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Regulation of leukocyte glass adherence and tube leukocyte adherence inhibition (LAI) reactivity by serum factors in dogs with progressing or spontaneously regressing canine transmissible venereal sarcoma (CTVS)*

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Summary. *We examined the regulation of leukocyte glass adherence and tube leukocyte adherence inhibition (LAI) reactivity by serum factors in dogs with regressing or progressing canine transmissible venereal sarcomas (CTVS). Both regressor and progressor peripheral blood leukocytes (PBL), draining and nondraining lymph node cells (LNC), and splenic leukocytes were significantly responsive to CTVS antigen extract in tube LAI. In contrast, a significant decrease in basal glass adherence of progressor PBL, draining and nondraining LNC, and splenic leukocytes was observed. Normal glass adherence was restored to progressor leukocytes by extensive washing with warm serum-free media, while significant tube LAI responsiveness to CTVS antigen extract was maintained. Preincubation of regressor PBL and LNC with progressor sera in two-stage tube LAI decreased the basal glass adherence of treated leukocytes. This effect of progressor sera was heat labile, a characteristic of CTVS antigen. Collectively, these findings suggest that progessor leukocytes and progressor sera treated regressor leukocytes were activated by interaction with serum CTVS antigen and thus behaved in tube LAI as stimulated cells, even in the absence of CTVS antigen. Regressor but not progressor sera were shown to contain anti-CTVS IgG with specific arming activity for normal dog PBL, but not LNC in two-stage tube LAI. The nonadherent response of peripheral blood neutrophils in two-stage tube LAI was proportional to the concentration of arming IgG, whereas no change was observed in glass adherence of PBL. The results of this study define the role of progressor and regressor serum factors in the mechanism of tube LAI and demonstrate a relationship between leukocyte glass adherence and the clinical course of CTVS. These findings show that tube LAI is a simple and reproducible measure of active factors in the immune response to a tumor.*

Introduction

The leukocyte adherence inhibition (LAI) phenomenon is based on the observation that antigen sensitized leukocytes are inhibited from adhering to a glass substratus following interaction with sensitizing antigen [9]. Glass test tube [6, 17] and microtiter plate [18] modifications of the original hemocytometer LAI method have been developed and all three assays have been applied in numerous studies of cellular immunity in experimental and human cancer systems (reviewed by Thomson [34]).

Recently, we applied a serum-free tube LAI assay in studies of specific tumor immunity in dogs with canine transmissible venereal sarcoma (CTVS); CTVS is a naturally occurring neoplasm which can be transplanted into nonpreconditioned allogeneic dogs where it will undergo spontaneous regression following a period of progressive growth, or metastasize in neonatally inoculated puppies or immunosuppressed dogs [2, 40]. We have identified a tumor antigen associated with CTVS [26] and have demonstrated a close correlation between the clinical course of CTVS and the magnitude of tube LAI [13, 14]; regressor leukocytes showed a significant LAI response to CTVS antigen extract whereas progressor leukocytes appeared to have a reduced basal glass adherence.

Using a competitive enzyme-linked immunosorbent assay (CELISA) we have also determined that tumor antigen shedding into the peripheral circulation is proportional to tumor volume, and that mean levels of circulating CTVS antigen increase significantly during the progressive growth of CTVS (Palker et al., unpublished work). Both cross-sectional and time-course studies showed an inverse relationship between levels of circulating CTVS antigen and the magnitude of LAI reactivity in tumor dog lymphoid tissues [16]. Collectively, these findings suggest that tumor antigen shedding may contribute to progressive tumor growth and that monitoring leukocyte glass adherence by tube LAI may provide a simple method for detecting serum tumor antigen.

The mechanism of tube LAI has two components: Fc receptor bearing peripheral blood monocytes appear to recognize antigen via a specific cytophilic antibody [8, 22, 35] and then release leukotriene-like substances which alter the glass adherence of bystander peripheral blood leukocytes (PBL) [39]. Sensitized T lymphocytes participate in tube LAI by direct interaction with antigen [15, 30, 31; our unpublished work] and by production of leukocyte adher-

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ence inhibition factor (LAIF) which alters the glass adherence of nonsensitized bystander mononuclear cells [15].

In the present study, we examined the effects of serum from dogs with CTVS at different stages of growth on leukocyte glass adherence and tube LAI reactivity. Results show that regressor, but not progressor sera contain anti-CTVS IgG with specific arming activity for normal dog PBL but not lymph node cells (LNC) in a two-stage modification of tube LAI. In contrast, progressor sera contain a heat labile component which acts to decrease the glass adherence of treated leukocytes. The role of serum antibody and antigen in the mechanism of tube LAI is discussed. These findings show that tube LAI is a simple and reliable measure of active components in the tumor immune response which may provide important prognostic information in clinical human cancer.

Materials and methods

Dogs

A total of 77 dogs, 44 Beagles (25 male and 19 female), 14 Collies (7 male and 7 female), and 19 Labrador-Collie cross-bred dogs (5 male and 14 female), ranging in age from 3 months to 4 years, were used in this study.

Serum

Blood samples were collected from normal dogs, dogs with regressing and progressing tumors, and dogs hyperimmunized to CTVS following spontaneous regression of the initial transplant. The blood was allowed to clot, serum was collected and stored at −70 °C for up to 1 year.

Transplantation

Canine transmissible venereal sarcomas were transplanted as previously described by Yang and Jones [40]. Briefly, following excision tumors were collected in Hanks's balanced salt solution (HBSS) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. Tumors were minced with scissors, and single cells, in suspension, were washed once in HBSS and adjusted to a concentration of 1.0×10^8 cells/ml. Dogs were inoculated SC in the interscapular region with 0.5 ml of the cell suspension. Tumor growth was monitored by weekly measurements of the mass and the status of tumor growth classified as follows: progressor – a tumor with a steadily increasing volume; and regressor – a tumor diminishing in size after having reached a maximum volume.

Preparation of tissue extracts. Water-soluble, 3 M KCl extracts of CTVS or normal dog leukocytes were prepared as described by Meltzer et al. [23]. Briefly, following excision CTVS tissues were minced in HBSS, washed three times, and resuspended to 3.0×10^8 cells/ml. For extraction of normal tissue antigens, blood samples were collected from 10 dogs covering a spectrum of dog leukocyte antigen types, buffy coat cells were harvested, washed twice and also resuspended to 3.0×10^8 cells/ml. Then 10 ml of 3 M KCl – 5 mM potassium phosphate buffer, pH 7.4, was added for each milliliter of cell suspension. Cells were extracted for 24 h at 4 °C and then centrifuged at 40,000 *g* for 60 min at 4 °C. Supernatants were dialyzed against 20 vol. of distilled water overnight and centrifuged at 40,000 *g* for 15 min. The resulting supernatants were dialyzed against 20 vol. of 0.1 M NaCl for 60 min. Protein concentrations were determined by the method of Markwell et al. [20], and adjusted to 2.0 mg protein/ml with phosphate-buffered saline (PBS), pH 7.4.

Preparation of leukocyte suspensions

Peripheral blood leukocytes. Venous blood (20 ml) was collected from dogs into tubes containing 300 units of preservative-free heparin, and centrifuged at 200 *g* for 10 min. Buffy coat cells were aspirated and resuspended in 9.0 ml distilled water for 15 s, followed by addition of 2.9 ml of 3.5% NaCl to restore isotonicity. This procedure was repeated twice, or until contaminating red cells were eliminated. Then PBL were washed three times with serum-free Medium 199 supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin, and buffered with 2.2 g sodium bicarbonate/l and resuspended at a concentration of 1.0×10^7 cells/ml. The PBL suspensions routinely contained 45% to 50% neutrophils, 5% monocytes, 5% eosinophils, and 35% lymphocytes, with no differences observed in the proportion of leukocyte types among regressors, progressors or normal dogs.

Lymph node and splenic leukocytes. Draining (prescapular) and nondraining (popliteal) lymph nodes and spleen tissue were excised, minced in Medium 199, and passed through wire mesh to obtain single cell suspensions. Contaminating red blood cells were lysed as described above, and cells were then washed three times in serum-free Medium 199 and resuspended at a concentration of 1.0×10^7 cells/ml. Spleen cell and LNC suspensions contained on average 22% to 28% B lymphocytes, 55% to 65% T lymphocytes, 6% to 8% null lymphocytes, and 4% to 10% monocytes. No differences were observed in the proportion of leukocyte types among tumor-bearing or normal dogs.

Antigen-induced leukocyte adherence inhibition

The tube LAI assay was performed as described by Harding and Yang [13, 14]. Briefly, 0.1 ml aliquots of a leukocyte suspension (1.0×10^6 cells/ml) were placed in quadruplicate 16 × 125 mm Kimax glass culture tubes. After addition of 0.025 ml CTVS or normal dog tissue (leukocyte) antigen (NTAg) extract, the total volumes were brought to 0.5 ml by the addition of 0.375 ml of serum-free Medium 199. Culture tubes were gently placed in a horizontal position so that medium evenly coated the glass surface of each tube. Following incubation for 2 h at 37 °C in 5% CO_2, tubes were uniformly returned to a vertical position. Nonadherent leukocytes in the fluid phase were gently agitated with a Pasteur pipette, samples introduced into a hemocytometer, and the number of leukocytes in the white cell grids counted. The use of a hemocytometer for quantitation enables expression of results as either (a) the mean percentage of nonadherent cells relative to the number of input (1.0×10^6) leukocytes, and (b) as a nonadherence index (NAI), where $NAI = (A-B)/B \times 100$, with A representing the mean number of nonadherent cells in the presence of CTVS antigen extract, and B representing the mean number of nonadherent cells in the presence of NTAg extract.

Table 1. Analysis of the nonadherent response of lymphoid tissues from normal and tumor-bearing dogs to normal tissue antigen (NTAg) and canine transmissible venereal sarcoma antigen (CTVS-Ag) extracts

	Peripheral blood leukocytes		Draining lymph node		Nondraining lymph node		Splenic leukocytes	
Status	NTAg	CTVS-Ag	NTAg	CTVS-Ag	NTAg	CTVS-Ag	NTAg	CTVS-Ag
Normal dogs ($n=6$)	15.8 ± 1.0[a]	24.1 ± 1.3	24.4 ± 2.9	36.8 ± 3.2	27.0 ± 0.8	34.9 ± 1.8	21.6 ± 2.2	30.5 ± 1.8
	NAI = 52.6		NAI = 51.0		NAI = 29.3		NAI = 41.4	
Regressors ($n=20$)	21.7 ± 1.5	58.0 ± 3.4	32.9 ± 1.5	83.8 ± 4.9	33.1 ± 1.4	82.3 ± 3.7	25.2 ± 1.3	66.7 ± 2.7
	NAI = 167.4		NAI = 154.7		NAI = 148.6		NAI = 164.8	
Progressors ($n=22$)	30.9 ± 2.0	39.3 ± 2.6	54.4 ± 2.6	76.3 ± 3.4	51.5 ± 2.4	75.6 ± 2.7	36.0 ± 2.1	51.5 ± 2.3
	NAI = 29.1		NAI = 40.3		NAI = 47.9		NAI = 43.0	
Significance:								
[b] N vs R	$p<0.05$	$p<0.001$	$p<0.05$	$p<0.001$	NS	$p<0.001$	NS	$p<0.001$
N vs P	$p<0.001$	$p<0.005$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
R vs P	$p<0.001$	$p<0.001$	$p<0.001$	NS	$p<0.001$	NS	$p<0.005$	$p<0.001$

[a] Mean percentage of nonadherent cells ± SEM.
[b] N = normal dogs; R = regressors; P = progressors; NS = not significant

Two-stage tube LAI for analysis of serum arming and blocking activity

A two-stage modification of the standard tube LAI assay was adapted for screening serum samples for arming and blocking activity. In the first stage, 1.0×10^7 PBL or LNC were resuspended in 1.0 ml of a 1:1 dilution of test serum in Medium 199. After incubation at 37 °C for 30 min to allow for binding of cytophilic antibody or CTVS antigen to leukocytes, cells were washed twice with serum-free Medium 199, and resuspended to 1.0×10^7 cells/ml. In stage 2, leukocytes were plated in tubes for LAI as described above. For analysis of arming activity in purified immunoglobulin fractions, samples were diluted in serum-free Medium 199, or RPMI 1640 containing 10% fetal calf serum (FCS) for stage 1.

Purification of serum arming immunoglobulin

To remove antibodies specific for normal dog histocompatibility antigens from hyperimmune and regressor sera, pooled sera were absorbed twice against normal dog spleen and liver cells and once against PBL collected from dogs covering a spectrum of known dog leukocyte antigen types at a 1:5 packed cell-serum volume. The immunoglobulin fraction was recovered by ammonium sulfate precipitation [4], and purified further by DEAE-cellulose chromatography [3]. Column fractions were analyzed by immunoelectrophoresis for identification of immunoglobulin isotypes [4].

Results

Analysis of the mean percentage of LAI reactive cells in normal and tumor-bearing dogs

In addition to analysis of LAI reactivity by the NAI value, it is important to directly examine the percentage of nonadherent leukocytes after stimulation with CTVS antigen or nonspecific NTAg extracts. Table 1 presents the mean percentage of PBL, draining and nondraining LNC, and splenic leukocytes in nonadherent fractions after tube LAI. Data show that both regressor and progressor PBL, draining and nondraining LNC and splenic leukocytes were significantly responsive to CTVS antigen extract in tube LAI; 58%–84% of tumor-bearer leukocytes were nonadherent in comparison to only 24%–37% of normal dog leukocytes ($p<0.001$, Table 1).

In contrast, however, a significantly greater percentage of progressor PBL, draining and nondraining LNC, and splenic leukocytes (31%–55%) were nonadherent in the presence of nonspecific NTAg extract in comparison to regressors (21%–33%, $p<0.001$) and normal dogs (16%–27%, $p<0.001$) (Table 1). Such a decrease in the basal glass adherence of progressor leukocytes suggests they were activated by interaction with serum CTVS antigen in vivo; progressor leukocytes behaved in tube LAI as stimulated cells even in the absence of specific antigen.

Analysis of CTVS antigen specific arming activity in tumor dog serum

Serum samples collected from dogs with progressing and regressing tumors were screened for CTVS antigen specific arming activity by two-stage tube LAI. Data in Table 2

Table 2. Analysis of canine transmissible venereal sarcoma antigen (CTVS-Ag) specific arming activity in tumor dog sera by two-stage tube leukocyte adherence inhibition

Normal dog PBL preincubated with:	Mean percentage of nonadherent cells		Nonadherence index (NAI)
	NTAg	CTVS-Ag	
Normal dog sera ($n=5$)	15.5 ± 4.1[a]	21.9 ± 4.6	41.1
Regressor sera ($n=10$)	18.9 ± 6.1	43.6 ± 10.1[b]	130.6
Progressor sera ($n=6$)	15.3 ± 7.3	20.1 ± 8.9	31.4

[a] Mean ± SD
[b] Significance: regressor vs normal $p<0.001$; regressor vs progressor, $p<0.001$

show that regressor sera armed normal dog PBL for a significant response to CTVS antigen extract (43.6% nonadherent cells) in comparison to normal dog sera (21.9%, $p<0.001$). Regressor and progressor sera did not arm PBL for a tube LAI response to NTAg extract (Table 2), indicating that arming activity was specific for CTVS antigen extract and that two-stage tube LAI did not detect antibody specific for normal dog histocompatibility antigens in tumor dog sera. Also, preincubation of normal dog PBL with normal dog sera did not have a nonspecific effect on the basal leukocyte glass adherence in two-stage tube LAI (Table 2).

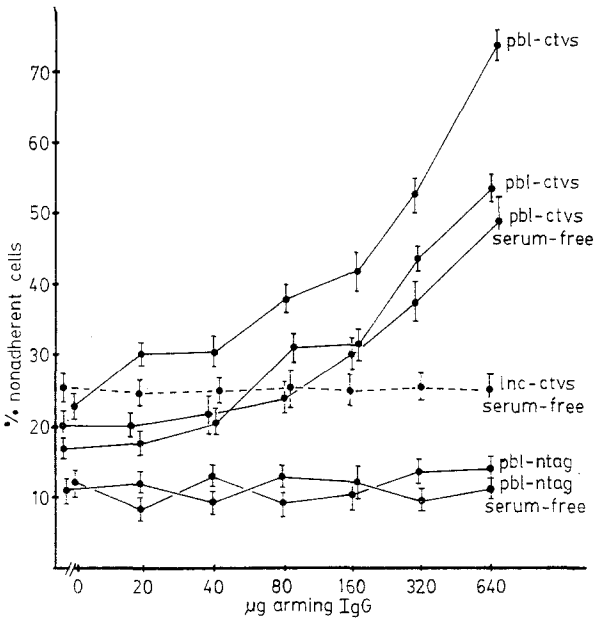

Fig. 1. Specific arming of normal dog PBL, but not LNC in tube LAI with purified anti-CTVS IgG. For stage 1, normal dog PBL or LNC were incubated with IgG dilutions prepared in Medium 199 (designated serum-free) or RPMI-1640 containing 10% FCS. After 30 min at 37 °C, leukocytes were washed twice with serum-free Medium 199, resuspended to 1.0×10^7 cells/ml, plated in tube LAI, and challenged with CTVS antigen or NTAg extract for stage 2.

To further identify the serum component with CTVS antigen specific arming activity, sera from regressor and hyperimmunized dogs were collected, pooled, and absorbed against normal dog liver and spleen cells, and PBL were collected from dogs covering a broad spectrum of known dog leukocyte antigen types. The immunoglobulin fraction was recovered by ammonium sulfate precipitation and purified further by DEAE-cellulose ion exchange chromatography. A single peak was recovered, and immunoelectrophoresis revealed the presence of IgG, but not IgM or IgA. Data in Fig. 1 show that purified IgG possessed significant arming activity for normal dog PBL in two-stage LAI. A linear relationship between the concentration of IgG and the CTVS antigen extract induced tube LAI response of PBL was observed, whereas no response of PBL to NTAg was noted. Significant arming activity was first detected at 80 μg IgG/1.0×10^7 normal dog PBL, with maximum activity at 640 μg IgG, the highest concentration tested (Fig. 1). It is of particular importance to note that similarly armed normal dog LNC did not show a tube LAI response to CTVS antigen extract (Fig. 1). Data in Fig. 1 also suggest that arming efficiency was slightly enhanced when IgG was diluted in RPMI-10% FCS, in comparison to arming with IgG diluted in serum-free Medium 199.

Data in Table 3 record the proportion of leukocyte types present in nonadherent fractions after CTVS antigen extract stimulation of IgG armed PBL. Results show an increase in the percentage of nonadherent neutrophils (1% to 35%–40%) which was proportional to the concentration of arming IgG (Table 3). By contrast, no change in the glass adherence of monocytes or lymphocytes was noted with increasing arming IgG concentrations (Table 3).

Analysis of blocking activity in CTVS progressor sera

Sera from dogs with progressively growing tumors were screened for CTVS antigen activity by two-stage tube LAI using CTVS regressor PBL and LNC as indicator cells.

Table 3. Differential cell count analysis of anti-CTVS IgG armed leukocytes in two-stage tube leukocyte adherence inhibition: neutrophils are the responsive cell type

Arming IgG (μg)	Experiment	Mean percentage of nonadherent cells	Mean percentage of nonadherent leukocyte subtypes		
			Monocytes	Neutrophils	Lymphocytes
0	1	23.4	0.6	1.2	21.3
	2	16.0	0.2	0.6	14.9
20	1	29.9	0.6	2.4	25.1
	2	18.3	0.3	1.3	16.4
40	1	30.4	0.9	2.4	26.5
	2	20.5	0.4	1.4	18.2
80	1	37.8	1.1	7.6	27.7
	2	31.8	1.6	9.9	19.1
160	1	43.6	2.2	13.5	26.2
	2	32.1	1.6	11.5	17.7
320	1	53.6	2.2	21.5	26.8
	2	44.5	2.2	19.1	20.0
640	1	75.8	2.1	41.7	30.3
	2	61.1	2.4	35.4	20.8

Table 4. Effect of progressor sera on the glass adherence of regressor leukocytes in two-stage leukocyte adherence inhibition

Cell type and treatment	Peripheral blood leukocytes Mean percentage of nonadherent cells		Draining lymph node cells	
	NTAg	CTVS-Ag	NTAg	CTVS-Ag
Regressor leukocytes ($n=7$)	15.2 ± 2.0[a]	45.6 ± 5.0	25.8 ± 2.8	78.2 ± 2.1
	NAI = 200.0		NAI = 203.1	
Regressor leukocytes preincubated with progressor sera ($n=7$)	30.7 ± 4.7	46.4 ± 5.4	51.2 ± 5.1	78.2 ± 1.7
	NAI = 51.1		NAI = 52.7	
Significance:	$p<0.025$	NS	$p<0.005$	NS

[a] Mean percentage of nonadherent cells ± SEM

Table 5. Effect of progressor sera on the glass adherence of regressor leukocytes

Cell type and treatment	Peripheral blood leukocytes Mean percentage of nonadherent cells		Draining lymph node cells	
	NTAg	CTVS-Ag	NTAg	CTVS-Ag
A Regressor leukocytes ($n=4$)	18.1 ± 3.4[b]	52.7 ± 4.8	29.7 ± 2.1	81.5 ± 2.8
	NAI = 191.2		NAI = 174.4	
B Regressor leukocytes preincubated with progressor sera	38.7 ± 7.0	53.9 ± 3.7	50.2 ± 5.1	76.5 ± 1.8
	NAI = 39.3		NAI = 52.4	
C Regressor leukocytes preincubated with heat-inactivated progressor sera	18.4 ± 3.0	52.1 ± 4.0	27.8 ± 1.4	75.9 ± 1.4
	NAI = 183.2		NAI = 173.0	
Significance:				
A vs B	$p<0.001$	NS	$p<0.001$	NS
A vs C	NS	NS	NS	NS

[a] Mean percentage of nonadherent cells ± SEM

Data in Table 4 show that progressor sera preincubated regressor PBL and LNC demonstrated significant tube LAI responses to CTVS antigen extract, as did untreated regressor leukocytes. However, progressor sera preincubated regressor PBL and LNC showed a significant decrease in basal glass adherence in the presence of NTAg extract (31% and 51%, respectively) in comparison to untreated regressor PBL (15%, $p<0.025$) and LNC (26%, $p<0.005$).

Data in Table 5 show that the active component in progressor sera is heat labile. As observed in Table 4, progressor sera preincubated regressor PBL (39%) and LNC (50%) showed a significant decrease in basal glass adherence in the presence of NTAg extract in comparison to untreated regressor leukocytes (18% and 30%, respectively, $p<0.001$). However, preincubation of regressor leukocytes with progressor sera which had been heated at 65 °C for 30 min had no effect on the basal glass adherence of PBL (18%) or LNC (29%) in the presence of NTAg (Table 5).

Data in Table 6 show that normal dog PBL armed with regressor sera may also serve as an indicator population for detecting progressor serum CTVS antigen activity. As shown, incubation of regressor sera armed normal dog PBL with progressor sera (29%) significantly decreased basal leukocyte glass adherence in the presence of NTAg extract in comparison to normal dog PBL exposed to only regressor sera (16%, $p<0.001$) or normal dog sera (18%, $p<0.001$). As observed in Table 2, regressor sera armed normal dog PBL for a significant response to CTVS antigen extract in two-stage tube LAI (Table 6).

Unblocking of progressor leukocytes

Data in Table 1 show the decreased basal glass adherence of progressor leukocytes in comparison to regressor and normal dog leukocytes. Data in Table 7 show that normal glass adherence of progressor PBL (11%, $p<0.001$) and LNC (14%, $p<0.001$) was restored by 30 min incubation at 37 °C and additional washing with warm serum-free Medium 199, whereas decreased basal glass adherence in the presence of NTAg extract was observed in untreated pro-

Table 6. The detection of progressor serum blocking activity with regressor sera armed normal dog PBL in two-stage leukocyte adherence inhibition

Normal dog PBL preincubated with:	Mean percentage of nonadherent cells		Nonadherence index (NAI)
	NTAg	CTVS-Ag	
Normal dog sera ($n=4$)	17.7 ± 4.7[a]	27.0 ± 0.6	52.5
Regressor sera ($n=5$)	16.5 ± 5.7	47.5 ± 12.2[b]	187.9
Regressor sera, washed twice, and progressor sera added ($n=10$)	29.4 ± 5.6[c]	43.9 ± 9.4[b]	49.3

[a] Mean ± SD
[b] Regressor and progressor vs normal $p<0.001$
[c] Progressor vs regressor and normal, $p<0.001$

Table 7. Restoration of progressor leukocyte glass adherence by extensive washing

Cell type and treatment	Peripheral blood leukocytes		Draining lymph node cells	
	Mean percentage of nonadherent cells			
	NTAg	CTVS-Ag	NTAg	CTVS-Ag
Progressor leukocytes ($n=6$)	25.5 ± 2.2[a]	45.9 ± 4.4	39.4 ± 2.0	66.4 ± 2.0
	NAI = 80.0		NAI = 68.5	
Progressor leukocytes extensively washed	10.8 ± 1.4	45.6 ± 4.5	14.1 ± 2.0	68.5 ± 2.4
	NAI = 331.5		NAI = 385.8	
Significance:	$p<0.001$	NS	$p<0.001$	NS

[a] Mean ± SE

gressor leukocytes (26% and 39%, respectively). Extensive washing did not affect the tube LAI response of PBL and LNC to CTVS antigen extract (Table 7), suggesting further that interaction with serum CTVS antigen decreases glass adherence as opposed to preferential sensitization of progressor leukocytes to normal canine histocompatibility antigens.

Discussion

This study has examined the relationship between the clinical course of CTVS, the glass adherence of tumor bearer leukocytes, and specific reactivity to CTVS antigen extract in tube LAI. Data in Table 1 show that both regressor and progressor PBL, draining and nondraining LNC, and splenic leukocytes were significantly responsive to CTVS antigen extract (40%–80% nonadherent cells) in comparison to normal dog leukocyte populations (15%–36%, $p<0.001$). The significant tube LAI reactivity of progressor leukocytes (Table 1) indicates there is no deficiency in CTVS antigen reactive cells or specific immunosuppression associated with progressive tumor growth. This observation is in contrast to reports by Raina et al. [27] and Mortensen and Elson [24] showing that T lymphocytes from mice with progressing murine colon adenocarcinoma 38 or sarcoma virus-induced tumors suppress specific antigen reactivity in microplate LAI.

Data in Table 1 also show that decreased basal glass adherence is a characteristic of progressor leukocytes which can be detected by tube LAI. More progressor PBL, draining and nondraining LNC, and splenic leukocytes (31–54%) were nonadherent in the presence of nonspecific NTAg extract than in the corresponding regressor (22%–33%, $p<0.001$) or normal (16%–27%, $p<0.001$) leukocyte populations (Table 1). By competitive enzyme-linked immunosorbent assay (CELISA), we have shown that shedding of CTVS antigen into the peripheral circulation is proportional to tumor volume and that shedding increases significantly during progressive growth of CTVS (Palker et al., unpublished work). Also, an inverse correlation was observed between serum CTVS antigen levels and the NAI value measurement of tumor dog leukocyte tube LAI responses in both time-course and cross-sectional studies [16; Palker et al., unpublished work]. Our interpretation is that serum CTVS antigen modulates basal leukocyte glass adherence; progressor leukocytes activated by interaction with CTVS antigen in vivo behave in tube LAI as specifically stimulated cells. A similar decrease in basal glass adherence of PBL from advanced colorectal cancer patients was coincident with high levels of serum carcinoembryonic antigen [29]. Thomson et al. [36, 37] have also reported that PBL from advanced breast cancer, colon cancer, and melanoma patients show decreased basal glass adherence in comparison to PBL from normal humans or early stage cancer patients.

Data in Table 6 show that normal glass adherence could be restored to progressor leukocytes by extensive washing with warm, serum-free media. This observation

suggests that the CTVS antigen coating acquired by progressor leukocytes in vivo had been removed. Extensive washing did not affect tube LAI response of progressor leukocytes to CTVS antigen extract (Table 6). Grosser and Thomson [7] and Thomson et al. [36] have also restored normal glass adherence to advanced cancer patient PBL by trypsinization to remove the tumor antigen coating acquired in vivo.

Modulation of basal leukocyte glass adherence by progressor sera was examined by two-stage tube LAI. Data in Table 4 show that preincubation of regressor PBL and LNC with progressor sera decreased basal glass adherence of leukocytes in the presence of nonspecific NTAg extract. Data in Table 5 show that the effect of progressor sera on glass adherence is labile to heat at 65 °C, a characteristic of CTVS antigen. In combination with data in Table 6, these findings suggest further that CTVS antigen in progressor sera is largely responsible for decreasing glass adherence of leukocytes and imply that progressors are not preferentially sensitized to normal canine histocompatibility antigens.

Grosser and Thomson [7] and Thomson et al. [37, 38] have reported similar findings. Preincubation of PBL from patients with limited breast cancer, colon cancer, or melanoma with sera from patients with advanced disease significantly decreased the basal glass adherence of treated PBL in an immunologically specific manner. In studies with hemocytometer LAI, incorporation of sera from advanced cancer patients blocked specific antigen-induced LAI but did not alter basal leukocyte glass adherence [10, 11]. Koppi et al. [19] and Halliday et al. [12] recently reported that serum blocking factors active in hemocytometer LAI possess antigenic determinants recognized by anti-I-J antisera and thus resemble suppressor factors. Differences in the mechanism of action of progressor serum factors in hemocytometer and tube LAI may largely reflect the molecular nature of the active compounds.

Two-stage tube LAI was also used to screen tumor dog serum for CTVS antigen specific arming activity. Data in Table 2 show that incubation of normal dog PBL with regressor sera armed leukocytes for specific reactivity to CTVS antigen extract (44% nonadherent cells) in tube LAI whereas progressor and normal dog sera did not (20% and 22%, respectively, $p < 0.001$). It appears that arming activity in regressor sera is specific for CTVS antigen and not canine histocompatibility antigens since armed PBL maintained normal glass adherence in the presence of NTAg extract (Table 2). Grosser and Thomson [7] and Marti and Thomson [21] have also reported that sera from patients with limited breast cancer or melanoma, but not sera from patients with advanced disease, contain antigen specific arming activity for normal human PBL in two-stage tube LAI. Similarly, Tanaka et al. [32] reported that sera from patients with limited breast disease, but not advanced disease, contain antigen specific arming activity for nonimmune PBL in hemocytometer LAI. Thus, there appears to be a correlation between tumor growth status and the ability of LAI to detect antigen specific arming activity in tumor bearer sera.

To identify the serum component with specific arming activity, pooled regressor and hyperimmune sera were absorbed against normal dog spleen cells, liver cells, and PBL to remove antibodies specific for normal dog histocompatibility antigens. The IgG fraction was purified and tested for arming activity by two-stage tube LAI. Data in Fig. 1 show a linear relationship between the concentration of IgG and the nonadherent response of armed normal dog PBL to CTVS antigen extract implying that cytophilic anti-CTVS IgG mediates tube LAI. Indeed, we have recently identified anti-CTVS antibody in regressor but not progressor sera by radioimmunoprecipitation of ^{125}I-CTVS antigen and resolution by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Furthermore, Taylor-Bennett et al. [33] and Beschorner et al. [1] have isolated an IgG2a subclass of immunoglobulin from regressor sera with specificity for CTVS cells in a colony inhibition assay. Our findings are in agreement with other studies.

Tumor antigen specific cytophilic IgG has been shown to arm normal monocytes and macrophages for reactivity in tube [35] and microplate LAI [25]. Other studies have shown that antigen stimulated B lymphocytes produce a soluble IgG mediator which programs monocyte reactivity in microplate LAI [5, 28]. Data in Table 3 show that tube LAI responses of neutrophils, but not lymphocytes increase in proportion to the concentrations of arming IgG, whereas monocytes contribute to only a small increase in the mean percentage of nonadherent cells. Thomson et al. [39] and Shenouda et al. [31] have recently reperted that armed monocytes release leukotriene-like substances after specific antigen binding and that lipoxygenation of arachidonic acid is required for T lymphocyte responses in tube LAI. It is possible that leukotriene-like compounds released by monocytes may inhibit the glass adherence of bystander neutrophils, however, the nonsaturating response of neutrophils suggests that individually armed cells respond after binding of CTVS antigen to cytophilic IgG. In fact, we have failed to detect any soluble activity mediating the nonadherent response of PBL in tube LAI [15]. We favor the hypothesis that lipoxygenation metabolites may be required for signal transduction in individual cells which couples the binding of CTVS antigen with biochemical changes leading to inhibited glass adherence. Data in Fig. 1 and Table 3 also show that LNC and peripheral blood lymphocytes armed with anti-CTVS cytophilic IgG fail to respond in tube LAI. Indeed, we have recently reported that LNC responses in tube LAI involve LAIF activity [15]. The association of IgG with PBL and LAIF with LNC tube LAI responses strongly suggests that the reactivity of cells from these different immunologic compartments proceed through different mechanisms.

In summary, serum factors from dogs with progressing or regressing CTVS contribute to the mechanism of tube LAI; CTVS antigen specific IgG recovered from regressor sera was associated with the response of neutrophils, but not lymphocytes, in tube LAI. Progressor sera were deficient in anti-CTVS antibody, but contained serum CTVS antigen capable of decreasing basal glass adherence of sensitized leukocytes. Direct analysis showed a significant decrease in basal glass adherence of progressor leukocytes in comparison to regressors or normal dogs. Collectively, these findings show that tube LAI is a simple and reliable measure of active components in the tumor immune response which may provide important prognostic information in clinical human cancer.

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