When these two IS agents were used together, they suppressed 99% of the anti-RTA response in normal mice receiving six courses of IT. Most importantly, suppression of the anti-RTA response in tumor-bearing mice led to an increase in the IT-mediated antitumor response.

Clinical trials using RTA-containing ITs have demonstrated that IT therapy induces the production of antibodies against both the murine immunoglobulin and the RTA moieties (20–24). These antibodies cause the rapid elimination of the IT from the circulation in subsequent courses, limiting the total dose of IT that can be administered, and therefore, the number of tumor cells that can be killed. The elimination, or at least reduction of the HAMA, but not the HARA response, can be achieved through chimerization (1) or humanization (2) of the murine immunoglobulin, but this does not solve the problem of the antibody response against a plant or bacterial toxin.

The generation of a TD immune response requires the interaction of several surface molecules on B cells with their ligands on T<sub>B</sub> cells. These include MHC class II/CD4, CD40/CD40L, B7/CD28, and B7/CTLA-4. Recent evidence suggests that antibodies or fusion proteins that disrupt these interactions are highly immunosuppressive *in vivo*. Thus, anti-CD4 inhibits the immune response after multiple courses of IT in mice (28). The hCTLA4Ig binds to B7-1 and B7-2 on B cells and interferes with CD28-mediated signaling in T<sub>B</sub> cells, thereby suppressing TD antibody responses and even blocking xenogeneic pancreatic islet cell graft rejection (25). Similarly, the administration of anti-B7–2 MAb suppresses TD immune responses (7) and increases graft survival (26). Finally, by preventing B-cell triggering through CD40, the anti-CD40L MAb prevents the development of CD4<sup>+</sup> T cell-dependent B-cell responses and facilitates the engraftment of allogeneic islet cells (27).

Using the rat-anti-β IT, JA12.5-RTA, as our immunogen, we evaluated a panel of the above-mentioned agents in normal mice. Our studies demonstrate that pretreating mice with hCTLA4Ig or anti-CD40L effectively suppresses the anti-RTA response without depleting splenic CD4<sup>+</sup> T cells. Furthermore,
in agreement with a recent report (29), we found that the combined use of the two IS agents, hCTLA4Ig + anti-CD40L, inhibited the anti-RTA response in normal mice receiving six courses of IT. This IS regimen also inhibited the immune response against the rat immunoglobulin portion of the IT. In addition, our results indicate that the decreased anti-IT response was not due to the induction of tolerance against RTA. This conclusion is in agreement with results of a study demonstrating the inability of hCTLA4Ig to induce tolerance against sheep RBCs in mice (30).

To determine whether suppressing the anti-RTA response would result in an increase in the therapeutic efficacy of the IT, we evaluated the combination IS regimen in BCL1 tumor-bearing mice receiving IT therapy. BCL1 is a sIgD+ B-cell lymphoma (31) that grows primarily in the spleens of BALB/c mice, with tumor burdens reaching 20% of their body weights (32). As demonstrated previously (17), JA12.5-RTA successfully eliminated large numbers of BCL1 lymphoma cells in vivo. Our studies further demonstrated that treatment with four doses of the combined IS agents during IT therapy resulted in an 88% reduction in tumor burden, representing an elimination of $1.1 \pm 0.1 \times 10^9$ tumor cells. In contrast, the IT alone resulted in a 63% reduction in tumor burden, i.e., the elimination of $0.8 \pm 0.08 \times 10^9$ tumor cells. Thus, the use of the IS regimen in conjunction with IT therapy resulted in a 1.5-fold improvement in tumor cell killing. Because treatment of the tumor-bearing mice with the IS agents alone did not have an antitumor effect, the improved antitumor response cannot be attributed to a direct effect of the IS agents on tumor growth. In patients, the enhanced antitumor activity could be even more dramatic because tumor burdens,
even in minimal residual disease, would be larger. Furthermore, in patients with more bulky chemorefractory disease, ITs are often highly effective in eliminating kilograms of tumor (22–24), but cures cannot be achieved because of subsequent HAMA/HARA responses. If these responses could be eliminated, otherwise refractory tumors might be curable with 5–10 courses of IT therapy.

The mechanisms by which induced or preexisting anti-IT antibodies diminish the antitumor effect of the IT are not yet clearly understood. Anti-IT antibodies could influence either the levels of the IT in the circulation, the ability of the antibody to bind to tumor cells, or the enzymatic activity of the RTA. There are several reports of a prolonged half-life of the less immunogenic chimeric molecules as compared to their more immunogenic counterparts (33–35). It has also been reported that HAMA dramatically altered the pharmacokinetics of an infused MAb by mediating its rapid clearance (36). Studies of IT administered to rhesus monkeys demonstrated that the presence of anti-IT antibodies reduced the serum half-life of the IT from hours to minutes.5

Antibodies that bind to an IT may also alter its ability to bind to target cells in vivo. For example, patients with colorectal cancer treated with an RTA-containing IT mounted an anti-IT humoral immune response, which inhibited the in vitro binding of the IT to target cells (37). A similar finding was reported using nude mice with human tumor xenografts, where the administration of anti-idiotypic antibody prevented tumor localization of the MAb (38).

Because RTA must assume a specific tertiary structure to maintain its active site and enzymatic activity, antibodies that bind to the RTA portion of the IT may inhibit its cytotoxic activity by altering the conformation of the molecule (39). Indeed, it has been shown that the presence of antitoxin antibodies does not necessarily prevent the binding of the IT to its target cells but can block RTA-mediated cytotoxicity in vitro (40, 41). Thus, even if the IT could bind to target cells and undergo appropriate intracellular routing, it may not retain its cytotoxic activity in the presence of anti-RTA antibodies.

One of the first attempts to use IS agents in humans to inhibit the anti-IT response was based on the results of studies in rats where a significant reduction in the anti-IT humoral immune response was observed following concomitant administration of one dose of cyclophosphamide (42, 43). Results using this combination of IT + cyclophosphamide in a Phase II trial in patients with metastatic malignant melanoma proved unsuccessful (3). Although the IS agents tested in this study target a more specific subset of cells, it is unclear whether our results will translate to humans. In addition, there is the concern that the IS agents used to treat cancer patients may promote tumor growth. Although this was not observed in the present study, there is evidence to suggest that immunosuppression favors the growth of at least some existing malignant cells (44).

In summary, the use of hCTLA4Ig + anti-CD40L in the treatment of cancer patients receiving multiple courses of IT is a viable option that should be further evaluated. Combination therapy should both facilitate the administration of multiple doses of IT and increase the antitumor activity of each dose, thus increasing efficacy.

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Note Added in Proof

While this paper was under review, a report by Siegall et al. (J. Immunol., 159: 5168–5173, 1997) demonstrated that hCTLA4Ig suppressed the production of anti-IT responses in mice, rats, and dogs. Furthermore, tumor-bearing rats treated concomitantly with IT + hCTLA4Ig could tolerate an additional course of IT, which induced greater antitumor efficacy.

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