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Tumor-blocking and -inhibitory Serum Factors in the Clinical Course of Canine Venereal Tumor[1]

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SUMMARY

An *in vitro* method for growing colonies of canine transplantable venereal tumor cells in a semisolid agar medium is described. Using autologous or pooled homologous normal dog sera as a feeder layer, 49.3 ± 3.5 and 47.5 ± 4.5 tumor colonies were obtained, respectively, when 2×10^4 tumor cells were plated. With this assay system, assessment of colony counts provided an accurate and rapid technique for monitoring serum factors in tumor-bearing dogs that inhibited colony formation, or blocked the inhibition of colony formation. In 52 dogs given tumor transplants, a direct correlation was demonstrated between serum activity tested *in vitro* and the *in vivo* growth characteristics of the tumor. Tumor cells preincubated with serum from dogs with active tumor growth consistently showed normal colony growth when cultured in agar containing colony-inhibitory sera (blocking effect). *In vivo* regression was characterized by loss of serum blocking and the development of serum colony-inhibitory activity in culture. In metastatic disease, only blocking activity could be identified, while persistent local invasive disease was characterized by low levels of both blocking and inhibitory serum activity. The sensitivity of this technique coupled with its *in vivo* predictive capabilities provides a model for monitoring serological responses to a naturally occurring neoplasm in a large, randomly bred animal.

INTRODUCTION

The canine TVT[3] is an undifferentiated round-cell neoplasm which is transmitted in nature by sexual contact (19). Current information suggests that the tumor is cellularly transmitted, and tumor growth regularly occurs following experimental inoculation of random dogs with tumor cells (5, 8). The neoplasm follows a variable clinical course under both natural and experimental conditions (8, 21). Following a period of logarithmic growth, spontaneous regression may occur within 6 months (8). Some animals maintain their tumor for extended periods of time without evidence of systemic metastasis, but with extensive local tissue invasion. A few animals succumb from disseminated metastatic disease (18). In the experimental situation, volumetric determination of tumor size has provided an accurate means to monitor clinical changes of tumor growth (4).

The apparent immunogenic characteristics of this canine tumor and its variable clinical course may provide a model for assessing the immunological regulation of tumor growth in a large randomly bred species (2, 6, 7).

Serum-mediated blocking as a mechanism for continued tumor growth in the presence of tumor rejection antigens has been studied by a variety of techniques in experimental animal systems (13). *In vitro* blocking of cell-mediated destruction of tumor cells has also been demonstrated in autochthonous human neoplasms (12). The relationship of these *in vitro* findings to the natural history of tumor growth remains to be defined.

The present studies were conducted to evaluate in the TVT system the presence of serum factors which, by their inhibitory or blocking characteristics, correlated with the clinical course of the tumor.

MATERIALS AND METHODS

Random mongrel dogs weighing between 6 and 15 kg, 4 months to 1 year of age, were used in this study. Dogs were routinely dewormed, immunized against distemper, hepatitis, and leptospirosis and isolated for 3 weeks before entering the colony. Dogs of both sexes were used. Females were judged to be nulliparous on the basis of being raised in the kennel or by physical examination.

Tumor Induction and Clinical Course

For the past 3 years, 2 tumor lines have been maintained in our laboratory. Both lines were originally acquired from naturally occurring clinical cases (4, 8). Following tumor inoculation at 4 s.c. sites with a suspension of 10^8 tumor cells, dogs were inspected daily until a measurable tumor was present and weekly thereafter. As previously described, tumors regularly developed at all injection sites and followed a similar course (8). Measurement of the tumor masses was carried out with a caliper in 3 perpendicular directions. The tumor volume in cu mm of one of the masses was calculated by the formula:

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[3] The abbreviations used are: TVT, transmissible venereal tumor; CFUa, colony-forming units in agar; NDS, normal dog sera.

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$$V = \frac{\pi}{6}\left(\frac{D_1 + D_2 + D_3 - 9}{3}\right)^3$$

and a tumor growth curve was prepared for the representative mass (4, 8). All animals were routinely examined for the presence of local invasive growth or metastatic spread. Regression was considered complete when no palpable tumor could be detected.

Tumor Colony Formation *in Vitro* (CFUa)

Tumor cells were cultured according to the CFUa technique of Marsh *et al.* (17). Sterile biopsy samples were minced with scissors and processed in Roswell Park Memorial Institute Medium 1640 on a magnetic stirrer. After approximately 10 min, the fluid containing separated tumor cells was transferred to 50-ml plastic Falcon test tubes (Falcon Plastics, Oxnard, Calif.). The cells were washed 3 times in a basic medium consisting of TC-199 supplemented with 1 mM sodium pyruvate, 40 μg L-asparagine per ml, and 75 μg DEAE-dextran per ml. The washed cells were combined in the basic media in 0.3% agar so as to yield a final plating concentration of 1 to 4 $\times$ 10^4 cells/ml. One-ml volumes were plated in 35-mm Petri dishes which had 0.1 ml of pooled NDS added to serve as a feeder layer for cell growth. The pool of NDS was obtained from 6 healthy 6- to 12-month-old mongrel dogs. Plates were prepared in duplicate and incubated at 37° in 5% CO_2. Following 48 hr of incubation, colonies consisting of 20 to 25 tumor cells were enumerated with an inverted phase microscope. Duplicate plates assessed for reproducibility showed an average variation of 2.5 $\pm$ 2.7 colonies in 50 random tests analyzed. Results are reported as average counts for duplicate plates. For morphological studies, colonies were removed from the agar plates with a finely drawn Pasteur pipet, smeared, and stained with Wright's-Giemsa.

Inhibition of Colony Formation

Serum samples were tested for inhibitory activity by substituting the sera under investigation for pooled NDS in the feeder layer. A serum was considered completely inhibitory when there was an absence of colony growth following incubation with the test sera. Partial inhibition was indicated by a reduction of colonies greater than 2 S.D. from normal control values for colonies cultured in autologous or pooled homologous NDS.

Blocking of Colony-inhibitory Activity

Serum blocking activity was assessed by incubating an equal volume of tumor cells and serum being tested at 37° for 30 min. The cells were then washed 3 times in the basic media, adjusted to the concentration needed, and plated in 0.3% agar media containing 0.1 ml of known completely inhibitory serum as the feeder layer. A serum was considered to have complete blocking activity if normal colony growth was obtained or to have partial blocking activity if colonies were present in reduced numbers (−2 S.D. from control).

Serum Samples

Serum samples were obtained from tumor-bearing dogs and stored at −70°. Preliminary studies comparing sera inactivated at 56° for 30 min to noninactivated sera failed to show differences in assays of CFUa or CFUa inhibition. Inactivated sera were routinely used in these investigations.

Experimental Groups

From an ongoing study of the immunogenetics of the canine TVT, 52 dogs, belonging to 3 clinical groups, were evaluated.

Group 1: Regressors. Twenty-eight animals showed clinical signs of tumor regression within 6 months of challenge. Eighteen were followed to complete regression, while 10 were sacrificed during the early stages of regression. Serum samples from 10 of these dogs were serially monitored throughout the course of the neoplastic process.

Group 2: Persistors (Locally Invasive Disease). This group was composed of 20 animals of which serum samples from 5 were serially monitored. The dogs were sacrificed from 5 to 18 months after transplantation with locally invasive tumor masses which often measured in excess of 10 cm in diameter.

Group 3: Metastatic Disease. This group consisted of 4 dogs that developed disseminated metastatic disease. In all of these animals, serial serum samples were monitored.

In animals of Groups 1 and 2 that were not serially monitored, selected serum samples were examined at specific times during the course of tumor growth.

RESULTS

Tumor Colony Formation in Semisolid Agar. Colony formation was evaluated on tumor cells obtained from 15 random dogs between 14 and 28 days posttransplantation during the log phase of tumor growth. Table 1 shows that when 2 $\times$ 10^4 cells were plated, 48 $\pm$ 5 colonies developed within 48 hr when pooled NDS was used as the feeder serum. When autologous sera were substituted for the NDS, 49 $\pm$ 3 colonies were present. Colonies consisted of well-circumscribed clusters of from 20 to 25 cells. Morphological identification of the tumor cells was confirmed by Wright's-Giemsa stain of representative colonies.

Colony formation was determined for 1 to 4 $\times$ 10^4 plated cells when feeder layers consisted of pooled sera from: (*a*) 6 normal dogs; (*b*) 4 tumor-bearing animals; and (*c*) 7 dogs following tumor regression. The results are shown in Chart 1. Colony formation increased linearly with cell dose. A marked difference in the colony counts was observed between NDS and regressor sera. At 2 $\times$ 10^4 plated cells, colony formation was completely inhibited by the regressor sera. Culture in autologous sera appeared to yield a slight, but consistent increase in colony formation.

Colony inhibition was studied in sera from 5 regressor dogs and compared with the cytotoxic activity of the same sera against tumor cells. Complement-dependent cytotoxicity testing was performed by the 2-stage technique of

Table 1

Colonies[a]/2 × 10⁴ plated tumor cells using pooled normal dog sera or autologous serum as the feeder layer

Tumor sample	Normal pooled sera	Autologous serum
1	40	52
2	50	48
3	40	46
4	54	49
5	50	53
6	53	51
7	50	53
8	48	51
9	47	54
10	50	45
11	44	47
12	45	43
13	49	45
14	41	54
15	51	49
Mean ± S.D.	47.5 ± 4.5	49.3 ± 3.45

[a] Average colony counts of duplicate plates incubated for 48 hr.

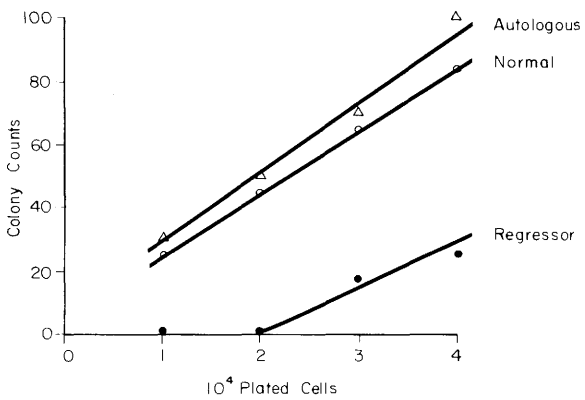

Chart 1. Colony counts at different concentrations of tumor cells. Feeder layers consisted of autologous, pooled normal, or regressor sera.

Terasaki and McClelland (24). Table 2 demonstrates that cytotoxicity was present with titers of 1 to 16 in the 5 sera tested, while colony inhibition occurred at titers between 512 and 2048.

Blocking of Colony Inhibition by Progressor Serum. Fifteen consecutive tumor samples were tested for colony formation following 30 min of incubation with 3 groups of sera: (*a*) individual sera from each of the 15 dogs prior to tumor challenge, (*b*) individual sera from the animals obtained during the period of tumor growth, and (*c*) a pool of 7 sera obtained from regressor animals. Each regressor serum had previously been tested separately and shown to be completely inhibitory to colony growth when used as a feeder layer in the culture system. Cells were plated in media containing either regressor or pooled normal dog sera. The results are shown in Table 3. Preincubation in autochthonous pretumor challenge or progressor serum and culturing with pooled normal dog sera in the media resulted in normal colony formation. Preincubation in pooled regressor sera completely inhibited tumor growth. When the agar media contained regressor serum, colony formation was prevented following preincubation with either normal or regressor sera. Complete blocking of colony inhibition was consistently demonstrated when TVT cells were preincubated in progressor serum. Blocking titers ranged from 128 to 1024.

To establish the time sequence of the blocking activity, daily serum samples were obtained from before and for 11 days following transplantation of the tumor. Blocking activity was first detected between Days 7 and 9.

Clinical Course Associated with Inhibitory and Blocking Factors. A series of experiments were performed to explore the correlation between the clinical course of the tumor and the presence of the serum factors observed *in vitro*. Serial serum samples were tested for the presence of blocking by preincubating the tumor cells in the test serum prior to plating in agar containing regressor serum. To recognize the appearance of colony-inhibitory factors, these same serum samples were used as the feeder serum with no preincubation of the tumor cells. The colony count curves for both blocking and inhibitory activity were then superimposed on the growth curves.

Table 4 depicts the serum blocking and inhibitory activities of 52 dogs measured 5 months posttransplantation. At this time during the course of tumor growth, 28 of the dogs showed either clinical signs of regression or had completely regressed, while 4 eventually developed systemic metastases. The remaining 20 dogs with locally invasive tumor masses showed no evidence of regression when sacrificed at up to 440 days. All sera from regressor animals showed complete colony-inhibitory activity and no evidence of blocking. In contrast, the sera from 4 animals with disseminated neoplasms showed complete blocking activity. Those 20 animals with persistent locally invasive neoplasms showed both

Table 2

Cytotoxic and colony-inhibitory titers of 5 regressor sera tested against tumor cells

Serum sample	Cytotoxicity	Colony inhibition[a]
1680	1:16	1:1024
1058	1:4	1:2048
1885	1:8	1:512
1713	1:8	1:2048
1666	1:1	1:512

[a] Titer at which colony formation was reduced by more than 50%.

Table 3

Effects of preincubation in normal,[a] progressor,[b] and regressor[c] sera on 15 tumor biopsies when cells were cultured in pooled normal or regressor sera

	Culture sera	
Preincubation sera	Normal	Regressor
Normal	47 ± 5.0	0
Progressor	47 ± 4.4	45 ± 2
Regressor	0	0

[a] Individual sera obtained prior to tumor challenge.
[b] Individual sera obtained from the tumor-bearing animal.
[c] Pooled sera from 7 regressor animals.

inhibitory and blocking activity. In these cases, colony formation was reduced by 10 or more when the test sera were used as the feeder layer as compared to pretumor challenge sera or pooled NDS (partial inhibition). Preincubation with the test sera resulted in colony formation in reduced numbers when completely inhibitory sera were used as feeder layer (partial blocking).

Chart 2 depicts the correlation between the assessment of *in vitro* progress of the tumor in a regressor animal. In this dog (No. 1680), serum inhibition was first detected between the 5th and 6th posttransplantation week and very rapidly increased until there was complete colony inhibition prior to regression.

In Chart 3, the correlation curves for Dog 2272 are illustrated. This animal developed large tumor masses which persisted for over 200 days and, at necropsy, was found to have metastases in the internal iliac nodes and the kidneys. Serum inhibitory activity failed to appear throughout the clinical course of the tumor. Blocking activity rose to high levels and remained unchanged from maximal levels until the day of sacrifice. This same pattern was demonstrated in 4 animals in which systemic metastases could be identified.

Table 4

Serum Blocking and Inhibitory activity in 52 dogs tested 5 months posttransplantation

Clinical course	No.	Blocking[a]	Inhibition[b]
Regression	28	0/28	28/28
Persistence			
Metastasis	4	4/4	0/4
Local invasion	20	20/20[c]	20/20[c]

[a] Normal colony counts when cells were cultured in inhibitory serum feeder layers.

[b] No colony formation when test sera were used as the feeder layer.

[c] Low levels of blocking or inhibitory activity as compared to normal or inhibitory sera.

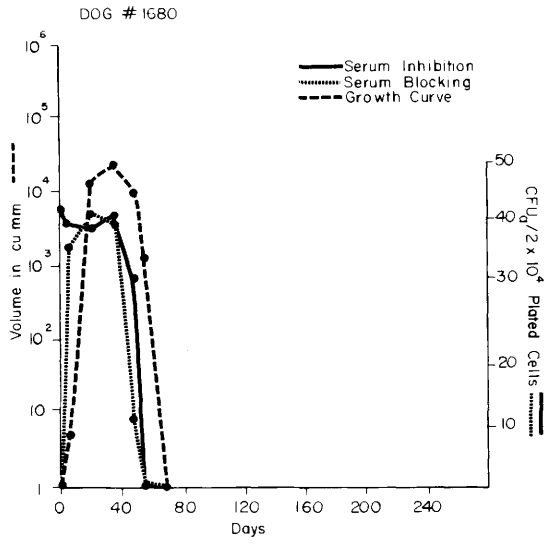

Chart 2. Serum factors during the clinical course of the TVT in Dog 1680. The parallelism between the regressive course of the tumor and the increased inhibitory and decreased blocking activity of the serum is depicted.

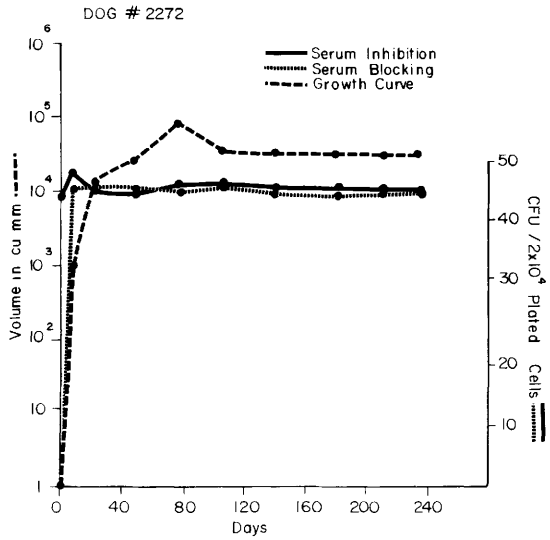

Chart 3. Serum factors during the clinical course of the TVT in Dog 2272. In this animal in which metastases occurred, the levels of blocking antibody remained high, while the absence of colony inhibition is evident.

In contrast to those animals that regressed or developed systemic metastases, 20 persistor dogs with large invasive tumors showed both inhibitory and blocking factors, as illustrated in Chart 4. Maximal levels of blocking activity were reached and then fell very slowly, but never disappeared. Maximal levels of inhibitory activity were never reached but slowly rose following their initial appearance in the serum.

DISCUSSION

The presence of serum factors that could modify the course of the canine TVT has been reported by several investigators (3, 20). In the present study, the role of the serum factors in the natural history of the TVT was clarified by the development of an *in vitro* colony assay system. Serum from dogs could be serially monitored for colony-inhibitory or -blocking effects. The test proved to be highly reproducible, was independent of heat-labile components of complement, and did not require variable cellular feeder layers. It could be rapidly performed (48 hr), thus avoiding long periods of *in vitro* culture. This contrasts with difficulties encountered in previously described colony assay techniques for cell-mediated cytotoxicity where marked variability from test to test and reproducibility in different laboratories have provided a source of controversy (11, 23). In the present studies, no direct comparisons of cell-mediated immunity by colony inhibition were made. Hellstrom *et al.* (9) have described inhibition of sera from regressor animals in the BALB/c mouse Moloney sarcoma system. The TVT appears to represent a model in which colony-inhibiting and -blocking factors can be evaluated in the absence of lymphocytes from the test system.

Using the CFUa assay, a striking effect of serum on the

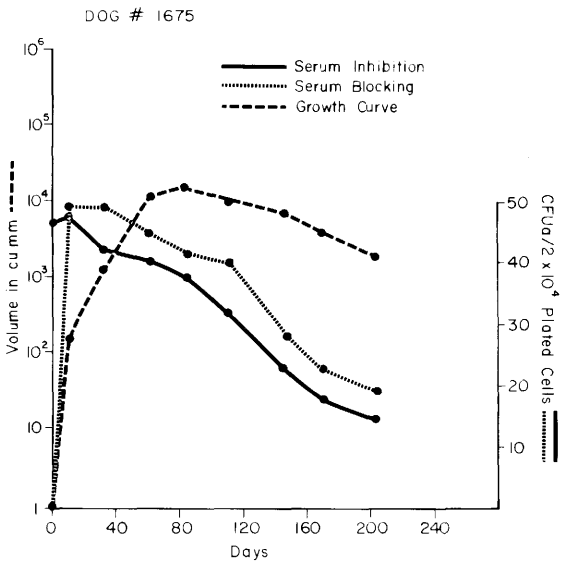

Chart 4. Serum factors during the clinical course of the TVT in Dog 1675. In this animal with local invasive disease, the prolonged low levels of both blocking and inhibitory activity are demonstrated.

growth of tumor cells *in vitro* was demonstrated. Normal dog serum incorporated in the media or used for preincubation served as a control to evaluate serum samples from dogs carrying tumors. Serum from tumor-bearing animals proved to have both inhibitory and blocking effects in high dilutions, depending upon when samples were taken during the evolution of the neoplastic process. A clear relationship between serum blocking activity *in vitro* and tumor growth *in vivo* was seen. Complete serum inhibitory activity was characteristic of regressive tumor changes. The blocking activity of the serum provides a tenable explanation for tumor growth in this system (13).

In the TVT, precise quantitative determinations of tumor size with sera activity throughout a long clinical course is an advantage not readily available in other models (8). Serum blocking activity was noted as early as 7 days following transplantation. The same sequence of events has been demonstrated in inbred rodent systems (1, 10, 22). This early appearance of blocking corresponds to the time when cell-mediated rejection might be expected to occur. The preliminary demonstration of intact cell response, prior to challenge as measured in mixed culture, suggests that the potential for initiating cell-mediated rejection exists (14). Serum-dependent blocking of responding cells thus appears to be of major importance. The persistence of blocking activity has been shown in dogs that develop aggressive tumor courses, and its disappearance in dogs undergoing regression is consistent with this hypothesis.

The development of inhibitory activity was invariably observed in CFUa assay prior to the onset of tumor regression, but the role of these serum factors in tumor regression is less clear than the role of blocking in tumor growth. Associated, cytotoxic antibody development is common in the rejection of normal tissue grafts but is not the mechanism of primary allograft rejection (15). The participation of antibodies in chronic rejection or the hyperacute rejection following hyperimmunization is better established (16). Of interest in the present studies was the ability to detect growth-inhibitory serum activity prior to the appearance of cytotoxic antibodies.

With the CFUa culture system, 3 distinct patterns of reactivity have been described which are correlated with the clinical courses previously reported (8). Such a system should be suitable for monitoring an animal's immune responses during immunotherapeutic manipulation of this neoplastic process.

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