Immunoendocrine Modulation and Stimulation of Hematopoiesis with the Ionophore Copolymer T150R1 (43091)

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Abstract. **T150R1, an 8000-dalton copolymer with sodium ionophore activity, has been shown to modulate cellular responses in multiple systems. In this article, we studied its effects on lymphoid and hematopoietic organs in the context of the adrenal-pituitary axis. When injected in mice as an oil in water emulsion, T150R1 caused a rapid, profound, and dose-dependent thymic involution accompanied by splenic hyperplasia. Time course experiments with a 2.5-mg dose revealed that the thymus size was minimal at Day 2, rose to normal by Day 14, then enlarged and gradually returned to normal by Week 6 postinjection. Thymic involution was due to cellular depletion of the cortical area, whereas thymic enlargment was due to cortical hyperplasia. Splenomegaly was seen as early as Day 4, peaked by Day 14, and gradually returned to normal by Week 6. The splenic enlargement was due to hyperplasia of the red pulp, with evidence of proliferating erythropoietic, myelopoietic, and megakaryopoietic precursors. In addition, the bone marrow was stimulated and extramedullary hematopoiesis was present in the liver. The effects of T150R1 on the thymus appeared to be mediated by corticosteroids while the effects on hematopoiesis were not. Corticosterone and ACTH levels were increased in treated animals. Adrenalectomy diminished the T150R1-induced thymic involution but enhanced the splenic hyperplasia. Hypophysectomy did not prevent thymic involution, suggesting that T150R1 has endocrine stimulatory effects. These data suggest that T150R1 represents a new class of ionophores which may act on excitable cells within the endocrine, immune, and hematopoietic systems.** [P.S.E.B.M. 1990, Vol 194]

Copolymer T150R1 is a novel synthetic nonionic block polymer surfactant (Fig. 1). It was originally studied as a candidate vaccine adjuvant because of its structural similarities to other polymers with known adjuvant activity (1). Its immunologic activities were found to be more complex than those of other block copolymers. T150R1 is now recognized as one of a series of ionophore copolymers which differ in the length of their polyoxyethylene and polyoxypropylene chains. The octablock structure of four closely approximated polyoxyethylene chains suggested the potential for forming an ion cage similar to that of the crown polyethers and other ionophores (2). Atkinson *et al.* (2) then demonstrated that T150R1 and its closely related copolymers have ionophore activity in artificial membranes and red blood cell systems with selectivity for sodium, potassium, and hydrogen, but not for calcium or magnesium.

T150R1 is a relatively weak ionophore, but also has relatively low toxic effects. It was found to modulate cellular reponses in multiple systems. *In vivo*, it suppressed the induction of experimental allergic encephalitis in a rat model, possibly by stimulation of suppressor T cells (3). *In vitro*, the closely related ionophore, T130R2, induced histamine release from basophils and mast cells (4, 5). Both T150R1 and T130R2 promoted the monocytic differentiation of HL60 promyelocytic leukemia cell cultures (6). In addition, preliminary experiments in mice showed that T150R1 induced pathologic changes in the thymus and spleen and stimulated hematopoiesis in all of its elements like no other known compound (7).

This article characterizes the previous observations and explores their relation to the pituitary-adrenal axis. The effects of copolymer T150R1 on the thymus were

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found to be corticosteroid mediated while those on the hematopoietic system were not, and a role for monokine involvement is suspected. Since cation concentrations and fluxes are crucial to cell excitability (8), we propose that T150R1 exerts its effects by modulating ion concentrations within cells of the immune, endocrine, and hematopoietic systems.

Materials and Methods

Mice. Four- to 8-week-old outbred (ICR) or inbred (BALB/c) mice were purchased from Charles River, Raleigh, NC. They were housed five per cage and given good and water *ad libitum*. All mice were allowed to acclimate from shipping stress for 1 week.

In the experiments in which adrenalectomized or hypophysectomized mice were used, surgery had been performed by the animal supplier. Adrenalectomized mice were given saline drinking water. Adrenal remains were found at autopsy in some of these animals, therefore we considered them to be partially adrenalectomized. Hypophysectomized mice were also examined post-mortem for evidence of complete hypophysectomy. Mice that had pituitary remains were excluded from the studies.

Treatment with T150R1. Intravenous routes were used in the dose-response studies. Groups of 6- to 8-week-old female ICR mice ($n = 4$–5) were given a single injection in the tail vein of various doses of T150R1 (BASF Wyandotte Corp., Parsippany, NJ) solubilized in 4°C phosphate-buffered saline (PBS). The doses used were: 1.25, 2.5, and 5.0 mg per an average 20-g mouse. Noninjected, age-matched mice served as controls. The animals were sacrificed by asphyxiation with CO_2 on Day 4 postinjection. Body weights were recorded and the thymus and spleens were removed, carefully freed from surrounding tissues, then weighed.

Oil in water emulsions of T150R1 were used in all footpad treatments. Each mouse was injected subdermally with an emulsion made with 2.5 mg of T150R1, 5.0 mg of mineral oil (Drakeol 6VR; Penreco Refining Co., Butler, PA), and 50 μg of bovine serum albumin in 0.1 ml of 0.01 *M* PBS containing 0.2% Tween-80 (Sigma Chemical Co., St. Louis, MO) was divided between two footpads.

For the time course study, groups ($n = 3$ each) of 6- to 8-week-old female BALB/c mice, injected with the emulsion described above, were compared with mice that received the emulsion without T150R1 (vehicle injected) and groups of noninjected controls. At these time points (Days 1, 2, 3, 4, 5, 7, 11, 14, 18, and 21, Weeks 4, 5, 6, 9, and 12), mice were sacrificed and their body, thymus, and spleen weights were recorded.

The studies of the effects of adrenalectomy and hypophysectomy were also done with footpad-injected (2.5 mg of T150R1) mice. Six- to 8-week-old surgically modified female BALB/c mice ($n = 3$/group) were

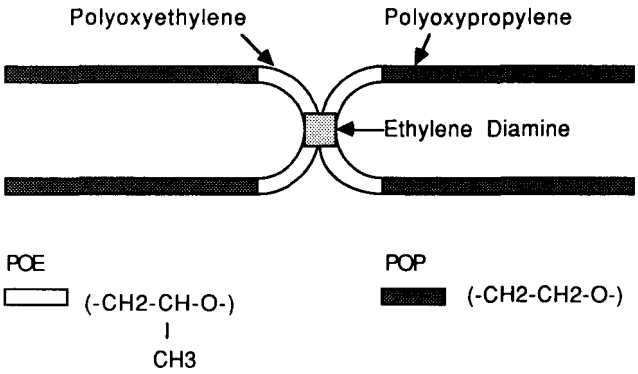

Figure 1. Structure of the synthetic copolymer T150R1 (M_r 8000). Four blocks (5 units each) of hydrophilic polyoxyethylene (POE) flanked by four blocks (32 units each) of hydrophobic polyoxypropylene (POP) are attached to a central ethylenediamine. This structure enables T150R1 to function as an ionophore by binding monovalent cations in its hydrophilic center then crossing the cell membrane via interactions of its hydrophobic peripheral blocks with the lipid bilayers.

used. At Day 3 or 7 posttreatment, body, thymus and spleen weights were compared in copolymer-injected animals versus noninjected surgically modified controls. In the hormone kinetic studies, retro-orbital blood was collected in heparinized capillary tubes at 2, 6, and 8 hr posttreatment. Sera were stored frozen at −70°C for future quantitation of corticosterone and ACTH levels. Mice were handled gently and bled in a distant area in order to minimize stressful situations that might affect the levels of these hormones.

Histology. All mice were subject to postmortem examination. Samples from the following tissues (thymus, spleen, liver, lung, kidney, small intestine, lymph node, and bone) were fixed in 10% neutral buffered formalin. Bone samples were subsequently decalcified with equal volumes of water solutions of sodium citrate (200 g/liter) and formic acid (45%). Tissues were then embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin. Slides were viewed under a light microscope and tissue sections from selected animals were photographed.

Hormone Measurements. Corticosterone and ACTH levels were determined by competitive binding radioassays using ^{125}I-labeled corticosterone and ^{125}I-labeled ACTH kits purchased from ICN Biomedicals, Inc., Diagnostics Division, Carson, CA. Assay procedures followed the manufacturer's instructions. The corticosterone kit is designed for measuring the hormone in rats and mice and is sensitive to 25 ng/ml. It is highly specific for corticosterone and has minimal cross-reactivity with other steroids (0.34% for desoxycorticosterone, 0.10% for testosterone, 0.05% for cortisol, and less than 0.03% for all the other steroids). The ACTH kit is designed to measure human ACTH, but since the antibody reacts with sites in residues 1–24 and 1–39, it reacts with mouse ACTH as well. It is sensitive to 10 ng/ml. It is highly specific for ACTH

and has minimal cross-reactivity with other peptide hormones (0.8% for β-lipotropin, 0.1% for α-lipotropin, and less than 0.1% for melanocyte-stimulating hormone and β-endorphin). All assays were performed in duplicate.

Statistical Analysis. Statistical analyses were carried out with Student's *t* test. Since comparison of thymus and spleen weights gave similar conclusions to comparison of organ to body weight ratios, we plotted organ weights in all graphs. Unless otherwise stated, all data are presented as mean ± SE. To calculate the significance of the differences in the mean percentage of change in organ weights with copolymer treatment in adrenalectomized or hypophysectomized versus normal animals, the standard deviation of each percentage of change was calculated (9) and the *t* test was applied.

Results

Dose-Response and Time Course Studies of T150R1 in Normal Mice. *Effects on the thymus.* The administration of T150R1 caused a rapid, dose-dependent thymic involution in mice. Four days after intravenous injection, the copolymer induced a significant decrease in thymus weights that was proportional to the amount injected (Fig. 2). A dose of 2.5 mg of T150R1 or higher was necessary to produce a significant ($P = 0.006$) thymic involution in mice of an average body weight of 20 g and all subsequent studies were performed using this concentration.

Time course studies showed that thymic involution, as measured by the decrease in thymic weight, occurred within 24 hr and reached its maximum at Day 2 (Fig. 3). The thymus returned to normal size by Day 14, then enlarged due to a rebound hyperplasia peaking at Day 28. By Week 6, thymus weights returned to normal again. Vehicle (emulsion without T150R1)-injected and untreated mice did not undergo thymic changes except for the age-related involution as reflected by the slight decrease in weight over the 3-month period of testing.

Histologically, there was a near total cortical lymphoid cell loss in the thymus at Day 1 (Fig. 4A), with evidence of cellular death among the cortical thymocytes. The picture was similar to that seen with the administration of massive doses of corticosteroids in sensitive species such as mice (10). By Day 14, the thymus had regenerated as demonstrated by the presence of many lymphoid cells in mitosis in the medullary area and increased cellularity with hyperplasia of the cortical area (Fig. 4B).

Effects on the spleen. T150R1 induced splenic hyperplasia in mice. An intravenous dose as small as 1.25 mg caused a significant ($P = 0.02$) increase in spleen size by Day 4 (Fig. 2). The time course study demonstrated a small transient decrease in spleen weight at Day 2 (Fig. 3). Then spleen weight gradually

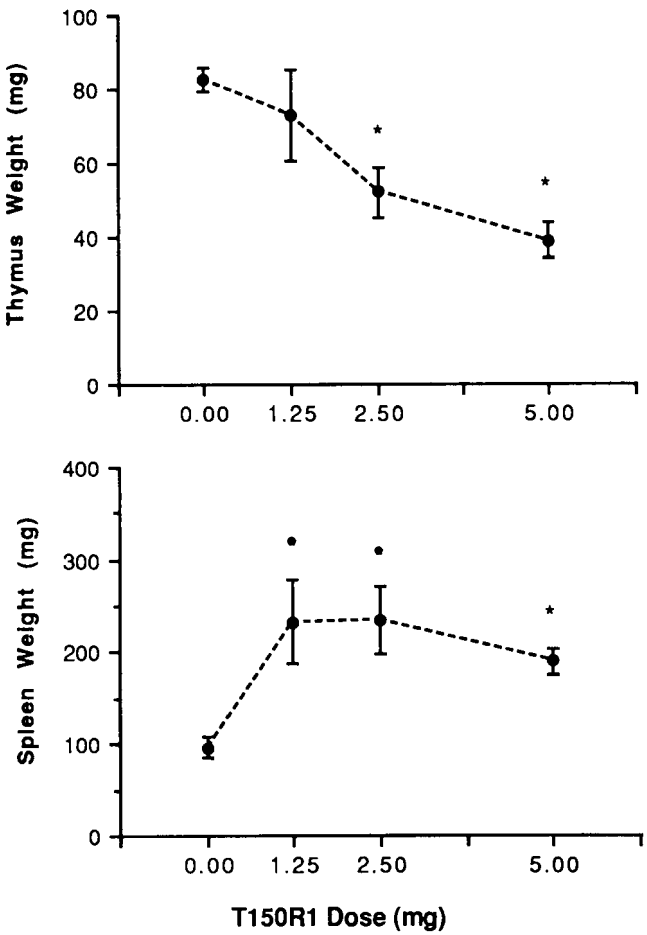

Figure 2. Dose-related thymic involution and spleen enlargement following a single T150R1 injection. Six- to 8-week-old female ICR mice were injected in the tail vein with PBS alone or with different doses of T150R1 solubilized in PBS. Four days later, mice were sacrificed and their thymuses and spleens removed and weighed. Each data point represents the mean of four to five organs ± SEM. Asterisks indicate data points where the organ weights from animals treated with T150R1 are significantly different from the 0 dose controls ($P < 0.05$).

increased, peaking at 14 days, when the size was more than double that of controls. It returned to normal by Week 6. Vehicle-injected mice did not exhibit changes in their spleen size.

Histologically, massive hyperplasia of the red pulp accounted for the weight increase. Colonies of myeloid, erythroid, and megakaryocytic precursors were present in the red pulp. The lymphoid tissue of the white pulp demonstrated relatively little change (Fig. 5).

Effects on other organs and tissues. T150R1 did not cause significant changes in other organs (liver, small intestines, lungs, and kidneys). However, extramedullary myelopoiesis was seen in the liver of copolymer-treated animals by Day 7 (Fig. 6). Myeloid precursors proliferating in focal areas throughout the liver parenchyma persisted for weeks before they subsided by Week 6. Sections from the bone marrow of T150R1-

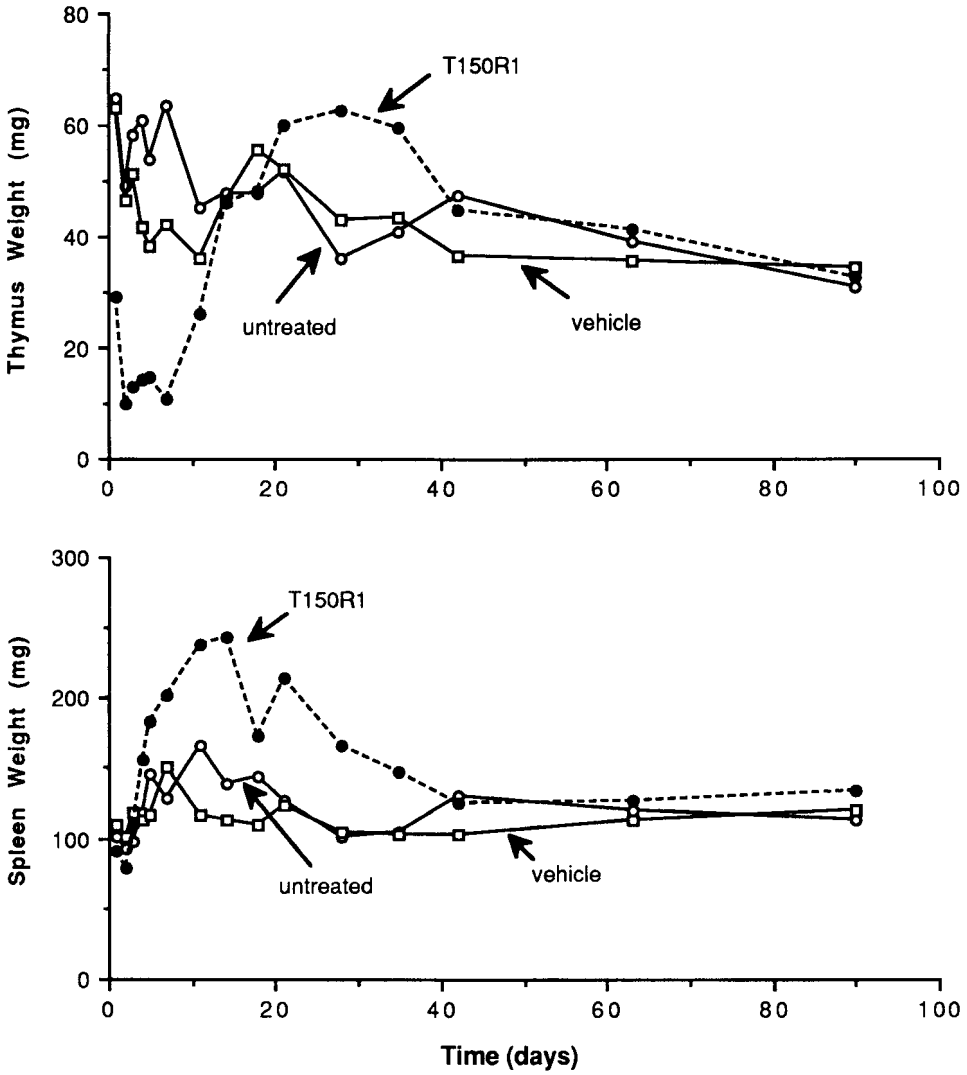

Figure 3. Time course of thymic modulation and spleen enlargement following a single T150R1 injection. A group of 6- to 8-week-old female BALB/c mice were injected in both footpads with an oil in water emulsion containing 2.5 mg of T150R1. Controls were a group of untreated mice and a group of vehicle (emulsion without T150R1)-injected mice. At different time points, mice were sacrificed and their thymuses and spleens removed and weighed. The data at each time point represent the mean of three different organs. Organ weights from uninjected and vehicle-injected controls were not statistically different. Thymus weights from T150R1-injected animals were significantly lower than those from vehicle-injected or uninjected animals at Days 1 through 7 and significantly higher at Days 21 and 28 ($P < 0.05$). Spleen weights from T150R1-injected animals were significantly higher at Days 4 through 35, except for Day 18. Standard errors are not shown, but in all cases represented <10% of the mean values.

treated mice also exhibited increased cellularity by Day 7. Myeloid and erythroid precursors were predominant.

Local lymph nodes draining the site of injections enlarged, especially when the copolymer was administered in oil in water emulsions. Paracortical hyperplasia and, in some cases, granulomas were seen in these nodes. This accompanied the inflammatory process at the site of the T150R1 injections. The injection of the oil in water vehicle without T150R1 did not induce any of the above changes.

Injection of T150R1 in adrenalectomized and hypophysectomized mice. *Effects on the thymus and spleen.* In order to determine whether corticosteroids were responsible for the rapid thymic involution caused by T150R1, preliminary measurements of serum corticosteroids were done. Their concentration was elevated within 24 hr. Therefore we explored the level at which T150R1 exerts its effects through the pituitary-adrenal axis by testing the copolymer in adrenalectomized and hypophysectomized mice.

When injected into adrenalectomized mice, T150R1 induced much less thymic involution by Day 3 than in nonadrenalectomized mice. The thymuses in normal mice treated with T150R1 decreased to 44 ± 2.5% of the untreated weights (from 86 ± 1.3 to 38 ± 2.6 mg in treated versus untreated animals) as seen in the experiments mentioned above. In contrast, thymuses in adrenalectomized animals decreased only to 75 ± 3.3% of the untreated weights (from 83 ± 1.8 to

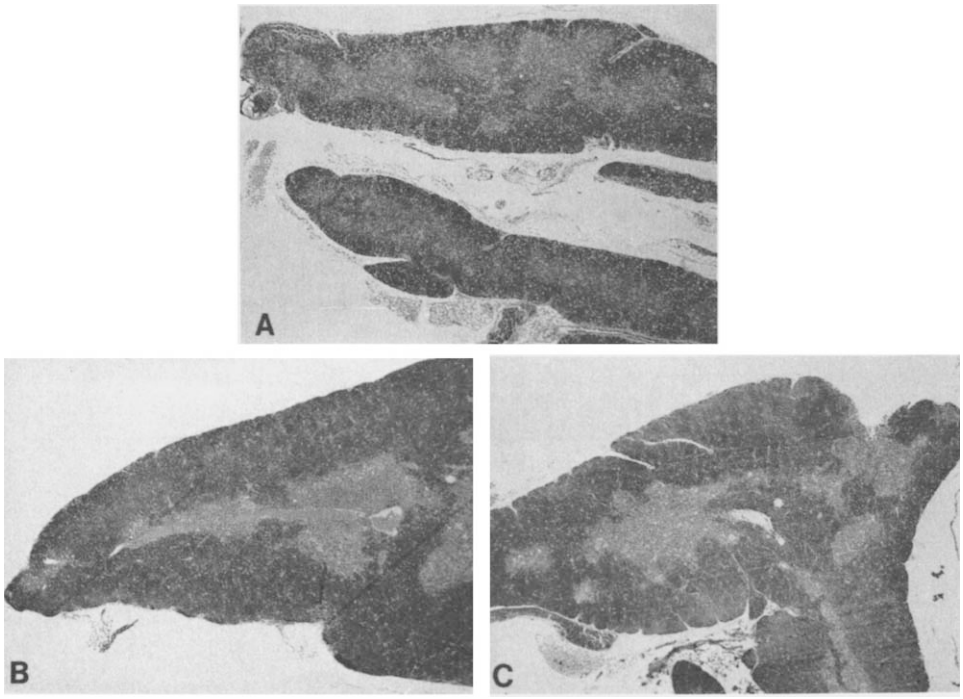

Figure 4. Histologic changes in the thymus of T150R1-treated mice. At Day 1 post-T150R1 (A), an extensive loss in the cortical area of the thymus was present, with signs of cellular destruction. At Day 14 post-T150R1 (B), cortical hyperplasia prevailed with large numbers of proliferating thymocytes. Normal age-matched thymus is pictured in C. Sections are hematoxylin-eosin stained (original magnification ×10).

62 ± 3.4 mg; P value for the difference in the percentage of change = 0.005).

T150R1 enlarged the spleens of normal mice to 125 ± 11.3% of the untreated weights by Day 3 (from 91 ± 8.2 to 114 ± 10.2 mg) as observed previously. Interestingly, it caused even greater splenic hyperplasia in adrenalectomized mice, 151 ± 22.7% (from 123 ± 19.8 to 186 ± 17.2 mg), although the difference in the percentage of change was not statistically significant (P = 0.1). These data, along with the histologic pattern, suggested that the corticosteroid hormones had mediated the effects of T150R1 on the thymus but not on the spleen.

When injected into hypophysectomized mice, T150R1 induced slightly less thymic involution by Day 3 than in nonhypophysectomized mice. The thymuses in normal mice treated with T150R1 decreased to 62 ± 12.5% of the untreated weights (from 55 ± 9.7 to 34 ± 7.9 mg in treated versus untreated animals). In contrast, thymuses in hypophysectomized animals decreased to only 75 ± 6.4% of the untreated weights (from 77 ± 4.2 to 58 ± 5.3 mg; P value for the difference in the percentage of change = 0.1).

Hypophysectomy delayed the onset of T150R1-induced splenic hyperplasia and diminished its magnitude. By Day 3, T150R1 enlarged the spleens of normal mice to 125 ± 13.0% of the untreated weights (from 84 ± 1.7 to 105 ± 7.2 mg), but hardly affected the spleens of hypophysectomized mice (from 96 ± 6.1 to 93 ± 0.1 mg). By Day 7, T150R1 enlarged the spleens of normal mice to 164 ± 22% (from 88 ± 4.3 to 144 ± 21.5 mg). The spleens in hypophysectomized mice were enlarged only to 145 ± 12.9%, (from 77 ± 10.5 to 112 ± 9.6 mg; P = 0.1 for the difference in the percentage of change). These preliminary data indicate that both pituitary and extrapituitary mechanisms might be involved in these processes.

Effects on serum corticosterone and ACTH concentration. To further investigate the role of the endocrine system in the modulation of the thymus and spleen by T150R1, serum corticosterone and ACTH concentrations were measured at various times after copolymer administration. In normal mice, T150R1 caused a 3-fold increase in corticosterone level within 2 hr (P = 0.01) (Fig. 7). The response peaked at 6 hr, with a 6-fold increase (P = 0.005) and remained elevated 3-fold over baseline at 8 hr (P = 0.001). The ACTH level was elevated at 6 hr and continued to rise at 8 hr. Vehicle-injected or untreated mice showed no increase in these hormones.

Discussion

T150R1 is one of a family of synthetic copolymers with ionophore activity for monovalent cations which have been shown to have a broad range of biologic activities (1–7). In this study of its effects on lymphoid and hematopoetic organs, we found that it caused a dose-dependent thymic involution followed by rebound hyperplasia. This process was mediated by corticoster-

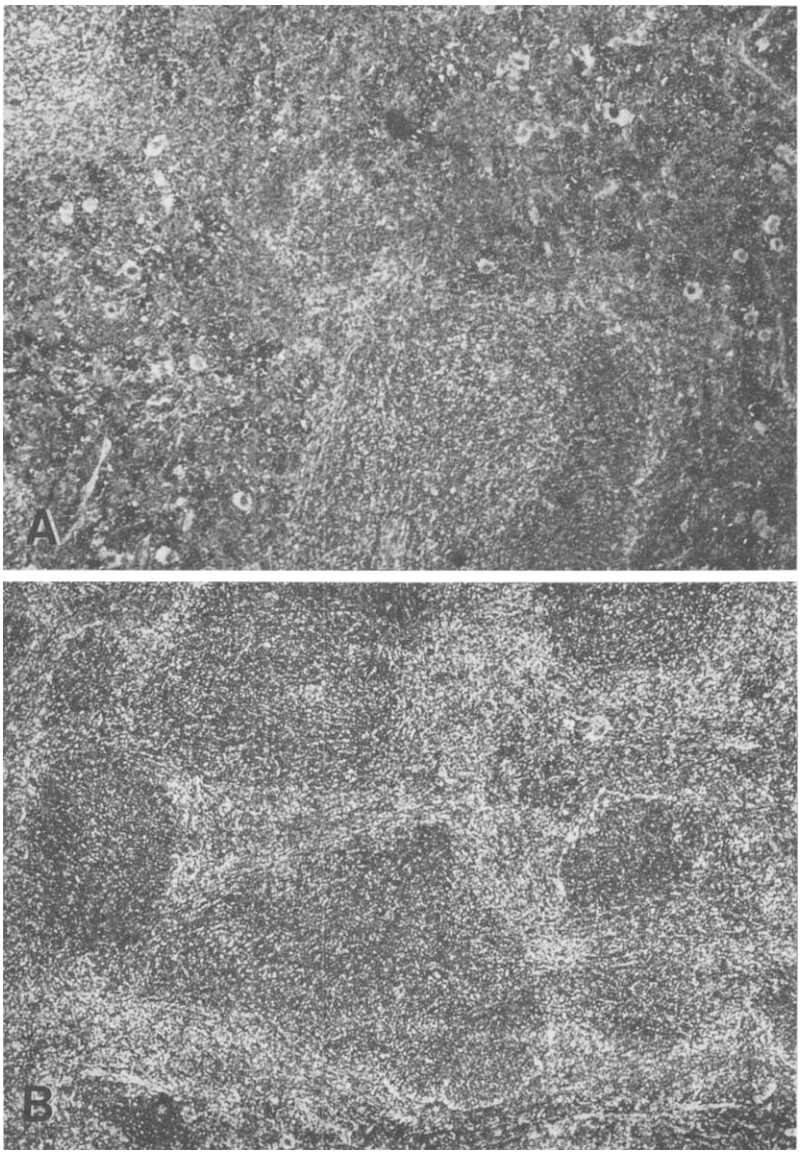

Figure 5. Histologic changes in the spleen of T150R1-treated mice. At Day 14 post-T150R1 (A), there was a profound stimulation of the myeloid, erythroid, and megakaryocytic precursors in the red pulp of the spleen. The marginal zone of the white pulp was prominent but few changes were seen in the lymphoid precursors. Normal age-matched spleen is pictured in B. Sections are hematoxylin-eosin stained (original magnification ×25).

oids, but did not require intact pituitary function. T150R1 also caused hyperplasia of the spleen and extramedullary hematopoiesis, with increased production of granulocytes, megakaryocytes, and erythrocytes. These effects were not explained by corticosteroid action.

We propose that T150R1, by altering sodium ion flux, stimulates the secretion of hormones and/or growth factors from excitable cells within the endocrine, immune, and hematopoietic systems. Elevated sodium ion concentrations have been reported to increase intracellular calcium by releasing it from storage pools (11) or by reducing calcium efflux by sodium/calcium countertransport (12), and there is evidence that alterations of intracellular calcium concentration play a major role in cell excitability (8). A homologous ionophore copolymer induced histamine release from mast cells (3) and demonstrated synergy with IgE, suggesting that the exchange of intracellular sodium for extracellular calcium triggered the increased excitability of these cells (4).

Because its effects on the thymus were reversible, T150R1 does not act like one of the many immunosuppressive drugs and toxic compounds such as dioxins which cause an irreversible involution of the thymus (13, 14). The histologic picture was different from that seen in animals treated with organotin compounds which also induce transient thymic involution (15), but

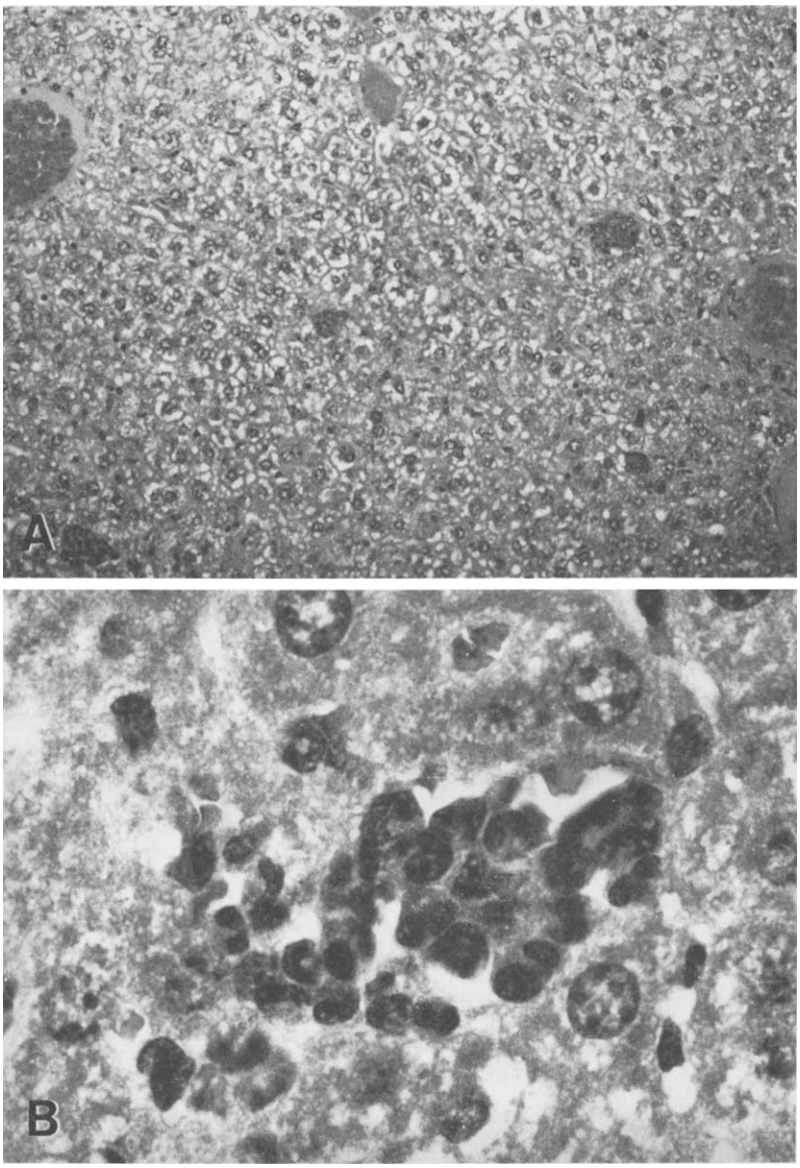

Figure 6. Histologic changes in the liver of T150R1-treated mice. Shown at Day 14 post-T150R1 (A), the liver demonstrated large numbers of granulopoietic precursors depicted at higher magnification in B. Sections are hematoxylin-eosin stained (original magnification at ×50 and ×300).

where there is not thymic cortical destruction. T150R1 caused cellular destruction in the cortical area of the thymus where mature T cells predominate. This pattern is most similar to that induced by massive doses of corticosteroids in sensitive species such as mice (10), although a direct effect of T150R1 on the thymocytes cannot be not excluded.

Adrenalectomy diminished the T150R1-induced thymic involution. This provides support for a role for corticosteroids in mediating its effects on the thymus. The hormones may be increased due to a direct stimulatory effect of T150R1 on the adrenal cortical cells. Alternatively, T150R1 may act indirectly on the adrenal by stimulating endocrine or immune cells to produce ACTH. Although other adrenal hormones such as catecholamines and mineralocorticoids might also be involved, they have not previously been implicated in thymic involution. Postmortem histologic examination demonstrated adrenal remains in some of these mice. This may explain why the effects on the thymus were not completely abolished. Interestingly, adrenalectomy enhanced spleen stimulation by T150R1 demonstrating that this effect is not mediated by corticosteroids and may even be antagonized by them.

Hypophysectomy diminished, but did not abolish, the effects of T150R1 on the thymus or the spleen, and, in all cases, surgical ablation of the pituitary gland was complete. Although these results are preliminary, the residual effects could be due to stimulation of ACTH

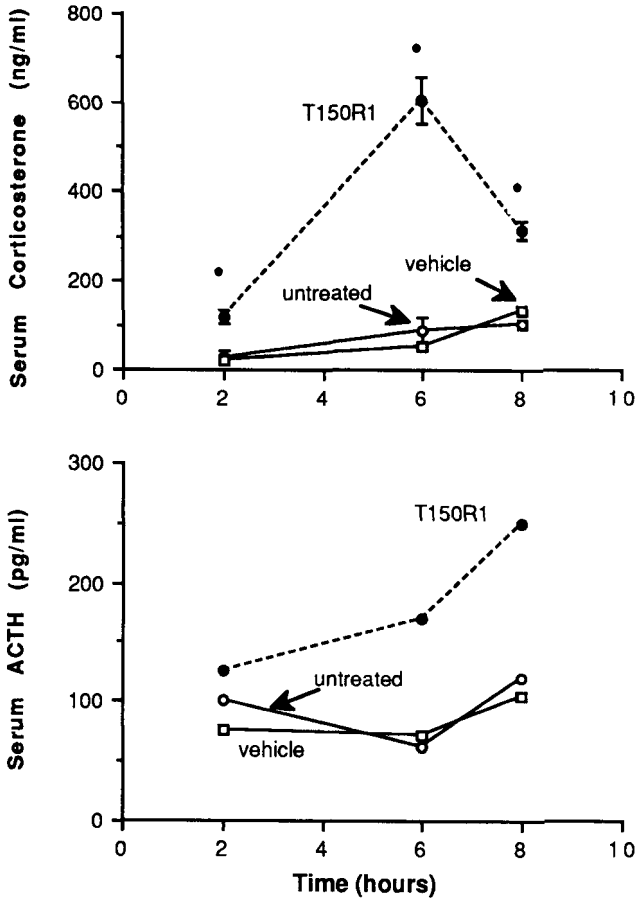

Figure 7. Kinetics of corticosterone and ACTH elevation with T150R1 treatment. Six- to 8-week-old male BALB/c mice were injected in both footpads with 2.5 mg of T150R1 in an oil in water emulsion, vehicle emulsion alone, or left untreated. At 2, 6, and 8 hr, retro-orbital blood was collected. Serum corticosterone and ACTH levels were determined by radioimmunoassays as described in Materials and Methods. Each corticosterone data point represents the mean values of three different sera, and asterisks indicate values which are significantly different from either control ($P < 0.05$). ACTH levels were determined on pooled sera from each group and P values could not be calculated.

release from extrapituitary sources (16, 17), although a direct effect on the adrenals cannot be ruled out.

More recently, we found that T150R1 can directly induce ACTH from the mouse anterior pituitary cell line AtT-20, propably via an ionophore mechanism. Childs *et al.* (18) demonstrated that sodium and calcium channels are involved in both basal and corticotropin-releasing factor-stimulated ACTH release from anterior pituitary cells. Further *in vitro* experiments, testing for synergy with other compounds and the effects of various inhibitors, are in progress to elucidate its mechanisms of action.

Although T150R1 might act at the hypothalamic level by stimulating the release of corticotropin-releasing factor and/or vasopressin, the signal pair that control ACTH secretion from the pituitary cells (19), our data suggest that it is more likely to work at a level below that of the hypothalamus. Furthermore, its effects on other hormones and mediators have not been evaluated.

T150R1 may also stimulate extrapituitary ACTH from macrophages in a manner similar to that proposed for the corticotropes (anterior pituitary cells). Macrophages have been found to secrete proopiomelanocortin-derived peptides including endorphins and ACTH when stimulated with corticotropin-releasing factor (20). There is evidence that T150R1 can induce macrophage activation (1), and an ionophore-mediated mechanism for this effect is also suspected.

While T150R1 clearly influences corticosteroid and ACTH levels, changes in these hormones could not explain its profound hyperplastic effects on the spleen. Corticosteroids generally cause a decrease in spleen weight (10), and although they can stimulate granulopoiesis in laboratory animals, they do not cause extramedullary granulopoiesis in the liver nor do they affect red cell and platelet precursors. Even if the inflammation at the site of T150R1 injections could account for the increase in granulopoiesis and megakaryocyte production, it could not explain the overwhelming erythropoiesis that was observed.

Just as the ionophore activity of T150R1 may lead to its induction of ACTH release, it may directly stimulate proliferation and differentiation of the progenitor cells or induce one or more factors which regulate hematopoiesis. It has been demonstrated that sodium transport pathways are implicated in the granulopoiesis induced by lithium (21) which acts on the granulocyte-macrophage colony-forming stem cells.

Alternatively, the stimulatory effects of T150R1 on hematopoiesis may occur via macrophage mediation and activation. Activated macrophages produce interleukin 1 as well as tumor necrosis factor, both of which stimulate endothelial cells, fibroblasts, and T cells to secrete most hematopoietic factors including the T cell factor interleukin 3 (22). Interleukin 3 supports the proliferation and differentiation of multipotential hemopoietic stem cells and lineage-committed hemopoietic cells that include progenitors of the granulocytic, monocytic, erythroid, megakaryocytic, and mast cell lineages (23, 24). Recently, Prpic *et al.* (25) demonstrated that the rapid exchange of sodium and hydrogen by means of the Na^+/H^+ antiporter mediated some of the genomic responses induced by physiologic doses of γ-interferon in murine macrophages.

T150R1 represents a new class of ionophores which act on excitable cells within the endocrine, immune, and hematopoietic systems. Since it is one of a series of structurally similar copolymers with various degrees of ionophore activity, it provides a useful tool for exploring the role of cation flux in these complex functions.

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