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# Glucocorticoid-mediated Control of the Activation and Clonal Deletion of Peripheral T Cells In Vivo

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## Summary

Poly- and oligoclonal T cell stimuli like anti-CD3ε monoclonal antibody or *Staphylococcus aureus* enterotoxin B (SEB), injected at doses that per se are not lethal, provoke acute death within less than 24 h, provided that endogenous glucocorticoids (GC) are depleted by adrenalectomy or by injection of saturating amounts of the GC receptor antagonist RU-38486 (mifepristone). Pharmacological doses of the GC agonist dexamethasone (DEX) alter the in vivo response of splenic $V\beta8^+$ T cells to SEB, thus impeding the expansion of such cells and causing their rapid (3 d) clonal deletion. In contrast, coadministration of RU-38486 counteracts a SEB-induced early (12 h) reduction of $V\beta8^+CD4^+$ and $V\beta8^+CD8^+$ spleen cells. In vivo T cell stimulation by injection of bacterial superantigen induces a rapid (peak at 90–120 min) increase in corticosterone serum levels, suggesting that endogenous GC might control early T cell activation. Accordingly, kinetic studies revealed that RU-38486 has to be administered within 2 h after superantigen administration to exert its lethal effect. Similarly, exogenous GC must be injected during this critical phase (2 h) to rescue animals from acute death induced by coinjection of SEB and D-galactosamine (GalN). Adrenalectomy, injection of RU-38486 and priming with GalN per se provoke the programmed death of peripheral $CD4^+$ and $CD8^+$ T cells. Thus, three manipulations that sensitize mice for the lethal effect of T cell stimulation also exert a proapoptotic effect on peripheral T cells. In synthesis, endogenous and exogenous GC regulate T cell responses and determine the propensity of peripheral T cells to undergo apoptosis.

Synthetic glucocorticoid (GC)[1] agonists are used for therapy of a broad spectrum of organ-specific and generalized autoimmune diseases. In the same way, endogenous GC secreted by the adrenal glands may act as immunosuppressive and antiinflammatory agents that contend life-threatening overreactions of the immune system, as well as autoaggressive responses (for reviews see references 1, 2). GC inhibit macrophage functions, including cytotoxic functions, processing, and presentation of antigen to T cells, inhibit the production of IL-2 and IFN-γ in T lymphocytes (3), shift T cell responses from the Th1 to the Th2 type (4), decrease the activity of NK cells, induce programmed cell death in a variety of different immunologically relevant cells, including immature T and B cell precursors (for reviews see references 5, 6) and mature T cells (7), and inhibit the synthesis of a variety of proimflammatory cytokines (IL-1, IL-6, and TNF-α) (8). Immune activation frequently is associated with an increase in ACTH and GC secretion, and the susceptibility of certain animal strains to develop autoimmune diseases is linked to a deficient GC-mediated inhibition of immune function (2, 9–12).

Recently, a synthetic steroid, RU-38486 (RU486, mifepristone), with potent antiprogestational and antiglucocorticoid activity has become available for clinical use as an abortifacient agent (13, 14) and for treatment of gestagen-dependent tumors and hypercortisolism (15). In the present study, we investigated the effect of RU-38486 on T cell activation in vivo. It is shown that RU-38486, present during the acute phase of T cell stimulation, dramatically enhances the lethality of in vivo T cell activation. Evidence is presented that administration of T cell activators provokes an acute transient release of corticosterone (CN) into the circulation. Administration of GC reduces the toxicity of T cell activation, while preventing the antigen-driven expansion and accelerating the deletion of the specific T cell subset. The data presented in this report support the contention that (endogenous) GC intervene in the regulation of the activation, expansion, antigen-specific deletion, and programmed cell death of peripheral T cells in vivo.

[1] *Abbreviations used in this paper:* CN, corticosterone; CsA, cyclosporine A; DEX, dexamethasone; GalN, D-galactosamine; GC, glucocorticoid; SEA/B, *Staphylococcus aureus* enterotoxin A/B; PCD, programmed cell death.

## Materials and Methods

*Animals and In Vivo Treatment.* Male 8–10-wk-old BALB/c mice were obtained from the local animal housing facility (Centro de Biología Molecular, CSIC, Madrid), whereas adrenalectomized BALB/c mice (maintained with 0.9% NaCl in the drinking water) and sham-operated BALB/c controls were obtained from a commercial source (Charles River, Lyon, France). Animals received injections of *Staphylococcus aureus* enterotoxin B (SEB) (Sigma Immunochemicals, St. Louis, MO), SEA (Sigma Immunochemicals), mAbs directed against CD3ε (145.C11; 16) or Vβ8 (F23.1 that recognizes Vβ8.1, Vβ8.2, and Vβ8.3; 17), RU-38486 (Roussel Uclaf, Romainville, France, kindly provided by Dr. Martini), cyclosporine A (CsA) (Sandoz, Basel, Switzerland), D-galactosamine (GalN) or dexamethasone (DEX; Sigma Immunochemicals). These reagents were suspended in 200 μl PBS and were injected either intravenously or intraperitoneally at the indicated dose. In one series of experiments, blood was taken 120 min after the injection of SEB, and corticosterone (CN) concentrations were determined by means of a commercial RIA (ICN Biomedicals, Inc., Costa Mesa, CA).

*Immunofluorescence Analysis.* Splenic monocellular cell suspensions were subjected to hypotonic erythrocyte lysis, washed and stained with mAbs directed against CD4 (HK1.5; Becton Dickinson & Co., Mountain View, CA), CD8 (H02.2), Vβ6 (RR4-7; 17), or Vβ8 (F23.1; 18) which were either FITC labeled or biotin conjugated and developed by means of streptavidin-PE as described (19, 20).

*Assessment of Apoptotic DNA Fragmentation.* $CD3^+$, $CD4^+$ or $CD8^+$ splenic T cells were prepared by negative selection using columns (Cellect™; Biotex, Edmonton, Canada). Contamination with $sIg^+$, $CD8^+$, or $CD4^+$ cells was <1% for $CD3^+$, $CD4^+$, and $CD8^+$ T cells, respectively. After a brief in vitro culture (2 h at 37°C in RPMI 1640 supplemented with 10% FCS), $2 \times 10^6$ cells were washed, pelleted, and incubated at 50% in 20 μl of 10 mM EDTA, 50 mM Tris-HCl, 0.5% sodium laurylsarkosinate, and 0.5 μg/ml protease K, followed by the addition of 10 μl of 10 μg/ml RNase A, incubation for 1 h at 37°C, and electrophoresis over a 1.2% gel containing 0.1 μg/ml ethidium bromide, as described (20, 21).

## Results and Discussion

*Endogenous GC Protect Mice against the Lethal Effect of Acute T Cell Activation In Vivo.* Normal BALB/c mice do not succumb to doses as high as 250 μg of the CD3ε-specific mAb 145-C11, an antibody that induces a cytokine release syndrome via the nonspecific polyclonal stimulation of T cells (22). However, two manipulations sensitize to the lethal effect of anti-CD3, namely coinjection of the monosaccharide GalN (22) and coinjection of the GC antagonist RU-38486 (Table 1), an agent that has been previously shown to sensitize mice to the lethal effect of TNF (23), one of the major intermediate effector molecules of polyclonal T cell stimulation (22, 24). The $ED_{50}$ of αCD3 combined with GalN (~2.5 μg αCD3) is lower than that of αCD3 combined with RU-38486 (>10 μg αCD3). The sensitizing effect of the anti-GC RU-38486 is abolished by coadministration of DEX and is not restricted to αCD3, but also concerns the superantigen SEB, which specifically stimulates $V\beta8^+$ T cells (about 30% of $CD3^+$ T cells in BALB/c mice). The toxic effect of SEB requires the presence of large numbers of superantigen-reactive $V\beta8^+$ T cells (25). Accordingly, SEA (which stimulates $V\beta3^+$ and $V\beta11^+$ T cells) is not toxic for BALB/c mice that contain <1% of SEA-reactive $V\beta3^+$ or $V\beta11^+$ cells among peripheral lymphocytes (but kills GalN-sensitized C57/B16 mice that contain >10% $V\beta3^+$ plus $V\beta11^+$ T cells). Surprisingly, the Vβ8-specific antibody F23.1 was not capable of killing GalN- or RU-38486-sensitized BALB/c mice (Table 1), indicating that the mode of stimulation exerted by this antibody must differ from that of the equally Vβ8-specific superantigen SEB. Accordingly, the signal transduction cascades triggered by superantigens and conventional stimuli exhibit an only partial overlap in vitro (26, 27). Moreover, F23.1 differs from SEB in the sense that its in vitro application fails to trigger an expansion of $V\beta8^+$ T cells but rather provokes an immediate programmed cell death-mediated depletion of $V\beta8^+$ T cells (data not shown).

Since pharmacological doses of RU-38486 might have GC-agonistic and nonspecific toxic effects (28), we tested whether adrenalectomy also would predispose BALB/c mice to the lethal effect of SEB. Indeed, adrenalectomy with the subsequent decrease in endogenous GC level rendered animals susceptible to the acute lethal effect of SEB. This phenomenon was readily reverted by administration of low doses of DEX (Table 1). Thus, endogenous GC control the acute response to T cell stimulation in vivo, and the effect of RU-38486 can be attributed to the blockade of GC receptors.

*Mutual Interaction between the Immune and the GC Systems during a Critical Phase of Several Hours.* As shown in Fig. 1, the injection of SEB induces an increase in circulating CN levels by at least one order of magnitude over the baseline (peak at 90–120 min). This increase in plasma CN drops off within 6 h after injection. The CN response induced by SEB

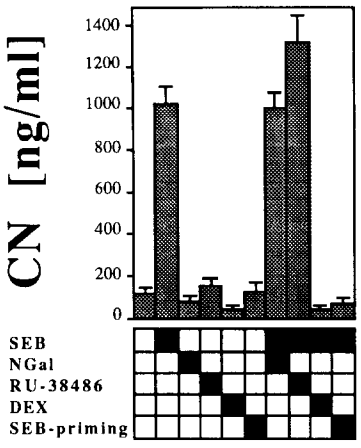



**Figure 1.** CN serum levels after injection of SEB alone or in combination with several immunomodulators. BALB/c mice received injections (*black squares*) of *S. aureus* enterotoxin B (*SEB*; 50 μg i.v.), D-galactosamine (*NGal*, 20 mg i.p.), RU-38486 (10 mg i.p. at the same time) dexamethasone (*DEX*, 1 mg, 2 h before SEB), or were primed with SEB (50 μg i.v. 6 h before the second injection of SEB). (*White boxes*) Appropriate PBS controls. After 2 h, animals were killed by cervical dislocation and heart blood was immediately removed. CN serum levels (three to four animals per group) were measured by RIA.

**Table 1.** *Endogenous GC Control the Lethal Effects of Polyclonal T Cell Stimuli*

| Toxin (Intravenous doses) | Sensitizing agent (Intraperitoneal doses) | Percent mortality (Deaths/total) |
|---|---|---|
| None | 10 mg RU486* | 0 (0/5) |
| None | 20 mg GalN | 0 (0/6) |
| 250 μg α CD3 | None | 0 (0/6) |
| 100 μg α CD3 | 10 mg RU486 | 100 (6/6) |
| 50 μg α CD3 | 10 mg RU486 | 100 (6/6) |
| 50 μg α CD3 | 10 mg RU486 + 1 mg DEX | 0 (0/5) |
| 25 μg α CD3 | 10 mg RU486 | 100 (3/3) |
| 10 μg α CD3 | 10 mg RU486 | 0 (0/3) |
| 5 μg α CD3 | 10 mg RU486 | 0 (0/3) |
| 25 μg α CD3 | 20 mg GalN | 100 (3/3) |
| 10 μg α CD3 | 20 mg GalN | 100 (6/6) |
| 5 μg α CD3 | 20 mg GalN | 100 (6/6) |
| 2.5 μg α CD3 | 20 mg GalN | 67 (2/3) |
| 1 μg α CD3 | 20 mg GalN | 0 (0/6) |
| 500 μg SEB | None | 0 (0/3) |
| 100 μg SEB | None | 0 (0/5) |
| 100 μg SEB | Sham-operated | 0 (0/5) |
| 100 μg SEB | Adrenalectomized | 100 (5/5) |
| 100 μg SEB | Adrenalectomized + DEX‡ | 0 (0/5) |
| 100 μg SEB | 10 mg RU486 | 100 (5/5) |
| 50 μg SEB | 10 mg RU486 | 100 (5/5) |
| 50 μg SEB | 5 mg RU486 | 25 (2/8) |
| 50 μg SEB | 1 mg RU486 | 0 (0/5) |
| 50 μg SEB | 10 mg RU486 + 1 mg DEX | 0 (0/5) |
| 25 μg SEB | 10 mg RU486 | 67 (2/3) |
| 10 μg SEB | 10 mg RU486 | 0 (0/6) |
| 50 μg SEB | 20 mg GalN | 100 (6/6) |
| 100 μg SEA | None | 0 (0/6) |
| 100 μg SEA | 10 mg RU486 | 0 (0/6) |
| 100 μg SEA | 20 mg GalN | 0 (0/6) |
| 250 μg α Vβ8 | None | 0 (0/6) |
| 250 μg α Vβ8 | 10 mg RU486 | 0 (0/6) |
| 250 μg α Vβ8 | 20 mg GalN | 0 (0/3) |

8–10-wk-old male BALB/c mice received the simultaneous injection of T cell–targeted stimuli (intravenously via the tail vein) and the indicated sensitizing agent (intraperitoneally) suspended in 200 μl PBS. Death rates were monitored during the following 5 d. In all cases, animals died within an interval of 12–48 h after experimental manipulation.

* RU486, RU-38486.

‡ Animals that had been adrenalectomized 5 d before received two 10 μg i.p. injections of DEX 6 and 2 h before injection of SEB.

was increased in the presence of RU-38486, but was completely prevented by pretreatment of animals with DEX (1 mg i.p. 2 h before the injection of SEB), suggesting the action of a GC-mediated feedback inhibition of the immune-stimulated CN release (1, 29). Injection of SEB into mice that had been primed 6 h earlier with SEB, a manipulation that leads to an exhaustion of T cell function and lymphokine release (30), fails to increment the level of plasma CN. GalN did not modulate the SEB-induced CN response, thus indicating that its mode of action differs from that of RU-38486.

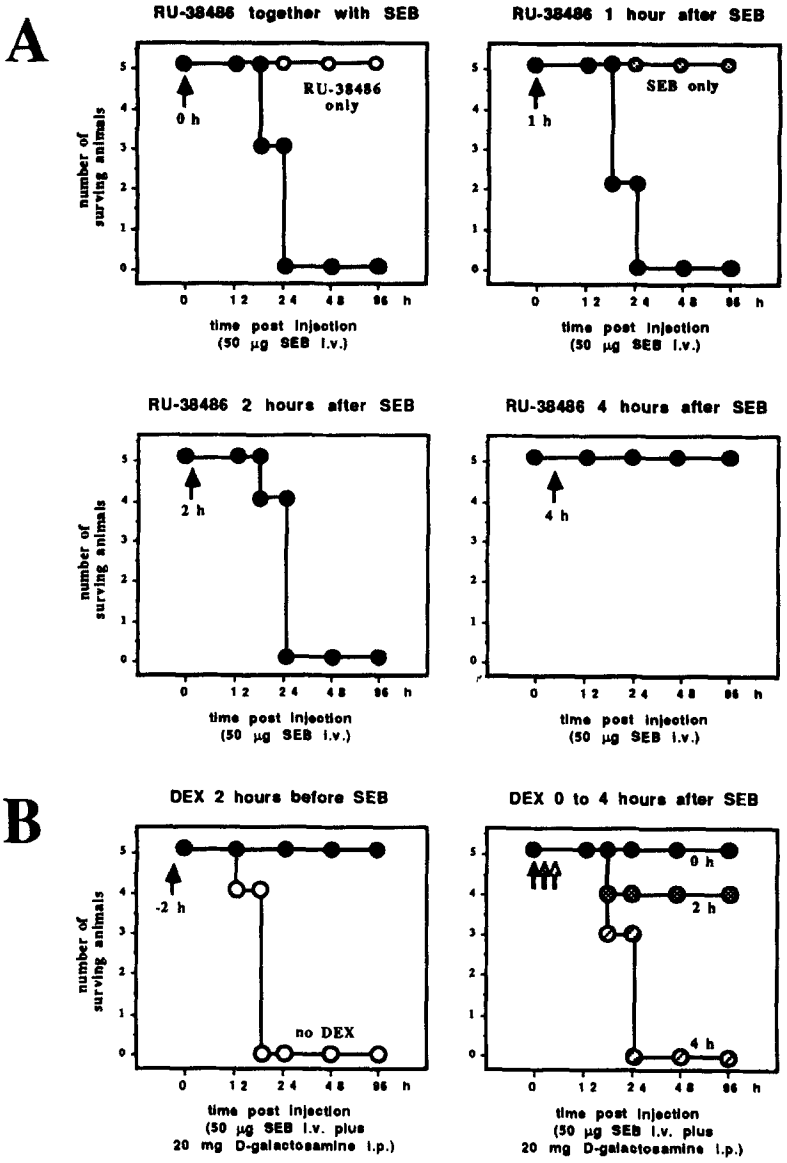



**Figure 2.** Effect of GC antagonists and agonists on SEB toxicity in vivo. (*A*) Effect of the anti-GC RU-38486 on SEB challenge. BALB/c mice received 50 µg SEB i.v. (0 h) followed by intraperitoneal injection of 10 mg RU-38486 0–4 h after SEB. Survival was monitored at 12, 18, 24, 48, and 96 h. (*B*) Effect of DEX on challenge with SEB plus D-galactosamine. DEX (1 mg i.p.) was given 2 h before and up to 4 h after the challenge with SEB (50 µg i.v.) plus D-galactosamine (20 mg i.p., given simultaneously with SEB). Dying animals exhibited hypothermia, pilierection, and diarrhea.

To test whether the SEB-triggered short-term elevation of CN is relevant to the outcome of SEB responses in vivo, the GC antagonist RU-38486 and the GC superagonist DEX were injected at different intervals into mice challenged with SEB. RU-38486 only increased the susceptibility to the lethal effect of SEB when given within 2 h after SEB challenge. Application of RU-38486 after 4 h failed to provoke death (Fig. 2 *A*). Along the same line, DEX only was effective in preventing the acute death of mice receiving simultaneous treatment with SEB and GalN, when administered within 2 h (Fig. 2 *B*). These observations suggest the physiological relevance of acute short-term elevations of GC in the regulation of the immune response.

*Effect of GC on Superantigen-induced Clonal Expansion and Deletion of Peripheral T Cells.* In vivo injection of SEB entails a phase of clonal expansion of V$\beta$8$^+$ T cells (maximum, 2–3 d), followed by a phase of clonal deletion (minimum, 10 d). These changes affect both the CD4$^+$ and the CD8$^+$ subsets (20, 21, 31, 32). To test the possible effect of endogenous GC on the SEB-driven sequential expansion and contraction of V$\beta$8$^+$ T cells, RU-38486 was administered to mice 4 h subsequent to the SEB challenge (as in Fig. 2), followed by two daily injections of RU-38486 throughout the experiment. This should be sufficient to reduce the effect of endogenous GC, as RU-38486 saturates the GC receptor within 1 h after in vivo administration and inhibits the transcription of GC-inducible genes for several hours (15, 33). Moreover, the fact that this protocol of RU-38486 administration and surgical adrenalectomy have similar effects in other experimental systems (vide infra) suggests that the GC receptor blockade is rather complete. In spite of this manipulation, no significant changes in the SEB-modulated expression of

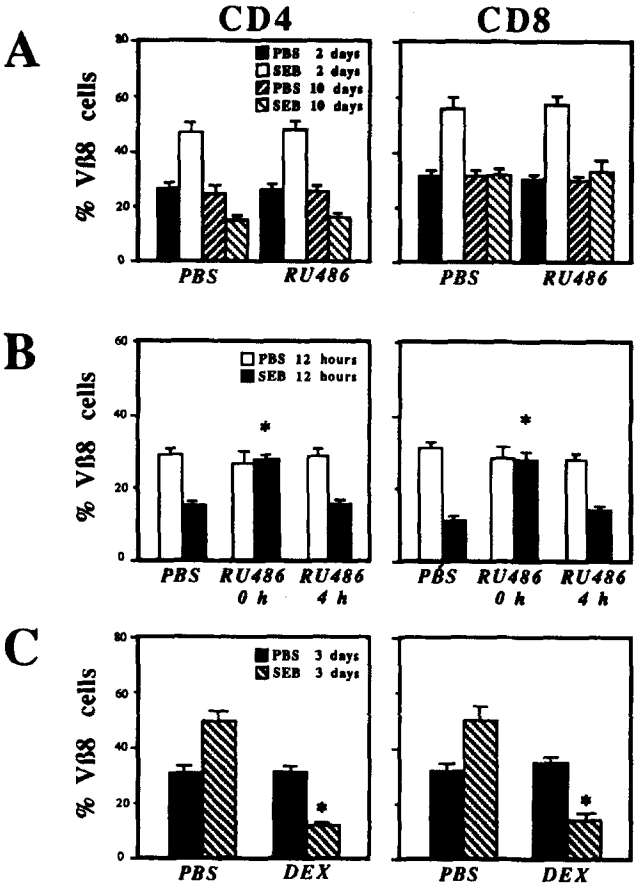
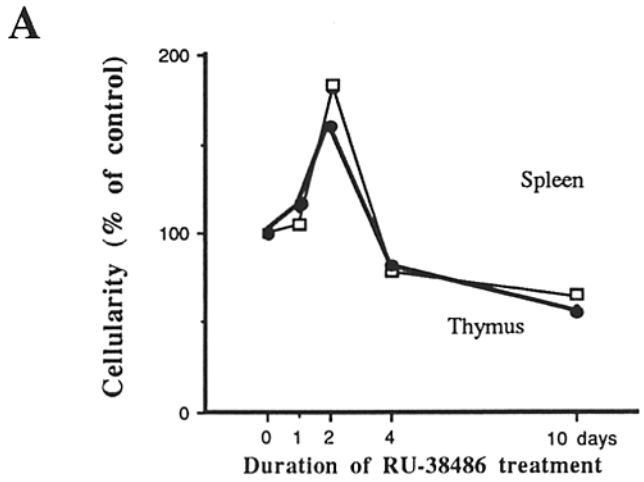



**Figure 3.** Modulation of the frequency of V$\beta$8$^+$ T cells by SEB in conjunction with RU-38486 (*RU486*) (*A* and *B*) and dexamethasone (*DEX*) (*B*). 2 or 10 d subsequent to the injection of SEB (50 $\mu$g i.v.), the frequency of V$\beta$8$^+$ T cells among CD4$^+$ (*left*) or CD8$^+$ (*right*) splenic T cells was determined by FACS® analysis as described in Materials and Methods. RU-38486 (10 mg i.p.) was administered starting from 4 h after SEB (50 $\mu$g i.v.) challenge, and then reinjected every 12 h (*A*). Alternatively, the frequency of V$\beta$8$^+$ T cells was assessed 12 h subsequent to SEB injection, and RU-38486 was injected once either together with SEB or 4 h later (*B*). In a further experiment, V$\beta$8$^+$ T cells DEX (1 mg i.p.) was given 2 h before the injection of SEB and was repeated once 24 h later (*C*). PBS served as vehicle control for RU-38486 and DEX injections, respectively. Mean values ± SEM for three to six animals are given. Data are representative for three independent experiments.

V$\beta$8 among total T cells was detected as compared to vehicle-injected controls (Fig. 3 *A*), indicating that baseline GC levels are not important for the regulation of clonal expansion and deletion.

Since RU-38486 coadministered with SEB is lethal within 24 h (Fig. 2 *A*), it cannot be determined whether the GC peak after SEB injection (Fig. 1) is relevant to the regulation of SEB responses that become detectable several days after SEB. We therefore evaluated the consequences of SEB injection on the repertoire of splenic T cells within a shorter interval. 4–8 h after intravenous administration of SEB the percentage of V$\beta$8$^+$ T cells begins to decline (data not shown) to reach significantly reduced levels after 12 h (Fig. 3 *B*), i.e., before the expansion of V$\beta$8$^+$ cells that is well detectable after 2 d (Fig. 3 *A*). This acute, transient, previously undescribed drop in V$\beta$8$^+$CD4$^+$ and V$\beta$8$^+$CD8$^+$ cells apparently is GC dependent, since it is fully abolished by coadministration of RU-38486. No such effects were observed when RU-38486 was given 4 h after SEB (Fig. 3 *B*), thus underlining the probable role of the GC elevation over baseline levels that immediately follows SEB injection.

High doses of exogenous GC present during the early phase of the SEB response provoke a major alteration in the behavior of the V$\beta$8$^+$ subset. Instead of exhibiting an expansion of V$\beta$8$^+$ T cells, DEX-treated animals display a highly significant rapid deletion of V$\beta$8$^+$ T cells that can be well



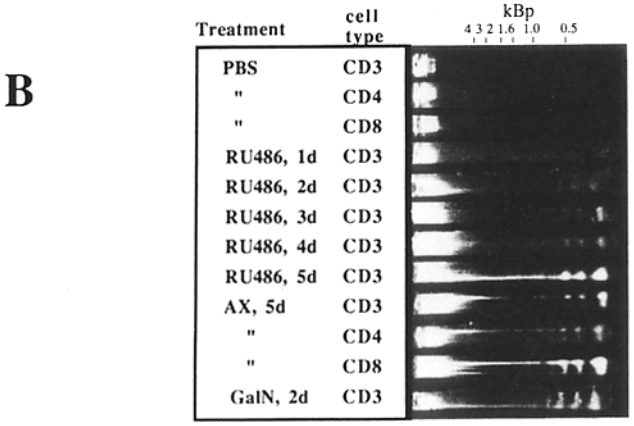




**Figure 4.** Impact of RU-38486 on the programmed cell death of splenic T cells. (*A*) Effect on splenic and thymic cellularity. Groups of age-matched BALB/c mice received injections of saturating amounts of RU-38496 (10 mg i.p.) in 12-h intervals, followed by determination of the overall cellularity (viable cells), expressed as the percentage of vehicle-injected controls. (*B*) DNA fragmentation patterns of purified CD3$^+$, CD4$^+$, or CD8$^+$ splenic T cells. Cells were recovered from animals that had been treated for 1–5 d with RU-38486 (20 mg i.p./d in 2 doses), adrenalectomized (*AX*; or sham operated) 5 d before, or that had been treated by injection of D-galactosamine (*GalN*, 20 mg i.p.).

detected 2–3 d after SEB challenge (Fig. 3 *B*). This in vivo finding contrasts with in vitro data that show that GC actually inhibit the CD3-stimulated activation cell death of T cell hybridomas (34) and thymocytes (35). It remains to be determined whether these discrepancies are due to rather disparate experimental systems (in vivo versus in vitro) or must be attributed to the nature of the trigger targeted to the TCR ($\alpha$CD3 versus superantigen). Although reminiscent of the effect of CsA, which also may enhance the SEB-driven deletion of V$\beta$8$^+$ T cells (36), DEX is much more efficient in this system, revealing its prodeletional effect after one single injection of SEB, whereas the effect of CsA is only observed after several rounds of SEB treatment (36).

*Withdrawal of Endogenous GC Induces Programmed Cell Death of Peripheral T Cells.* Shortly after treatment with RU-38486, the cellularity of the thymus and the spleen increases by up to 100%, reaching its maximum 48 h after the initiation of RU-38486 injections in 12-h intervals (Fig. 4 *A*). Simultaneously, the oligonucleosomal DNA fragmentation pattern typical of programmed cell death becomes detectable among splenic T cells (Fig. 4 *B*). Whereas vehicle controls do not show signs of apoptosis, peripheral T cells from RU-38486 treated mice display a DNA fragmentation that increases with the duration of RU-38486 exposure and is accompanied by a relative depletion of splenic T cells and thymocytes. DNA fragmentation was also observed in adrenalectomized mice. This phenomenon concerned both CD4$^+$ and CD8$^+$ T cells. Surprisingly, GalN per se also induced PCD of purified CD3$^+$ splenic T cells (Fig. 4 *B*). Thus, two manipulations that sensitize mice to the lethal effect of T cell–targeted polyclonal stimuli, withdrawal of endogenous GC, and injection of GalN, per se induce apoptosis in peripheral T cells. Whether a massive apoptotic decay of lymphocytes contributes to the lethal effect of polyclonal stimuli, however, remains to be elucidated. In this context, it appears intriguing that the immunomodulator linomide, a substance that displays a marked antiapoptotic effect on peripheral T lymphocytes, also counteracts the lethal effect of bacterial endotoxins and exotoxins in vivo (Gonzalo, J. A., A. González-García, T. Kalland, G. Hedlund, C. Martínez-A., and G. Kroemer, manuscript submitted for publication).

*Concluding Remarks.* As shown in this article, endogenous GC are involved in the regulation of the cellularity of lymphoid organs, programmed cell death of peripheral T cells, and specific superantigen responses. Mice treated with RU-38486 become susceptible to the fatal effect of T cell–targeted stimuli, anti-CD3$\epsilon$, and superantigen, i.e., substances that are not lethal per se and act via the stimulation of lymphokine release in T cells (22, 24, 25). It has been shown previously that adrenalectomy enhances the mortality provoked by injection of other agents mimicking septic shock, namely LPS, TNF, and IL-1 (37). Like SEB (Fig. 2), LPS, IL-1, and TNF are potent inducers of GC in vivo (1, 38). Thus, GC produced during acute immune stimulation may constitute an important feedback mechanism for contending the potentially lethal hyperactivation of the immune system. Accordingly, the presence of RU-38486 early after SEB injection, when endogenous GC rise over baseline levels, will have fatal consequences and interfere with the SEB/GC–driven reduction of V$\beta$8$^+$ T cells. If these data were to be extrapolated to the human system, the treatment of persons at risk of sepsis with the antiglucocorticoid RU-38486 should be avoided.

A further potentially important observation concerns the kinetics with which exogenous GC will exert beneficial effects in this model of septic shock. To prevent acute death, GC have to be applied early after providing a polyclonal stimulus, i.e., within a few hours, thus mimicking the kinetics of endogenous GC elevation. This may be related to the fact that some critical events occur early after stimulation, e.g., the massive, GC-inhibitable secretion of TNF (22, 24), and might explain why patients with established septic shock will scarcely profit from (late) GC medication (39).

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