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Increased Growth Rate of a Benzo(*a*)pyrene-induced Transplantable Tumor in Bursectomized Chickens[1]

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SUMMARY

A benzo(*a*)pyrene-induced transplantable tumor was used to study the effects of bursectomy and thymectomy on tumor development in recipients that were isogenic with the donor for the major histocompatibility locus. Surgical bursectomies and thymectomies were done in different groups of chickens on the day of hatching, with no adjunct treatment. The following day, the experimental and control animals were given 10^3 live tumor cells s.c. Tumor frequency and size were followed weekly. With the experimental protocol used, thymectomy did not significantly affect tumor frequency or growth. Bursectomy did not affect tumor frequency, but the transplanted tumors grew significantly more rapidly in bursectomized than in control animals. Thus, bursectomy and, consequently, the antibody-forming system influence not only virus-induced tumors, but also the development of a chemical carcinogen-induced transplantable tumor line. The data underscore the host-protective role of the antibody-forming system in oncogenesis.

INTRODUCTION

The influence of the immunological system on tumor development seems complex. Immunity has been suggested to have not only host-protective functions (1, 40), but also tumor-enhancing functions (6, 10, 33). The biological significance of immunological influences on tumor development remains unclear, and further clarification in this area is necessary. The immunological system of the chicken offers the opportunity to study the separate influences of cell-mediated and of humoral immunity on tumor development because of the fairly clear-cut delineation of immunological responsiveness in this species (2, 47). In the chicken, the thymus is the central lymphoid organ involved primarily in cell-mediated immunity, while the bursa of Fabricius, a hindgut lymphoid organ unique to birds, is essential for normal antibody-producing capacity but does not significantly influence cell-mediated immunological functions (2, 4). Thus, by removing the thymus or the bursa of Fabricius early in life and combining this treatment with tumor inoculation, it is possible to evaluate the effects of impaired cell-mediated immunity and of impaired humoral immunity, respectively, on tumor development.

Such studies have been performed with RNA and DNA virus-induced tumors, with varying results. Avian reticuloendotheliosis is a malignancy of the reticuloendothelial system caused by the reticuloendotheliosis virus, strain T (39, 45), a C-type virus belonging to the reticuloendotheliosis virus group (20, 30, 43, 44). Our studies have demonstrated that thymectomy significantly increases tumor mortality and causes progression of normally regressing tumors in animals inoculated systemically or locally with reticuloendotheliosis virus (17, 18, 46).

Studies of the effects of thymectomy on development of other tumors in the chicken have also been carried out. Thymectomy increases tumor frequency, growth rate, and mortality in chickens (37) and in Japanese quail (49) infected with Rous sarcoma. Similar data have recently been obtained in our laboratory in chickens inoculated with XC cells (13), a tumor cell line carrying the Rous sarcoma genome (41, 42). Thymectomy does not seem to significantly change the development of Marek's disease, a lymphoproliferative disease caused by a DNA virus (3), or of avian leukosis, caused by RPL 12 virus (31). Thus, some, but not all, thymectomy data in birds support the concept of a surveillance function of thymus-dependent, cell-mediated immunity in cancer. Similar data are available for many tumors in mammals (1, 40).

Bursectomy combined with reticuloendotheliosis virus inoculation has consistently resulted in increased tumor mortality (17, 18, 46) and progression of normally regressing reticuloendotheliosis tumors (17, 18). These results have been obtained both after surgical bursectomy in the newly hatched period and after chemical (cyclophosphamide) bursectomy, which more profoundly affects antibody-forming and immunoglobulin-producing capacity (14, 15), without measurably affecting cell-mediated immunological functions (15). In no case have we obtained data compatible with a tumor-enhancing function for the antibody-forming system.

In Marek's disease, the data on effects of bursectomy on tumor development are conflicting and controversial (3, 11, 22, 27, 35). Bursectomy at hatching or during the first weeks of life interferes with the development of avian leukosis, caused by the RPL virus, demonstrating that bursa cells are target cells for this virus, but not elucidating the question of the function of the antibody-producing system in tumor development (31, 32). Reports obtained with Rous sarcoma also vary, maybe reflecting biological variation in tumors

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caused by different strains and variation in experimental protocols. Earlier studies in chickens (21, 36) and in Japanese quail (49) have not demonstrated a significant host-protective or tumor-enhancing effect of bursectomy on tumor development. However, it has recently been demonstrated that when a Rous sarcoma genome-bearing cell line (XC) is used for inoculation in newly hatched, bursectomized, and control chickens, tumor growth rate is significantly faster in bursectomized than in control chickens (13).

Thus there are data from 2 different tumor systems, both reticuloendotheliosis tumors and XC cells, a tumor cell line derived from Rous sarcoma, indicating that the bursa-dependent, antibody-forming system plays a role in host protection in cancer.

This paper presents data on immunological control of tumor development in a 3rd tumor system, namely a benzo(*a*)pyrene-induced transplantable tumor line. The data demonstrate that also, in this system tumor, growth is more rapid in bursectomized than in control animals. Similar findings from 3 tumors of widely different genesis give firm support to the earlier raised postulate (15, 18, 46) that the antibody-forming system has a host-protective function in oncogenesis.

MATERIALS AND METHODS

Animals. WC line chickens (Hy-Line International, Des Moines, Iowa) were obtained as fertile eggs, incubated, and hatched in a Jamesway Model 252B egg incubator-hatcher under standard conditions. After hatching, the chicks were transferred to a brooder, controlled by thermostat, and were kept under standard conditions with free access to feed and water. At 3 to 4 weeks of age, they were transferred to cages. The thymectomy group consisted of 17 animals, and 26 birds were their controls; 18 were bursectomized and 20 served as their controls. The experiments were ended when the largest tumors started breaking through the skin.

Operations. Thymectomies, bursectomies, and sham operations were done on the day of hatching. Thymectomies were done from a dorsal midline incision, using the method described by Peterson *et al.* (31). All distinguishable thymic tissue was removed. However, it is realized that complete removal of thymic tissue is virtually impossible in the chicken (26). In the sham operations, the neck incision was made and the thymic area exposed on both sides, but no tissue was removed. The wound was closed with wound clips. The bursectomies were also done by means of standard surgical techniques (31) from an incision dorsal to the cloacal vent. The bursa, including the bursal stalk area, were dissected free and removed and the wound closed with a clip. The operations were done in Combuthal (Abbott Laboratories, Chicago, Ill.) anesthesia.

Tumor. The tumor line used in this study was obtained from a WC chicken, thymectomized in the newly hatched period. At 5 weeks of age, the animal received an i.m. injection of 1.0 ml of 1% (w/v) benzo(*a*)pyrene (Fisher Scientific Co., Philadelphia, Pa.) in trioctanoin (Fisher). Five months later, a tumor with the histological characteristics of a fibrosarcoma was harvested. The tumor was cut into small fragments with scissors, and a single-cell preparation was made after the tumor was rubbed against a 60 mesh stainless steel wire screen in Hanks' balanced salt solution. Transfer of 4×10^3 tumor cells resulted in 100% tumor takes in less-than-1-week-old chickens when the donors and recipients were isogenic for the major *(B)* histocompatibility locus. Tumor take frequency was variable when outbred recipients were used. With 4×10^6 cells/recipients, tumors occurred in about 40% of outbred chickens. Tumor homogenates, preparations containing heat-killed (56°, 30 min) cells, and supernatants of tumor cell preparations were unable to induce tumor formation. One thousand live (0.1% trypan blue-excluding) cells, derived from the transplantable tumor, were given to each recipient s.c. in these experiments on the day following surgery. Thymectomized animals and their controls were given cells from one transplanted tumor preparation, and the bursectomized animals and their controls from another, but both were from the same transplant generation. All cells were given s.c. The cells used for transfer were in the 4th transplant generation in both experiments.

Assessment of Tumor Growth. Tumor growth was assessed by weekly measurements with a caliper. Tumor volume was calculated from these values, assuming spheroid tumor shape. Tumor frequencies and sizes were recorded. Differences in tumor frequencies between experimental and control animals were evaluated with Fisher's exact test for 4-fold tables, and differences in tumor sizes between experimental and control animals were determined with Student's *t* test and with multivariate profile analysis for 2 independent groups (23).

RESULTS

Tumors could be observed somewhat earlier in the bursectomized chickens than in the control animals. However, on no occasion was tumor frequency significantly higher in bursectomized than in control animals (Table 1). Approximately one-half of the animals developed tumors in both the bursectomized and the control groups. Most tumor-bearing animals in both groups developed progressively growing tumors. The tumors were significantly larger in bursectomized than in control animals at the end of the experiment, as evaluated by Student's *t* test (Table 1). When the rate of tumor growth was further evaluated between the 2 groups with the multivariate profile analysis, tumor growth rate was significantly ($p < 0.005$) faster in the bursectomized than in the control group.

The thymectomized animals also developed tumors somewhat earlier than their controls, but the frequency was not significantly different between experimental and control animals on any occasion (Table 2). All thymectomized and control animals developed progressively growing tumors in this experiment, indicating that the actual tumor cell dose may have been larger than in the thymectomy study. There was no significant difference in tumor size between experimental and control animals at any observation point in this study (Table 2), as evaluated by Student's *t* test or by multivariate profile analysis.

Table 1

Tumor frequency and volume in bursectomized and control chickens

There were 18 bursectomized and 20 control animals.

Time (wk) after inoculation with tumor cells	Bursectomized animals		Controls	
	Tumor frequency (%)	Tumor volume (cu mm)	Tumor frequency (%)	Tumor volume (cu mm)
3	17	0.2 ± 0.1[a]	0	0 ± 0
4	39	2 ± 1	20	0.8 ± 0.6
5	44	5 ± 2	20	4 ± 4
6	44	34 ± 23	25	9 ± 4
7	44	258 ± 188	40	16 ± 8
8	50	1497 ± 824	40	73 ± 47
9	50	2947 ± 1396	40	322 ± 167
10	50	10044 ± 4097[b]	40	1329 ± 671

[a] Mean ± S.E.

[b] $p < 0.05$ determined by Student's t test.

Table 2

Tumor frequency and volume in thymectomized and control chickens

There were 17 thymectomized and 26 control animals.

Time (wk) after inoculation with tumor cells	Thymectomized animals		Controls	
	Tumor frequency (%)	Tumor volume (cu mm)	Tumor frequency (%)	Tumor volume (cu mm)
2	12	0.1 ± 0.0[a]	8	0.0 ± 0.0
3	24	0.6 ± 0.3	19	0.4 ± 0.2
4	58	9 ± 4	62	8 ± 3
5	100	96 ± 22	88	68 ± 19
6	100	348 ± 67	88	350 ± 115
7	100	2146 ± 355	92	1903 ± 511
8	100	9590 ± 1753	100	7649 ± 1410
9	100	34279 ± 5433	100	23735 ± 4321

[a] Mean ± S.E.

DISCUSSION

Chemical carcinogens have been used much less extensively in studies of tumor formation in chickens than in mammals, especially rodents. However, the ability of chemical carcinogens to form tumors in chickens was demonstrated a long time ago (24, 25, 28). Also, the capacity of benzo(*a*)pyrene to induce formation of sarcomas and fibrosarcomas in fowl was demonstrated a long time ago by Rothbard and Herman (38). Extremely few of the tumors studied by them were transplantable, possibly due to histocompatibility differences between tumor donors and recipients. The benzo(*a*)pyrene-induced tumor used in this study was about 100% transplantable when 4×10^3 cells or more were used in transfers to histocompatible recipients, but much less frequently in transfers to outbred recipients. Cell-free preparations of the tumor were unable to induce tumor formation, indicating that the tumor is indeed a transplantable tumor, and not one caused by virus present in the original tumor.

In this study, the immunological manipulations were limited to surgical bursectomy or thymectomy in the newly hatched period. These treatments alone cannot be expected to result in a profound deficiency of antibody-forming capacity or of cell-mediated immunity. In our experience and that of other laboratories, surgical bursectomy in the newly hatched period does not significantly alter IgM or IgG levels in most such treated birds. However, the birds have a marked decrease in the ability to respond to antigens such as sheep erythrocytes or killed *Brucella abortus* organisms, demonstrating an impairment of antibody-producing capacity. A more profound immunodeficiency could be expected if sublethal irradiation were added to surgery (2). A high frequency of agammaglobulinemic birds (14, 15), with normal cell-mediated immune responses (15), could also be expected if the birds were treated with high doses of cyclophosphamide in the newly hatched period, with or without the addition of bursectomy. However, since these treatments can potentially damage functions other than bursa- and thymus-dependent immunity, this study was limited to the simple ablation treatments.

The thymectomy study failed to reveal differences in tumor frequency or growth rate between thymectomized and control animals with this experimental protocol. This may well be due to the insufficient depression of cell-mediated immunity obtained by newly hatched surgical thymectomy without adjunct irradiation. The tumor cells were given on the day of hatching in this study, *i.e.*, when the immunological system of the recipients had not yet matured, in order to obtain tumor take with the smallest possible cell number. Tumor cell administration, somewhat later, might have resulted in a difference in tumor-take frequency between ex-

perimental and control birds. The data in this study do not lend themselves to comparisons between the influence of thymus- and bursa-dependent immunity on tumor development, and cannot be taken to indicate that thymus-dependent immunity might play a lesser role than bursa-dependent immunity in tumor defense. Since all animals in the thymectomy study developed larger tumors than in the bursectomy study, a possible effect of thymectomy may have been masked by the larger tumor load. In our laboratory, thymectomy alone has resulted in a significantly increased tumor frequency and mortality rate, and in a significant impairment of tumor growth control mechanisms in the case of reticuloendotheliosis virus-induced tumors (17, 18, 46) and XC cell-induced tumors (13). As mentioned earlier, similar data are available for other Rous sarcoma lines in chickens and quail (37, 49) and for many antigenic tumors in mammals (1, 40).

In the present study, surgical bursectomy in the newly hatched period did not significantly change tumor incidence, but this relatively modest damage to the antibody-forming system increased tumor growth rate significantly. In the case of reticuloendotheliosis tumors, surgical bursectomy affects tumor mortality after systemic virus administration, but not as much as cyclophosphamide bursectomy, which results in a more profound deficiency of the antibody-forming system (17, 18, 46). When the reticuloendotheliosis virus is given locally to surgically or cyclophosphamide-bursectomized birds, tumor progressors are much more frequent than regressors in the bursectomized groups than among their controls (17, 18). After XC cell inoculation, tumor growth rate is significantly more rapid in surgically bursectomized than in control birds, but there was no clear difference in tumor frequency or in tumor mortality between bursectomized and control birds (13). Thus, these studies demonstrate a host-protective function for the bursa-dependent, antibody-forming system in 3 widely different cancers, *i.e.*, 1 caused by an RNA virus of the reticuloendotheliosis group, 1 by a Rous sarcoma-derived cell line, and the 3rd being the tumor in this study, a chemical carcinogen-induced transplantable tumor line.

The precise mechanism by which bursectomy influences surveillance against cancer is not clarified by these studies. The possibility that antibodies would be instrumental in this respect, either by the classical complement pathway or by the antibody-dependent cell-mediated cytotoxicity mechanism (19, 29), seems closest at hand. However, it cannot be excluded that there could be an indirect effect on another killer cell population, such as the "natural" killer cells demonstrated in the mouse (7, 12).

These findings support the idea that the biological function of the antibody-forming system in cancer may well be in protection of the host, not of the tumor. This is further underscored by the curative effect of immune serum, even after absorption of antiviral antibodies, in the reticuloendotheliosis virus-induced cancer (9, 16), as well as by similar results in other tumor systems (5, 8, 48). While our findings do not contradict the existence of tumor-enhanced antibodies in the classical sense (10), they do indicate that if such antibodies arise during tumor formation, their effect may be overshadowed by the host-protective influences of the antibody-forming system.

This study and the study on the effects of bursectomy on XC cell-induced tumors show that bursectomy influences tumor growth rate, but not necessarily frequency of tumor takes. Thus, it is possible that the bursa-dependent, antibody-forming system may primarily influence tumor-growth rate without having significant influence on the initial events leading to tumor formation. However, this conclusion cannot be safely drawn, since surgical bursectomy alone in the newly hatched period does not completely abolish antibody-producing capacity, and tumor cell administration occurs early in life, when the control animals have not completely matured. Experiments with cyclophosphamide-bursectomized, more profoundly immunodeficient animals are necessary before a firmer conclusion in this respect can be drawn with confidence. It is of interest in this connection that Proctor *et al.* (34) have found an immunologically specific serum factor that can inhibit formation of metastases in experimental rat sarcoma but that does not noticeably influence the primary tumor.

While the concept of a host-protective influence for the antibody-forming system in oncogenesis has firm support in the data presented in this study and elsewhere, the validity of the idea that this part of immunity should mainly influence tumor growth rate must await further experimentation.

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