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Active Specific Chemoimmunotherapy of Lymph-node Metastasis from a Poorly Immunogenic Murine Fibrosarcoma

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The fibrosarcoma MCA-SP, which was recently induced with methylcholanthrene (MCA) in C3H/HeJ mice, displays poor immunogenicity in *in vivo* prophylaxis. A cell variant MCA-SPN1, which bears a tumor-specific transplantation antigen (TSTA) cross-reactive with the parental line MCA-SP, was selected because of its proclivity for axillary lymph-node metastases. Although these lymph-node metastases were resistant to sinecomitant (post-excisional) immunity, they were susceptible to combined active and passive specific chemoimmunotherapy, using tumor-specific, 1-butanol-extracted, preparative isoelectric focusing-purified, TSTA (1 μg weekly sc injections), cyclophosphamide (CY, a single intraperitoneal 20 mg/kg dose), and adoptive transfer of immune splenic T lymphocytes, which had been re-stimulated *in vitro* with extracted TSTA and interleukin-2. This triple regimen both reduced the incidence of spontaneous lymph-node metastases, and prolonged the survival of tumor-bearing, as well as tumor-resected hosts. The results from local adoptive transfer assay using T-lymphocyte subpopulations of spleen and lymph nodes in these treated hosts suggested that Lyt 2^+ cytotoxic T-lymphocytes (CTL) mediated *in vivo* tumor-neutralization. Thus TSTA/CY/CTL therapy activates tumoricidal host responses effective against the poorly immunogenic MCA-SP tumor and its lymph-node metastases.

Key words: Active specific chemoimmunotherapy — Tumor antigen — Murine fibrosarcoma — Lymph-node metastasis

Distinctive, polymorphic, tumor-specific transplantation antigens (TSTA)[1] have been extracted from methylcholanthrene(MCA)-induced fibrosarcomas, using either 3 *M* KCl[2] or a single-phase solution of 1-butanol.[3,4] Not only do the extracts induce primary, prophylactic host resistance against supralethal, subcutaneous (sc) neoplastic challenges,[5] but also they evoke delayed-type hypersensitivity (DTH) reactions in syngeneic hosts immunized with irradiated neoplastic cells.[6] Extracted TSTA administered alone decrease, rather than augment, host resistance in tumor-bearing mice, apparently due to stimulation of suppressor T cells.[7,8] However, the combination of cyclophosphamide (CY), which retards suppressor T-cell induction,[9,10] with TSTA inhibits local neoplastic recurrence after incomplete surgical resection,[11] and decreases the number of spontaneous pulmonary metastases following amputation of a tumor-bearing limb.[12] Addition of a third modality, namely adoptive transfer of immune cytotoxic T lymphocytes (CTL) expanded *in vitro* with extracted TSTA and interleukin-2 (IL-2), potentiates the therapeutic effect of TSTA/CY against primary tumors and their spontaneous pulmonary metastases.[13] However, lymph-node metastases, which occur commonly during cancer progression in man, may be more difficult to eradicate by immunotherapy. The experiments presented herein demonstrate that the triple modality of TSTA/CY/CTL reduces the incidence of spontaneous lymph-node metastases of a neoplastic cell variant selected from the poorly immunogenic MCA sarcoma, MCA-SP, and prolongs the host survival.

MATERIALS AND METHODS

Animals and tumors Three non-cross-reactive sarcomas (MCA-SP, MCA-F and MCA-2A) induced by sc injection of 3-MCA[2] were maintained by cryopreservation, and serial sc transplantation for not more than 7 passages in 10- to 15-week-old female C3H/HeJ mice, which were obtained from the Jackson Laboratory (Bar Harbor, ME).

Selection of lymph-node metastatic sublines A subline of the MCA-SP parental tumor was selected by a modification of the method of Fidler.[14] Twenty-eight days after sc inoculation of 10 viable MCA-SP cells suspended in 0.2 ml of Hanks' balanced salt solution (HBSS, GIBCO, Grand Island, MA) into the flank of a C3H/HeJ mouse, ipsilateral axillary lymph nodes were isolated aseptically and single cell suspensions were placed *in vitro*. The confluent monolayers, which emerged upon *in vitro* culti-

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vation, were re-inoculated sc to produce primary neoplasms. After this cycle was repeated three times, the subline MCA-SPN1 displayed a marked proclivity to lymph-node metastases.

Antigen extraction Tumor cell extraction with 1-butanol and partial purification by isoelectric focusing were performed as previously described[4]; protein concentrations were estimated by the Bradford method.[15]

***In vitro* expansion of CTL with soluble TSTA and IL-2** *In vitro* re-stimulation of immune CTL with extracted TSTA and IL-2 was performed as previously described.[13] Briefly, 1×10^5 nylon-wool-nonadherent lymphocytes harvested from the spleens of mice, which had been preimmunized with 12000 R irradiated MCA-SP cells, were grown in 24-well, flat-bottomed tissue culture plates (Linbro, Flow Laboratories, VA) in the presence of 5 U/ml purified rat IL-2 (Collaborative Research, Lexington, MA) and 0.01 μg/ml 1-butanol-extracted MCA-SP TSTA. The culture medium containing RPMI 1640 (GIBCO) was supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT), 5×10^{-5} *M* 2-mercaptoethanol (Sigma), 100 U/ml penicillin (GIBCO), and 100 μg/ml streptomycin (GIBCO). Three days later the cultured cells were transferred to new plates which contained adherent monolayers of syngeneic, 2000 R irradiated splenic antigen-presenting cells, and re-fed every 4 days. After 14–21 days of cultivation, immune lymphocytes were recovered for passive transfer, as described below.

Immunotherapy model After 10^6 MCA-SPN1 cells injected sc into the right flank had grown to the size stipulated by the experiment, mice were randomized into groups receiving weekly sc injections of 1 μg of MCA-SP TSTA, a single ip dose of 20 mg/kg CY (Cytoxan; Mead Johnson, Evansville, IN), and/or adoptive transfer of *in vitro* re-stimulated T cells (5×10^7/0.8 ml HBSS, iv). Thereafter tumor size, as measured by vernier calipers, and host survival were serially monitored. The statistical significance of differences in tumor sizes between groups was determined by Student's *t* test; and in host survival, by the Gehan modification of the Wilcoxon test.[11] Metastases were diagnosed by the criterion of twice enlarged ipsilateral right axillary lymph nodes at autopsy 42 days after tumor inoculation; this index correlated with extensive histopathologic involvement. The statistical significance of differences in the incidence of lymph-node metastases between groups was assessed by the Kruskal-Wallis test to calculate the Q statistic.[12]

Cytotoxicity assay A 4-h ^{51}Cr-release assay was used to estimate *in vitro* cytotoxic activity.[13] Various numbers of effector cells in RPMI 1640-10% FCS were added to 96-well, round-bottomed plates (No. 3739 Costar, Cambridge, MA). Each well contained 10^4 viable, tumor target cells, which had been prepared from sc neoplasms by a method modified from that of Rong *et al.*,[16] using digestion for 2 h at room temperature with constant stirring in HBSS medium containing 0.1 mg/ml deoxyribonuclease (DNAse, Sigma, St. Louis, MO), 2.5 mg/ml collagenase (Type V-S; Sigma), and 2 units/ml hyaluronidase (Type VI-S, Sigma) and labeled *in vitro* with ^{51}Cr. After a 4-h incubation of lymphocytes with targets at 37°C, an aliquot of cell-free supernate was counted for ^{51}Cr release, using a scintillation apparatus. Maximum ^{51}Cr release, as assessed by detergent lysis, ranged from 2,000 to 10,000 cpm ^{51}Cr; spontaneous release in the presence of medium was less than 20% of the maximal values. Cytotoxic activity was calculated from the average of triplicate cultures according to the formula: % specific lysis = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. The statistical significance of differences between ^{51}Cr release by spleen cells from treated versus control groups was assessed using Student's *t* test.

Local adoptive transfer assay (LATA) For *in vivo* tumor neutralization tests, mixtures of 5×10^5 effector (E) with 5×10^3 target (T) cells (E:T = 100:1) in 0.2 ml of HBSS were inoculated sc into normal, syngeneic C3H/HeJ recipients. In order to ascertain the effector cell phenotype in the lymphocyte population, negative selection was performed using monoclonal antibody coating and complement-mediated lysis for depletion of specific T-cell subsets. Plastic-dish non-adherent lymphocytes were harvested from the spleens or axillary lymph nodes of MCA-SPN1 tumor-bearing mice treated with TSTA/CY/CTL. Single cell suspensions in RPMI 1640 medium were incubated for 45 min at 4°C with monoclonal antibodies directed against mouse T-cell subsets, namely anti-Thy 1.2 (1/400 dilution; New England Nuclear, Boston, MA), anti-Lyt 1 (1 μg/10^6 cells; Becton Dickinson, Mountain View, CA), or anti-Lyt 2 (0.2 μg/10^6 cells; Becton Dickinson). After washing, treated cells were exposed to 100 μl of rabbit complement (1/10 dilution; Cedarlane Laboratories, London, Ontario, Canada) for 45 min at 37°C. After three further washes, the depleted spleen cell or lymph node populations were admixed with MCA-SPN1 cells for LATA. Diameters of the nodules containing effectors and tumor cells were serially monitored with vernier calipers.[17]

RESULTS

Immunogenicity of index MCA-induced fibrosarcomas The immunogenicity of the parental line MCA-SP, of MCA-F and of MCA-2A sarcomas was tested in terms of the capacity of immunization with 1×10^6 irradiated (12000 R) homotypic tumor cells to confer immunoprotection (Table I). The minimum tumorigenic dose (MTD),[5] namely the number of inoculated neoplastic

Table I. Tumorigenicity and Immunogenicity of Methylcholanthrene-induced Fibrosarcomas

Challenge load[a]	Tumor incidence[b]					
	MCA-SP		MCA-F		MCA-2A	
	Normal	Immunized[c]	Nomal	Immunized	Normal	Immunized
10^2	6/10	0/5	5/10	0/5	3/7	0/5
10^3	9/10	0/5	10/10	0/5	10/10	0/5
10^4	10/10	4/5	10/10	0/5	10/10	0/5
10^5	5/5	5/5	5/5	0/5	5/5	0/5
10^6	5/5	5/5	5/5	1/5	5/5	2/5

a) Number of viable tumor cells inoculated into groups of mice.
b) Tumor incidence determined 42 days after challenge was expressed as number of tumor-bearing mice/total number of challenged mice.
c) Immunized hosts received 10^6 irradiated MCA-SP, MCA-F, or MCA-2A cells ten days prior to challenge with various numbers of tumor cells.

Table II. Immunologic Specificity of Irradiated Cell Prophylaxis against MCA-induced Tumors[a]

Challenge[a]	Tumor incidence[b]				
	Immunizing tumor: MCA-SP	MCA-SPN1	MCA-F	MCA-2A	None[c]
MCA-SP	3/10	3/10	7/10	8/10	5/5
MCA-SPN1	1/10	2/10	9/10	8/10	5/5
MCA-F	9/10	10/10	1/10	9/10	5/5
MCA-2A	10/10	10/10	8/10	0/10	5/5

a) Groups of ten mice were immunized with 10^6 irradiated MCA-SP, or MCA-SPN1, or MCA-F, or MCA-2A cells. Ten days later 10 MTD (10^4 cells) of MCA-SP, or MCA-SPN1, or MCA-F, or MCA-2A cells were incoculated SC.
b) Tumor incidence determined 42 days after challenge was expressed as number of tumor-bearing mice/total number of challenged mice.
c) These recipients did not receive an immunizing injection.

cells required to uniformly produce neoplasms in C3H/HeJ hosts, was 10^3 for MCA-SP, MCA-F, and MCA-2A. Mice immunized with 10^6 irradiated MCA-SP cells resisted challenge with 10^2 or 10^3 (0.1 and 1.0 MTD), but not with $\geq 10^4$ (10 MTD), homotypic tumor cells. In contradistinction, mice immunized with either MCA-F or MCA-2A cells uniformly rejected homotypic tumors up to the neoplastic load of 10^6 (1000 MTD) cells. Thus MCA-SP is far less immunogenic than MCA-F or MCA-2A. The sc preimmunization injection induced specific host resistance toward homotypic MCA-SP sarcoma cells (Table II): the outgrowth of 1 MTD (10^3 cells) MCA-SP challenge was inhibited only by pre-immunization with MCA-SP or its variant MCA-SPN1, but not with MCA-F or MCA-2A cells.

Incidence of spontaneous lymph-node metastases during primary tumor progression The incidence of axillary lymph-node metastases progressively increased during the outgrowth of 10^6 MCA-SPN1 cells inoculated sc into the right flank (Table III). While few hosts bearing MCA-SPN1 primary tumors for 14 days showed lymph-node metastases, the incidence increased by 21 days to 55%, and was uniform by 28 days, at which time these hosts bore tumors of more than 20 mm in diameter.

Table III. Effect of Tumor Progression upon the Appearance of Lymph-node Metastases

Days after challenge[a]	Tumor size[b]	Incidence of lymph-node metastases (%)[c]
7	2.3	0/5 (0)
14	9.8	2/14 (14)
21	15.0	6/11 (55)
28	19.3	11/11 (100)
35	23.1	10/10 (100)

a) Fitty-one mice were injected sc with 1×10^6 MCA-SPN1 cells into the right flank. Thereafter, groups of hosts were sacrificed in order to determine axillary-node metastases.
b) Mean tumor diameter (mm) measured all surviving hosts.
c) Incidences determined as number of mice showing lymph-node metastases/number of challenged hosts (with percentage in parenthesis).

Table IV. Influence of Primary Tumor Resection upon the Appearance of Spontaneous Axillary Lymph-node Metastases

Treatment[a]	Day after treatment[b]	Tumor-bearing days[c]	LN metastatic incidence (%)[d]
Resection			
day 7	28	7	0/5 (0)
day 14	21	14	2/10 (20)
day 21	14	21	6/10 (60)
day 28	7	28	9/10 (90)
day 35	7	35	9/9 (100)
Sham			
day 7	7	14	1/5 (20)
day 7	14	21	2/5 (40)
day 7	21	28	5/5 (100)
day 14	7	21	3/5 (60)
day 14	14	28	5/5 (100)
day 21	7	28	5/5 (100)

a) Groups of mice injected sc with 1×10^6 MCA-SPN1 cells into the right flank underwent tumor excision 7, 14, 21, 28, or 35 days later. Groups of mice with sham operation received only skin incision 7, 14, or 21 days after tumor challenge.
b) Day after treatment on which hosts were killed to assess axillary lamph-node metastases.
c) Tumor-bearing days from tumor challenge to tumor resection or sacrifice.
d) Incidence determined on the day of sacrifice and expressed as number of metastasis-bearing mice/total number of challenged mice (with percentage in parenthesis).

In order to determine the impact of the primary neoplasm upon the incidence of axillary lymph-node metastases, tumors were excised at various times after MCA-SPN1 inoculation (Table IV). There was no difference in the incidence of lymph-node metastases between resected hosts and non-resected tumor-bearing animals. Thus in contradistinction to pulmonary metastases,[18] lymph-node metastases occur and progress independently of the primary neoplasm by 7 days after tumor establishment. These lymph-node metastases are resistant to post-excisional immunity.

Therapeutic effect of TSTA/CY/CTL on 14-day-established MCA-SPN1 tumors Mice bearing 14-day-established tumors were randomized into groups receiving weekly 1 μg MCA-SP TSTA sc, a single ip dose of 20 mg/kg CY, and/or adoptive transfer of immune T lymphocytes re-stimulated *in vitro* with extracted TSTA and IL-2 (Table V). Treatment with TSTA, CY or CTL alone altered neither the size of primary tumor outgrowths, nor the incidence of axillary lymph node metastases. Dual TSTA/CY and CY/CTL, but not TSTA/CTL, regimens significantly inhibited primary tumor outgrowth, and modestly prolonged host survival, but did not affect the incidence of lymph-node metastases. Only the triple TSTA/CY/CTL regimen markedly reduced the size of primary tumor outgrowths ($P<0.001$), significantly prolonged host survival ($P<0.001$), and decreased the incidence of lymph-node metastases ($P<0.01$).

Table V. Effect of Active Specific Chemoimmunotherapy with TSTA/CY/CTL against Mice Bearing 14-day-established sc MCA-SPN1 Tumors

Treatment[a]	Tumor size[b]	P[c]	LN metastatic incidence (%)[d]	MST[e]	P[f]
None	18.8±1.2	—	10/10 (100)	39.6±2.4	—
TSTA	19.4±1.1	NS	9/10 (90)	38.8±3.0	NS
CY	17.0±1.0	NS	9/10 (90)	40.3±3.5	NS
CTL	17.8±1.4	NS	10/10 (100)	48.6±3.9	NS
TSTA/CY	13.3±1.0	<0.005	8/10 (80)	52.1±4.9	<0.05
CY/CTL	13.6±0.9	<0.005	7/10 (70)	51.9±5.0	<0.05
TSTA/CTL	15.9±1.2	NS	9/10 (90)	41.1±5.1	NS
TSTA/CY/CTL	10.3±0.9	<0.001	1/10 (10)	62.2±4.3	<0.001

a) Groups of ten mice inoculated sc with 1×10^6 MCA-SPN1 cells on day 0 received weekly injections of TSTA (μg, sc) beginning on day 14; a single ip treatment with 20 mg/kg CY on day 14; and/or 5×10^7 *in vitro*-expanded T cells (CTL) iv on days 17 and 21.
b) Tumor sizes as mean tumor diameter (mm)±standard error of mean (SEM) were compared on day 28.
c) Statistical differences between tumor sizes were determined by Student's *t* test.
d) Incidence of axillary metastases/total number of mice determined at 42 days after challenge with the percentage in parenthesis.
e) Mean survival days±SEM.
f) Statistical significance of differences in MST was determined by means of the Gehan modification of the generalized Wilcoxon test.

Table VI. Therapeutic Effect of TSTA/CY/CTL against Spontaneous Lymph-node Metastases after Resection of Primary MCA-SPN1 Tumors

Day of resection[a]	Treatment[b]	LN metastatic incidence (%)[c]	MST[d]	P[e]	
21	None	6/10 (60)	48.8±2.9	—	<0.001
	TSTA/CY	3/10 (30)	61.9±3.3	<0.01	<0.005
	TSTA/CY/CTL	1/10 (10)	76.2±2.9	<0.001	—
28	None	10/10 (100)	43.3±3.5	—	<0.001
	TSTA/CY	7/10 (70)	52.6±3.9	NS	<0.01
	CY/CTL	5/10 (50)	54.5±4.6	NS	<0.05
	TSTA/CY/CTL	2/10 (20)	69.8±4.1	<0.001	—

a) Mice were inoculated with 1×10^6 MCA-SPN1 cells sc into the right flank and the tumors were excised either 21 or 28 days later.
b) Treatment group included combinations of weekly TSTA (1 μg, sc) beginning on the day of resection, a single (20 mg/kg, ip) injection of CY on the day of resection, and/or 5×10^7 *in vitro*-expanded immune T cells (CTL) twice iv on days 24 and 28 against the 21-day-resected group, or days 31 and 35 against the 28-day-resected group.
c) Incidences expressed as number of axillary tumor-bearing mice/total number of mice at 42 days after challenge.
d) Mean survival days±standard error of mean (SEM).
e) Statistical differences between survival times determined by means of the Gehan modification of the generalized Wilcoxon test against either untreated controls (left column) or the triple modality regimen (right column).

Effect of TSTA/CY/CTL on spontaneous lymph-node metastases after tumor resection In spite of resection of the primary tumor 21 or 28 days after inoculation of 10^6 MCA-SPN1 cells, untreated hosts displayed progressively enlarging, axillary lymph-node metastases, and their mean survival times (MST) were within 50 days. Triple therapy, combining TSTA/CY/CTL, augmented host resistance toward these spontaneous metastases (Table VI). In contradistinction to the dual regimens of TSTA/CY or CY/CTL which had only modest effects, triple TSTA/CY/CTL therapy reduced the incidence of spontaneous axillary lymph-node metastases. While the dual TSTA/CY regimen only prolonged the survival of hosts whose tumor was resected at 21 days ($P<0.01$) but not at 28 days, triple TSTA/CY/CTL therapy improved host survival after resection at both times. Thus the TSTA/CY/CTL regimen potentiates the therapeutic effects of its component modalities against postoperative spontaneous lymph-node metastases from the weakly immunogenic MCA-SPN1 tumor.

***In vitro* cytotoxicity of spleen cells from hosts receiving TSTA/CY/CTL** In the setting of another newly induced MCA tumor, MCA-F, whose clone 9 displays spontaneous pulmonary metastases, the triple regimen augments not only *in vivo* neoplastic resistance, but also *in vitro* splenic lymphocyte cytotoxicity against ^{51}Cr-labeled homotypic neoplastic targets.[13] Similarly, the TSTA/CY/5×10^7 CTL regimen augmented the specific cytotoxicity of spleen cells harvested from MCA-SPN1 tumor-bearing hosts to 73.3% toward homotypic MCA-SP compared with 11.3% toward heterotypic MCA-F, and 9.3% toward MCA-2A cells in 4-h ^{51}Cr release assays (Table VII). Treatment with the dual MCA-SPN1 extracted TSTA/CY regimen significantly enhanced *in vitro* cytotoxicity toward MCA-SPN1 targets to 42.6% with little effect on the lysis of heterotypic MCA-F (6.1%) or MCA-2A (2.9%) cells. Adoptive CTL therapy alone also increased *in vitro* tumoricidal activity of spleen lymphocytes particularly against specific MCA-SP (21.2%), but

Table VII. Cytotoxic Specificity of Spleen Cells from Tumor-bearing Mice Treated with TSTA/CY/CTL Chemoimmunotherapy

Treatment[a]	% ^{51}Cr release[b]		
	Target cells: MCA-SP	MCA-F	MCA-2A
None	1.0	1.6	−0.1
TSTA/CY	42.6	6.1	2.9
CTL	21.2	9.9	10.3
TSTA/CY/CTL	73.3	11.3	9.3

a) Spleen cells were harvested 42 days after tumor challenge from MCA-SPN1-bearing mice treated with TSTA (1 μg, sc) on days 14 and 21, CY (20 mg/kg, ip) on day 14, and adoptive transfer of 5×10^7 immune T cells expanded *in vitro* on days 17 and 21.
b) Percent specific lysis was determined in a 4-h ^{51}Cr-release assay using an effector-to-target ratio of 50:1.

Table VIII. Characterization of *in vivo* Tumor-neutralizing Activity in Splenic and Axillary Lymph Nodes of Tumor-bearing Mice by LATA

Effector cells[a]	Treatment[b]	Mean tumor diameter[c]	P[d]
None	—	16.8±1.3	
Spleen cells	Unfractionated	10.3±1.0	—
	C alone	9.9±1.1	NS
	Thy 1.2+C	15.6±1.2	<0.005
	L3T4+C	10.0±0.9	NS
	Lyt 2+C	14.0±1.1	<0.025
	L3T4/Lyt 2+C	15.6±1.4	<0.01
Lymph-node cells	Unfractionated	8.9±0.8	—
	C alone	8.3±0.7	NS
	Thy 1.2+C	12.9±1.1	<0.01
	L3T4+C	7.2±0.7	NS
	Lyt2+C	12.1±1.0	<0.025
	L3T4/Lyt 2+C	13.3±1.2	<0.01

a) Nonadherent lymphocytes were prepared 42 days after tumor challenge from spleen or axillary lymph nodes of MCA-SPN1 tumor-bearing mice treated with TSTA (1 μg, sc) on days 14 and 21, CY (20 mg/kg, ip) on day 14, and adoptive transfer of 5×10^7 immune T cells expanded *in vitro* on days 17 and 21.
b) Negative selection was performed by treatment with a variety of monoclonal antibodies (Thy 1.2, L3T4, or Lty 2) and complement (see the text for details).
c) Mean tumor diameters (mm±SEM) determined 28 days after admixtures of 5×10^5 effector cells and 5×10^3 MCA-SP tumor cells (effector:target ratio=100:1) were inoculated sc into 10 naive C3H/HeJ recipients.
d) Statistical significance of differences determined by using Student's t test versus unfractionated controls.

to a lesser extent against nonspecific MCA-F (9.9%) or MCA-2A (10.3%) cells.

In order to identify the T-cell subpopulation of the spleen and lymph node mediating the immunotherapeutic effect of TSTA/CY/CTL, local adoptive transfer assays were performed after selective cell depletion of plastic-dish nonadherent lymphocytes, using Thy 1.2, L3T4 or Lyt 2 monoclonal antibodies and complement (Table VIII). The tumor-neutralizing activity of both splenic and lymph-node populations was diminished by treatment with either anti-Thy 1.2 or anti-Lyt 2, but not with anti-L3T4, monoclonal antibodies, suggesting that the triple therapeutic regimen augments an Lyt 2^+ subpopulation which neutralizes homotypic MCA-SP tumor growth.

DISCUSSION

Previously it was demonstrated that active specific chemoimmunothrapy with 1-butanol-extracted TSTA, CY, and adoptive transfer of CTL, which had been re-stimulated *in vitro* with soluble TSTA and IL-2, retard the primary outgrowth and spontaneous pulmonary metastases of the MCA-F sarcoma.[13] The experiments presented herein document that this regimen also reduces the incidence of spontaneous regional lymph-node metastases of the poorly immunogenic, recently induced, sarcoma MCA-SP and prolongs the host survival. This tumor displays reduced susceptibility to immunoprophylaxis with irradiated tumor cells compared with two other MCA-induced fibrosarcomas. Although primary tumor outgrowth was significantly inhibited by the double regimen TSTA/CY, the incidence of lymph-node metastases was affected.

Using the line 10 transplantable hepatocellular carcinoma in strain 2 guinea pigs, in which progressive regional lymph-node metastatic outgrowth occurs, Smith *et al.*[19] demonstrated adoptive transfer of immune peritoneal exudate cells to overcome metastases to superficial distal axillary nodes. However, the effect was obviated either when the tumor burden was increased 6-fold or when therapy was delayed from day 7 to day 14. The present experiments utilizing active specific immunotherapy with TSTA/CY/CTL documented efficacy in 14-day tumor bearers. Further experiments are under way to assess therapeutic activity in settings of even larger tumor burdens, and to assess the dose-response relation of the number of transferred cells within the triple regimen. The lymph-node metastasis model presents a stringent test for immunotherapy. In contradistinction to spontaneous lung metastases, where at least a portion of the effect of TSTA/CY/CTL may be potentiation of post-excisional immunity by amputation,[18] the lymph-node metastasis presented herein, using the poorly

immunogenic MCA-SP tumor, showed no susceptibility to this mechanism. Whether the refractoriness of MCA-SPN1 nodal metastases relates to an immunologic difference between MCA-F clone 9 and MCA-SPN1, or to variable sensitivities of nodal versus pulmonary metastatic sites, is under study, utilizing a lung-proclivity, MCA-SP cell variant and an MCA-F cell variant with proclivity for lymph nodes.

Although the mechanism of antigen-specific chemoimmunotherapy to retard neoplastic outgrowth is not known, one explanation suggests that extracted TSTA induces specific activation of both helper ($L3T4^+$) T cells and suppressor T ($Lyt\ 2^+$) cells.[12] Upon addition of low-dose CY, in order to dampen suppressor T-cell generation,[9, 10] the vectorial outcome activates specific L3T4 effector cells, blunting tumor progression. On the one hand, this hypothesis is concordant with reports that an $L3T4^+$-positive T-cell subset serves as the effector population to mediate regression of established tumors, probably by functioning as helper cells.[20, 21] On the other hand, $Lyt\ 2^+$ CTL appear to be important effectors in the regression of sarcomas and leukemias in mice.[22, 23] The addition of the CTL arm, which is comprised of $Lyt\ 2^+$ cells stimulated *in vitro*,[13] may reinforce $Lyt\ 2^+$ effector generation in lymph nodes and spleen, as documented by the monoclonal antibody depletion experiments presented herein. These findings are consistent with the therapeutic effects adoptively transferred cloned CTL,[24, 25] and of $Lyt\ 1^+$, $Lyt\ 2^+$ immune T cells recovered after secondary *in vitro* stimulation with irradiated C57BL/6 MCA sarcoma cells.[26] The present studies, documenting the effect of systemic transfer of *in vitro* restimulated lymphocytes in the difficult model of lymph metastases, suggest that this modality may, in combination with active specific immunotherapy, offer an important adjunct in difficult clinical situations of nodal micrometastases.

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