The Immunosuppressant 1,5-deoxyspergualin Reveals Commonality between PreT and PreB Cell Differentiation

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Summary

1,5 deoxyspergualin (DSG) is a potent immunosuppressant whose mechanism of action is still somewhat of a mystery. We have studied the generation of lymphocytes in mice treated with this drug. The differentiation of T cells in the thymus was blocked at an important early control point: the $CD4^-8^- \rightarrow CD4^+8^+$ transition, known to depend on the expression of a preTCR complex that includes the variable TCR-β, but not TCR-α, chain. In clear contrast, a later control point, the $CD4^+8^+ \rightarrow CD4^+8^-$ or $CD4^-8^+$ transition, dependent on the display of a conventional α:β TCR complex, appeared unaffected, as did activation of mature T cells both in vitro and in vivo. Interestingly, preB cell differentiation in the bone marrow was blocked at a precisely equivalent point: the A-C → C′ transition, controlled by expression of a pre-receptor complex containing the Ig heavy, but not light, chain. Mature B cells seemed unperturbed. These findings have theoretical implications, suggesting common signaling pathways in early lymphocytes that are distinct from those employed by more mature cells, and are also of practical interest, to be considered in the design of DSG treatment protocols.

Recent results with 1,5 deoxyspergualin (DSG)[1] have aroused considerable interest in the transplantation field (for review see reference 1). This drug, a synthetic derivative of the antibiotic spergualin, is a potent immunosuppressant in a variety of contexts. It can prolong the survival of, even induce tolerance to, tissue grafts in several animal models. These include transplants of various organs in diverse donor–host combinations, involving either allo- or xeno-disparities. It can also enhance survival of patients' tissue grafts, particularly renal transplants, often those of an intransigent nature. DSG is frequently more effective than the popular immunosuppressants cyclosporin A, FK506, or rapamycin, and routinely elicits fewer side effects. Therefore, its use is presently being extended to chronic autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis, which may require long-term administration.

It is rather surprising, then, that so little is known about the mechanism of action of DSG. Several findings indicate that it operates very differently from the cyclosporin A family of drugs (1, 2). However, there is no consensus on whether its immunosuppressant properties are primarily due to effects on T cells, B cells, monocytes, or some combination of the three, perhaps varying in different contexts. Alterations in T cell proliferation (3–6), CTL generation (4, 7, 8), B cell maturation (9), Ab formation (9–12), antigen presentation (13), and cytokine production (12, 14–16) have all been described, but these reports are at times contradictory and are difficult to assemble into a consistent framework.

The goal of our study was to evaluate the effect of DSG on the generation of lymphocytes, an important consideration for long-term treatment protocols. We began by analyzing T cell differentiation in the murine thymus; the results obtained prompted us to perform a parallel analysis on B cell differentiation in the bone marrow.

Materials and Methods

Mice. 6–8-wk-old C57BL/6 (B6), Balb/c, DBA/2, and NOD/Lt mice were raised in our specific pathogen-free animal facility. Most experiments were performed with B6 mice, but identical results were obtained with Balb/c, NOD, or DBA/2 animals. Mice deficient for the recombination activating gene (RAG)-2 (17) were obtained from Dr. H. Mossmann (Max Planck Institut für Immunbiologie, Freiburg, Germany).

DSG Administration. Unless stated otherwise, DSG was dissolved in PBS and administered by intraperitoneal injections of 75 μg/adult mouse (3.75 mg/kg) body weight daily for different durations, as indicated for individual experiments in the figure legends. Lymphoid tissues were usually analyzed 24 h after the last injection. For treatment of pregnant mice, 150 μg/d of DSG was

[1] *Abbreviations used in this paper:* DSG, 1,5 deoxyspergualin; B6, C57Bl/6; RAG, recombination activating gene; HSA, heat stable antigen; FTOC, fetal thymic organ culture; DN, double negative ($CD4^-8^-$); SP, single positive ($CD4^+8^-$ or $CD4^-8^+$); DP, double positive ($CD4^+8^+$); BCR, B cell receptor.

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injected intraperitoneally (two injections), starting on embryonic day 11 (the day of discovery of the vaginal plug being termed embryonic day 0). Thymi were removed from embryos at day 16 or 17 of gestation.

Histological Analysis. Mice were treated with DSG as described above. Thymi were excised after various injection protocols, fixed in Bouin's solution, and then embedded in paraffin. 5 μm-thick sections were cut and stained with hematoxylin and eosin.

Cytofluorimetric Analysis. The following antibodies and reagents were used for cell-surface staining: anti-CD4 and anti-CD8 (phycoerythrin-, fluorescein-, or biotin-conjugated; CALTAG Labs., South San Francisco, CA); anti-CD3 (KT3) (18); anti-CD44 (1M7) (19); anti-B220 (RA3-6B2; American Type Culture Collection, Rockville, MD [ATCC], reference 20); anti-HSA (M1/69; ATCC, reference 21); anti-CD25 (7D4, biotin), anti-Mac-1 (M1/70, fluorescein), anti-c-kit (2B8 biotin), anti-CD43 (S7, phycoerythin), anti-CD69 (H1.2 F3, fluorescein; PharMingen, San Diego, CA); and anti-mouse IgM (Texas-red-conjugated; Jackson ImmunoResearch Labs., Inc., West Grove, PA). Cy5-conjugated streptavidin was used to reveal biotin-conjugated antibodies.

Most thymocyte analyses were performed as described (22, 23). To distinguish subsets of $CD4^-8^-$ cells by CD44 and CD25 expression, three-color staining of thymocytes was performed essentially as described (24). This involved staining with FITC-conjugated reagents against CD3, CD4, CD8, Mac-1 and CD45R (B220), and detecting CD44 and CD25 with Texas Red and PE, respectively. BrdU labeling and analysis has been described recently (25, 26).

For the analysis of bone marrow cells, single cell suspensions from femur and tibia were prepared by flushing with Dulbecco's modified Eagle's medium, followed by gentle pipetting in a small volume. To subdivide bone marrow cells of the B lineage, four-color staining was performed. Cells were first stained with anti-CD45R supernatant on ice for 15 min, and Cy-5-conjugated goat anti–rat IgG was used to reveal the first antibody. Free rat Ig-reactive sites were then blocked by normal rat serum, and the cells stained with anti-CD43 PE, anti-mouse IgM Texas red– and biotin-conjugated anti-HSA (M1/69). The latter was revealed with FITC-conjugated streptavidin. Cells were analyzed on a flow cytometer (Coulter Corp., Hialeah, FL); fluorochromes were excited by the 488-nm line of an Argon laser (FITC and PE), and by the 600-nm line of a dye laser (Texas Red, Cy5).

In Vitro Thymus Cultures. Fetal thymus organ cultures (FTOC) were performed basically as described (27). Briefly, fetal thymi were obtained from day 14 B6 × DBA/2 embryos, lobes were placed on Nucleopore filters (0.8 μm; Costar Corp., Cambridge, MA) floating on 2 ml Iscove's modified Dulbecco's medium with 10% heat-inactivated fetal bovine serum and gentamicin at 50 μg/ml, and incubated at 37°C in 5% CO_2 humidified air atmosphere (28). DSG was added at 25 μg/ml as indicated in the figure legends. Cell suspensions were prepared from individual lobes and stained with anti-CD4 PE and anti-CD8 FITC.

In Vivo Antibody Treatment. Anti-CD3 ascites from the mAb KT3 (18) was first titrated on RAG-2-deficient mice; the optimum condition was determined to be 50 μl of ascites injected intraperitoneally. Experimental animals were injected with KT3 ascites plus or minus DSG (3.75 mg/kg) according to protocols detailed in the figure legends.

For the studies on peripheral T cell activation, DSG-treated or control mice were injected with 50 μl of KT3 ascites and, lymph nodes were taken 24 h later. Single-cell suspensions were prepared and stained with anti-CD4-PE, anti-CD8-biotin and anti-CD69-FITC, followed by Cy5-conjugated streptavidin.

Ex Vivo and In Vitro T Cell Proliferations. For ex-vivo T cell proliferations, B6 mice were treated with DSG as described above for 7 d. Single-cell suspensions were prepared from the spleens of these mice. Various numbers of cells were stimulated with 5 μg/ml ConA in 200 μl RPMI-1640 medium supplemented with 10% FCS, antibiotics, 1 mM sodium pyruvate, glutamine, and 50 μM 2-ME in 96-well flat-bottomed plates. 1 μCi [^{3}H]thymidine was added to each well in the last 8 h of a 36-h incubation, and uptake of label measured. All data points were in triplicate; standard deviations (not shown) were consistently less than 10% of the mean.

For the in vitro assay, 4×10^5 spleen cells from untreated B6 mice were stimulated with different concentrations of ConA, or of purified anti-CD3 antibody coated on 96-well flat-bottomed plates, in the presence of 0 or 25 μg/ml of DSG and 20 μg/ml of aminoguanidine (13). After 24 h of culture, 1 μCi of [^{3}H]thymidine was added and uptake of label measured.

Results

DSG Has Severe Effects on T Cell Differentiation in the Thymus. We first focussed on the effect of DSG on thymus architecture. B6 mice were injected daily with 3.75 mg/kg for varying times, thymi were removed 24 h after the last injection, and sections were cut and stained with hematoxylin/eosin. As illustrated in Fig. 1, drug treatment profoundly perturbed the morphology of the thymus, nearly dissolving the cortical regions. This effect was first observed after 4 d of injections (*C*) and was extreme after 8 d (*D*). Interestingly, the subcapsular region could still be distinguished even after the longest treatment time (*D, arrow*). The morphological perturbation was fully reversible: after 8 d with treatment and then 10 d without, the cortex was fully regenerated and thymus architecture appeared normal (*E*).

To evaluate DSG effects on differentiating T cells, we performed a cytofluorimetric analysis of thymocytes prepared from mice injected for different times. Profiles of anti-CD4 versus anti-CD8 staining revealed a progressive increase in the proportion of double-negative (DN) and single-positive (SP) cells at the expense of double-positives (DP) (Fig. 2 *A*). Like the changes in thymus architecture, these alterations were fully reversible 10 d after drug withdrawal (Fig. 2 *A*, right-most panels). The changing proportions of thymocyte subsets were mainly due to a drastic decrease in the number of double-positive cells beginning after 2 d of injections; there were also reductions in the three other compartments, but they were considerably less severe (Fig. 2 *B*).

Reduced numbers of DP thymocytes are commonly observed with sick or otherwise stressed animals, a phenotype commonly attributed to glucocorticoid-mediated apoptosis. To rule out this and other indirect mechanisms for the DSG effects, we studied the consequences of treating FTOCs with this drug. When thymi were removed from 14-d-old fetuses and cultured for 8 d, the last three in the presence of drug, they also showed a depletion of DP cells (Fig. 2 *C*).

DSG Blocks the DN to DP Thymocyte Transition. The drastic reduction in double-positive thymocytes provoked by

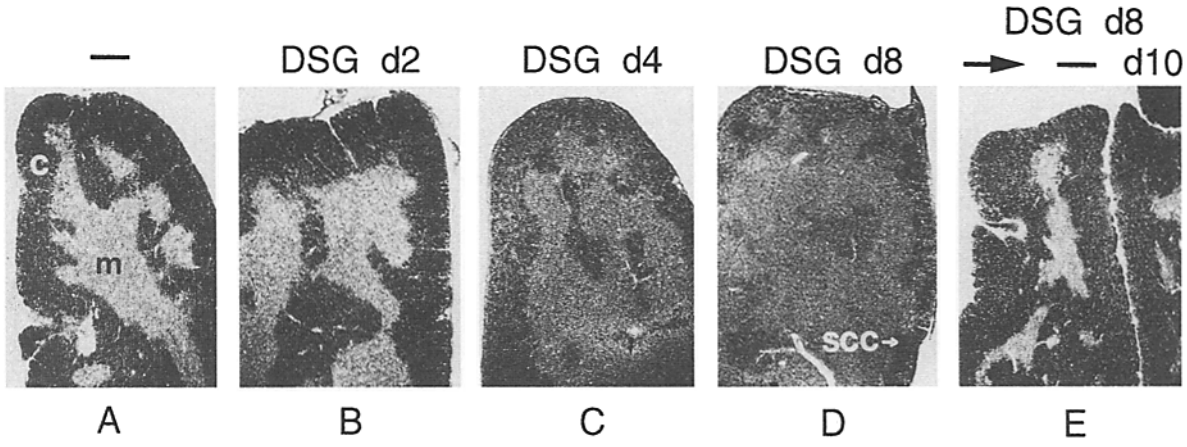
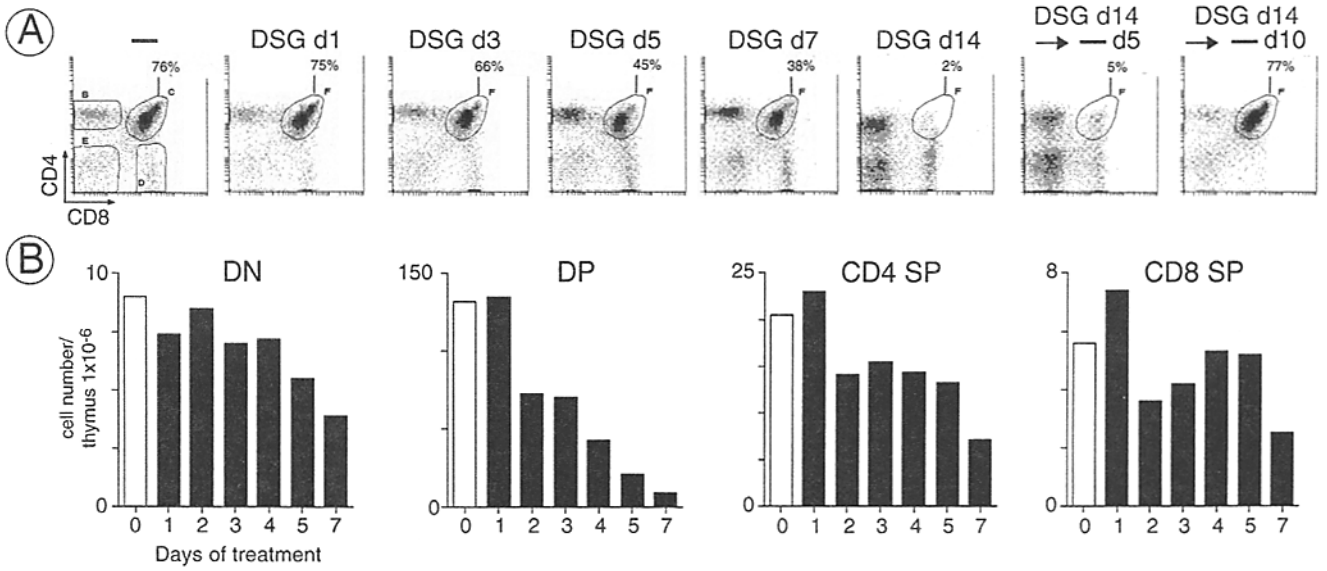


Figure 1. DSG induces reversible depletion of the thymic cortex. Mice were treated with DSG by daily intraperitoneal injection for 2, 4, and 8 d (*B–D*), treated for 8 d and then rested for 10 (*E*), or treated with PBS only (*A*). Thymi were removed 24 d after the final injection, sectioned and stained with hematoxylin and eosin. *c*, cortex; *m*, medulla; *ssc*, subcapsular cortex.

DSG treatment might be explained by either (*a*) a direct effect on the DP cells themselves or (*b*) an indirect effect resulting from depletion of a population of precursor cells or from a block in their further maturation. Certainly, the bulk of double-positive thymocytes was affected—both the small resting cells and the less mature blasts (Fig. 3 *A*). Nevertheless, the second explanation seemed more likely because we found that the number of $CD4^-8^+$ $CD3^{lo}$ cells, the immediate precursors of the DP population, was also severely reduced in animals injected with the drug (Fig. 3 *B*).

Focussing one step further back, we performed a more detailed cytofluorimetric analysis of the double-negative compartment in untreated versus DSG-treated mice. Many past studies have used anti-CD44 and anti-CD25 reagents to characterize DN cells, revealing the following differentiation sequence: $CD44^+25^- \rightarrow CD44^+25^+ \rightarrow CD44^-25^+ \rightarrow$



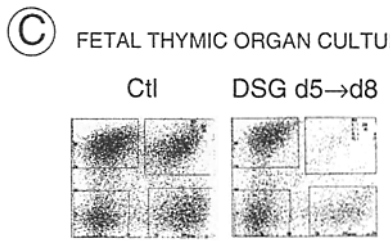


Figure 2. DSG preferentially affects immature double-positive thymocytes. (*A*) B6 mice aged 6 wk were treated with DSG (3.75 mg/kg body weight) by daily injection (i.p.) for 1, 2, 3, 4, 5, 7, and 14 d. Thymocytes were harvested 24 h after the final injection, stained with anti-CD4 and anti-CD8 and analyzed by cytofluorimetry. The proportion of double-positive cells in the thymi of this representative experiment is shown. The left-most panel shows the thymus of a mouse injected with PBS only. For the two right most panels, mice were injected with DSG for 14 d, treatment was discontinued and the mice analyzed after another 5 or 10 d. (*B*) Quantitation (expressed as total number of cells per thymus) of the four thymic subpopulations, gated as shown in *A*. DN: 4^-8^- double negatives. DP: 4^+8^+ double positives; CD4SP and CD8SP: $CD4^+CD8^-$ and $CD4^-CD8^+$ single positives, respectively. (*C*) Fetal thymi from day 14 embryos was cultured in vitro at the air-medium interface for 8 d. DSG was added in some of the cultures (*right*) at the fifth day of culture, a time at which substantial differentiation has occurred into the double-positive compartment.

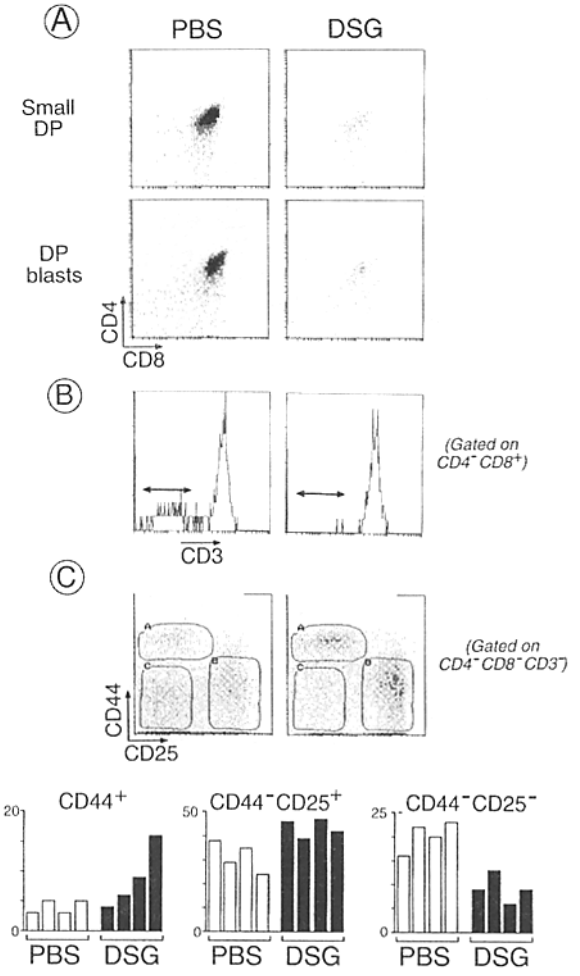


Figure 3. DSG blocks T cell differentiation at the double-negative stage. A. Mice were treated with daily intraperitoneal injection of DSG for 8 d, or with PBS only. Thymocytes were analyzed by three-color flow cytometry after staining with anti-CD4, -CD8, and -CD3. In *A*, the histograms portray small double-positives and double-positive blasts (gated on scatter profiles and on $CD3^{-/lo}$ phenotypes). The dot plots are standardized by total thymocyte number and display an identical proportion of the thymi (3×10^{-5}), to illustrate the DSG-induced depletion of both populations. (*B*) Anti-CD3 staining histograms of gated $CD4^-CD8^+$ cells. The arrow highlights the immature $CD4^-CD8^+CD3^-$ cells whose loss is provoked by DSG. (*C*) Thymocytes were labeled with FITC-conjugated anti-CD3, -CD4, -CD8, -CD11b, and -CD45RO (B220) and counterstained with anti-CD44 (Texas Red) and anti CD25 (PE). Gated FITC-negative cells were analyzed for expression of CD44 and CD25. CD44/CD25 profiles of PBS-injected mice (*left*) and DSG-treated mice (*right*) of representative experiment are shown. The histograms show the percentage of the three major subpopulations of double-negatives, gated as shown, in PBS injected mice (*white bars*) and DSG treated mice (*black bars*). Each bar represents an individual animal.

$CD44^-25^-$ (29). As indicated in Fig. 3 *C*, drug injection led to a reduction in the proportion of the most mature DN subset, the $CD44^-25^-$ cells. This was due mostly to a decrease in their number, but there were also slight increases in the numbers of cells in the other subsets.

A block in differentiation might be more obvious with the quasi-synchronous cohort of thymocytes maturing in a fetal thymus than with the cells residing in the steady-state adult organ. Hence, we injected DSG daily into pregnant females starting on day 11 after fertilization and analyzed thymocytes on days 16 and 17. The drug strongly blocked thymic development, preventing the rapid expansion of thymocytes that normally occurs. Only very rare cells in the drug-treated mice differentiated to the DP stage: on average only ~0.1% of the normal numbers of $CD4^+8^+$ cells were observed with thymi from day 17 fetuses taken from DSG-injected mothers (Fig. 4 *D*). The remaining DN population was highly enriched in $CD25^+$ cells (Fig. 4 *E*). It is possible that the block here is even more extreme than at first appreciated: many of the cells in the $CD44^-25^-$ gate might actually be very immature, $preCD44^+25^-$ recent immigrants.

The DN $CD44^-25^+$ stage is an important control point during thymocyte differentiation (for review see reference 30). At this stage, the preTCR complex is first expressed and this event seems pivotal for further maturation: turn-off of TCR-β gene rearrangement, turn-on of TCR-α rearrangement, population expansion and progression to the double-positive stage. Consequently, in mice which do not express the preTCR, for example those with a null mutation at the RAG locus, many fewer thymocytes are produced, and they are enriched in those at the DN $CD44^-25^+$ stage. Recent experiments revealed that the preTCR can be bypassed by crosslinking CD3 in vivo: injection of anti-CD3ε mAb into RAG-deficient animals provoked the transit of a large number of cells into the double-positive compartment (31–34). Given our observation that DSG blocked thymocyte differentiation rather specifically at the DN $CD44^-25^+$ stage, we wondered whether it could influence the artificial transition promoted by CD3 cross-linking. Thus, we injected mAb with or without the drug into RAG-deficient animals and stained thymocytes after 3 d. Clearly, DSG inhibited the differentiation provoked by anti-CD3ε treatment, evident from a diminished DP population (Fig. 5 *A*, *upper panels*) and a reduced DN $CD44^-25^-$

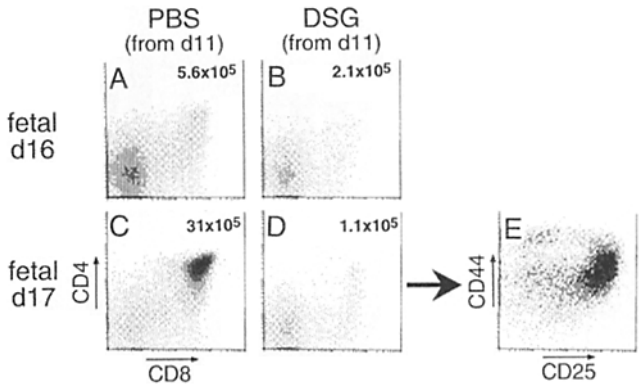


Figure 4. DSG blocks differentiation in the embryonic thymus. Pregnant DBA/2 females (impregnated by B6 males) were injected twice daily with DSG or PBS starting at day 11 of gestation, and pooled thymi of the developing embryos were analyzed at gestational day 16 or 17 by cytofluorimetry. Total cell numbers per thymus (an average of 4 to 8 thymi) are shown on each panel. (*A–D*) CD4/CD8 staining; (*E*) CD44/CD25 staining of the DSG-treated day 17 sample.

subset (Fig. 5 *A*, *lower panels*). The drug could be added as late as 24 h after the mAb and still inhibit the appearance of DP cells (Fig. 5 *B*).

DSG Does Not Inhibit the DP to SP Thymocyte Transition, nor the Stimulation of Peripheral T Cells. A second important control point during thymocyte differentiation is at the transition between the double-positive and single-positive stages. Positive selection into the $CD4^+8^-$and $CD4^-8^+$ compartments involves specific recognition of MHC molecules on stromal cells by the α:β TCR on $CD4^+8^+$ T cells (see reference 35 for review). Positive selection can be monitored by tagging replicating DNA in dividing immature cells with a pulse of the thymidine analogue BrdU and following the flow of label into the mature single-positive compartments. It routinely takes ~3–5 d before label incorporated by dividing DP, immature SP and DN precursors is detected to a significant degree in the population of mature SP cells (23, 25, 36, 37). As indicated in Fig. 6, DSG did not influence the flow of BrdU label into either $CD3^+$ single-positive compartment. The kinetics and degree of BrdU staining were indistinguishable in control and DSG-treated mice.

This result brought up the issue of whether DSG influences fully mature peripheral T cells. Total numbers of T cells in spleen and lymph node, and their subpopulation distribution, were not significantly affected (data not shown). As indicated in Fig. 7 *A*, the drug had little effect

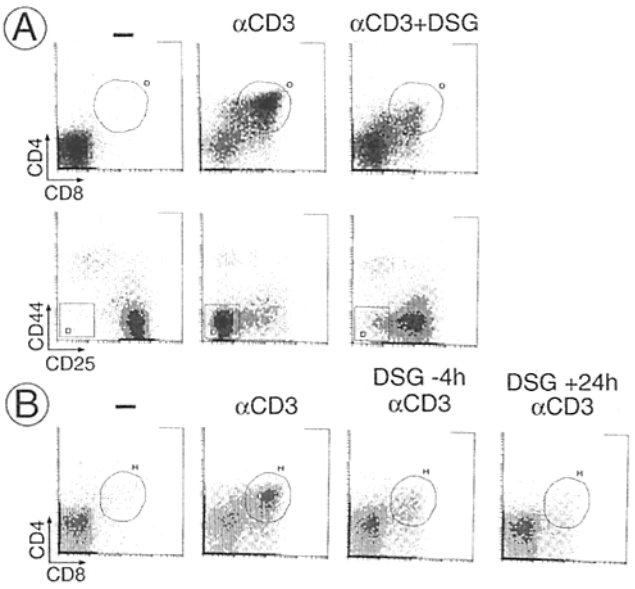


Figure 5. DSG blocks anti-CD3 induced differentiation in the RAG°/° thymus. (*A*) Knockout mice deficient in the RAG-2 gene were injected once with anti-CD3 ascites to induce the bypass of the differentiative block at the double-negative stage. Control mice were injected with PBS only (*left panels*); DSG (*right panels*) was administered at −2, 0, 12, 24, and 36 h relative to the anti-CD3 injection. Thymi were analyzed 48 h after the injection of anti-CD3, by staining with anti-CD4 and -CD8 (*top panels*), or with anti-CD44 and -25 (*bottom panels*). (*B*) RAG-deficient mice were injected as in *A*, except that DSG treatment was initiated either 4 h before or 24 h after the anti-CD3 treatment, and repeated every 12 h thereafter. Stainings were performed 60 h after anti-CD3 injection.

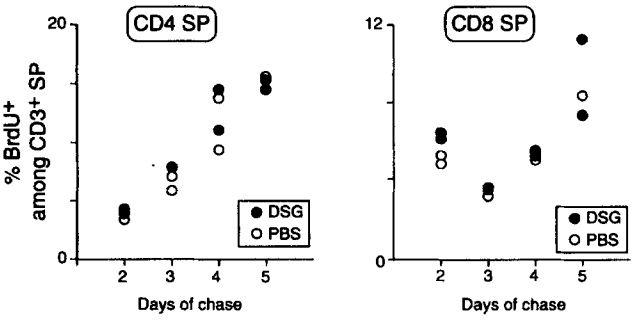


Figure 6. DSG does not impair selection into the mature single-positive compartments. Cycling thymocytes from B6 mice were pulse-labeled by injection of BrdU, which was allowed to chase into the $CD4^+8^+$ double-positive compartment. The flow of BrdU label into the mature $CD3^+CD4^+8^-$ and $CD3^+CD4^-8^+$ compartments was examined every day thereafter by cytofluorimetric analysis. DSG treatment was applied in the last 48 h of the chase for each sample. The data shown here represent the percentage of BrdU-positive cells in the $CD3^+CD4^+8^-$ and $CD3^+CD4^-8^+$ single-positive compartments (CD4SP and CD8SP, respectively) in DSG-treated or control mice.

on in vitro T cell proliferation provoked by anti-CD3 or ConA stimulation. It seemed more relevant, however, to measure the reactivity of peripheral T cells from animals treated under the conditions which had led to a block in differentiation of immature thymic T cells. Therefore, we injected DSG-treated or untreated mice with anti-CD3ε mAb (the same amount used for the experiments in Fig. 5) and evaluated the drug's effect on T cell activation by monitoring expression of the early activation marker CD69. DSG did not prevent T cell activation by anti-CD3 in vivo (Fig. 7 *B*). Finally, we assayed ex vivo the reactivity to anti-CD3 or ConA of peripheral lymphocytes from mice treated for 1 wk with the drug. No impairment of reactivity was detected (Fig. 7 *C*, and data not shown).

Differentiation of Immature B Cells Is Blocked at an Equivalent Stage by DSG. The differentiation of immature B cells in the bone marrow, like that of T cells in the thymus, is strongly regulated at the point where the first of the two variable chains of the antigen receptor is expressed (for review see references 38 and 39). This control seems to be exerted through a preB cell receptor (BCR) that includes the variable Ig heavy chain and the invariable chains λ5 and VpreB. In mice that are unable to make one of these components, for example RAG-deficient animals, B cell maturation is arrested just before stages characterized by rapid expansion and Ig light chain rearrangement, and few cells are exported from the bone marrow.

Given the above-described effects on thymocyte differentiation and the obvious parallels between early T and B cell maturation, we explored the influence of DSG on B cell differentiation. Mice were injected with the drug for various lengths of time and, 24 h after the last treatment, bone marrow cells were isolated and analyzed by cytofluorimetry. The effects were quite dramatic. First, drug administration led to a depletion of cells exhibiting the size characteristics of the lymphoid lineage, whereas granulocytes and other

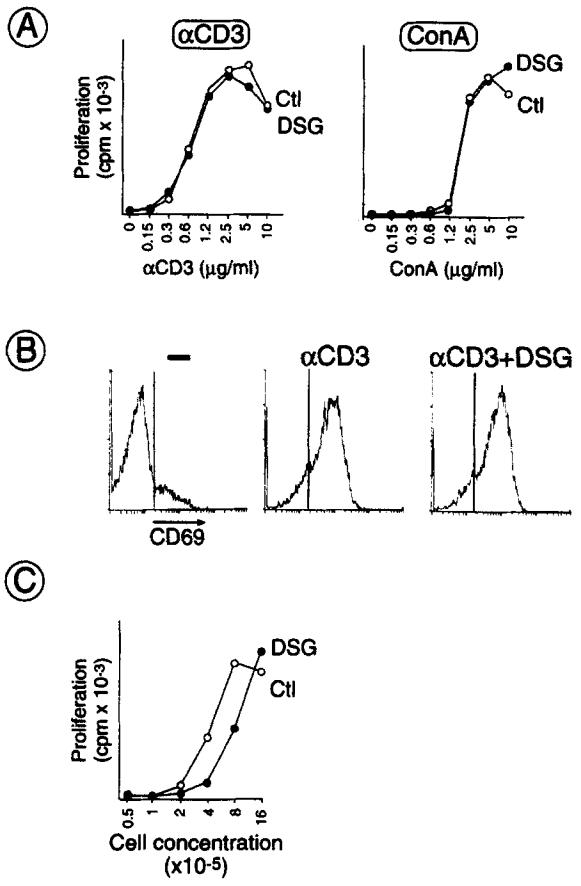


Figure 7. DSG does not grossly affect the reactivity of peripheral T cells. (*A*) Lymph node T cells from B6 mice were stimulated in vitro with anti-CD3 or ConA, as indicated, and the proliferative response measured 24 h later as incorporation of [^{3}H]thymidine. (*B*) B6 mice were injected with anti-CD3 ascites as in Fig. 5, and CD69 expression on T cells (gated on CD4- or CD8-positive) was measured by cytofluorimetry. Control mice were injected with PBS only (*left panel*); DSG (*right panel*) was administered at −2, 0, and 12 h relative to the anti-CD3 injection. (*C*). B6 mice were treated for 1 wk with DSG (or PBS as control) and T cell reactivity was assessed by ConA stimulation in vitro. The proliferative response was measured 36 h later by incorporation of [^{3}H]thymidine.

large myeloid cells appeared unaffected (Fig. 8 *A*). The time course of depletion paralleled that for lymphoid cells in the thymus, significant reductions only becoming apparent after several days of treatment.

Multiparameter staining with reagents specific for IgM, CD45R (B220), CD43, and HSA allowed us to follow the fate of cells at different stages of B cell differentiation. The more mature subsets are illustrated in Fig. 8 *B*, a plot of anti-B220 versus anti-IgM staining of cells gated as CD43-negative. DSG treatment gradually eliminated the preB and immature B cell populations (fractions D and E according to the nomenclature of Hardy and colleagues (38 and references therein), but spared the most mature B cells expressing IgM and the highest levels of B220 (fraction F). Earlier B cell subsets are depicted in the top panels of Fig. 8 *C*, where anti-B220 versus CD43 staining of IgM-negative cells is displayed. The preB cells of fraction D ($B220^{++}$, $CD43^{lo/-}$) were completely eliminated after 12 d of drug treatment, but a substantial proportion of the proB cells in fractions A-C′ remained. These latter subsets were further separated according to anti-HSA staining levels (Fig. 8 *C*, *bottom panels*). Administration of DSG eliminated the more mature cells with the highest HSA and B220 levels (fraction C′), but appeared to spare fractions A-C. The patterns observed with bone marrow cells isolated from RAG-deficient mice and stained in parallel were quite similar, with an absence of subsets C′ and D, as anticipated (see reference 38 and references therein). The cells which accumulated in $RAG^{o/o}$ mice did have a phenotype slightly different from those which remained in the drug-treated animals, i.e., slightly higher B220 and HSA levels.

Overall, the picture we observed for the B cell lineage in the bone marrow was strikingly reminiscent of that seen for the T lineage in the thymus: DSG eliminated the incompletely mature populations undergoing expansion and selection, but spared both the most immature and most mature subsets. Drug susceptibility appeared to coincide with expression of a pre-receptor composed of a single Ig or TCR variable chain paired with an invariant surrogate partner.

Discussion

Our studies have explored the effect of DSG on the generation of lymphocytes in the mouse. This drug interfered with the differentiation of early T cell precursors in the thymus but not with the maturation/selection nor the activation of T cells at later stages. It also blocked, in a quite parallel manner, the differentiation of early B cell precursors in the bone marrow. These findings bear on two important issues concerning lymphocyte development.

Early- versus Late-Stage T Cells. DSG blocked T cell differentiation at an early stage, effectively and precisely: most DN $CD44^-25^+$ cells did not mature any further. The effect was most obvious with thymus cells taken from fetuses exposed to the drug in utero (Fig. 4 *E*), but was also clear with thymocytes from animals treated as young adults (Fig. 3, *C* and *D*). In addition, the drug could block the anti-CD3-induced progression of DN cells in $RAG^{o/o}$ mice (Fig. 5 *A*). Quite in contrast, DSG did not have a measurable effect on late-stage T cells. Positive selection of DP thymocytes into the SP compartments was unperturbed in drug-treated animals (Fig. 6), fully mature thymocytes and peripheral T cells were spared (Fig. 2, *A* and *C* and data not shown), and activation of peripheral T cells by anti-CD3 and ConA either in vitro or in vivo was unaffected (Fig. 7).

One explanation for this dichotomy is that DSG inhibits signaling through the preTCR but not the conventional α:β TCR. Although no existing data establish that the preTCR complex, itself, transduces signals during thymocyte differentiation, this would seem a likely explanation for the controls on gene rearrangement, population expansion and cell differentiation exerted in coincidence with its expression. Signal-transducing CD3 subunits are known to be components of the preTCR complex, and it has been reported that the zeta chain binds more weakly than it does to the α:β

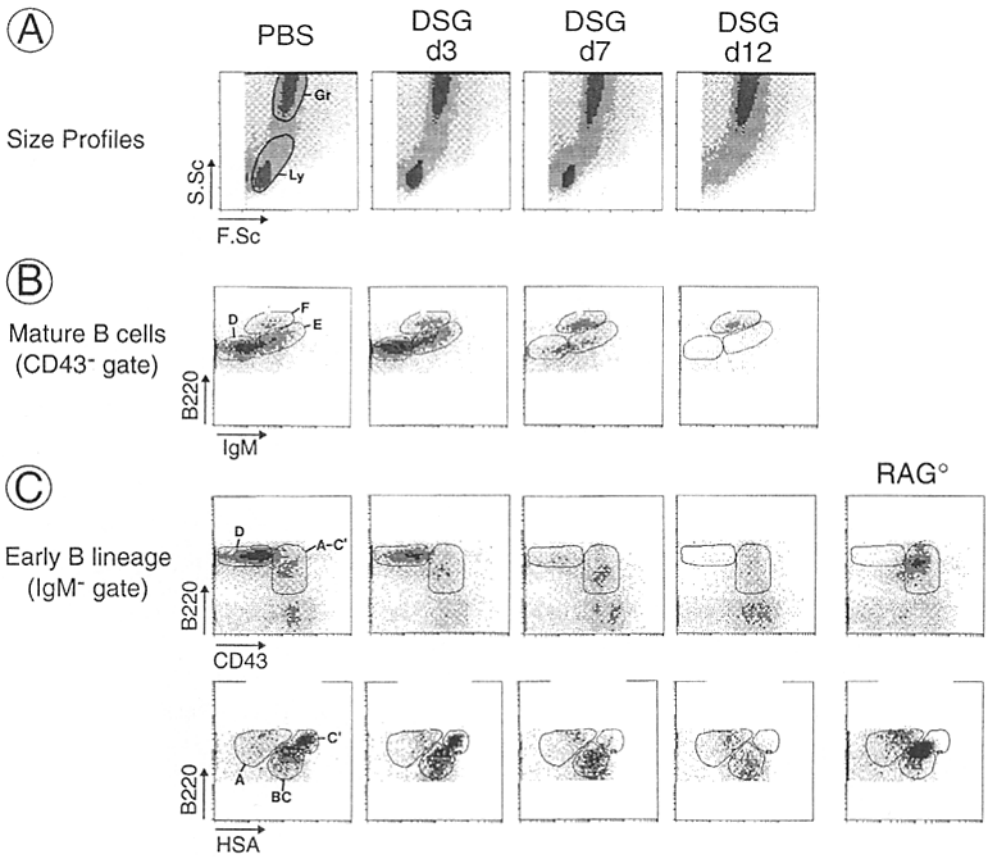


Figure 8. DSG blocks B cell differentiation at an early stage. BALB/c mice were treated with DSG or PBS as a control for varying times, as indicated. Bone marrow cells were analyzed by four-color cytofluorimetry after staining with anti-IgM, anti-CD45R (B220), anti-CD43, and anti-HSA. (*A*) Side vs forward scatter two-dimensional profiles; the granulocyte and lymphoid populations are shown on the PBS panel. (*B*) Mature B cells are gated as CD43-negative, and their IgM/B220 profiles displayed; populations D (pre-B), E (immature B), and F (mature B), according to the nomenclature of Hardy and colleagues (38) are indicated. (*C*) The pre- and pro-B stages of differentiation are displayed after gating on IgM-negative cells, to eliminate the B populations. Populations D (pre-B) and A-C′ (pro-B) are shown on the top panels. The CD43-positive cells, gated as shown, are further resolved on the lower panels.

TCR complex (40), suggesting a possible basis for differential signaling. Nonetheless, in vitro experiments with transfected cell lines have not so far been able to distinguish signaling events set in motion by mAb engagement of the two receptors. As yet, only a few parameters have been investigated, however (40, 41). On the other hand, in vivo or ex vivo experiments with mice have revealed differences between the early and late control points in the thymus. Mice deficient in ZAP70 (42), interferon regulatory factor (IRF)-1 (43), and CD45 exon 6 (44) all had defects in the DP → SP transition, mediated by the α:β TCR, but the DN → DP transition, dependent on the preTCR, appeared quite normal. Conversely, tyrosine kinase inhibitors, such as herbimycin A, blocked the early transition but not the late one (28).

It is also possible that the DSG effect on DN thymocytes is independent of signaling through the pre-TCR. Indeed, we did not observe any effect of the drug on pre-TCR signaling elicited in vitro: when a thymocyte line derived from a SCID mouse and transfected with the TCR-β gene (40) was stimulated with anti-TCR-β or anti-CD3, there was comparable Ca^{++} mobilization and CD69 induction in the presence or absence of DSG (data not shown). One could question, however, how closely antibody stimulation of cultured cells in vitro mimics receptor-ligand interactions during thymocyte differentiation in vivo. Or it could be that any differences just lie further downstream.

Whichever explanation proves correct, one is left with the problem of explaining the immunosuppressive effects of DSG in transplant and autoimmune situations, which must ultimately depend on silencing mature T cells in the periphery. It is possible that effects on mature T cell activation more subtle than those measured by ConA or anti-CD3 stimulation are involved. It might also be that the immunosuppressive effects are more indirect involving, for example, antigen presenting cells. This has been suggested in a previous study (13) but we have not been able to find support in similarly designed experiments (data not shown).

PreT and PreB Cell Differentiation. DSG blocked T cell differentiation at the stage where one of the variable receptor genes has been rearranged and its product displayed at the cell surface as part of a pre-receptor complex. Expression of this preTCR is a prerequisite for population expansion and further maturation. DSG halted B cell differentiation at an equivalent stage, the (A-C)→C′ transition. Progression beyong the (A-C) stages generally requires expression of a pre-receptor composed of the Ig heavy chain and two nonvariable chains, and is marked by a period of rapid cell division and most likely some kind of cellular selection (38, 39). The analogous blocks in B and T cell differentiation were perhaps most evident in comparisons with RAG-deficient mice. These animals can not make the first receptor variable gene rearrangement and thus do not express either a preTCR or preBCR. T cell differentiation was

blocked at a very similar stage in DSG-treated and RAG-deficient mice, as was B cell differentiation. The slight differences that were seen, particularly with B cells, probably reflect the longer times of precursor accumulation in RAG-deficient animals.

What could be the root of this commonality? The simplest interpretation again invokes signaling: there could be some shared element in signaling events associated with the "checking" of the preTCR and preBCR. No such element has so far been identified, but would not be surprising given the highly analogous scenarios which seem to be emerging. It may be relevant that DSG was found to inhibit LPS-induced Ig κ expression in the 70Z/3 preB cell lymphoma by reducing nuclear translocation of the transcription factor NF-κB (45) and that anti-oxidant inhibitors of NF-κB arrested α:β thymocyte differentiation at the DN stage in FTOC (46). It is thus conceivable that DSG interferes with the action of an NF-κB family member whose action is specifically required at parallel stages in B and T differentiation.

It is also possible that the DSG-sensitive event is not directly tied to the pre-receptor complex. We think it unlikely that RAG-mediated gene rearrangements are inhibited, as transgenics expressing prerearranged TCR genes also show DSG-induced loss of DP thymocytes (data not shown). Other potential targets are interactions between adhesion molecules and their receptors or cytokines and their receptors. Signals emanating from such interactions might be required accompaniments for pre-receptor signals to effectively initiate or maintain the DN → DP transition. DSG could act directly on the immature T cell precursor or could just as well affect a stromal cell, preventing it from delivering a signal essential for T precursor maturation. IL-7/IL-7R and c-kit/stem cell factor interactions would at first seem attractive possibilities because they are known to influence both B and T cell differentiation at an early stage. However, ablation of the IL-7R gene, treatment in vivo with an anti-IL-7 mAb and mutation of the c-kit locus all appear to block thymocyte differentiation at an earlier DN stage than DSG treatment does, after the $CD44^+25^-$ rather than the $CD44^-25^+$ stage (47–49). Another possible target is CD81, a molecule expressed on thymic stromal cells and recently shown to be crucial during the DN → DP transition (50).

Finally, the leakiness observed with DSG treatment of adult animals suggests a third possibility. Perhaps it is not so much differentiation that is blocked as cell division. Thus, there could be maturation from the A-C to C′ stage for B cells, or $CD44^-25^+$ to $CD44^-25^-$ for T cells, but these products would remain a relatively minor fraction in the absence of the massive cell expansion that normally takes place at this time. However, BrdU-labeling experiments did not support this interpretation: division of DP blasts and mature SP cells appeared normal in mice given short-term DSG treatment (data not shown). Similarly, cell proliferation in vitro was not affected by DSG (Fig. 7 and data not shown). In addition, injection of mice in utero with DSG led to a much cleaner phenotype, only very few cells, if any, escaped the block at the DN $CD44^-25^+$ stage.

Practical Implications. Whatever the explanation for the DSG-induced block and its commonality, our observations have practical implications. In a clinical context, they argue that long-term treatment, as proposed for amelioration of autoimmune diseases, may be a problem because of its potential to prevent the normal replacement of B and T lymphoid compartments. In an experimental context, our findings provide a means to produce a cohort of T cell precursors arrested at an important control point, which might be very useful in attempts to address the many outstanding questions concerning early thymocyte differentiation.

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