

Detection of Soluble Tumor-associated Antigens in Serum of Tumor-bearing Rats and Their Immunological Role *in Vivo*¹

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SUMMARY

Circulating soluble tumor antigens were detected in the serum of tumor-bearing rats. Sublethally irradiated W/Fu rats inoculated with syngeneic C58(NT)D Gross virus-induced lymphoma served as the source of tumor antigens. Soluble antigens were assessed by specific inhibition of the complement-mediated cytotoxicity of isogenic W/Fu anti-C58(NT)D antibodies against ⁵¹Cr tumor target cells. With a s.c. inoculum of 5×10^7 tumor cells, circulating tumor antigens were first detected at Day 8, and a maximum concentration was reached by Day 13 to 14, which coincided with the peak of tumor growth and was followed by the sudden death of the animals. Pooled serum from tumor-bearing rats was fractionated on Sephadex G-150 and resulted in one peak that contained all of the antigenic activity. The molecular weight of this fraction was estimated to be 50,000 to 60,000 daltons. Presensitization of normal rats with soluble tumor antigens resulted in a specific acceleration of tumor growth and delay in tumor rejection. Specificity was shown by lack of C58(NT)D tumor enhancement in rats presensitized with serum containing tumor antigens from a syngeneic but antigenically unrelated WR-6 lymphoma. The biological significance of circulating soluble tumor antigen mediating specific immunosuppression against an immunogenic tumor is discussed.

INTRODUCTION

In previous reports we have shown that sensitization of animals against chemically solubilized membrane preparations containing transplantation antigens or tumor-associated antigens results in specific enhancement of graft survival and tumor growth, respectively (21, 22). The demonstration of enhancement of tumor growth by soluble tumor antigen suggested that naturally derived tumor antigens shed from the tumor, like the chemically solubilized membrane preparations, may also impair the immune response to the tumor.

Several reports have implied an important biological role for soluble tumor antigens (1, 2, 6, 7, 12, 25, 27, 28). A

direct proof of the *in vivo* immunological role of circulating tumor antigens and their influence on tumor growth is lacking. This paper reports the detection of the presence of soluble tumor antigens in the serum of tumor-bearing rats and demonstrates that presensitization of W/Fu rats with circulating tumor antigen results in specific enhancement of tumor growth.

MATERIALS AND METHODS

Animals. Inbred W/Fu rats and BALB/c mice were obtained from The Mammalian Genetics and Animal Production Section, National Cancer Institute, Bethesda, Md.

Tumors. The C58(NT)D (Gross virus-positive) tumor was originally obtained from Dr. R. B. Herberman, NIH, Bethesda, Md. This tumor was maintained in ascites form in weaning female W/Fu rats by serial i.p. passage of 1×10^7 cells suspended in 0.2 ml of minimal essential medium. The WR-6 (Gross virus-negative) lymphoma was originally obtained from the Mason Research Institute, New York, N.Y., in solid form in young W/Fu rats and maintained by serially implanting a small, nonnecrotic tumor piece s.c. at 10-day intervals. The G-35 (Gross virus-positive) leukemia in BALB/c mice was maintained in ascites form by weekly serial i.p. passage in BALB/c mice with the use of 5×10^6 cells in 0.2 ml of minimal essential medium.

Anti-C58(NT)D Hyperimmune Sera. C58(NT)D cells were collected from tumor-bearing female W/Fu rats and passed through fine stainless steel mesh to obtain a uniform suspension of cells. Adult male W/Fu rats were hyperimmunized as detailed earlier (21). Heat-inactivated hyperimmune serum was used in the present studies.

Preparation of Soluble Antigen Extract from C58(NT)D Tumor. Soluble antigen extract from C58(NT)D was prepared by the 3 M KCl extraction method (21). The protein content of this extract was estimated by the method of Lowry *et al.* (14).

Preparation of Serum Containing Tumor Antigen(s). W/Fu rats were given 500 rads total-body irradiation (32 R/min) from a ⁶⁰Co source. One day postirradiation, the rats were given s.c. injections of 5×10^7 C58(NT)D cells. Blood samples were collected at various time intervals from the tumor-bearing animals through cardiac puncture, and sera were heat inactivated at 56° for 30 min. Sera from irradiated W/Fu rats bearing WR-6 tumors were obtained similarly.

Partial purification of the tumor circulating antigen was done by Sephadex G-150 chromatography, with the use of 80 mM phosphate-buffered saline, pH 7.2. The peaks were

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concentrated by Amicon PM 10 Diaflo ultrafilters. Protein content of each concentrated fraction was determined by the method of Lowry *et al.* (14).

Inhibition of Complement-dependent Cytotoxic Antibody by Tumor Circulating Antigen. The presence of tumor circulating antigens was determined by inhibition of the cytotoxic activity of syngeneic anti-C58(NT)D serum on ^{51}Cr -labeled G-35 leukemia in the presence of rabbit complement (1:10) as described previously (21). In this test, the antiserum dilution used caused 60% of maximum release of the isotope in the presence of complement with no inhibitor present.

Measurement of Tumor Growth. Ten days after the last injection of either tumor circulating antigen or soluble antigen, all groups of W/Fu rats were inoculated with 5×10^7 C58(NT)D cells s.c. on the right flank. Normal W/Fu rats inoculated with C58(NT)D cells served as controls. Tumor size was measured as detailed earlier (21). The means of the individual values for both experimental and control groups were compared with the use of Student's *t* test.

RESULTS

Detection of Tumor-specific Circulating Antigens in Irradiated W/Fu Rats with Tumors. The kinetics of C58(NT)D tumor growth in sublethally irradiated (500 R) rats is shown in Chart 1. Irradiated adult W/Fu rats given s.c. injections of 5×10^7 tumor cells developed a tumor at the site of injection that was palpable on Day 4. The tumor reached its maximum size of 25 mm in diameter by Day 14 and the rats subsequently died. In nonirradiated W/Fu rats, the tumor appeared on Day 4, reached its maximum size at Day 8, and regressed completely by Day 14. Tumor-bearing irradiated rats were not immune to the tumor because of the immunosuppressive effect of irradiation. Neither complement-dependent cytotoxic antibody nor antibody-dependent cellular cytotoxicity against ^{51}Cr -labeled C58(NT)D was detected.

The presence of soluble tumor antigens was tested for by competitive inhibition of the complement-dependent cytotoxic activity of immune anti-C58(NT)D serum to ^{51}Cr -labeled G-35 target in serum samples collected from C58(NT)D tumor-bearing rats (Table 1). Serum inhibitory activity was first detected at Day 8 and was maximal at Day 13. The extent of inhibition was dependent on the concentration of antigen present in the serum and was absent in high dilutions of serum. The inhibition was specific since sera obtained from either irradiated normal rats or irradiated rats inoculated with an unrelated tumor, WR-6, syngeneic in W/Fu rats, were not inhibitory. These results demonstrate that tumor-bearing rats have tumor antigens in their serum that can bind specific antibodies to the tumor. The concentration of the circulating tumor antigens increases with tumor enlargement and seems to correspond directly to the growth pattern of the tumor.

Isolation and Partial Characterization of Circulating Tumor Antigen(s): Pooled serum samples obtained at Day 13 from C58(NT)D tumor-bearing irradiated rats were fractionated on Sephadex G-150. Three major peaks were obtained (Chart 2). Each peak was pooled, concentrated, and tested

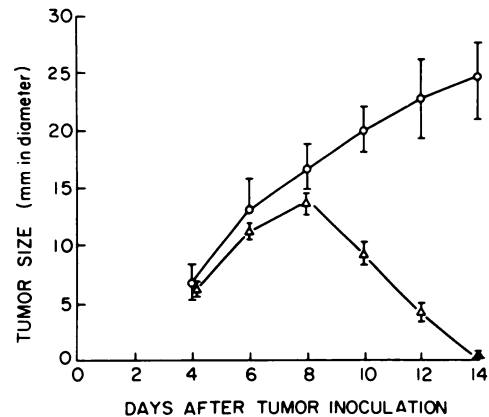


Chart 1. Growth of C58(NT)D tumor in normal W/Fu rats (Δ) and sublethally irradiated (500 R) W/Fu rats (\circ) following inoculation of 5×10^7 C58(NT)D tumor cells s.c. Each point represents the tumor mean diameter (\pm S.D.) of 4 individual rats. Irradiated W/Fu rats succumb with the tumor at 12 to 14 days after the tumor inoculation.

for the presence of tumor antigens by the inhibition assay (Table 2). Antigenic activity was found only in Peak 3. Peaks 1 and 2 showed little or no antigenic activity. Fractionation of normal serum from 500-R-treated rats did not show antigenic activity in any of the peaks. The fact that Peak 3 eluted with serum albumins suggested that the tumor antigen has a molecular weight of $\approx 6.5 \times 10^4$ daltons.

Enhancement of C58(NT)D Tumor Growth in Syngeneic W/Fu Rats Sensitized with Circulating Tumor Antigen. Chart 3 shows the kinetics of tumor growth in groups pre-sensitized with serum containing tumor antigens. Rats inoculated with circulating tumor antigen before tumor inoculation showed a statistically significant enhancement of tumor growth [19.2 ± 0.38 (S.D.) mm on Day 8]. The enhancement was dependent on the antigen concentration used, since rats pretreated with low doses of antigen did not exhibit tumor enhancement. The enhancement was tumor specific since rats pretreated with serum from 500-R-irradiated rats or from 500-R-irradiated and WR-6 tumor-bearing rats behaved similarly to controls. Groups sensitized with 600 μg of soluble tumor antigen from C58(NT)D tumor, given in 6 multiple injections or in a single injection, showed a significant enhancement of tumor growth (23 ± 0.52 mm on Day 8), in agreement with our previous findings (21). Administration, after tumor inoculation, of serum containing tumor antigen, did not influence tumor growth (Table 3). The above results demonstrate that rats inoculated with serum containing tumor antigens show enhancement of tumor growth. The enhancement is specific, dose dependent, reproducible, and observed only when the serum circulating antigen is injected before tumor inoculation.

DISCUSSION

This paper reports the detection and characterization of circulating tumor antigens, free of antibodies, from C58(NT)D tumor-bearing rats. Presensitization of normal rats against soluble tumor antigen impaired the host immune response against a subsequent inoculum of the tumor and resulted in enhancement of tumor growth and rejection.

Table 1

Circulating tumor antigens in tumor-bearing W/Fu rats

Anti-C58(NT)D serum was raised in W/Fu rats immunized s.c. with 1×10^8 tumor cells weekly for 4 weeks. Ten days after the last injection, the animals were bled by cardiac puncture. The separated serum was heat inactivated at 56° for 30 min. The complement-dependent cytotoxic titer of immune serum was determined with the use of ^{51}Cr -labeled G-35 target cells and rabbit complement (1:10). For the inhibition experiments, a constant amount of immune serum at a 1:4 dilution was used. This dilution yielded 60% specific ^{51}Cr release from the G-35 targets.

Tumor inoculation	Dilution ^a	% specific inhibition of serum collected on ^b				
		Day 4	Day 6	Day 8	Day 10	Day 13
C58(NT)D	1:2	0.59 ± 0.31	2.8 ± 0.5	13.3 ± 2.5	26.9 ± 5.2	37.4 ± 4.8
	1:4	0.16 ± 0.05	1.2 ± 0.28	8.6 ± 1.5	18.7 ± 3.4	26.6 ± 5.1
	1:8	0.21 ± 0.03	1.35 ± 0.41	6.0 ± 1.2	8.6 ± 2.7	13.3 ± 2.2
	1:10	0.12 ± 0.01	3.1 ± 0.5	6.33 ± 1.3	5.3 ± 1.8	8.5 ± 1.8
	1:20	0.4 ± 0.08	0.9 ± 0.12	2.0 ± 0.42	1.1 ± 0.18	2.8 ± 0.6
WR-6	1:2	0.79 ± 0.31	1.1 ± 0.51	2.4 ± 1.6	3.2 ± 3.4	2.4 ± 2.8
None	1:2	0.65 ± 0.29	2.4 ± 0.48	1.9 ± 0.31	2.6 ± 2.1	3.8 ± 1.8

^a Serum was derived from tumor-bearing and sublethally irradiated (500 R) W/Fu rats.

^b Mean ± S.D. of 4 sera samples that were collected from 4 tumor-bearing rats and tested separately. The experiments were repeated 3 times.

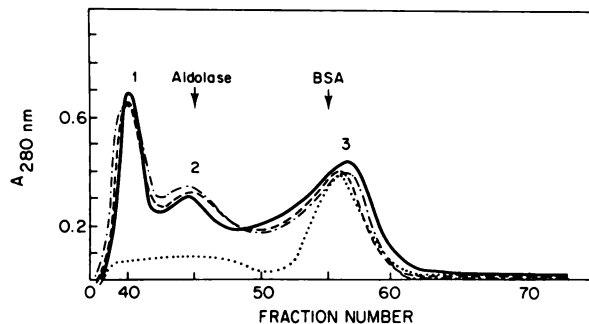


Chart 2. Pooled serum (5 ml) from W/Fu rats was used for fractionation on a Sephadex G-150 column. The eluate used was 80 mM phosphate-buffered saline, pH 7.2, and 8-ml samples were collected per tube. The tubes under each peak were pooled: Peak 1, Tubes 38 to 42; Peak 2, Tubes 43 to 49; Peak 3, Tubes 50 to 62. The eluates were concentrated to one-fifth the original volume of the serum. This is a representative pattern of 1 of 2 similar experiments performed. ·····, rat serum albumin; —, normal rat serum; ---, serum from 500-R-irradiated rats; -·-·-, serum from 500-R-irradiated rats bearing C58(NT)D tumor collected on Day 13. BSA, bovine serum albumin.

The detection of soluble tumor antigen in the serum of C58(NT)D tumor-bearing rats was facilitated by (a) abrogation of the natural host immune response to the tumor by sublethal irradiation, thus preventing antigen-antibody complex formation, and (b) availability of specific isogeneic antitumor antibodies. With the use of a complement-dependent cellular cytotoxic system with ^{51}Cr -radiolabeled target cells and by competitive inhibition of radioactive release by soluble antigen, we were able to detect circulating tumor antigen and to quantitate the amount of antigen present in the serum. By these means we noticed that the concentration of circulating tumor antigens was a function of tumor size. Maximum concentration of antigens was observed at maximum tumor size, at which point the animals succumbed.

Several controls were run in parallel to substantiate further our claim that the system studied revealed specific tumor-associated antigens. Normal serum and serum derived from rats bearing WR-6 tumor, antigenically unrelated to C58(NT)D, did not inhibit the cytotoxic anti-C58(NT)D

Table 2
Fractionation of serum containing tumor antigen on Sephadex G-150

Source of serum ^a	Peak ^b	% inhibition ^c	% recovery of inhibitory activity
<i>Experiment 1</i>			
C58(NT)D tumor-bearing irradiated rats	Before fractionation	35.2 ± 4.5 ^d	
	1	1.6 ± 0.3	
	2	2.5 ± 0.6	
	3	95.8 ± 8.3	54.4
Irradiated rats	Before fractionation	4.4 ± 0.9	
	1	0.8 ± 0.1	
	2	1.7 ± 0.3	
	3	10.6 ± 1.8	48.2
<i>Experiment 2</i>			
C58(NT)D tumor-bearing irradiated rats	Before fractionation	32.4 ± 3.8	
	1	1.3 ± 0.2	
	2	5.6 ± 2.1	
	3	91.8 ± 7.9	56.6
Irradiated rats	Before fractionation	7.2 ± 1.9	
	1	1.5 ± 0.3	
	2	3.2 ± 0.8	
	3	17.9 ± 3.1	52.4

^a Serum was collected on Day 13 from normal and C58(NT)D-bearing W/Fu rats after 500 R whole-body irradiation.

^b Serum pool from 3 rats was collected on Day 13, and 5 ml were fractionated through a Sephadex G-150 column, with the use of 80 mM phosphate-buffered saline, pH 7.2. The volume collected per tube was 8 ml. The tubes corresponding to all the 3 peaks are: Peak 1, Tubes 38 to 42; Peak 2, Tubes 43 to 49; Peak 3, Tubes 50 to 62. The eluates of each peak were concentrated to one-fifth the original volume of the serum.

^c Percentage of inhibition of the complement-dependent cytotoxic activity of isogeneic anti-C58(NT)D serum against ^{51}Cr -labeled G-35 target cells was calculated as described in "Materials and Methods."

^d Mean ± S.D.

serum. Our findings in the C58(NT)D system agree with several reports dealing with other tumor systems that described the presence of circulating tumor antigens either in a free form or in a complex with antibody (1-4, 25).

Bowen *et al.* (4) detected tumor antigens in the serum of hepatoma-bearing rats 7 to 10 days after tumor challenge, when no immune complexes can be detected. In our system, tumor antigen was also first detected in the serum at Day 6 to 8 after tumor implantation. Thompson *et al.* (27, 28) have also reported rapid release of tumor antigens in the serum of methylcholanthrene-induced rat sarcoma. In the guinea pig system, tumor antigen in serum has been reported by Smith and Leonard (26), and soluble tumor antigen in the ascites fluid has been reported recently by Detrick-Hooks *et al.* (8). Other studies have also reported the presence of soluble tumor antigen in the circulation (9, 19).

Naturally circulating tumor antigen was shown to be solu-

ble and retained in the supernatant of the serum following ultracentrifugation at 100,000 × g for 90 min. The molecular weight of the tumor antigen was estimated to be 50,000 to 60,000 daltons by gel filtration on Sephadex G-150. All the antigenic activity was found in 1 peak. It is not clear whether 1 or several different molecular forms of the antigen are present in this peak. The molecular size of the native tumor antigen cannot be ascertained since the circulating tumor antigen may represent a proteolytic fragment of the original molecule. Our study supports the notion that metabolically active tumor cells shed surface proteins from their membrane into the surrounding circulating system (11, 23, 24). This observation has also been shown in the HL-A system whereby circulating antigen was detected by Charlton and Zimjewski (5).

The *in vivo* biological role of circulating soluble tumor antigens is not well understood. We have examined this question by investigating the effect of antigen sensitization on tumor growth. Our data show that rats presensitized with soluble tumor antigen and subsequently inoculated with tumor showed a significant delay in tumor rejection and an increase in tumor diameter over controls. This enhancement was specific to the C58(NT)D tumor, inasmuch as rats presensitized with syngeneic but antigenically unrelated tumor antigen from WR-6 rejected C58(NT)D tumor as a control. Our results obtained with naturally soluble tumor antigen corroborate our previous finding with 3 M KCl extract of tumor antigen from C58(NT)D (21).

Both enhancement and immunity to tumor were observed following sensitization with soluble tumor antigens. Enhancement was suggested by Currie and Alexander (6) and by Vaage (29, 30), and, recently, Pellis and Kahan (18) reported accelerated tumor growth in animals sensitized with high doses (4.2 mg) of soluble tumor antigen. Other investigators have shown immune protection against a lethal dose challenge of a tumor in animals sensitized to tumor antigens (13, 15-17). Enhancement or protection may be 2 different expressions common to all tumors. Certain parameters, general and specific, of the tumor system may dictate the outcome of the sensitizing regimen. Such parameters may include antigen dose, time interval between sensitization and challenge, the route and molecular form

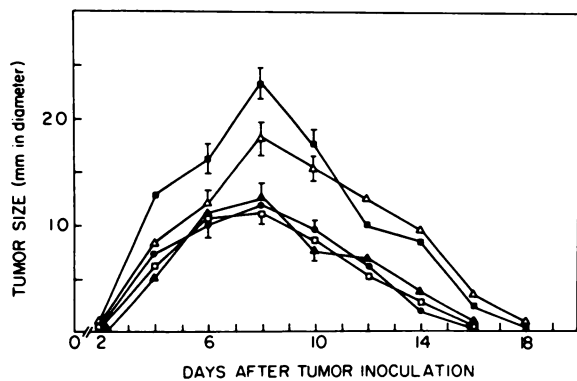


Chart 3. Specificity of C58(NT)D tumor growth enhancement in syngeneic W/Fu rats presensitized with serum containing tumor antigens. Normal adult W/Fu rats were given i.p. injections of a total of 2.4 ml serum-C58(NT)D given in 6 injections on Days -20, 18, 16, 14, 10) before tumor inoculation. The sensitized rats were subsequently inoculated s.c. with 5×10^7 C58(NT)D or control WR-6 tumor cells on Day 0. Each point represents the tumor mean diameter of 4 rats measured with calipers in 2 bisecting diameters of the tumor mass (bars, S.D.). The statistical significance between experimental and control groups was calculated as described in the text. The *p* values on Day 8 are shown in parentheses: ●, rats given 0.9% NaCl solution; ▲, rats given serum from 500-R-irradiated rats (*p*, not significant); □, rats given serum from WR-6 tumor-bearing irradiated rats (*p*, not significant); △, rats given serum from C58(NT)D tumor-bearing irradiated rats (*p* < 0.005); ■, rats given soluble antigen extract from C58(NT)D tumor (*P* < 0.005).

Table 3

C58(NT)D tumor growth in syngeneic W/Fu rats inoculated with C58(NT)D tumor cells and subsequently inoculated with serum containing tumor antigen

Group	Serum used for sensitization ^a	Tumor size (mm) measured on ^b					
		Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
1	0.9% NaCl solution	5.8 ± 0.25	12.2 ± 0.75	14.1 ± 0.69	9.6 ± 0.43	4.1 ± 0.28	ND ^c
2	Normal serum	5.2 ± 0.24	11.8 ± 0.62	14.8 ± 0.81	10.6 ± 0.58	4.9 ± 0.25	ND
3	500-R C58(NT)D serum	6.5 ± 0.38	13.4 ± 0.58	15.8 ± 0.75	10.6 ± 0.72	4.8 ± 0.23	ND
4	500-R serum	6.2 ± 0.29	13.1 ± 0.87	15.2 ± 0.62	11.5 ± 0.63	5.2 ± 0.38	ND
5	500-R WR-6 serum	6.4 ± 0.31	11.5 ± 0.58	14.0 ± 0.62	11.2 ± 0.38	5.1 ± 0.21	ND

^a Serum collected on Day 13 from normal cells, sublethally irradiated W/Fu rats (500-R serum), and from WR-6 or C58(NT)D tumor-bearing irradiated W/Fu rats [500 R WR-6 and 500 R C58(NT)D]. Different groups of W/Fu rats were inoculated with 5×10^7 C58(NT)D tumor cells s.c. on Day 0 and 1 day after were sensitized with 0.4 ml serum each day for 4 days. Different groups of W/Fu rats were also tested with lower doses, *i.e.*, 0.1 or 0.2 ml 500-R C58(NT)D serum. No significant difference in tumor growth was observed compared to the control groups.

^b The values represent the mean diameter of tumor ±S.D. from 4 rats used in each group. Tumor size was calculated by measuring tumor growth with calipers in 2 bisecting diameters.

^c ND, not detectable.

of the antigen, the presence or absence of adjuvants, etc. Such parameters have been shown to play an important role (18). More important is the immunological effect of the soluble tumor antigen. Several possibilities may arise. Sensitization can positively activate the immune system, which will then facilitate tumor rejection upon challenge. Alternatively, sensitization can elicit blocking antibody selectively (23) by itself or complexed with antigens that interfere with immune rejection. Likewise, soluble antigens can alter the immune balance by selectively activating suppressor cells which in turn will suppress the antitumor response (10). These last 2 possibilities may be the basis of tumor enhancement observed in our studies, following sensitization with soluble tumor antigen.

In our studies, enhancement can be obtained only if the rats had been presensitized with the tumor antigen. Administration of the antigen at the time of tumor inoculation had no effect on tumor growth characteristics. These results clearly indicated that the antigen acts on virgin precursor immunocompetent cells. Once the immune system is activated, under the conditions used, the soluble antigen cannot deviate the ongoing response against the tumor. Clearly, different doses and schedules of soluble antigen administration may also alter the ongoing antitumor immune response. The demonstration here, however, that sensitization to soluble tumor antigen may lead to adverse effects on tumor rejection cautions against the generalized use of soluble antigen in antitumor immunotherapy.

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