Tumor Res. 24, 75—86 (1989)

Autologous Tumor-Specific Transplantation Antigens and Cyclophosphamide Provide Superior Postsurgical Combination Immunotherapy

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SUMMARY

The purpose of this study was to determine whether autologous tumor-specific transplantation antigens (TSTA) were more effective than syngeneic TSTA for immunotherapy in a murine tumor model of local recurrence. We also examined whether combination chemoimmunotherapy with autologous TSTA and cyclophosphamide (CY) afforded better protection against postsurgical tumor growth than did antigen or CY alone. Two weeks after s. c. inoculation of 1×10^6 MCA-F clone cells into C3H/HeN (MTV^-) mice, the primary growing tumors were resected and each tumor was maintained as a completely individual cell line *in vitro*. Subsequently, crude butanol extracts (CBE) were obtained from cultured cells and on day 7 postsurgery 50 μg of autologous or syngeneic CBE were injected s. c. On day 14 after resection, the mice were challenged on the contralateral flank with 5×10^4 autologous or syngeneic cultured MCA-F cells. By measurement of tumor growth, the antitumor efficacy of CBE was determined. Autologous TSTA provided the most effective antitumor immune response against autologous tumor cell challenge, compared with other combinations of extract, host, and challenge cells ($p<0.005$ or $p<0.001$). Furthermore, combination chemoimmunotherapy with autologous CBE and CY (20 mg/kg CY i. p. on the day of immunization) produced more favorable sinecomitant immunity against secondary autologous tumor cell challenge after excision of the pri-

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[3] The abbreviations used are: TSTA, tumor-specific transplantation antigens; CY, cyclophosphamide; MCA, 3-methylcholathrene; CBE, crude 1-butanol extracts; PBS, phosphate-buffered saline; ST_{50}, the 50% survival time in days; MHC, major histocompatibility complex; CTL, cytotoxic T-lymphocytes.

mary tumor. These results suggest that in each tumor-bearing mouse slightly different tumor cell populations are selected for, which despite sharing the common TSTA express a unique collection of immunoprotective antigens to which the host responds in an individually specific manner.

Key words: Autologous tumor-specific transplantation antigens, Crude butanol extracts, Cyclophosphamide, Sinecomitant immunity, Chemoimmunotherapy

INTRODUCTION

The development of a method for the selective extraction of immunologically unique TSTA[3] from chemically induced experimental tumor cells using 2.5% single-phase 1-butanol has provided a new insight into the immunological reactivity and molecular properties of TSTA(9, 11-15). Many investigators have confirmed the usefulness of butanol extraction for the immunological analysis of "rejection-type" common tumor antigens from murine colon tumor cells(38, 40, 41, 43), common or tissue-specific immunoprotective antigens from rat colon tumors(39), biologically active, cross-protective embryonic antigens from Syrian golden hamster sarcomas(2), specific tumor-associated antigens from murine mammary tumors(33), common immunoprotective antigens from murine melanoma(5), and organ-specific cancer neoantigen from human cancer cell lines and primary breast cancers(16). Furthermore, more recently, we succeeded in clarifying TSTA extracted from a human breast cancer cell line that were involved in the cytotoxicity of autologous T-lymphocytes(44).

The fate of the host after tumor resection depends on the sinecomitant immunity, or ability to resist a second tumor challenge after excision of the primary tumor. The design and success of chemoimmunotherapy protocols utilizing tumor antigens may thus depend on the level of the host's sinecomitant immunity (6).

The therapeutic efficacy of butanol-extracted antigens in active-specific immunotherapy against postsurgical metastatic recurrence has been assessed in mice(6, 7, 45), demonstrating that combination therapy with extracted TSTA and CY was superior to either material alone(26). CY is a potent, versatile, and widely used antitumor agent(49). When high doses of CY are administered, the immune responsiveness of the host is totally suppressed(17, 19). In contrast, low doses of CY (20−100 mg/kg) have been reported to potentiate the host immune response by selectively eliminating supressor T-cells or their precursors(3, 18, 24). By combining TSTA and low-dose CY, a higher secondary antitumor immune

response was obtained(26).

From a purely immunological point of view, there still remains an unsettled question with respect to TSTA. In experimental models of immunotherapy that use TSTA extracted from syngeneic tumors, the spectrum of antigens to which an individual has been sensitized may be different from that of the syngeneic population as a whole. Recently, tumor heterogeneity has been demonstrated, wherein phenotypic traits of tumor cells, including tumor antigen expression and oncogene expression, were observed to vary(1, 20, 32). Antigenic heterogeneity may have a direct bearing on experimental models of tumor immunotherapy, where investigators have classically assumed that the use of syngeneic systems would ensure uniformity of response. We have directly tested this assumption through the comparison of completely autologous chemoimmunotherapy with semi-autologous and syngeneic models. We use the term "syngeneic" to denote extracts and challenge cells derived from an unselected parental MAC-F cell line propagated *in vitro*, while "autologous" materials are those derived from a single tumor resected from an individual mouse. Our results demonstrate the superiority of the totally autologous model and suggest that a greater degree of antigenic idiosyncrasy, or uniqueness, exists in individual tumors than was previously appreciated.

MATERIALS AND METHODS

1. *Mouse and tumor*

The antigenically distinct fibrosarcoma MCA-F cell line was induced in female C3H/HeJ mice with MCA(29) and was used in the sixth or seventh *in vivo* passage generations. The minimum tumorigenic dose of this tumor cell is 10^2. Tumors were maintained by serial s. c. passage in 4 to 6-week-old specific-pathogen-free female C3H/HeN (MTV$^-$) mice (Charles River, Kingston, NY) as previously descrived(9). By single cell cloning using a limiting dilution(42), MCA-F cell clones were established and propagated *in vitro* before use.

Single cell suspensions were prepared from individual tumors using 0.25% trypsin as described(9, 13). Usually, $1-5\times10^6$ viable cells were obtained from one 8 to 10 mm MCA-F tumor that was free of necrosis and bleeding. One-fifth of the dissociated cells were placed into culture to provide cells for challenge. The remaining cells were cultured separately for 5 days to provide a source of CBE.

2. *Butanol treatment and extraction of TSTA*

Tissue culture-propagated cells were released from the flask by brief incubation in 0.05% trypsin. Cells were washed 3 times in PBS prior to extraction

with 2.5% (v/v) 1-butanol in PBS, as previously described(9, 13). The viability of the extracted cells was routinely determined by trypan blue dye exclusion and was never less than 90%. The yield of protein obtained following butanol extraction of the cultured tumor cells was 10 μg/10^6 cells, Protein was determined using the Pierce protein assay (Pierce Chemical Co., Rockford, IL) with ovalbumin as standard.

3. *Experimental protocol*

In all experimental models secondary tumors were produced in mice by s. c. injection of either autologous or syngeneic cultured tumor cells into the contralateral flank following surgical resection of the initial tumors.

Experimeent I: Assay for CBE efficacy in the autologous tumor recurrence model.

Five groups of 10 C3H/HeN mice were challenged with 1×10^6 viable MCA-F cells in the right flank, and growing tumors were curatively resected when they reached a 10 mm diameter (counted as day 0).

After culturing for 5 days, the tumor cells were harvested and used to prepare CBE. On day 7, the mice received 50 μg of CBE protein s. c. into the distant site previously resected. The remaining cultured tumor cells were used for autologous challenge of 5×10^4 viable cells on day 14,. In control experiments, mice received either parental CBE or parental MCA-F challenge cells or both. As a "no-therapy" control group, we used a single s. c. injection of PBS.

Experiment II: Assay of chemoimmunotherapy with autologous CBE and CY in the autologous tumor recurrence system.

In order to examine the therapeutic efficacy of autologous CBE combined with CY, we used the same autologous tumor recurrence system described abobe. The CY (Cytoxan, Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water at a concentration of 20 mg/m*l*. Individual mice were weighed and given i. p. injections of CY and s. c. injections on day 7. The individual tumor of each mouse was measured 15, 21, and 27 days after challenge (29, 35, and 41 days after resection) as the average of 2 perpendicular measurements of tumor diameter with the use of a metric dial caliper, and the mean tumor diameter of each experimental group were calculated. Significant differences in mean tumor diameters of control and therapy groups or between therapy groups were determined by the Student-Newman-Keulus multiple comparison test. In addition, we monitored the median survival of individual mice in both experiments I and II, and obtained the 50% survival time (ST_{50}) of each group, in days. The ST_{50} was determined by observation of the percentage of mice surviving in each group, converted to probits and plotted against the logit of the days after challenge.

Intersection of probit value 5.0 with the regressed survival line yielded the ST_{50} as described previously(30). Statistical differences in ST_{50} were determined by Chi-square analysis. Tumor growth rate (mm/day) was also calculated from these data and converted to the tumor growth ratio using the following formula: Tumor growth ratio=experimental rate/control rate.

RESULTS

1. *Tumor growth suppression using autologous CBE*

The antitumor efficacy of fully autologous CBE immunotherapy against post-surgical secondary tumor challenge was compared with other combinations of extracts, challenge cells, and hosts (Table 1). Groups of 10 mice were treated s. c. with 50 μg of autologous or syngeneic CBE 7 days after resection of 10 mm tumors, and the mice were rechallenged s. c. on the contralateral flank with autologous or syngeneic MCA-F cells 1 week later. Although syngeneic CBE afforded some protection against autologous tumor challenge ($p<0.005$), the most potent immunotherapeutic effect was observed using the fully autologous

Table 1 *Effect of immunotherapy with extracted TSTA from autologous tumor cells in autologous tumor recurrence system.*

Groups of 10 mice were given s. c. injections into the right flank on day −14. On day 0, growing tumors were resected and tumor cells were divided into two groups; One for CBE and the other for challenge cells. One week following tumor resection, mice were treated with 50 μg CBE s. c. into the right flank. Mice were challenged s. c. with 5×10^4 cultured MCA-F cells in the left flank on day +14.

Therapy	Challenge cell	Host	Mean tumor diameter (mm±S. E.)[a]	p[b]		ST_{50}[c]	p[d]
PBS	Auto.	Auto.	23.3±1.3	—	<0.001	34.4	—
Auto. CBE	Auto.	Auto.	11.0±0.9	<0.001	—	53.3	<0.025
Auto. CBE	Auto.	Syn.	14.3±0.7	<0.001	NS	46.6	NS
Auto. CBE	Syn.	Auto.	14.5±0.6	<0.001	NS	40.4	NS
Syn. CBE	Auto.	Auto.	17.2±2.0	<0.005	<0.01	46.0	NS

[a] Mean tumor diameter (mm±S. E.) at 27 days after challenge.

[b] Statistical difference was determined using the Student-Newman-Keuls multiple comparison analysis. Left column: Mean tumor diameter in the control group was compared with that in immunotherapy qroups; Right column: Measurements in the autologous CBE, challenge cells and mouse group was compared with that in the other groups. NS, not significant.

[c] ST_{50}, the 50% survival time in days.

[d] Statistical difference was determined by Chi-square analysis. ST_{50} in the control group was compared with that in immunotherapy groups.

materials ($p<0.001$ v. s. PBS control). Immunization with autologous CBE followed by challenge with the syngeneic parental tumor yielded an intermediate reduction in tumor growth, as did the combination of autologovs CBE and challenge in a mouse in which a different MCA-F neoplasma had been resected (Table 1).The importance of the autologous CAE in boosting the immune response of mice is shown by the relatively good prognosis for these mice, compared with mice treated with syngeneic extract ($p<0.01$).

The ST_{50} of the fully autologous treatment regimen was also significantly increased, when compared with that of no treatrent or semi-autologous therapy (Table 1). Furthermore, the tumor growth rate and growth ratio was also significantly reduced in the fully autologous therapy system (Fig. 1). The 60% reduction in growth rate from 1.3 to 0.5 mm/day is consistent with an ongoing cytotoxic immune response that continually reduces the proliferating fraction within the challenge population (31). Thus, in the lacal tumor recurrence model used here, the fully autologous combination of extracted TSTA and challenge cells appeared to provide superior stimmulation of protective immunity.

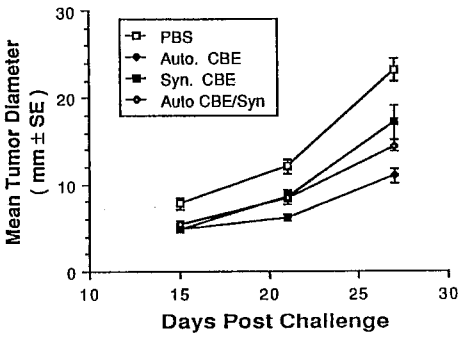

Fig. 1 Autologous tumor antigen therapy: Mice received either PBS or 50 μg of autologous or syngeneic CBE. Mice were challenged with autologous tumor cells, except for one group that received parental MCA-F tumor cells. Mean tumor diameter was determined at 15, 21, and 27 days after challenge. Growth rates were obtained by linear regression.

2. *Combination chemoimmunotherapy in the autologous tumor recurrence model*

Treatment of mice i. p. with 20 mg/kg CY on the same day that they recieved 50 μg of autologous CBE significantly inhibited the growth of secondary autologous tumor challenge (Table 2), when compared with atologous extracts alone ($p<0.05$) or syngeneic extract in combination with CY ($p<0.05$). This dose of CY without concurrent antigen therapy had no effect on tumor growth. Combination chemoimmunotherapy with autologous CBE and CY significantly increased the ST_{50} ($p<0.01$). The tumor growth ratio for the autologous CBE without CY was similar to that observed in the previous experiment (0.35), while autologous chemoimmunotherapy reduced the growth ratio to 0.27, the lowest value for any experimental group that we have studied (Fig. 2). Again, the observation of a flattened growth curve for the chemoimmunotherapy group

Table 2 *Effect of combination chemoimmunotherapy with CBE and CY in autologous tumor recurrence system.*

Groups of 6 mice were given s. c. injections of tumor cells in the right flank on day −14. On day 0, tumors were resected and cultured for CBE and challenge cells. One week following tumor resection, mice were injected with 50 μg CBE s. c. alone, 20 mg/kg CY i. p. alone, or a combination of autologous or syngeneic CBE and CY in the right flank. Mice were challenged s. c. with 5×10^4 cultured MCA-F cells in the left flank 7 days later.

Therapy	Mean tumor diameter (mm±S. E.)[a]	p[b]		ST_{50}[c]	p[d]
PBS	18.4±1.0	—	<0.001	33.4	—
Auto. CBE	9.0±0.5	<0.001	<0.05	52.3	<0.05
CY	15.9±0.6	<0.05	<0.001	41.9	<0.05
Auto. CBE+CY	6.8±0.8	<0.001	—	59.6	<0.01
Syn. CBE+CY	10.8±11.0	<0.001	<0.05	49.2	<0.05

[a] Mean tumor diameter (mm±S. E.) at 27 days after challenge.

[b] Statistical difference was determined by the Student-Newman-Keuls multiple comparison analysis. Left column: Mean tumor diameter in the control group was compared with that in chemoimmunotherapy groups; Right column: The measurements in the autologous CBE plus CY group was compared with the other therapy groups.

[c] ST_{50}, the 50% survival time in days.

[d] Statistical difference was determined by Chi-square analysis. ST_{50} in the control group was compared with that in the other therapy groups.

is consistent with a strong antitumor immune response continuing over the course of several weeks, engendered by a single treatment with 50 μg autologous CBE and 20 mg/kg CY.

DISCUSSION

Chemically and virally induced tumors possess not one, but several antigens on their surface capable of eliciting protective immune responses in syngeneic hosts(10, 47). Progress in the purification of tumor cell surface antigens has yielded information on the biochemical characteristics of tumor-associated antigens, such as common tumor rejection antigens(5, 39, 40), oncofatal antigens(2), and

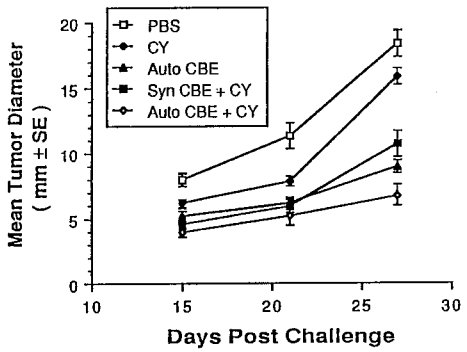

Fig. 2 Combination chemoimmunotherapy of autologous tumors: Mice were treated with 50 μg of autologous CBE, either alone or in combination with 20 mg/kg CY. Controls consisted of mice treated with PBS, with CY alone, or with syngeneic CBE plus CY. Tumor growth was measured at 15, 21 and 27 days after challenge.

individual TSTA(9, 23, 34). Chemically induced tumors express both individvally distinct TSTA and cross-reactive common antigens(8, 41). In addition to common and unique tumor rejection antigens, tumors frequently express antigens that can engender specific unresponsiveness to tumor challenge through the elicitation of suppressor T-lymphocytes(12, 25, 48). Thus, the goal of immunotherapeutic protocols is both the facilitation of protective responses against common or unique tumor antigens and the inhibition of suppressogenic reactivities.

In the same tumor model used here, others(6, 7, 45) demonstrated the immunotherapeutic effect of 3 M KCl-extracted TSTA against postsurgical tumor recurrence. Experiments using butanol-extracted TSTA suggested the possibility of immunotherapy for cancer in human as a means of enhancing sinecomitant immunity(9, 11-15, 26, 40, 41, 43). The relatively high specific immunoprotective activity of CBE, and the absence of MHC components, have also served to recommend butanol-extracted materials for immunotherapy(11, 26).

More recently, it has become clear that tumor antignes are not uniformily distributed on all tumor cell surfaces, but rather that individual tumor subpopulations may differentially express sets of antigens. The origin of the MCA-induced tumors is thought to be multi-focal(35) and antigenically heterogeneous(28, 31, 37). Thus, the anti-tumor immune response is not a limited response to a single, uniformly expressed epitope, but the result of various antigenic stimuli.

The antigenic heterogeneity of tumor cells might be useful for cancer therapy (4). Evidence for heterogeneity in the T-cell lineage that recognized tumor antigens has been reported(22, 46), and the enhancement of cellular immunity is thought to be one of the most powerful means of tumor rejection. Therefore, it is quite important to confirm the individually and specificity of TSTA in the tumor-bearing host.

This is the first preliminary report of autologous TSTA immunotherapy against tumor recurrence. Combination chemoimmunotherapy with autologous TSTA and low-dose CY proved even more effective against tumor recurrence, suggesting that postsurgical sinecomitant immunity might be complemented by combination therapy using autologous tumor cell extracts.

The question of why autologous TSTA was more effective than the syngeneic TSTA remains perplexing. It is possible that 1) the response of memory T-cells that recognized the antigens of the primary tumor were better able to protect the mice after the secondary antigenic stimulation, resulting in propagation and potentiation of specific CTL or other effectors, or 2) the spectrum of tumor antigens expressed by each tumor is affected by the antigenic stimuli and effector responses. There remain several unsolved problems concerning host immunity. As Nomi *et al.*(27) mentioned, the relationship between concomitant

immunity and tumor metastasis may depend upon factors such as in the amount of tumor antigen, the degree of tumorigenicity, and the duration of tumor growth. One would also expect the potency of sinecomotant immunity after tumor resection would also differ between individuals.

Finally, we have shown that combination chemoimmunotherapy with autologous TSTA and low-dose CY yielded potent inhibition of autologous tumor growth. The effect of CY in this study was probably attributable to its effect on suppressor T-cells(3, 18, 24, 49), rather than a directly cytotoxic antitumor effect (36, 49). This conclusion is supported by the observation that low-dose CY without antigen did not diminish tumor growth. Although superior antitumor effectiveness with low rather than high doses of CY have been reported(21), the data presented here demonstrate that the combination of low-dose CY and butanol-extracted autologous TSTA produced the best antitumor response in our autologous tumor recurrence model. More fundamental studies *in vivo* are needed to improve therapeutic efficacy of chemoimmunotherapy combined with autologous TSTA and antitumor drug before aplication for clinical use in human cancer.

ACKNOWLEDGEMENTS

The authors thank Drs. M. L. Kripke and N. R. Pellis for their helpful discussions, and Ns. Helen Farr and Ms. Keiko Honma for their expert secretarial assistance. This study was supported by NCI Grant CA 38500, and by a grant from the University Cancer Foundation.

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