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Enhancement of chemically induced bladder carcinoma by cyclosporin A

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Abstract. The effects of cyclosporin A (CsA) on the induction of bladder carcinoma were investigated in Wistar rats using *N*-butyl-*N* (4 hydroxybutyl) nitrosamin (BBN) as a known initiator of carcinogenesis. Rats treated with 0.05% BBN + 5 mg/kg per day CsA or 0.05% BBN + 12.5 mg/kg per day CsA developed a dose-dependent (two to fourfold) enhancement of bladder tumor expansion and infiltration as compared to those treated with 0.05% BBN alone. In control rats receiving CsA at doses of 5 mg/kg per day or 12.5 mg/kg per day, no bladder carcinoma occurred. All CsA-treated groups, with or without 0.05% BBN, displayed slight or moderate medullary atrophy of the thymus. The results indicate that immunosuppression with CsA enhances the induction of bladder tumors by BBN. Furthermore, the immunosurveillance theory that effective expression of the immune response may be important in the control of tumor development was confirmed in the carcinogenesis of epithelial cell tumors.

Key words: Immune system - Bladder tumor - Cyclosporin A - *N*-butyl-*N* (4 hydroxybutyl) nitrosamin.

Cyclosporin A (CsA), a powerful immunosuppressive drug, is used for the prevention of allograft rejection in organ transplant patients [21, 29]. It is relatively specific for T lymphocytes and is thought to act by inhibiting the expression of antigen-induced signals from T cells necessary for the subsequent recruitment, proliferation, and maturation of T-cell-dependent immune responses [18, 28]. Besides nephrotoxicity and hepatotoxicity, possible complications of CsA therapy in the lymphoid system are reported. Lymphoproliferative disorder occurs and lymphoreticular neoplasm is increased [29]. It is suggested that they originate with viral infection in conjunction with a compromised immune status. The Epstein-Barr virus (EBV) specifically infects B lymphocytes and induces their proliferation. This initial polyclonal benign proliferation is thought to escape control by the regulatory T cells [11, 17, 30]. Except in the case of the EBV-related lymphomas, there has thus far been little evidence to support a role for CsA in the genesis of cancer.

Treatment with CsA has been shown to enhance the probability of tumor metastasis in a variety of immunogenic animal tumors, but no effect on the growth rate of primary tumors has been noted [7, 27]. It is not known whether CsA has any effect on common human carcinogenesis. The long latent period between exposure to many known carcinogens and the development of human cancers represents another practical obstacle. Furthermore, current understanding of human carcinogenesis emphasizes the probable multifactorial nature of this process. For these reasons, we chose to investigate this topic using a well-established model of bladder carcinoma, in which the cancer is chemically initiated by *N*-butyl-*N* (4 hydroxybutyl) BBN. The characteristics of this malignancy are similar to those of human transitional cell carcinoma. We attempted to determine whether CsA affects the processes of bladder carcinogenesis, tumor growth, and metastatic spread.

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Table 1. Amount of 0.05% BBN intake (in drinking water) per day and over 8 weeks in the different groups. CsA blood levels were determined by radioimmunoassay

Group	1	2	3	4	5
0.05% BBN (µl/day)	17.3 ± 0.3	16.6 ± 0.2	18.7 ± 0.3	0	0
0.05% BBN (g/8 weeks)	5711 ± 549	5621 ± 534	6190 ± 491	0	0
CsA (ng/ml)	0	251 ± 106	1238 ± 505	203 ± 93	1340 ± 580

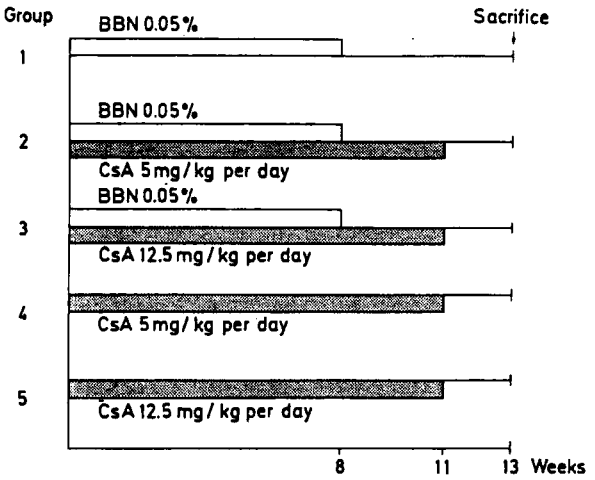

Fig. 1. Schematic representation of the protocol used for the induction of bladder carcinoma in Wistar rats. BBN was added to the drinking water, and CsA was administered orally once daily

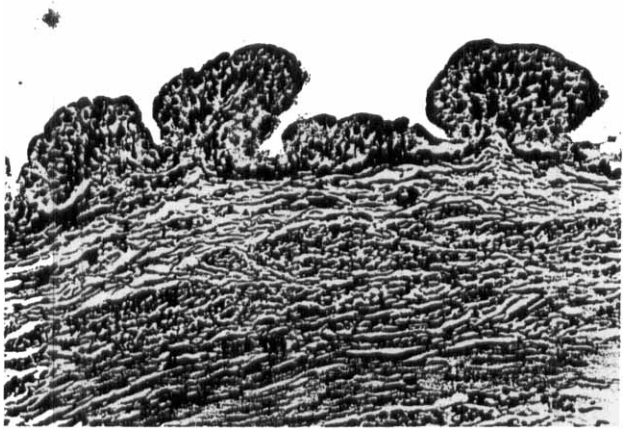

Fig. 2. Exophytic bladder tumors with slight-to-moderate cellular irregularity. Under immunosuppression with CsA, these BBN-induced bladder lesions increased dependent on the dose

Materials and methods

A total of 175 male Wistar rats (Hag farm, Aachen, FRG) with an average body weight of 200 g were randomized into five groups, each consisting of 35 animals. Rats were housed in wire cages with four rats per cage. Twelve-hour light/dark cycles, a constant temperature of 20° ± 2°C, and a humidity of 50% ± 10% were maintained. The carcinogen BBN was administered as a 0.05% solution in drinking water over a period of 8 weeks. Intake of 0.05% BBN over such a period has been known to result in preneoplastic lesions which inevitably lead to cancer [14]. All rats received commercial stock diet (Eggersman, Rinteln, FRG). Cyclosporin A (Sandoz, Basle, Switzerland) was dissolved in olive oil and ethyl alcohol and administered orally to experimental animals at doses ranging from 5 mg/kg per day to 12.5 mg/kg per day over a period of 11 weeks. Details of the different experiments carried out in the five groups are shown in Fig. 1. Group 1 was treated exclusively with the carcinogen BBN over a period of 8 weeks. Group 2 received 0.05% BBN for 8 weeks in addition to 5 mg/kg per day CsA for 11 weeks. Group 3 differed from group 2 in that it received a higher dose of CsA (12.5 mg/kg per day). Control groups 4 and 5 received only CsA in doses of 5 mg/kg per day and 12.5 mg/kg per day over a period of 11 weeks. All animals were weighed every 4 days. CsA blood levels were sampled in half of each group using radioimmunoassay (RIA). Sample checks were made on creatine and ureate levels in the blood specimens and the blood count was analyzed. All animals were sacrificed 2 weeks after the end (13th week) of CsA administration to exclude a direct toxicological influence. Complete autopsies were carried out. For histological examination, the bladder, ureter, kidney, liver, lung, and thymus were fixed in 8% formalin, embedded in paraffin, and stained for microscopic study with hematoxilin and eosin. The bladder was dissected into four rings, each of which was divided into 16 segments. The types of change in the bladder epithelium were determined for each of the 64 segments, and a score of one point was given to the most pronounced type of change in each segment, with reference to infiltrative tumor growth, to exophytic tumor growth, or to no cancer lesions. Percentages for the different types of change were determined and statistical comparisons performed using two-tailed Student's *t*-tests. The error probability of the first order was set to 0.05% for each test.

Results

One animal in group 2 and one in group 5 died during the experimental period due to olive oil aspiration. In groups 1 and 3, there were two deaths of unknown origin with no visible tumors. The intake of 0.05% BBN in groups 1, 2, and 3 was similar throughout the experimental period (Table 1). It was highest in group 3, with an intake of 18.7 ± 0.3 µl daily, followed by group 1 with 17.3 ± 0.3 µl and group 2 with 16.6 ± 0.2 µl. No BBN was given to groups 4 or 5. The total intake of groups 1, 2, and 3 amounted to 5711 ± 549 g, 5621 ± 534 g, and 6190 ± 491 g, respectively. CsA serum levels ranged from low values, common in human transplantation, of 251 ± 106 ng/ml

Table 2. Percentage of bladder tumor expansion. There are statistically significant differences between the various groups: exophytic: $P<0.004$ (I-II, II-III, I-III), infiltrative: $P<0.001$ (I-II, II-III, I-III)

Group	1[a]	2[b]	3[c]	4	5
Exophytic	4.2±0.4%	8.0±0.9%	11.6±1.4%	0	0
Infiltrative	0.7±0.08%	2.5±0.3%	3.3±0.4%	0	0

[a] Treated with BBN only
[b] Treated with BBN+5 mg/kg per day CsA. There was an enhancement of bladder tumors in this group
[c] Treated with BBN+12.5 mg/kg per day (high-dose) CsA. Tumor expansion was greatest in this group

(group 2) and 203±93 ng/ml (group 4) to toxic values of 1238±505 ng/ml (group 3) and 1340 ±580 ng/ml (group 5). Increases in body weight for all rats were equal throughout the experimental period. After CsA administration was stopped in groups 2, 3, 4, and 5 at the end of the 11th week, there was a temporary increase in food intake.

Microscopic findings

Exophytic tumors displayed extensive epithelial proliferations with a tendency to formation of papillomas. Cellular irregularity was slight to moderate, and a few mitotic figures were detected in the proliferative areas of the bladder epithelium (Fig. 2).

Infiltrative tumors crossed the lamina propria and were more irregular in appearance than exophytic bladder tumors. Using the previously described points system, the percentage of the bladder affected by tumors was determined (Table 2). For animals receiving only 0.05% BBN, there were percentages of 4.2±0.4% exophytic and 0.7± 0.08% infiltrative tumors on the bladder. By contrast, rats treated with 0.05% BBN and low-dose CsA (group 2) developed an incidence of exophytic urothelial tumors that was twice as high as that for group 1 (8.0±0.9%) and an incidence of infiltrative transitional cell tumors that was three times as high (2.5±0.3%). Tumor enhancement was greatest in group 3, which received 0.05% BBN+12.5 mg/kg per day CsA. In this group, 11.6±1.4% of the bladder surface was covered with exophytic urothelial tumors, three times more than in group 1. The proportion of infiltrative tumors in group 3 amounted to 3.3±0.4%, a four-and-a-half-fold increase as compared to that in group 1.

All pairwise comparisons between groups 1, 2, and 3 were statistically significant, with $P<0.004$ for the exophytic tumors and $P<0.001$ for the infiltrative urothelial carcinoma. Neither bladder tumors nor hyperplasia were detected in groups 4 or 5. None of the animals displayed any metastasis.

After 13 weeks, the thymuses of all CsA-treated animals were considerably smaller than those in group 1. The thymuses of this group remained unchanged throughout the period of the study. The CsA-treated rats displayed slight to moderate medullary atrophy, irrespective of whether 0.05% BBN had been administered.

Discussion

The results of our experiments demonstrate that CsA exerts a marked enhancing effect on BBN-induced bladder tumors. The enhancing effect on primary epithelial tumors not likely to have been induced by viral infection is described for the first time. No statistically significant effects of CsA on the growth rates of primary tumors have yet been observed, possibly because CsA has always been administered over too short a period of time [7, 15, 27]. Intensification of metastasis has been observed, however. Our results represent increased tumor induction, depending on the degree of immunosuppression with CsA.

Cancer induction is now considered to be a multistep process that can be separated into at least three distinct phases: initiation, promotion, and progression [9, 23]. Initiation appears to be an irreversible process involving interaction of ultimate carcinogens with the target cells. In our study, BBN met the criteria for a classic tumor initiator. BBN is known to produce bladder tumors selectively in great numbers after a short induction period [2, 6, 14]. No direct influence of BBN on the immune system has yet been described. Our own data revealed no changes in blood count or interleucine 2 levels during the 8-week period in which BBN was administered in this study [24].

Promoting agents are generally expected to be noncarcinogenic. The continuous administration of CsA in our control groups (4 and 5) produced no apparent tumorigenic effect. Thus, CsA partially meets the criteria for a promoter.

How may CsA create an environment with an enhancing effect on tumor development? CsA is known to mediate through its systemic effects on immunosuppression, which lead to a decrease in immunosurveillance. The main points of action are a decreased activation of T helper cells through inhibition of interleucine 1 secretion and diminished interleucine 2 production in T helper cells [3, 4, 16, 28]. This is followed by a reduction in cytotoxic T cells and diminished γ interferon secretion in T cells [25, 26]. γ-Interferon is known to inhibit tumor growth through direct inhibition of tumor cells, direct cytolysis of tumor cells, and activation of effector natural killer (NK) cells [10]. NK cells are characterized by their reactivity against a variety of tumors, affecting nonimmunogenic as well as highly antigenic tumors. They have been found to be strongly augmented by interleucine 2, the secretion of which is also diminished by CsA [13, 19]. An NK cell deficiency under CsA may thus be postulated as the reason for the observed tumor induction. The previously described reduced activation of cytotoxic T cells may also be responsible for higher tumor induction under CsA. Experimental studies have shown that chemically induced tumors are slightly more immunogenic than spontaneously developed carcinoma [1]. In rats with transitional cell carcinoma, a specific "disease-related" cytotoxic T cell activity has been demonstrated [20]. Despite development of this specific activity, a quantitative reduction in cytotoxic T cells has also been demonstrated in rats with transitional cell tumors [5]. A more pronounced deficiency of cytotoxic T cells under CsA may, therefore, also be partly responsible for the enhanced tumor induction. Other unknown CsA mechanisms affecting tumor development must also be suspected.

Medullary atrophy of the thymus was found to be a sign of CsA activity in our experiments. This may represent disturbances of T cell maturation, although the functional significance of such morphological alterations is not known. CsA blood levels certainly reached immunosuppressive values.

Clinically, there is a known higher incidence of transitional cell carcinoma in patients following long-term phenacitin intake. Whether this drug acts as an initiator or as a promoter in the multistep nature of carcinogenesis is unknown. In patients with transitional cell carcinoma, decreased effector T lymphocyte activity has been documented [8, 22]. Assuming that effective expression of the immune response may be significant in the control of tumor development [13, 17], regular clinical follow-ups may be of great importance in transplant patients with renal insufficiency due to phenacitin. Recently published observations describe an enhancement of tumor progression after administration of CsA in transplant patients initially presenting a superficial and well-differentiated transitional cell tumor [12].

The findings from these experiments support a role for CsA in the development of chemically induced epithelioid cell tumors in rats. Whether these results can be extrapolated to humans is not known, but the importance of immunosurveillance theory should be stressed. Further analysis of CsA action is necessary to achieve a better understanding of cancer development in allograft recipients.

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