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The Activity of Regional Nodes in the Evolution of Immune Responses to Allogeneic and Isogeneic Tumors[1]

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SUMMARY

The evolution of immune responses of C57BL/6 mice to allogeneic tumor Sarcoma 180 and of A/J mice to isogeneic tumor Sarcoma 1 was investigated by colony and cell inhibition (CI) assays. The immune response of lymphocytes from regional popliteal nodes, distant nodes, and spleens was examined at varying times after s.c. implantation of known numbers of *in vitro*-grown tumor cells in the hind feet. In the allogeneic system, only regional node lymphocytes produced CI activity, maximum response appearing at Day 14 and gradually diminishing thereafter with tumor regression. Serum-blocking activity was not observed until Day 21 and increased to significant levels by Day 39 when no lymphocyte CI activity was detectable. In the isogeneic system, CI activity was tumor-dose dependent. Responses to low-dose inocula were confined to regional nodes, whereas with high-dose inocula, initial responses were provided by regional nodes, but by Day 21 the spleen had become the primary source of CI activity. Examination of blocking activity in this system was not possible due to nonspecific serum cytotoxicity. Lymph nodes other than regional showed no CI response at any time in either tumor system. These studies demonstrate the importance of regional nodes in the development of immune responses to both allogeneic and isogeneic tumors.

INTRODUCTION

The activity of regional lymph nodes in the initiation and maintenance of the immune response was first demonstrated in experimental animal models, using allogeneic tumors (5, 20). Injection of alum-precipitated nontumorous antigen such as keyhole limpet hemocyanin has also been shown to evoke strong regional node immune responses maintained at levels higher than those of distant nodes (22). Several investigations of isogeneic animal tumors have shown that regional nodes are not strictly essential for either initiation or maintenance of host tumor immunity (1, 2, 11). Histological studies of nodes draining isogeneic tumors have shown morphological changes that may be indicative of a diminishing cellular immune response during tumor growth (8). It has been suggested that both duration of tumor growth and initial dosage of tumor are factors responsible for changes in regional node competence (5). Tumor antigenicity has also been cited as a major determinant of regional node function (10). Evaluation of regional node responses to human breast cancer have shown contrasting results which may relate to the above factors (7, 14, 23).

The purpose of this study was to investigate the role of regional nodes in the evolution of immunity to tumors under conditions of varying tumor antigenicity, dosage, and duration of growth. Two different models were chosen: Sarcoma 180, in allogeneic tumor in C57BL/6 mice, and Sarcoma 1, an isogeneic tumor in strain A/J mice. Because previous comparative studies of regional node function have almost exclusively used *in vivo* assays (1, 2, 5, 9, 10), it was decided to evaluate responses by *in vitro* techniques, which are now being used extensively in demonstrating immunity to experimental and human tumors (13, 18).

MATERIALS AND METHODS

Male A/J and C57BL/6 mice were obtained from The Jackson Memorial Laboratory, Bar Harbor, Maine, and were kept on a standard diet and water throughout the experiments.

Sarcoma 1, a fibrosarcoma of strain A/J origin, was obtained from Dr. Rolf F. Barth (University of Kansas Medical Center, Kansas City, Kans.). Sarcoma 180 from CFW mice, was obtained from Flow Laboratories, Rockville, Md. Both tumors were maintained as *in vitro* cell cultures in Falcon 3024 plastic flasks. Culture medium was composed of Hank's minimal essential medium (90%), fetal calf serum (10%), penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (292 μg/ml), and mycostatin (100 units/ml) (Grand Island Biological Company, Grand Island, N. Y.). Cultures of normal murine fibroblasts were prepared from minced, trypsinized skin fragments and maintained in medium through 4 subcultures. All trypsinizations of monolayers or tissue were done with 0.25% trypsin (1:250), Difco Laboratories, Inc., Detroit, Mich.).

Mice were given injections of viable tumor cells obtained from trypsinized monolayers. In all cases, viability of cells as determined by trypan blue was at least 95%. Total injection volume was 0.05 ml given s.c. into the dorsum of each hind foot. Tumor weight measurements were made by weighing amputated tumor-bearing feet and subtracting the weight of normal hind feet.

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Lymphocytes were obtained from spleens and axillary, inguinal, and popliteal (regional) lymph nodes of tumor-bearing and normal mice. Organs were dissected and handled separately. Cell suspensions from lymph nodes were prepared by gently mincing and aspirating the nodes in culture medium. Viability of node cells varied but was greater than 70% in all cases. Cell suspensions from spleens were layered onto Ficoll:Hypaque density gradients (3), after which the lymphocyte-rich cell layer was removed and washed in medium. Viability of spleen cells was greater than 90% in all experiments. Blood was collected from the retroorbital sinus. Serum was inactivated at 56° for 30 min and stored at −20°.

CI[2] and microCI assays were essentially the same as those described by Hellström (12). Lymphocyte concentrations used in the assays were established by prior testing. At lymphocyte doses greater than those indicated, the high degree of target cell destruction frequently seen prohibited comparison of normal and experimental groups. Incubation of all assays was performed at 37° in an atmosphere of 5% CO_2 and 95% air. For the CI test, 100 to 200 target cells were seeded into Falcon 3002 16-mm plastic Petri dishes in 5 ml medium. After 24 hr, medium was removed and 15×10^6 lymphoid cells in 1.0 ml medium were added. Following 45 min of incubation, 4 ml of fresh medium were added, and the dishes were reincubated for 6 days. In assays for blocking activity, 0.2 ml test or control serum was added and the dishes were incubated 45 min prior to the addition of lymphoid cells. Three or 4 replicate dishes were set for each variable tested.

In the microCI assay, 100 target cells in 0.2 ml medium were seeded into each well of a Falcon 3040 microtiter tray. After 24 hr, medium was removed and 1×10^5 lymphoid cells in 0.1 ml medium were added. Fresh medium (0.1 ml) was added after 45 min, and the trays were reincubated for 24 hr. For an assay of blocking activity, 0.1 ml of test or control serum was added, and trays were incubated 45 min prior to the addition of lymphoid cells. Six to 8 replicate wells were set for each variable tested. Assay controls included A/J or C57BL/6 normal skin fibroblasts and both Sarcoma 1 and Sarcoma 180 target cells.

Both assays were terminated by 1% crystal violet staining. Petri dish colonies were counted macroscopically; target cells in microtiter tray wells were counted under low-power microscopy. The %CI was calculated by comparing experimental groups exposed to sera and lymphoid cells from tumor-bearing donor nodes or spleens with controls treated with sera and lymphoid cells from the respective organs in normal mice. All data were analyzed by t test; a significant difference between test and control was defined as $p < 0.05$.

RESULTS

Allogeneic Tumor Experiments. Forty C57BL/6 mice were given injections of 7.5×10^6 viable Sarcoma 180 cells on each hind foot. At Days 7, 14, 21, and 39 after implant, 10 mice were killed, and sera, spleens, axillary, inguinal, and popliteal nodes were evaluated separately by CI assay.

In a total of 12 experiments, summarized in Table 1, only popliteal (regional) nodes demonstrated cytotoxic activity. No CI responses were observed with distant nodes or spleens at any time studied. Significant responses were seen only against Sarcoma 180 target cells. Measurement of tumor weights indicated virtually complete rejection by Day 39 (Table 1). In 2 separate CI experiments done to determine the blocking ability of serum, significant levels of blocking were not detected until Day 39 (Table 2). This rise of blocking activity coincided with the decrease of a regional node response as well as tumor rejection (Chart 1).

Isogeneic Tumor Experiments. Two preliminary CI experiments were performed to examine Day 12 regional node and spleen responses to variations in isogeneic Sarcoma 1 inocula ranging from 5×10^4 to 5×10^6 tumor cells/mouse. From these assays it was determined that inocula of 1×10^5 and 3×10^6 cells provided maximal and minimal regional node responses, respectively. No spleen responses were observed with any inoculum at the time studied. Parallel time studies of 2 groups of mice receiving either a high or a low dose of tumor inocula were then undertaken.

Two groups of 40 mice received either 1×10^5 cells (LD group), or 3×10^6 (HD group) of Sarcoma cells. One-half the total inoculum was injected s.c. into each hind foot. At Days 7, 14, 21, and 28 after implantation, 10 mice from each group were killed, and spleens, axillary, inguinal, and popliteal nodes were examined for cytotoxic activity by both CI and microCI assays. Sera were examined for blocking activity in separate experiments. Time-sequence experiments were repeated at least 3 times with both LD and HD groups.

No significant cytotoxic responses were observed with axillary or inguinal node lymphocytes from either the LD or HD group in a total of 23 of 24 assays. Significant reactivity was observed most frequently (18 of 30 assays) with regional nodes from both groups and with spleens from the HD group (Table 3). A comparison of tumor weights of both groups showed rapid achievement of logarithmic growth by HD inocula (Chart 2). Growth of LD tumors occurred more slowly, and tumor weights in this group were consistently less than those of the HD group. Approximately 90% of LD group mice survived to Day 28, compared with only 50% of the HD group.

Mean CI values (Chart 3) indicated both LD and HD regional node responses appeared to peak at Day 14 and decline to undetectable levels by Day 28. HD group spleens demonstrated a peak response at Day 21, but this activity was not paralleled by those of the LD group. Serum-blocking activity could not be determined because of nonspecific toxicity exhibited by A/J sera (including normal) at concentrations from undiluted to that diluted 1:50. No significant cytotoxicity was observed by lymphoid cells from either group against normal A/J skin fibroblasts or Sarcoma 180 tumor cells.

In 12 CI assays, normal and immune regional node cells of both LD and HD groups were plated alone in Petri dishes without target cells. Normal cells produced 3.7 ± 2.1

[2] The abbreviations used are: CI, colony inhibition; microCI, cell inhibition by microcytotoxicity; %CI, percentage colony or target cell reduction; LD, low dose; HD, high dose.

Table 1

CI responses of normal and immune C57BL/6 lymphocytes to allogeneic tumor, Sarcoma 180

Immune mice were inoculated with 7.5×10^6 Sarcoma 180 cells on each hind foot and sacrificed at Days 7, 14, 21, and 39 after implant. The popliteal nodes are the regional nodes.

Time implant	Tumor wt	No. of surviving Sarcoma 180 colonies after exposure to: Normal node lymphocytes	Immune regional node lymphocytes	%CI by immune lymphocytes	p^a
Day 7	95.0 ± 5.9[b]	88.5 ± 0.0	97.1 ± 0.0	0.0	
		171.5 ± 3.5	164.5 ± 17.1	4.1	>0.05
		152.3 ± 2.0	82.5 ± 6.6	45.8	<0.005
Day 14	141.0 ± 8.5	85.0 ± 2.1	41.3 ± 0.6	51.4	<0.001
		72.0 ± 3.1	44.5 ± 0.5	38.2	<0.001
		124.7 ± 3.1	31.7 ± 0.0	74.6	<0.001
Day 21	149.3 ± 17.7	90.3 ± 3.2	36.0 ± 7.1	60.1	<0.001
		90.1 ± 5.1	51.5 ± 6.6	42.8	<0.025
		87.5 ± 7.6	91.5 ± 10.6	0.0	
Day 39	21.0 ± 6.2	92.0 ± 1.8	98.3 ± 1.6	0.0	
		90.7 ± 4.5	97.5 ± 4.5	0.0	
		97.0 ± 11.1	125.5 ± 4.5	0.0	

[a] As determined by *t* test.
[b] Mean ± S.E.

Table 2

Effect of serum from normal and immune C57BL/6 mice bearing allogeneic Sarcoma 180 tumors on CI response of immune Day 14 popliteal (regional) node lymphocytes

Two CI assays of serum-blocking activity of mice inoculated with 7.5×10^6 Sarcoma 180 cells on each hind foot and bled at Days 7, 14, 21, and 39 after implant. Immune regional node lymphocytes were collected at Day 14 after implant.

Immune serum donors	No. of Sarcoma 180 colonies surviving after exposure to: Normal node lymphocytes	Immune lymphocytes + Normal serum	Immune lymphocytes + Immune serum	%CI by immune lymphocytes	p^a	% blocking activity by immune serum	p^a
			Experiment 1				
None	79.0 ± 2.1[b]	53.5 ± 1.5		32.3	<0.001		
Day 7			49.0 ± 0.0	38.0	<0.001	None	
Day 14			43.5 ± 6.6	45.0	<0.001	None	
Day 21			59.5 ± 2.5	24.7	<0.01	23.5	<0.05
Day 39			61.5 ± 0.4	22.2	<0.01	31.4	<0.01
			Experiment 2				
None	92.0 ± 5.0	51.0 ± 0.2		44.6	<0.01		
Day 7			50.0 ± 6.1	45.7	<0.01	None	
Day 14			52.5 ± 0.1	42.9	<0.01	3.8	>0.05
Day 21			58.0 ± 0.2	36.9	<0.05	17.3	>0.05
Day 39			76.0 ± 9.1	17.4	>0.05	61.0	<0.05

[a] As determined by *t* test.
[b] Mean ± S.E.

macroscopic colonies/10^7 lymphoid cells. LD group regional node cells formed 1.3 ± 0.3 colonies/10^7 cells for all times after implant, whereas 33.6 ± 7.3 colonies/10^7 cells resulted from plating of HD group regional nodes. Colonies from either group, but not from normal mice, were capable of producing palpable tumors when trypsinized and injected into the hind feet of A/J mice. No grossly involved regional nodes were included in the experiments. The maximal number of involved nodes seen was 3 out of 12 of a HD group killed at Day 28.

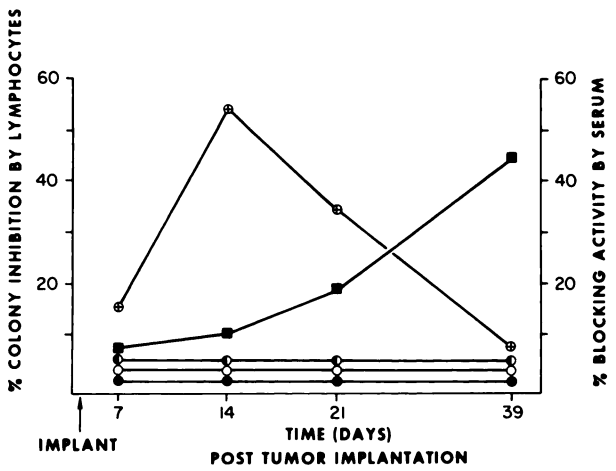

Chart 1. Summary of CI assays of immune responses of C57BL/6 mice to allogeneic tumor Sarcoma 180. Mice were inoculated with 7.5 × 10^6 Sarcoma 180 cells on each hind foot and killed at Days 7, 14, 21, and 39 after inoculation. ●, axillary node lymphocytes; ○, inguinal node lymphocytes; ⊕, popliteal (regional) node lymphocytes; ◐, spleen cells; ■, serum-blocking activity. *Left ordinate*, mean %CI of 2 experiments; *right ordinate*, mean %CI of 3 experiments.

DISCUSSION

Cell inhibition to allogeneic tumor Sarcoma 180 was strictly localized in the regional nodes. Such regional node dominance of immune reactivity has been observed previously with allogeneic tumors under *in vivo* assay conditions (5, 20). A study of draining and contralateral node responses to localized injections of nontumorous material (alum-precipitated keyhole limpet hemocyanin) demonstrated strict localization of primary immune responses for up to 43 days in the regional nods (22). It was observed that contralateral nodes acted like those from nonimmune animals (22). This finding is comparable to the negative results obtained with distant nodes and spleens in our experiments. The emergence of serum-blocking activity coinciding with waning cellular response had been noted previously in early studies (15) that labeled this serum activity "enhancing antibody." The nature of the factor-producing blocking activity in the present study is not clear at this time.

The experiments with Sarcoma 1 demonstrated a marked influence of initial tumor dose on host immune response.

Table 3

CI and cell inhibition assays of A/J response to varied doses of isogeneic tumor Sarcoma 1

Immune mice received either 5 × 10^4 Sarcoma 1 cells on each hind foot (LD) or 1.5 × 10^6 cells on each hind foot (HD). All mice were killed at Days 7, 14, 21, and 28 after inoculation. The popliteal nodes are the regional nodes. Lymphocyte controls for immune groups are from the corresponding organs in normal mice.

Time after implant	Dose	Immune group	No. of Sarcoma 1 colonies or cells surviving after exposure to: Normal lymphocytes	Immune lymphocytes	% cytotoxicity by immune lymphocytes	p^a
Day 7	Low	Regional nodes	83.0 ± 7.1[b]	83.8 ± 8.2	None	
			69.2 ± 3.1	63.0 ± 2.1	9.0	>0.05
			144.6 ± 3.1	140.0 ± 4.1	3.2	>0.05
	Low	Spleens	63.6 ± 4.0	82.0 ± 2.5	None	
			65.3 ± 2.8	52.3 ± 0.9	19.9	<0.01
			68.6 ± 8.6	59.3 ± 3.9	13.6	>0.05
	High	Regional nodes	75.3 ± 5.1	95.5 ± 10.6	None	
			83.0 ± 7.1	85.3 ± 7.0	None	
			82.6 ± 4.8	84.6 ± 4.4	None	
	High	Spleens	68.2 ± 2.9	74.0 ± 3.1	None	
			63.6 ± 4.0	89.2 ± 2.4	None	
			60.3 ± 6.3	71.0 ± 10.8	None	
Day 14	Low	Regional nodes	38.8 ± 2.8	26.2 ± 1.3	32.5	<0.001
			105.2 ± 5.1	64.6 ± 4.8	38.6	<0.001
			57.3 ± 2.6	40.5 ± 4.3	29.3	<0.005
			51.6 ± 0.9	35.3 ± 4.6	31.6	<0.025
	Low	Spleens	45.0 ± 2.9	36.8 ± 5.7	18.2	<0.05
			54.8 ± 2.5	58.7 ± 2.1	None	
			146.6 ± 2.8	138.2 ± 4.4	5.7	>0.05
	High	Regional nodes	38.8 ± 2.8	25.4 ± 1.3	34.5	<0.001
			57.3 ± 2.6	45.3 ± 7.3	20.9	<0.05
			58.5 ± 3.5	41.3 ± 3.9	29.4	<0.05
			43.3 ± 3.1	47.3 ± 0.5	None	

Table 3—*Continued*

Time after implant	Dose	Immune group	No. of Sarcoma 1 colonies or cells surviving after exposure to: Normal lymphocytes	Immune lymphocytes	% cytotoxicity by immune lymphocytes	p^a
	High	Spleens	45.0 ± 2.9	34.3 ± 1.3	23.8	<0.001
			146.6 ± 2.8	143.0 ± 7.7	2.5	>0.05
			54.8 ± 2.5	56.9 ± 1.8	None	
Day 21	Low	Regional nodes	44.0 ± 2.1	30.0 ± 2.0	31.8	<0.001
			103.3 ± 4.5	73.6 ± 1.9	28.8	<0.001
			16.6 ± 1.5	16.3 ± 0.9	None	
	Low	Spleens	48.6 ± 1.6	38.3 ± 1.8	21.2	<0.001
			74.1 ± 7.7	111.4 ± 6.1	None	
			15.3 ± 1.4	16.5 ± 1.3	None	
	High	Regional nodes	44.0 ± 2.1	39.0 ± 1.9	11.4	<0.05
			58.6 ± 2.7	50.1 ± 3.9	14.5	<0.05
			70.0 ± 4.1	100.8 ± 2.0	None	
			84.3 ± 5.4	56.5 ± 1.5	32.7	<0.025
	High	Spleens	48.6 ± 1.6	36.4 ± 1.8	25.9	<0.001
			56.0 ± 8.3	31.1 ± 3.5	44.1	<0.005
			49.3 ± 3.1	0.3 ± 0.2	99.4	<0.001
			80.2 ± 6.1	2.3 ± 0.8	97.1	<0.001
			74.1 ± 7.7	30.5 ± 1.5	59.0	<0.001
Day 28	Low	Regional nodes	39.4 ± 2.9	43.0 ± 2.3	None	
			59.7 ± 4.5	73.3 ± 1.6	None	
			40.2 ± 3.6	54.0 ± 3.8	None	
			63.0 ± 2.9	82.3 ± 4.3	None	
	Low	Spleens	40.0 ± 3.0	42.2 ± 3.8	None	
			13.5 ± 2.6	21.0 ± 1.0	None	
			47.9 ± 4.7	68.7 ± 6.5	None	
			39.3 ± 2.8	59.0 ± 6.2	None	
	High	Regional nodes	39.4 ± 2.9	54.5 ± 3.8	None	
			36.1 ± 4.8	18.0 ± 0.0	50.1	<0.025
			40.2 ± 3.6	70.4 ± 4.4	None	
			63.0 ± 2.9	83.8 ± 5.3	None	
	High	Spleens	40.0 ± 3.0	31.2 ± 2.8	22.0	=0.01
			15.0 ± 0.6	15.3 ± 2.6	None	
			47.9 ± 4.7	58.8 ± 2.7	None	
			39.3 ± 2.8	43.7 ± 2.8	None	

[a] As determined by *t* test.
[b] Mean ± S.E.

The recruitment of the spleen as the major source of CI activity by the HD group probably indicated a spread of tumor or related antigens beyond regional control. A number of murine studies (1, 14, 19) have noted spleen function to be more critical than that of regional nodes in expressing host immunity to isogeneic tumors. In addition to the unique invasive characteristics of each tumor, dosage and period of tumor growth may play important roles in changing the nature of host response from regional to systemic. Weak antigenicity of some tumors has also been cited as a prime factor in promoting tumor proliferation and metastasis (16). The capacity of regional nodes to effectively control smaller tumor loads is evidenced by comparison of Sarcoma 1 growth curves for both groups (Chart 2). LD regional response appeared adequate to delay tumor growth and possible spread of antigen. No micrometastases were visible in nodes of the LD group, and this may indicate that sufficient proliferation of immunocompetent cells had time to occur before significant numbers of tumor cells infiltrated the nodes. The lack of spleen response observed in the LD group may indicate that regional control was in effect. However, the spleen response of the HD group may reflect an antigen overflow.

The possibilities of desensitization of responsive clones (24) or the activation of suppressor cell populations (17) must also be considered. A state of nonresponsiveness to

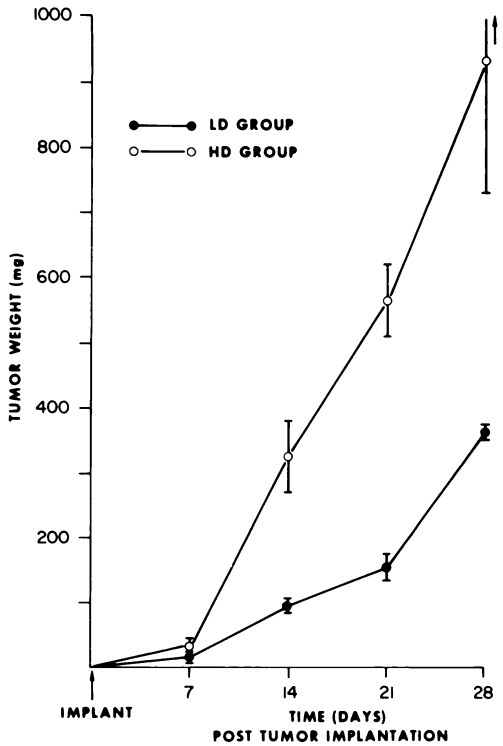

Chart 2. Growth of isogeneic tumor Sarcoma 1 in A/J mice receiving 2 different sizes of inoculum; LD group received 5×10^4 Sarcoma cells on each hind foot. O, HD group received 1.5×10^6 cells on each hind foot. Mice were killed at Days 7, 14, 21, and 28 after inoculation.

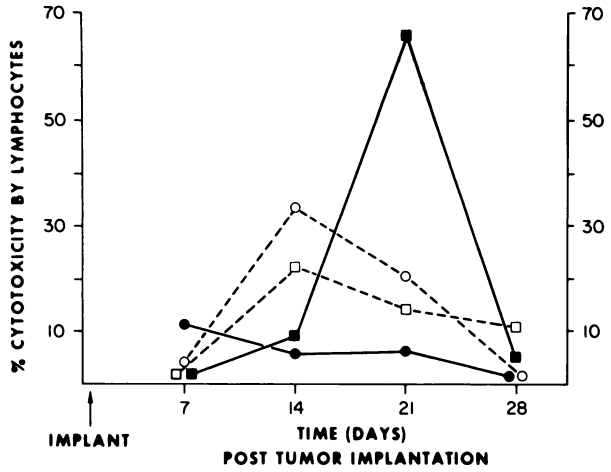

Chart 3. Summary of immune responses of A/J mice to varying isogeneic tumor Sarcoma 1 inocula. Mean cytotoxicity calculated from results of all CI and cell inhibition assays shown in Table 3. Inoculum of the LD group was 5×10^4 cells on each hind foot; that of the HD group was 1.5×10^6 cells on each hind foot. Mice were killed at Days 7, 14, 21, and 28 after inoculation. O, LD popliteal (regional) node lymphocytes; ●, LD spleen cells; □, HD popliteal (regional) node lymphocytes; ■, HD spleen cells.

Sarcoma 1 was shown to exist in both HD and LD groups by Day 28. Studies of induced tolerance have demonstrated different threshholds for T- and B-cells and the ability of nonresponsive population to influence another (4). Nonreactivity of T- or B-cells has also been attributed to the functioning of an unknown suppressor cell population (17). T-cells of both LD and HD groups appeared to have reached nonresponsive status at the same time.

Since A/J serum activity could not be assessed, we cannot comment upon the existence of blocking factors in the form of either free or antibody-bound antigen, as has been proposed in other systems (21).

Cell-mediated tumor immunity to either HD or LD inocula appeared to be initiated by regional nodes. With small inocula, maintenance of immune response was a function of regional nodes whereas, with large tumor loads, this was not the case. Multiple factors appear to be operative in the tumor-host relationship, and additional models are needed to provide more data relevant to the surgical treatment of human cancer. The critical issue of regional node removal or retention in breast cancer must be considered in light of the functional capacity of immunologically committed lymph nodes (6). The complex determinants of tumor growth and metastases and the role of the immune process must be better understood if effective therapeutic measures can benefit man.

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