Actions of Immunosuppressor Drugs on the Development of an Experimental Ovarian Tumor[1]

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Immunosuppression has been related to the incidence of tumor apparition, including endocrine tumors. The intrasplenic ovarian tumor (luteoma) is a typical benign endocrine tumor that develops under high gonadotropin stimulation and, from the immunological perspective, is located in a critical organ involved in immune response. To establish if immunosuppression could alter the development of this experimental tumor, the effects of cyclosporin A (CsA) and dexamethasone (Dex) were evaluated. After surgery, tumor-bearing and sham animals were kept without treatment for 4 weeks; thereafter, they were distributed into CsA (25 mg/kg), Dex (0.1 mg/kg), or vehicle (75:25 castor oil:ethanol) groups and were injected on alternate days for 50 days. Body weight was evaluated weekly. Animals were sacrificed after a jugular vein blood sample was obtained. Thymi were weighed. Tumors were measured and placed in formaline for histological studies. Serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL), and estradiol were measured by radioimmunoassay. Hematological parameters were determined. CsA induced a significant decrease in survival rates both in tumor-bearing and sham animals ($P < 0.01$). Dex significantly impaired weight increase in both groups of animals. CsA induced a significant weight loss in sham animals, not observed in tumor-bearing animals. Dex induced thymus weight loss in both groups, whereas CsA induced thymus weight loss only in sham animals. Only Dex induced a decrease in lymphocyte number in both groups. CsA induced an increase in monocyte number only in sham animals. Treatments did not alter LH, FSH, or estradiol, whereas PRL was increased by CsA only in sham rats. Neither Dex nor CsA induced any significant variations in tumor volume, nor did they alter tumor histology. In addition, no visible metastases or alterations in other organs were observed. We conclude that, though immunological parameters were altered by the treatments, immunosuppressor drugs did not condition tumor development. In addition, tumors secrete one or more factor/s that counteract CsA effect.

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Immunodeficiency, whether primary, or secondary to immunosuppressive drugs, radiation, infection, or other causes, may increase the incidence of tumor apparition (1). There are evidences in the human that the incidence of tumors is significantly increased in immunosuppressed individuals (2). Nevertheless, although a slight increase in the frequency of most tumors is observed, in some kind of tumors, a disproportionate increase is determined. The relative risk of suffering some rare kinds of tumors, such as Kaposi's sarcoma or brain lymphoma, may be enhanced 1000-fold in immunosuppressed individuals; for endocrine tumors, this relative risk is enhanced 320-fold (1).

The experimental intrasplenic ovarian tumor, selected for the following experiments, was developed initially by Biskind and Biskind (3), with later modifications, including those of our laboratory (4). It is a typical benign endocrine tumor that secretes various hormones including estradiol, progesterone (5, 6), and inhibin (7), as well as growth factors such as insulin-like growth factor (IGF)-1 (8). The tumor develops when an ovary is grafted into the spleen of an adult bilaterally castrated female rat. In this new anatomic location, the steroids produced by the ovary reach the hepatic portal vein and are metabolized in the liver. In this way, the negative feedback normally exerted by the steroids on the hypothalamic-pituitary unit is abolished, and hypergonadotropinemia is established. The grafted ovary turns into a highly luteinized tumor (luteoma) under the constant stimulation of gonadotropins, which induce growth and hormonal activity (5, 9, 10). The dependency of this experimental luteoma on gonadotropin hyperstimulation for its growth is similar to the one observed in different ovarian pathologies as in certain ovarian carcinomas (11–13), granulosa-cell tumors after ovarian stimulation in treatments of infertility (14), or in the polycystic ovary syndrome (15, 16) among others.

The question concerning tumor localization from the immunological perspective is a very interesting point. The site of the ovarian graft was selected to ensure steroid me-

tabolism by the liver and, as a consequence, tumor development under hypergonadotropinemia, as described above.

From the immunological point of view, this is a critical site of antigen presentation, taking into account the hypothesis proposed by Zinkernagel *et al.* (17), which postulates that antigen localization, dose, and time kinetics form a three-dimensional integral that determines the immunological response. Thus, the localization of the antigen (luteoma) in a secondary lymphoid organ (spleen) is a decisive component in this paradigm.

In view of this, and due to the lack of information regarding the compromise of the immune system in the development of luteoma, the study of tumor progress under immune suppression was an attractive approach.

To establish if immunosuppression could alter the development of this experimental tumor, the effects of cyclosporin A (CsA) and dexamethasone (Dex) were evaluated in tumor-bearing animals. These drugs exert their actions on the immune system by different mechanisms. CsA is a microbial product with potent immunosuppressing properties that result from a selective inhibition of T lymphocyte activation. CsA interacts with the intracellular signaling triggered by the activation of the T cell receptor, forming a complex with cyclophilin and calcineurin, which blocks the translocation of the transcription factor NF-AT to the nucleus, inhibiting the synthesis of many cytokines. This selectively suppresses the adaptive response of T lymphocytes as well as their *de novo* development in the thymus (18). Because of its capacity of interfering with T cell activation, this drug is used as a potent immunotherapeutic agent in autoimmune diseases and also to prevent transplanted organ rejection (19). On the other hand, Dex is a synthetic glucocorticoid 10- to 20-fold more active than cortisol and corticosterone. It is a potent anti-inflammatory and immunosuppressor that works by inhibiting transcription factors as activation protein-1 (AP-1) and NF-κB, which in turn suppress cytokine, receptor, and adhesion molecule induction involved in the activation, migration, and cell recruiting. As a consequence, an inhibition of peripheral T lymphocyte proliferation is produced, accompanied by an inhibition of cell migration to inflammation sites and control of leukocyte recirculation (20–22). Furthermore, it induces $CD4^+CD8^+$ thymocyte apoptosis, inducing thymus atrophy (23, 24).

In sum, our aim was to analyze the effect of immunosuppressor drugs, CsA and Dex, on the development of a hormone-dependent ovarian tumor grafted into the spleen after 7 weeks of treatment in comparison with vehicle-treated animals. For this purpose, various endocrine and immune parameters were evaluated as well as survival rates, body weight, and tumor volume and histology at the end of the experimental procedures.

Methods

Materials. CsA (Sandimmun Neoral, 50 mg/ml, a gift from Sandoz, Buenos Aires, Argentina) was diluted in castor oil:ethanol (75:25); Dex (4 mg/ml, Sidus, Buenos Aires, Argentina) was diluted in sterile phosphosaline solution.

Animals. Adult female virgin Sprague-Dawley rats (200–250 g) from the Instituto de Biología y Medicina Experimental colony were housed in groups in an air-conditioned room, with lights on from 0700 to 1900 hr. They were given free access to laboratory chow and tap water. To obtain tumor-bearing animals, surgical procedures were performed as previously described (4–6). Briefly, animals were anesthetized with ketamine (100 mg/kg body wt ip), both ovaries were removed, and one gonad was cleared from the adherent fat and oviduct and was inserted into the spleen. Sham animals had a piece of abdominal muscle inserted into the spleen after being bilaterally ovariectomized.

Treatments. Tumors were left to develop for 1 month. Thereafter, tumor-bearing animals were randomly distributed into the different groups that were treated subcutaneously with CsA (25 mg/kg, following the protocols of Murphy *et al.* [25] and Hojo *et al.* [26]), Dex (0.1 mg/kg), or vehicle (75:25 castor oil:ethanol) in the morning on alternate days for 50 days. Body weight was determined once a week to adjust the dose. Each group comprised seven to 12 animals.

Sampling. After finishing the treatments, a jugular vein blood sample was taken with heparin under ether anesthesia. Thereafter, animals were sacrificed by cervical dislocation according to protocols for animal use approved by the Institutional Animal Care and Use Committee, which follows National Institutes of Health guidelines. Autopsies of animals were performed, searching for tumor-induced disorders or metastatic propagation. A macroscopic observation of several organs and tissues, including lungs, heart, liver, kidneys, adrenal glands, intestines, uterus, spleen, adipose tissue, and peritoneum was performed to establish the presence of any anomalies.

Thymi were dissected and weighed. Tumors were measured in two dimensions with a caliper, and tumor volumes were calculated based on an ellipsoid tumor shape ($V: 4/3\pi . r_1^2 . r_2$, where r_1 is the minor radius (10, 27). Some tumors of each group were placed in formaline for histological studies.

Hematological Parameters. To determine the immunological status of the animals, a leukocyte differential count was performed, and the number of lymphocytes, segmented neutrophils, and monocytes in heparin-treated blood was quantified with a cell counter. In addition, red blood cell number and hemoglobin content were also determined.

Hormonal Determinations. Serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin (PRL) were determined by radioimmunoassay (RIA) using kits provided by the NIDDK. Results were expressed in terms of RP_3 rat LH, FSH, and PRL standards. Assay sensitivities were 0.015 ng/ml for LH and 0.1175 ng/ml for FSH. Intra- and interassay coefficients of variation for LH were 7.2% and 11.4%, respectively, 8.0% and 13.2%, re-

spectively, for FSH, and 8.1% and 11.4%, respectively, for PRL.

Serum estradiol was also quantified by RIA using a specific antiserum kindly provided by Dr. G. D. Niswender. Labeled estradiol was purchased from New England Nuclear (Boston, MA). Assay sensitivity was 1.7 pg and intra- and interassay coefficients of variation were 9.3% and 11.4%, respectively.

Histology. Tumors from CsA-, Dex-, or vehicle-treated animals were kept in 10% formaline, embedded in paraffin wax, and sectioned at 4 μm using a microtome, as in previous studies (7). For light microscopic examination, sections were stained with hematoxylin and eosin using standard procedures. Three or four different tumors in each experimental group were subjected to blind evaluation by an independent pathologist. Several slides were examined from each tumor.

Statistical Analysis. Results are shown as the mean ± SEM. Differences in means among groups were analyzed with Statistica v 5.0 software by multiple variance analysis (two-way ANOVA), followed by Newman-Keuls test for interaction factors, when the interaction was significant ($P < 0.05$), or for the main effects, when the interaction was nonsignificant (28). Differences between percentages were evaluated by test of comparison of two percentages (29). $P < 0.05$ was considered significant.

Results

Survival Rate. Total survival rate was 91% (48/53), with deaths exclusively occurring in CsA-treated animals. Seventy-five percent of CsA-treated animals survived in either the tumor group (9/12) or the sham group (6/8), significantly less than in the Dex- or vehicle-treated groups ($P < 0.01$; Table I). Deaths occurred 6–7 weeks after treatments were started.

Body Weight. Body weight in vehicle-treated animals increased significantly from the 1st week in sham animals ($P < 0.05$) and the 3rd week in tumor-bearing animals ($P < 0.05$; Fig. 1).

Both tumor-bearing and sham animals responded in a similar way to Dex administration, showing a significant inhibition of weight increase from the 2nd week of treatment when compared with vehicle-treated controls ($P < 0.05$). In sham Dex-treated animals, a significant loss of weight was observed in the 6th and 7th weeks of treatment ($P < 0.05$ and $P < 0.005$, respectively), whereas no significant variations in body weight were observed in tumor-bearing Dex-treated rats.

In CsA-treated tumor-bearing animals, body weight increased from the 3rd week of treatment and continued to increase until the end of the experiment, similar to vehicle-treated animals ($P < 0.05$). In CsA-treated sham animals, body weight increased already in the 2nd week ($P < 0.001$), continued to increase up to the 5th week, and then abruptly fell ($P < 0.001$), reaching initial levels.

Immunological Parameters. With the purpose of assessing the immunosuppressor effect of the treatments (CsA and Dex), some immunological indicators were determined. Dex induced a significant decrease in thymus weight in both tumor-bearing ($P < 0.005$) and sham ($P < 0.001$) animals when compared with vehicle-treated animals (Fig. 2). CsA induced thymus weight loss only in sham animals ($P < 0.005$), and no effect was observed in tumor-bearing rats. The same results were observed when thymus

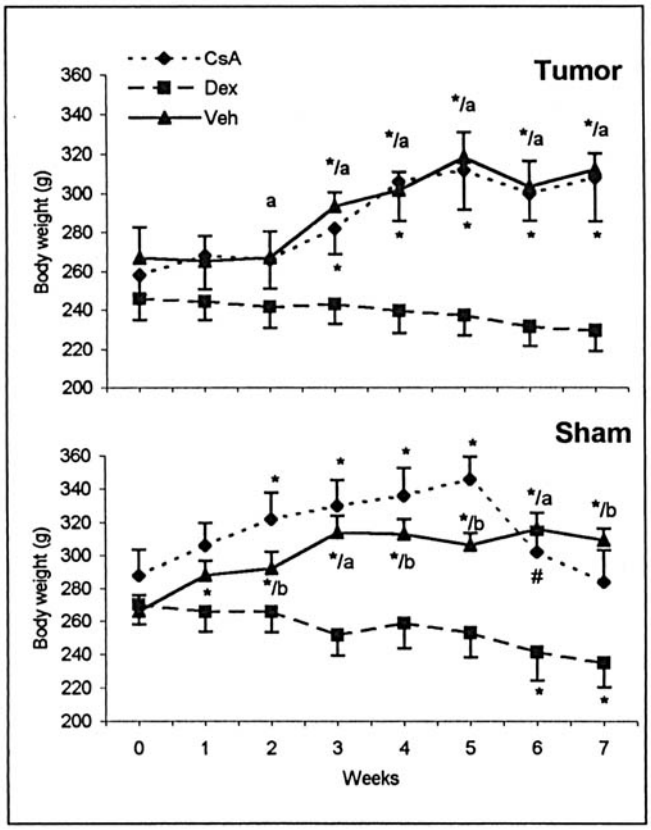

Figure 1. Weekly body weight variations in tumor-bearing animals (top panel) and sham animals (bottom panel) treated with CsA (25 mg/kg/every other day), Dex (0.1 mg/kg/every other day), or vehicle for 50 days after 1 month of initial tumor development (Week 0 indicates initiation of treatments). ANOVA: significant interaction, $P < 0.01$. Significance within each treatment: * vs initial value $P < 0.05$; # vs 5th week, $P < 0.001$. Significance between treatments at each time interval: a, vs Dex $P < 0.05$; b, vs CsA and Dex, $P < 0.05$.

Table I. Survival Rates in Tumor-Bearing and Sham Animals Treated with Immunosuppressor Drugs

Survival rate (%)	CsA	Dex	Veh
Tumor	75 (9/12)	100 (12/12)	100 (7/7)
Sham	75 (6/8)	100 (7/7)	100 (7/7)
Total	75[a] (15/20)	100 (19/19)	100 (14/14)

Note. Survival rates were determined after 7 weeks of treatment. Vehicle, Veh.
[a] CsA vs Veh or Dex: $P < 0.01$.

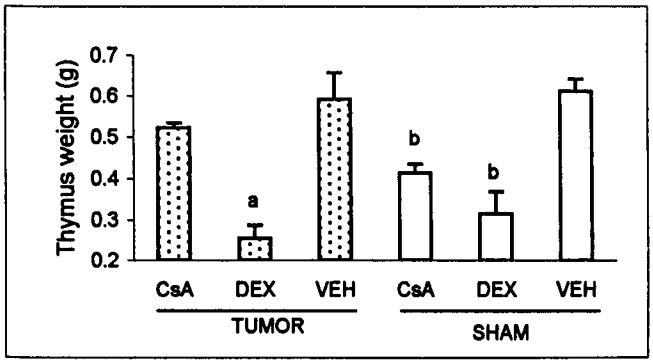

Figure 2. Thymus weight (in grams) in tumor-bearing and sham rats treated with CsA, Dex, or vehicle as above. ANOVA: significant interaction, $P < 0.05$. Significantly different from vehicle: a, $P < 0.001$; b, $P < 0.005$.

weight was expressed relative to body weight (data not shown).

When analyzing hematological parameters, hemoglobin concentration in CsA-treated animals was significantly lower than in either vehicle- ($P < 0.01$) or Dex- ($P < 0.01$) treated rats (Fig. 3A). Neither CsA nor Dex altered red blood cell number (not shown). Segmented neutrophil number/mm^3 did not vary with treatment, but was higher in sham animals than in tumor-bearing animals ($P < 0.05$; Fig. 3B). Lymphocyte number/mm^3 was significantly decreased in Dex-treated rats with regard to vehicle ($P < 0.005$) and to CsA ($P < 0.001$) injected animals (Fig. 3C). CsA had no effect on lymphocyte number. CsA, on the other hand, induced an increase monocytes/mm^3 only in sham animals ($P < 0.005$), whereas Dex had no effect (Fig. 3D).

Hormonal Levels: Gonadotropins, PRL, and Estradiol. As expected (5), both LH and FSH levels were significantly higher in sham animals than in tumor-bearing rats ($P < 0.005$ and $P < 0.001$, respectively; Fig. 4, A and B), and no differences due to drug treatments were apparent. With regard to PRL, CsA induced a significant increase only in sham animals ($P < 0.05$; Fig. 4C); the different treatments did not alter PRL secretion in tumor-bearing rats. No variations in estradiol levels under any treatment or in any group were observed (Fig. 4D).

Tumor Volume. Neither Dex nor CsA induced any significant variations in tumor volume with regard to vehicle treatment animals (Fig. 5). In addition, no visible metastases or alterations in other organs were observed.

Tumor Histology. The histological analysis showed that 86% of the tumors presented highly luteinized tissue, whereas 11% showed granulosa cell predominance (Table II). No signs of corpora lutea involution were observed, in agreement with lack of TUNEL-positive cell detection in these tumors (30). Immunosuppressor treatments did not alter tumor histology, and no signs of malign transformation were observed. A collagen interphase was observed in three of 10 tumors with a focalized pattern.

Discussion

Here, we evaluated the effect of two immunosuppressor drugs on the development of an experimental ovarian tumor, as it has been postulated that immunosuppression may increase the incidence of tumor apparition and the intrasplenic localization of the tumor is critical for antigen exposure to the immune system.

With regard to survival rates, only CsA induced a significant decrease in this parameter both in sham and tumor-bearing animals, suggesting a noxious effect of this drug at the present protocol of administration.

In sham vehicle-treated rats, the expected postcastration weight increase was observed. Tumor presence delayed this weight increase in both vehicle- or CsA-injected animals. This may imply that tumors produce some factor(s) that is inhibitory on weight increase or that the postcastra-

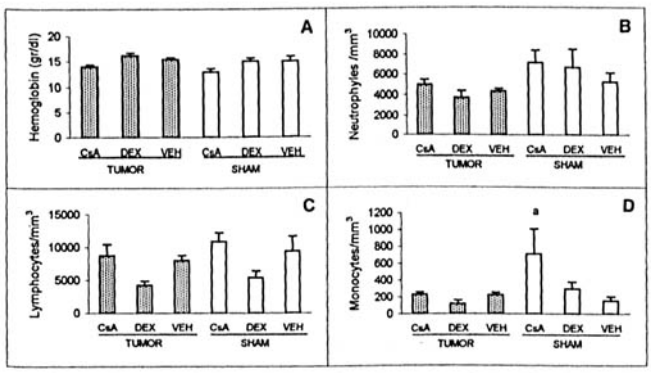

Figure 3. Serum hemoglobin concentration (grams per decaliter; A), segmented neutrophyle (B), lymphocyte (C), and monocyte (D) number per blood mm^3 of tumor-bearing and sham rats treated with CsA, Dex, or vehicle as above. Serum hemoglobin: ANOVA, nonsignificant interaction. CsA treatment significantly different from vehicle, $P < 0.05$. Segmented neutrophyle number/mm^3: ANOVA, nonsignificant interaction. Tumor-bearing animals significantly different from sham animals, $P < 0.05$. Lymphocyte number/mm^3: ANOVA, nonsignificant interaction. Dex treatment significantly different from vehicle, $P < 0.005$. Monocyte number/mm^3: ANOVA, significant interaction $P < 0.05$. a: significantly different from sham vehicle, $P < 0.01$.

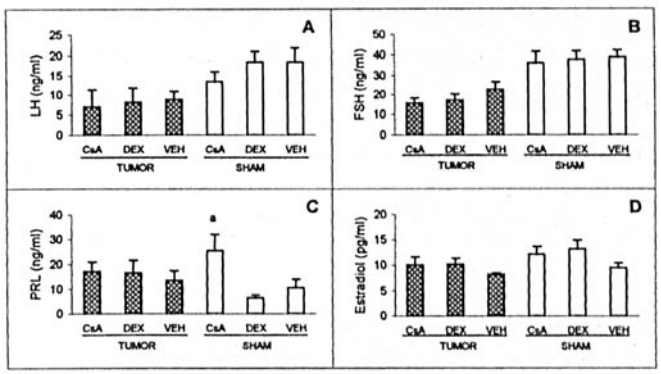

Figure 4. Serum LH (nanograms per milliliter; A), FSH (nanograms per milliliter; B), PRL (nanograms per milliliter; C), and estradiol (picograms per milliliter; D) levels in tumor-bearing and sham animals treated with CsA, Dex, or vehicle as above. a: significantly different from vehicle, $P < 0.05$. LH and FSH: ANOVA, nonsignificant interaction. Tumor-bearing animals significantly different Sham rats, $P < 0.005$. Estradiol: ANOVA, nonsignificant. PRL: ANOVA, significant interaction, $P < 0.05$. a: significantly different from sham vehicle, $P < 0.01$.

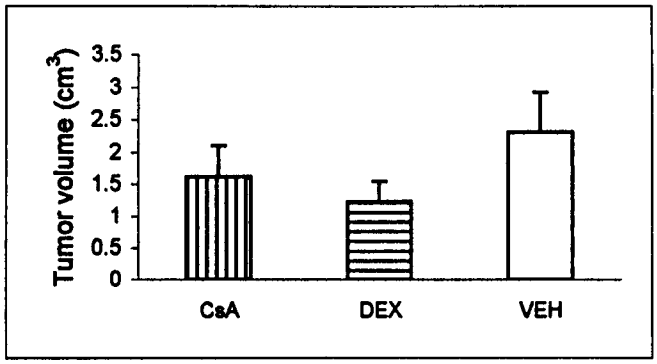

Figure 5. Tumor volume (cm^3) in tumor-bearing rats treated with CsA, Dex, or vehicle as described above. ANOVA, nonsignificant.

tion state is not established in tumor-bearing animals to the same degree as in sham animals (in agreement with lower gonadotropin levels, see below). The CsA-treated tumor-bearing animals that survived until the end of the experiment showed a mean body weight similar to vehicle-treated animals, whereas in sham animals, CsA induced an important loss of weight after 6 weeks of treatment, suggesting a protective effect in tumor-bearing animals with regard to CsA action. On the other hand, Dex induced considerable body weight loss starting in the 2nd week of treatment in both sham and tumor-bearing animals, in agreement with the catabolic effect extensively described for this drug (31).

The effects of CsA on survival rates and body weight could be due to side effects of the drug. In this context, Whiting and colleagues (32) described that rats treated for 7 weeks with CsA presented structural and functional renal anomalies as well as liver damage.

When analyzing thymus weight, Dex induced the expected reduction (approximately 50%) in both sham and tumor-bearing animals (23). On the other hand, CsA induced a 30% decrease in sham animals, in agreement with previous data (33), whereas no effect was observed in tumor-bearing rats. This once again points to the presence of some factor secreted by the tumor that protects the thymus from CsA action. Previous studies from our laboratory have shown that these tumors secrete inhibin (7, 8). Hedger and colleagues (34) have shown that inhibin stimulated [H^3]thymidine incorporation in adult, male rat thymocyte nonstimulated or mitogen-stimulated cultures. Therefore, a proliferative action of inhibin could oppose the antiproliferative effect of CsA, preventing thymus weight loss in tumor-bearing animals.

To establish if immunosuppressor-treated tumor-bearing animals were still able to produce inhibins, mRNA for inhibin subunits was studied by Northern blot. The results indicated that tumors still had the capacity to synthesize inhibins in vehicle-treated and immunosuppressed animals, though β subunits were expressed in low amounts (unpublished results from this laboratory). In contrast to the results from Hedger and colleagues (34), inhibin could not counteract the CsA-induced inhibition of proliferation in ovariectomized female rat thymocyte cultures stimulated with phytohemagglutinin in our experimental conditions (unpublished observations from this laboratory).

Therefore, under our conditions, inhibin does not seem to be responsible for counteracting CsA actions in tumor-bearing animals. Various other proteins not inactivated by the liver, such as IGF-I, which is produced by these tumors (8) and has been shown to induce proliferation of thymocytes (35), may be involved in these effects. Moreover, IGF-I has been shown to accelerate reconstitution of the rat thymus after CsA-induced involution (36).

When analyzing other immunosuppression parameters, a Dex-induced decrease in lymphocyte number was observed, as expected (20), whereas CsA had no effect. The lack of CsA effect on lymphocyte number may be due to insufficient dosage, which is improbable because with the dose of CsA used, animals showed side effects. This response may also be conditioned to the strain of animals used. With regard to monocytes, in sham animals, CsA induced an increase monocyte number, in agreement with data from Whiting *et al.* (32). Similarly to what occurs with thymus and body weight, some factor produced by the tumor also blocked CsA action on monocyte number in tumor-bearing animals. Though red blood cell numbers were not modified by any treatment, a low hemoglobin concentration was observed in CsA-treated animals, also in agreement with previous data (32); this parameter did not seem to be altered by the putative tumor-secreted factor, as it occurred in both sham and tumor-bearing animals.

The hormonal environment in these animals was also determined. In agreement with previous data from our laboratory (5), in tumor-bearing rats, LH and FSH were markedly increased with regard to estrous animals ($LH_{estrous}$: 1.3

Table II. Histological Analysis of Vehicle, Dex, or CsA-Treated Intrasplenic Ovarian Tumors

Animal treatments	Percentage of tumor luteal versus granulosa cells	Cellular alterations (signs of malign transformation)	Presence of collagen deposits in interphase between luteoma and spleen
Vehicle	96% vs 4% (3)	None	1/3 (up to 26μm width, focalized)
CsA	83% vs 17% (3)	None	0/3
Dex	75% vs 25% (4)	None	2/4 (up to 40μm width, focalized)

Note. Three of four different tumors in each experimental group were subjected to blind evaluation by an independent pathologist; number between parenthesis = number of tumors per group. Signs of malign transformation: mitotic index, cytological atypies, necrosis, and hematic or lymphatic vascular invasion.

± 0.2 ng/ml; $FSH_{estrous}$: 6.6 ± 0,4 ng/ml), but were significantly lower than in sham animals. Immunosuppressor drugs did not alter gonadotropin secretion either in tumor-bearing or in sham animals. In contrast, an CsA-induced increase in PRL levels was observed in sham animals, although not in tumor-bearing rats, an observation that endorses our hypothesis of a factor secreted by the tumor that impedes some of CsA actions. A serum PRL increase due to CsA administration had already been shown by Esquifino *et al.* (37). CsA-induced effects on monocytes and PRL secretion could be related events, as it has been shown that human monocytes and T and B lymphocytes express the PRL receptor mRNA and protein (38, 39). In addition, PRL enhances absolute numbers of splenic granulocyte-macrophage colony-forming units in immunosuppressed mice (40).

Histological analysis of tumor samples showed a high degree of luteinization, which was not modified by immunosuppressor drug treatment. It is interesting to note that the percentage of tumors with predominance of luteinized cells (corpora lutea or luteinized follicles) over granulosa cells (well-developed follicular structures) was similar to the one observed in tumors after 1 year of development (7).

Our main aim had been to evaluate if immunosuppressor drugs could alter the development of this ovarian tumor, specially taking into consideration that, because of its intrasplenic localization, there was a possibility that the graft could have been limited in its development by the immune system (17). Neither CsA nor Dex significantly affected tumor volume or histological parameters, and no visible metastases could be observed in the tissues analyzed. Neither drug, in the present experimental conditions, induced tumor exacerbation or malignization. This lack of exacerbation under immunosuppressive therapy could be due to tumors presenting differentiation antigens and thus inducing a very weak immune response; in this case, immunosuppression would not substantially affect their development. In addition, one mechanism of malignant transformation is the reduction of major histocompatibility complex (MHC) class I molecules expression. Van Niekerk et al. (41), studying the marker profile of normal human ovarian tissues and their derived tumors, have shown a loss of MHC class I molecules expression in areas of tumoral cells. As our tumor did not present malignant transformation, the tissue probably expressed normal levels of MHC class I molecules presenting no foreign antigens, and as a consequence, the immune system may not have been induced. Furthermore, in a recent work, Ochsenbein *et al.* (42) described that tumor cells injected directly into the spleen but separated from T cells by barriers including collagen or hemostasis factors did not induce priming of cytotoxic T cells and were thus not rejected. From histological observations, a collagen interphase seems to be formed around some intrasplenic ovarian tumors (three of 10 tumors analyzed, see Table II), which may, in part, explain the lack of differences in tumor development between control and immunosuppressant-treated animals.

It is interesting to note that although Hojo *et al.* (26) have shown that CsA promotes cancer progression by a direct cellular mechanism inducing morphological alterations in tumor cells such as membrane ruffling and acquirement of exploratory pseudopodia, no cell alterations were observed in intrasplenic ovarian tumors under CsA treatment.

We conclude that immunosuppression induced by CsA or Dex, although effective on immunological parameters, did not affect luteoma development. In contrast to tumor-bearing animals, in sham animals, CsA induced several significant effects on body weight, thymus weight, monocyte number, and PRL levels, suggesting the presence of tumor-secreted factor(s) that impede or counteract some of CsA actions. Future experiments will be designed to verify this hypothesis.

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