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Corticosterone binding globulin regulation and thymus changes after thermal injury in mice

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D'Elia, Michele, Julie Patenaude, Claudine Hamelin, Dominique R. Garrel, and Jacques Bernier. Corticosterone binding globulin regulation and thymus changes after thermal injury in mice. *Am J Physiol Endocrinol Metab* 288: E852–E860, 2005. First published December 14, 2004; doi:10.1152/ajpendo.00407.2004.—Thermal injury is extremely stressful, and data characterizing the systemic endocrine stress response to this injury are sparse. The objective of this study was to measure the effects of thermal injury on mice on corticosterone (Cort) levels in relation with corticosteroid-binding globulin (CBG) and thymus cell populations. The endocrine stress response was determined by measuring total Cort, free Cort, CBG binding capacity, liver CBG mRNA, and circulating CBG levels at 1, 2, 5, and 10 days postburn. Thymus cell populations were also analyzed. After thermal injury, a rapid increase of total Cort was observed in the first 48 h. This was associated with a decrease of hepatic CBG mRNA, protein levels, and binding capacity. Percentage of free Cort in the burn group peaked at *day 2* postburn with a dramatic (+500%) increase. This correlated with a significant decrease of thymus cellularity (50% less). Phenotypic analyses showed that corticosensitive cells were significantly altered. After treatment (5 days), both endocrine and immune parameters returned to control levels. Our results demonstrate that, after a thermal injury, CBG is mainly responsible for Cort's action on corticosensitive immune cells.

glucocorticoids; corticosteroid-binding globulin; burn injury; immune system

IT IS WELL ESTABLISHED that severe injuries, including a burn injury, can suppress the immune system, predispose injured patients to infection, and increase morbidity and mortality (1, 25, 36). Immune suppression is caused mainly by alterations in T cell subpopulations from blood and lymphoid organs (3, 14). Increased apoptosis in the thymus early after burn injury has been reported and may be caused by stress mediators such as FasLigand (CD95L, FasL, Apo1L) interactions, heat shock proteins, tumor necrosis factor-α (TNF-α), and glucocorticoids (37, 43, 51, 54, 55). Increased circulating levels of glucocorticoids in the serum measured after a burn injury are caused by the activation of the hypothalamic-pituitary-adrenal (HPA) axis (16). Depending on the nature of the stimulus, the HPA axis can be triggered by various factors such as TNF-α, interleukin (IL)-1, and IL-6 released mainly from immune cells (42, 49). The thymic response to high levels of glucocorticoids is a reversible loss of thymic mass (2). In fact, glucocorticoids have been known to induce programmed T lymphocyte cell death (apoptosis) at physiological levels (19). Glucocorticoid action is mediated by its interaction with glucocorticoid receptors (GR) present in the cytoplasm of T cells. Glucocorticoids also have an impact on T cell proliferation and maturation in the thymus (17, 44). Glucocorticoids are mainly produced by the adrenal glands. However, recently it was demonstrated that thymic epithelial cells can produce glucocorticoids, thus supporting the hypothesis that glucocorticoids are important in regulating T cell differentiation (53, 31, 32, 40). Moreover, it was shown that the balance between glucocorticoid receptor (GR)- and T cell receptor (TCR)-delivered signals in the thymus was important in the generation of mature and effective T cell populations (52). Although double-positive immature $CD4^+CD8^+$ thymocytes are highly sensitive to glucocorticoid-induced apoptosis, mature T cells that bare a TCR^{high} surface receptor are resistant to glucocorticoids (56).

The action of glucocorticoids is regulated by their binding to plasma carrier proteins that sequestrate the hormone and inhibit its activity on target cells. Corticosteroid-binding globulin (CBG) is the specific transport protein for serum corticosterone (Cort). CBG carries >80% of total Cort, whereas 10% is generally bound to albumin. The free fraction of Cort, which represents ~5% of total Cort, is the biologically active fraction that is available for tissues (47). CBG is primarily produced in the liver, and changes in circulating CBG levels occur after stress because glucocorticoids play an inhibitory role in the control of hepatic CBG synthesis (9, 15). Moreover, it was demonstrated that IL-6, a cytokine that plays an important role in the hepatic acute-phase response, can inhibit CBG synthesis in human hepatoma-derived cells (HepG2) probably by decreasing CBG mRNA stability (5). Also, high IL-6 levels in burn patients correlated with decreased CBG serum concentrations (7). Initially, it was demonstrated that hormone-binding globulins, like CBG, underwent serpin conformational change during inflammation, thus reducing their binding capacity (41). It was then shown that CBG was sensitive to protease activity, specifically neutrophil elastase, which considerably reduces CBG binding capacity (23). Knowing that proteases are released in elevated levels during inflammation, CBG stability could be compromised after a burn injury. Moreover, changes in CBG synthesis or binding capacity for Cort during inflammation may be important in regulating Cort-driven activities in numerous tissues and organs after a burn injury. The importance of total Cort, free Cort, the CBG concentrations, and their binding capacity related to thymic atrophy is not documented at this day.

The purpose of this study was to assess the relationship between the form of circulating Cort, CBG regulation and

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binding activity, and immune system modulations after a severe burn injury. For these purpose, we have evaluated several parameters of both systems at different time intervals postburn injury. In the present study, we first established at different time points postburn injury total Cort, free Cort, and percentage of free Cort. Second, we established the effect of burn injury on CBG concentrations, its binding capacity in serum, and its hepatic mRNA expression for the same time interval points. Next, we correlated endocrine effects to changes in thymus homeostasis. To do so, phenotypic analyses of thymic subpopulations were carried out. Using this strategy, we were able to demonstrate that, after a thermal injury, CBG is the primary regulator of Cort availability and, consequently, its effect on corticosensitive T cells.

MATERIALS AND METHODS

Animals. All experiments were performed on 6-wk-old male mice (C57BL/6; Charles River Laboratories, St. Constant, Quebec, Canada). The mice were acclimated for a period of 2 wk before the initiation of any procedures and were housed in a central animal facility under strictly controlled temperature, relative humidity, and a 12:12-h light-dark cycle. They were kept in cages, each containing five mice. Standard chow (Richmond Standard Lab Diet; Lab Diet, Richmond, IN) and water were provided ad libitum. The Institutional Animal Care Committee reviewed and approved all procedures performed in accordance with the Canadian Council on Animal Care guidelines.

Alzet microosmotic pump. Microosmotic pumps were installed as described previously (27). On *day 0* of the experiment, mice were anesthetized with Isoflurane gas (CDMV; St. Hyacinthe, Quebec, Canada), and the upper sternal region of each mouse was shaved. Animals were randomly assigned to a control buprenorphine group or burn group, each group containing five animals per day of experiment. An Alzet microosmotic pump (Durcet, Cupertino, CA) was inserted subcutaneously by an incision made on the upper sternal skin and pushed carefully down to the peritoneal cavity. Surgical sutures were used to close the wound. All animals survived these procedures. Control group buprenorphine- and burn-treated mice received a pump delivering 0.1 ml/kg buprenorphine (Reckitt and Coleman Pharmaceuticals, Richmond, VA) per 12 h. For a 20-g mouse, that is equivalent to 2.0 μg/12 h buprenorphine (0.2 mg·kg body $wt^{-1}\cdot day^{-1}$). This dose of buprenorphine is optimal for pain relief (26, 27). We performed a thermal burn injury on mice representing 20% total body surface area using a hot water bath (90°C for 7 s). Flamazine (Smith and Nephew, Montreal, Quebec, Canada), a topical antiseptic cream, was directly applied on the wound to reduce the risk of infection.

Euthanasia and organ preparation. On *days 1*, *2*, *5*, and *10* after treatment, the animals were anesthetized with isoflurane and killed by cardiac puncture. Peripheral blood was collected in a heparinized coated tube and kept on ice. Plasma was collected after centrifugation and kept at −20°C. Livers were frozen immediately in liquid nitrogen after dissection and kept at −80°C until assays were performed. Thymuses were removed and prepared immediately for study.

Preparation of thymus cell suspensions. Thymuses were prepared individually as single-cell suspensions by teasing apart the matrix in Hank's balanced salt solution (HBSS; Sigma-Aldrich, Oakville, Ontario, Canada). The suspensions were washed three times in HBSS and depleted of red blood cells by osmotic shock in Gey's solution supplemented with 0.155 M NH_4Cl. The cells were resuspended in RPMI 1640 medium (Biomedia, Drummundville, Quebec, Canada) supplemented with 50 μM 2-mercaptoethanol (Sigma-Aldrich), 10% heat-inactivated FBS (Biomedia), and antibiotic solution (100 IU/ml penicillin and 100 μg/ml streptomycin; Biosource, Montreal, Quebec, Canada). Cell viability was determined by trypan blue exclusion and was consistently >90%.

Flow cytometry. Cells were analyzed by three-color flow cytometry with a FACScan (Becton-Dickinson), as previously described (28). Thymocytes (10^5 cells/100 μl) were incubated for 30 min on ice in the presence of 1 μg fluorescein-conjugated monoclonal rat anti-mouse anti-CD4, 1 μg rat anti-mouse anti-CD8 conjugated to R-phycoerythrine (eBioscience, San Diego, CA), and 1 μg at anti-mouse biotin anti-CD24 (Cedarlane Laboratories, Hornby, Ontario, Canada) or 1 μg rat anti-mouse biotin anti-Qa.2 (B-D Biosciences, Mississauga, Ontario, Canada), all conjugated to streptavidin-Pe/Cy5 molecule (Cedarlane). The cells were washed two times with cold HBSS before FACScan analysis. Results were analyzed with WinMdi software (Scripps Research Institute, La Jolla, CA).

Total Cort assay. Total Cort was assessed by dextran-coated charcoal (DCC) RIA, as first described by Newsome et al. (38). Briefly, 100 μl serum sample was heated at 60°C for 30 min to denature CBG and obtain the free form of Cort (34). Total Cort concentrations were determined by adding 500 μl diluted antiserum containing 0.3 pg anti-Cort (Sigma-Aldrich) to either the heated serum sample or to standard Cort (0–1,000 pg/100 μl; Sigma-Aldrich). After 30 min at room temperature, [^{3}H]Cort (25 μCi; Amersham Biosciences, Baie-D'Urfe, Quebec, Canada) was added to each tube and then incubated for 1 h at 37°C. Samples were cooled to 4°C, and 200 μl DCC suspension were added for 10 min at 4°C. After centrifugation, the amount of radioactivity present in the supernatant (bound radioactivity) was determined with a β-scintillation counter (LKB Wallac).

Free Cort assay. Plasma free Cort was measured using a procedure previously described by Fleshner et al. (15). Briefly, 100 μl plasma samples were loaded in 10,000 mol wt Millipore filter tubes (Fischer Scientific) and labeled with 10 μl diluted [^{3}H]Cort tracer (25 μCi/ml; Amersham Biosciences). Tubes were then vortexed, incubated 1 h at 37°C, and spun at 3,500 rpm for 20 min at room temperature. Filtrate (10 μl) and 10 μl retentate were removed from each sample and placed in scintillation counting tubes. Radioactivity was counted for 1 min. Free Cort was determined using the following equation: free Cort = [cpm filtrate (free Cort)]/[cpm retentate (bound Cort)] × total Cort (prior RIA).

CBG binding capacity assay. The measurement of CBG binding capacity was adapted from a method previously described by Hammond and Lahteenmaki (21). Briefly, a saturation binding experiment was conducted with each mouse plasma sample: 20 μl plasma was incubated with 2 ml DCC suspension (0.5% Norit A, 0.05% dextran T-70, and 0.9% NaCl in 0.01 M PBS, pH 7.4) for 60 min at 37°C with intermittent shaking to remove endogenous steroids. DCC was removed by centrifugation (3,000 *g* for 15 min at room temperature), and the supernatant was further diluted 1:5 in 0.01 M PBS (plasma dilution: 1:500). [^{3}H]Cort stock solution was freshly prepared to a concentration of 1.6 pmol/100 μl in PBS buffer. Stock solution was further diluted in PBS to obtain standard solutions of the following concentrations: 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, and 0.0125 pmol/100 μl PBS. Duplicate tubes were prepared for total binding, each containing 100 μl diluted plasma, 100 μl PBS, and 100 μl [^{3}H]Cort standards (0.0125–1.6 pmol/100 μl PBS). A set of tubes for nonspecific binding was prepared; these contained 100 μl diluted plasma, 100 μl cold Cort (85.8 pmol/100 μl PBS), and 100 μl [^{3}H]Cort standards (0.0125–1.6 pmol/100 μl PBS). After thorough mixing, all tubes were incubated for 1 h at room temperature and then placed on ice for 15 min. Cold DCC suspension was added (750 μl), and the tubes were vortexed and incubated for an additional 10 min on ice. DCC was removed by centrifugation at 3,000 *g* for 5 min at 4°C. The supernatant was decanted from each tube and placed in a scintillation vial to which 3 ml Scinti-Safe (Fisher Scientific) scintillation fluid were added. The tubes were capped and mixed, and the radioactivity was counted for 1 min in a LKB Wallac counter with a counting efficiency of 65%.

The concentration of specifically bound Cort was calculated by subtracting the nonspecific binding from the mean value of total Cort binding. We then obtained a saturation curve for each sample, where the saturation point (B_{max}) equals the CBG binding capacity. All saturation binding curves and Scatchard plot analyses were performed on PRISM 3.0 software (Graphpad, San Diego, CA). CBG binding capacity is expressed as picomole [^{3}H]Cort bound.

CBG mRNA expression. CBG gene expression in the liver was assayed by Northern blot analysis. RNA was extracted using the guanidium thiocyanate-phenol-chloroform method and quantified by normalizing the amount of RNA loaded on the gel. Samples were run on a 2.5% agarose gel and transferred to a nylon Hybond N^+ membrane (Amersham Biosciences). The CBG cDNA probe was graciously provided by Dr. G. L. Hammond (University of Western Ontario, London, Ontario, Canada) and used as described in Scrocchi et al. (46). Redi-Prime beads (Amersham Biosciences) were used to incorporate [α-^{32}P] dCTP (Amersham Biosciences) into CBG probe. Following hybridization and revelation, membranes were stripped and rehybridized with an 18S rRNA probe that was used as a loading control. The CBG signal for each sample was then divided by its corresponding 18S signal. CBG and the 18S-probed membranes were analyzed and quantified using a PhosphorImager apparatus (Molecular Dynamics, Amersham Biosciences).

Western blot analysis of CBG in mouse serum. Western blot analysis of CBG in mouse serum was assessed using previously described techniques (46). Serum samples were diluted 1:100 in 1× PBS, and the protein concentrations of diluted samples were measured using the Bradford assay kit (Bio-Rad reagents) and BSA as the standard. Protein (50 μg) was diluted in sample buffer, subjected to SDS-PAGE, and electroblotted on nitrocellulose membranes (VWR, Montreal, Quebec, Canada). The membranes were washed in Tris-buffered saline (TBS; 50 mM Tris, pH 8.0, and 150 mM NaCl) and blocked overnight at 4°C in TBS plus 5% nonfat dry milk (Fisher Scientific). The membranes were then incubated for 4 h at room temperature with rabbit anti-mouse CBG antiserum (gift from Dr. G. L. Hammond, University of Western Ontario) in TBS containing 5% nonfat dry milk (Fisher Scientific). Specific antibody-antigen complexes were identified using a horseradish peroxidase-labeled anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL Western blotting detection reagents (Amersham Biosciences).

Statistical analysis. All data were expressed as means ± SE. Data were analyzed by one-way ANOVA using the Statistica 6.0 software program (Statsoft, Tulsa, OK). Differences between groups were considered statistically significant at $P < 0.05$. CBG binding capacity analyses were performed on Graphpad Prism 3.0 software. All experiments were performed in triplicates.

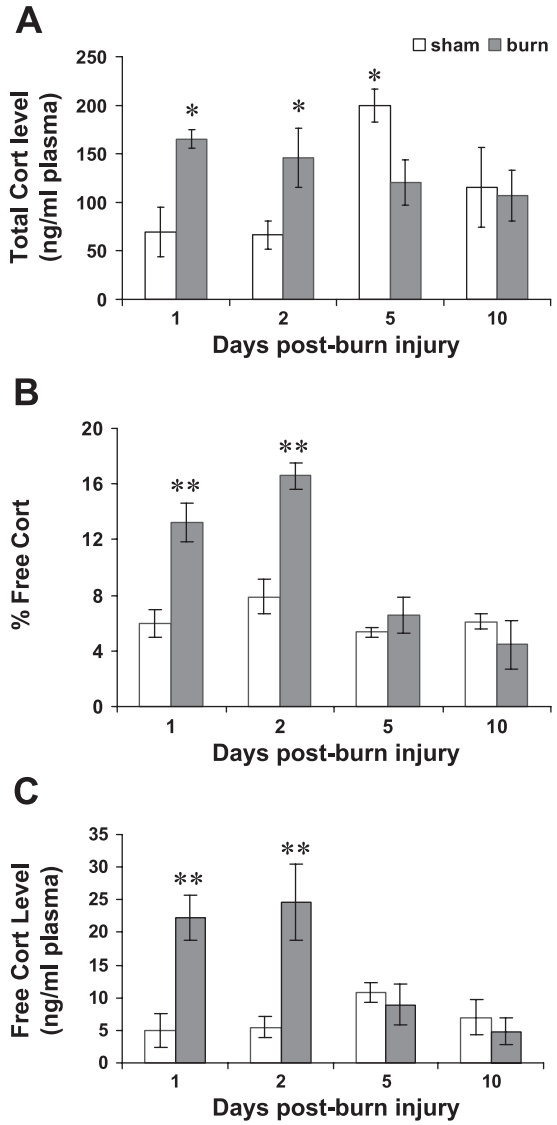

Fig. 1. Serum total corticosterone (Cort; *A*), %free Cort (*B*), and free Cort (*C*) levels at selected time intervals after sham (open bars) or burn (filled bars) injury. *A*: on *days 1* and *2*, burn injury significantly increased total serum Cort concentration, whereas buprenorphine alone increased total Cort on *day 5*. On *day 10*, values were similar for both groups. *B*: %free Cort was significantly increased for the first 2 days after burn injury. *C*: on *days 1* and *2* postburn, free Cort levels were significantly higher in the burn group and then returned to control values past this period. Statistical analyses indicated a significant difference between sham and burn groups: $^{**}P < 0.01$ and $^{*}P < 0.05$. Data represent means for a group ± SE of three replicate determinations from a representative experiment ($n = 5$ mice/group).

RESULTS

Burn injury increases total and free Cort levels in serum. Burn injury induced an increase of total Cort (Fig. 1*A*). Because only the free form of Cort has biological activity (Fig. 1, *B* and *C*), we determined their levels after burn injury. Total serum Cort levels in burn-treated mice were significantly increased by 237 and 220% at *days 1* and *2*, respectively (Fig. 1*A*). We subsequently observed a gradual decrease in total Cort levels in burn-treated mice, and values were similar with the sham burn group 10 days postburn. Interestingly, a significant increase (165%) in total Cort was measured in sham-treated mice compared with burn group mice 5 days after the installation of the microosmotic pumps. This increase is solely induced by buprenorphine, since we have previously demonstrated that the level of total Cort in the saline microosmotic pumps group remained lower (10). Negative regulation of Cort production induced by burn injury after the initial increase can explain the absence of an opioid effect in burned mice. Thus burn injury rapidly increased total Cort, with an maximal effect observed at *day 1*.

Considering that only free Cort can be biologically active, we determined their levels after burn injury. The burn injury induced a significant increase by 221 and 209% in free Cort on *days 1* and *2*, respectively (Fig. 1*B*). The maximum values recorded for free Cort were 17% in burned mice, whereas the levels in sham burn-treated mice were at 8%. After the second day postburn, percent free Cort levels were comparable in both burn- and sham-treated mice, with values ranging between 5 and 7%. Burn injury caused a significant and major increase in

free plasma Cort by 447 and 450% on *days 1* and *2* postburn (Fig. 1*C*). Both percent free Cort and the amount of free Cort were found to be similar between the two groups on *day 5*. The same phenomenon was also noted when we compared with the sham-saline group (data not shown). These results indicate that the increase of total Cort levels noted in the sham group on *day 5* does not result in biologically active Cort. This disparity can be explained by an increase in Cort binding to plasma transporters such as CBG or albumin. Overall, our results indicate that the total and the free form of Cort increase in the first 2 days after burn injury.

Burn injury decreases CBG binding capacity. To verify if Cort transport in the plasma was modulated by the burn injury, we did a saturation binding assay of plasma proteins and calculated the B_{max}. Saturation experiments clearly show in Fig. 2*A* for sham group and in Fig. 2*B* for burn group that the binding capacity of plasma proteins for Cort was altered 2 days after burn injury. In fact, the saturation point was reached at a lower level in the burn-treated mouse compared with a typical sham-treated saturation binding assay curve. Because CBG is the main Cort carrier in the plasma, we assume that a change in the binding capacity was mainly caused by a modulation of CBG binding properties. Evaluation of B_{max} for each experimental day shows a decrease of CBG binding capacity on *days 1* and *2* (Fig. 2*C*). On *day 5*, the value of CBG binding capacity returned to control levels. Because no change in free Cort was observed at *day 5* in the sham group, it was possible that the increase of total Cort was associated with an increase of binding to another carrier, such as albumin. Overall, our results have shown a decrease of CBG binding capacity by ~50% for the first two days after the injury.

Burn injury decreases hepatic CBG mRNA expression. Next, we wanted to test the hypothesis that the burn injury had an effect on CBG mRNA expression in the liver, where the protein is primarily produced. Northern blot analyses were conducted to determine the effect of burn injury on hepatic CBG mRNA expression. As shown in Fig. 3*A*, we found a significant reduction in CBG mRNA expression 24 h after a burn injury. Similar results were observed 12 h after burn injury (data not shown). However, CBG mRNA expression in the liver reached control levels by *day 2*, and we did not observe any significant differences between sham- and burn-treated mice for the remainder of the study period (Fig. 2*B*). These data suggest that high serum free Cort levels noted after a severe burn injury can be associated with decreased hepatic CBG mRNA expression.

Plasma CBG levels revealed by Western blot. Having established that CBG binding capacity was decreased on *days 1* and *2* after burn injury, Western blot analyses of mice serum were conducted to determine changes in serum CBG concentrations. Figure 4*A* clearly demonstrates that a rapid and drastic decrease of CBG in burned mice serum occurred at *days 1* and *2* after injury, whereas serum CBG levels were normal between both groups 12 h after the injury (data not shown) and at 5 and 10 days postburn.

Thymus phenotypic analysis after burn injury. It is well documented that high Cort levels can affect thymocyte proliferation, causing thymus atrophy. Thus, to demonstrate this, we had to determine thymus cellularity at different time intervals after burn injury. Figure 5*A* shows that thymus cellularity is affected by the burn injury and resulted in a significant reduction of 41 and 50% in the number of thymocytes on *days 1* and *2* postburn. The decrease was caused mainly by a reduction in double-positive $CD4^+CD8^+$ immature thymocytes, which constitute the major thymic subpopulation. This is exemplified by the concomitant expression of CD4 and CD8 cell surface markers. In fact, Fig. 5, *B* and *C*, shows that the percent of $CD4^+CD8^+$ T cells was significantly reduced to levels representing 60 and 65% of the total thymocyte population on *days 1* and *2* postburn. In contrast, in sham-treated animals, we observed values ranging between 80 and 85% for each day. Considering the increase in total Cort in the sham group on *day*

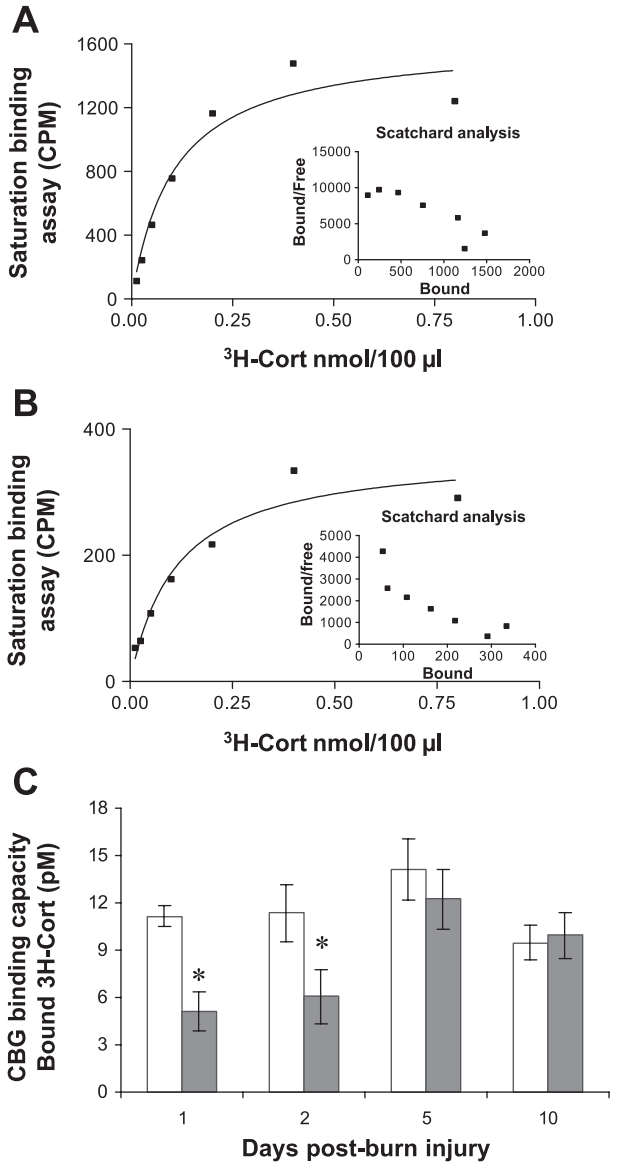

Fig. 2. Cort-binding globulin (CBG) binding capacity in C57BL/6 male mice serum after sham (open bars) or burn (filled bars) injury. *A* and *B*: typical saturation binding assay results; one sample from each group analyzed on Graphpad Prism is presented. Scatchard conversion is used to extrapolate the *x*-intercept, which represents the binding capacity (B_{max}) or the saturation point. *C*: CBG binding capacity is significantly reduced in serum between *days 1* and *2* postburn injury. Statistical analyses indicated a significant difference between sham and burn groups: $*P < 0.05$. Data represent means for a group ± SE of three replicate determinations from a representative experiment ($n = 5$ mice/group).

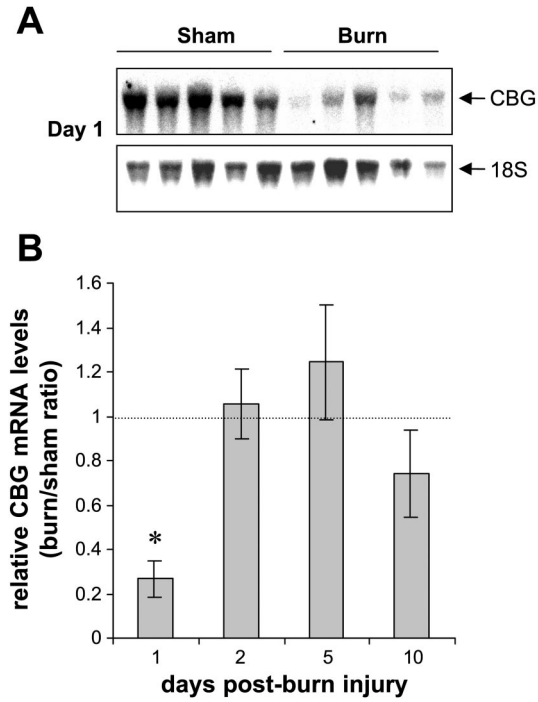

Fig. 3. CBG gene expression in the liver at selected time intervals after sham or burn injury. *A*: on *day 1* postburn injury, a significant decrease in the expression of CBG gene in the liver was measured. There were no significant differences observed between the two groups after *day 1* postburn. *B*: quantitation results for CBG mRNA expressed as a ratio between burn- and sham-injured mice. Statistical analyses indicated a significant difference between sham and burn groups: $*P < 0.05$. Bars represent the burn vs. sham ratio ± SE of three replicate determinations from a representative experiment ($n = 5$ mice/group).

5, we observed no differences between saline- and buprenorphine-treated mice, indicating that the level of total Cort does not represent the biologically active form (data not shown).

In absolute numbers, this reduction is equivalent to a significant loss of 40×10^6 and 38×10^6 of double-positive $CD4^+CD8^+$ cells for *days 1* and *2* postburn. To confirm our results, we did a specific thymocyte analysis by targeting both the CD24 and Qa.2 cell surface markers; the former is expressed only on immature T cells, whereas the second is expressed by the most mature thymocytes. It is evident in Fig. 6, *A* and *B*, that both $CD4^+$ and $CD8^+$ T cells expressing the CD24 marker were depleted after burn injury. In fact, at 1 day postburn, we observed an important decrease of $CD4^+CD24^+$ T cells (45×10^6 cells, representing 50% less cells), although this was not significant ($P = 0.09$). But, at 2 days postburn, the same differences existed between buprenorphine and burn-treated animals with respect to $CD4^+CD24^+$ T cells, with a significant 50% reduction in cell numbers. This represents a loss of 35×10^6 immature cells. When we looked at T cells expressing $CD8^+CD24^+$ surface markers, we observed similar results as those seen with $CD4^+CD24^+$, with a highly significant loss of cells (42×10^6 and 30×10^6) representing a 50% reduction of cells for both *days 1* and *2* postburn. However, the changes observed with immature T cells were not seen when phenotyping mature thymocytes. In fact, there were no significant differences observed in both $CD4^+Qa.2^+$ and $CD8^+Qa.2^+$ mature T cell subpopulations when comparing thymus from sham- and burn-treated mice at *days 1* and *2* postburn.

Having now established that both the endocrine and the immune system were affected by burn injury, we then wanted to correlate the two parameters that changed after this injury. Thus Fig. 6*E* shows the result of a multiple-regression analysis between free Cort and the absolute number of immature double-positive T cells expressing both CD4 and CD8 markers. A strong correlation exists between the two parameters, and the reduction of immature cells is explained mainly by the modulation of free Cort levels in the plasma.

DISCUSSION

A severe burn injury causes an elevation in total serum Cort by activating, using different pathways, the HPA axis. In the present study, we demonstrated that CBG is the main regulator for Cort availability in the plasma and tissues and that decreased hepatic CBG mRNA expression followed by its depletion in the plasma are primarily responsible for the action of high Cort levels, which cause a direct effect on thymic involution.

Induction of Cort production was rapid. High rising Cort levels were seen as early as 24 h after the injury but lasted only for another 24–48 h and then gradually decreased below control values by *day 5* and returned to control levels 10 days after the burn injury. The fact that we observed higher total Cort levels on *day 5* for the control group can be explained by the administration of buprenorphine, an analog of morphine. This opiate, in contrast to morphine, does not immediately activate the HPA axis but represses Cort production early (1–6

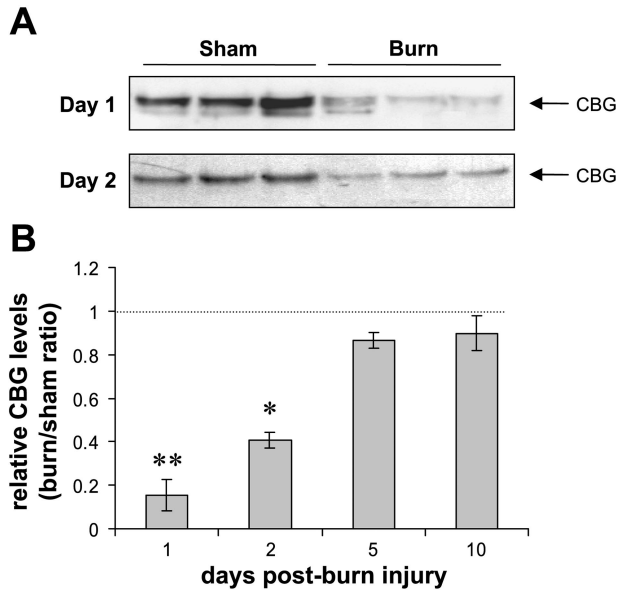

Fig. 4. *A*: Western blots of mouse serum proteins at different time intervals after sham or burn injury. Serum was diluted 1:100 and assayed for protein content by Bradford colorimetric assay. Serum was furthermore diluted 1:10 to equally adjust protein concentrations to be loaded. Western blot results demonstrate that CBG protein concentration is markedly decreased in the serum of mice at *days 1* and *2* after a burn injury. *B*: quantitation results presented as the ratio between burn- and sham-injured mice. Statistical analyses indicated a significant difference between sham and burn groups: $**P < 0.01$ and $*P < 0.05$. Bars represent the burn vs. sham ratio ± SE of three replicate determinations from a representative experiment ($n = 5$ mice/group).

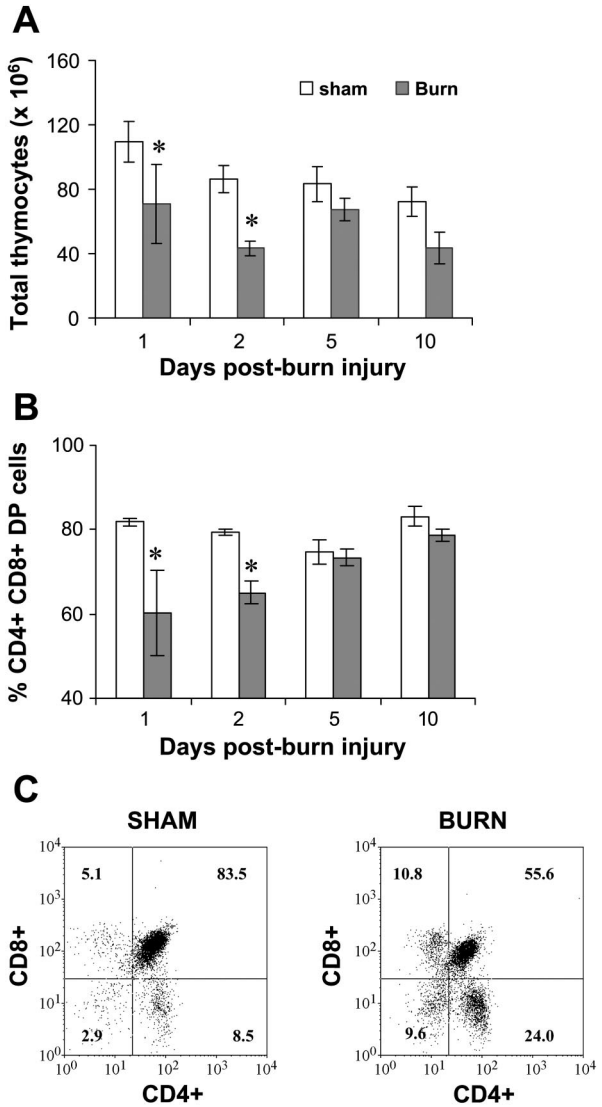

Fig. 5. Thymus analysis after sham (open bars) or burn (filled bars) injury. *A*: thymus atrophy was observed at *days 1* and *2* postburn injury ($P < 0.05$). *B*: variations of cellularity observed at *days 1* and *2* postburn are accompanied by a marked reduced expression of $CD4^+/CD8^+$ thymocytes. *C*: typical dot-blot analysis with one sample from each group. Statistical analyses indicated a significant difference between sham and burn groups: $^*P < 0.05$. Data represent means for a group ± SE of three replicate determinations from a representative experiment ($n = 5$ mice/group).

h) after its administration (18, 34, 45). Indeed, it has a more slow-going activity observable essentially at 5 days after a burn injury. Incidentally, total Cort levels observed for burned mice 5 days postburn would have been lower if it was not for the stimulatory effect of the drug on the HPA axis at that time (10). The absence of a rise in free Cort levels suggests an increase of binding to plasma proteins. However, our CBG results have shown no marked changes for this specific carrier. LeVier et al. (33) have reported that morphine sulfate increases the serum levels of albumin. Because albumin binds only a minor portion of Cort, it is possible that buprenorphine could induce an increase in albumin in the absence of burn injury and that the associated small change in CBG level can increase the sequestration of Cort at *day 5* in the sham group. This latter possibility remains to be determined.

Free Cort percentage increased rapidly and reached peak values 2 days after burn injury. These levels progressively returned to control values and remained at ~5–7% throughout the experimental period. Variation in percent free Cort was the biggest contributor to free Cort level variations because it amplified the variations previously seen in total Cort values. Indeed, we observed that free Cort levels were 400 to 500% higher in burned mice between *days 1* and *2* after burn injury and that these reached normal values on *day 5* (4–10 ng/ml serum). The increase observed in the degree of freedom of Cort can be explained by a rapid and sustained decrease in hepatic CBG mRNA expression between 12 and 24 h after burn injury. In fact, decreased CBG mRNA levels in the liver preceded the increase in total Cort. This created a gap in time between increasing Cort levels and decreasing plasma CBG concentrations, which explains the spectacular increase in free Cort levels observed between *days 1* and *2* postburn. High circulating Cort levels are believed to act negatively and transiently on the CBG gene expression in the liver immediately after the burn injury, thus increasing free biologically active Cort levels (24, 48). Although no response element for glucocorticoids was found in the murine CBG gene, it is believed that GR may repress transcription via physical interactions with other transcription factors such as hepatic nuclear factor 1 (57). In addition to the Cort effect on CBG gene expression, increasing IL-6 levels, which arise after burn injury (20, 39), could explain the sustained repression of CBG production. Several studies from our laboratory or others have pointed out a relationship between increasing IL-6 levels and decreasing CBG levels (6, 7). Thus IL-6 may act directly on a specific binding site (NF-1IL6) found in rat and human CBG promoter or in association with transcription factors to regulate hepatic CBG production (13). Bartalena et al. (5) suggested that IL-6 could affect CBG mRNA stability by a posttranscriptional mechanism.

It is believed that plasma CBG goes through a serpin conformational change during inflammation (41). In fact, CBG is cleaved by elastase, a protease released at high concentrations by neutrophils at inflammation sites (30). This, in addition to reduced hepatic CBG mRNA expression, may explain a low binding capacity of CBG for Cort occurring 24 to 48 h postburn injury, resulting in high free biologically active Cort.

It is important to point out that the decrease in CBG observed in burn mice should not have resulted in an increase in free Cort if the negative feedback on adrenocorticotropin (ACTH) secretion was normal. This impaired negative feedback was observed in patients with sepsis during dexamethasone infusion; the same situation can be hypothesized for our mice (35).

Glucocorticoids are secreted in large amounts in response to stressful stimuli and have a potent action on the thymus. In fact, although no observable changes occurred 12 h after the burn injury, we have observed a transient thymic involution (reversible loss of thymus mass) at *days 1* and *2*. This loss of 50% of total thymocytes was mainly caused by a depletion of double-positive $CD4^+CD8^+$ T cells. Indeed, at *days 1* and *2* postburn, a significant drop of double-positive cell number (60–65%) was noted in burned mice, which later reached control levels that varied between 80 and 85% during the experimental period. Furthermore, phenotyping results indicate a selective depletion of immature T cells positive for the CD24

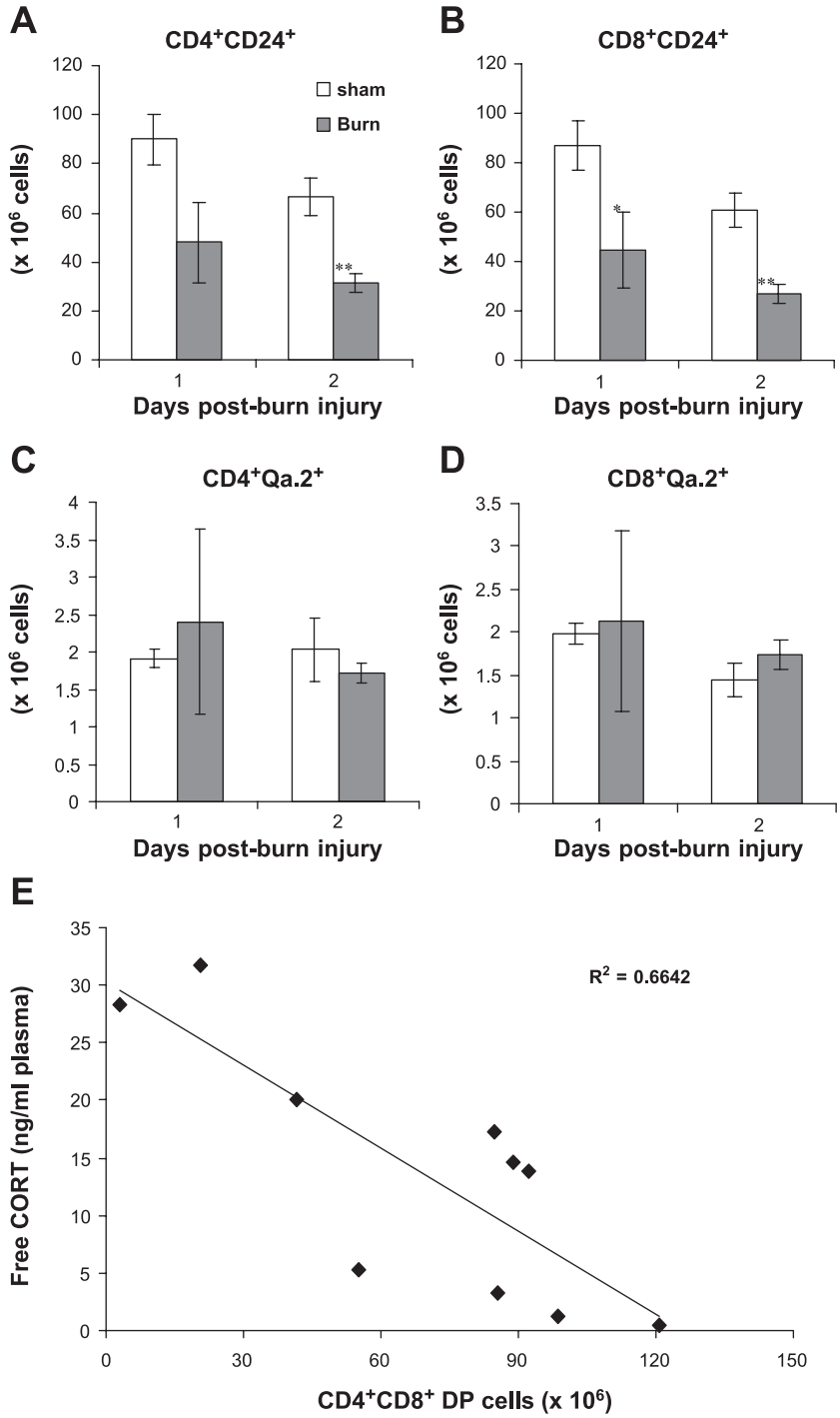

Fig. 6. Thymus subpopulation analysis 1 and 2 days after sham (open bars) or burn (filled bars) injury. *A* and *B*: a significant decrease of immature $CD24^+$ T cells for both $CD4^+$ and $CD8^+$ T cell populations was observed. *C* and *D*: no significant differences were observed for the most mature thymic cells characterized by the Qa.2 cell surface marker expression. *E*: multiple-regression analysis between free Cort and total double-positive $CD4^+/CD8^+$ immature T cells 1 day after burn injury. DP, double positive. Statistical analyses indicated a significant difference between sham and burn groups: $^{**}P < 0.01$ and $^{*}P < 0.05$. Data represent means for a group ± SE of three replicate determinations from a representative experiment ($n = 5$ mice/group).

cell surface marker or heat-stable antigen, which include the double-positive T cell population and a variety of intermediate immature T cell subpopulations (50). On the other hand, no changes between control and burn groups were observed on mature T cells characterized by the Qa.2 cell surface marker.

Several causes can explain the transient thymic involution that occurs between *days 1* and *2* postburn. First, double-positive T cells are known to be corticosensitive. Indeed, double-positive T cell numbers are negatively correlated with increasing free biologically active Cort at *day 1* postburn. Normally, T cell selection in the thymus occurs when both TCR- and glucocorticoid-mediated signals antagonize each other, thus establishing a T cell repertoire effective against nonself antigens (52). High endogenous Cort levels can disrupt T cell selection and direct immature thymocytes to apoptosis. Glucocorticoid-induced thymocyte apoptosis is mediated by the mitochondrial pathway and requires Apaf-1 complex formation and capsase-9 (2). Fukuzuka et al. (16) observed that thymus atrophy after burn injury was accompanied by increased FasL and caspase-3 activity and was greatly reduced by pretreatment with mifepristone, a Cort receptor blocking agent. Second, a reduced repopulation of thymus by T cell

precursors from the bone marrow could explain the selective loss of immature cells. In fact, in a chronic immobilization stress situation, it was previously demonstrated that the reduction in the number of T cell precursors and in thymic T cell chemoattractants was partially responsible for thymic involution (11). Third, a reduced proliferation of double-positive T cells could explain low numbers of immature cells at that moment. In fact, double-positive thymic T cells are known to be highly proliferative, and it was previously established that thymus involution was associated with glucocorticoid treatment, which decreased thymocyte proliferation (4, 30).

The increase in Cort can explain the thymic atrophy observed. Importantly, proinflammatory cytokines, which activate the HPA axis, play a central role in the amplification of Cort production and CBG downregulation. Indeed, high circulating levels of IL-1β, IL-6, and TNF-α were increased early after a thermal injury (7, 12, 29). Moreover, parallel self-induction of TNF-α and apoptosis in the thymus of mice after burn injury suggests an active role of this cytokine in thymic atrophy (8). Other factors can also contribute to thymocyte depletion early after burn injury. Hobson et al. (26) pointed out the role of transforming growth factor (TGF)-β production in burn-induced thymic apoptosis. It was observed that there is an increase in TGF-β expression in the thymus from 1 to 7 days postburn injury. The increased levels of this cytokine are correlated with increased thymocyte apoptosis.

Overall, our results demonstrate that CBG is the primary in vivo regulator of Cort action on corticosensitive thymocytes after burn injury. Burns affect the CBG level and free Cort level shortly after injury, with a window of 2 days where changes are observed. It is clear that the stress induced by the injury had a negative impact on hepatic CBG mRNA expression, which preceded the depletion of circulating plasma CBG levels, resulting in a reduced binding capacity for Cort.

Glucocorticoids are also known to contribute to T cell selection, and high Cort levels could impair negative and positive selection occurring in the thymus (52). This would result in the selection and maturation of autoreactive or defective T cell clones that could later impair the recovery of patients suffering from burn injury.

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