Allogeneic Cell Therapy for a Murine Mammary Carcinoma

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ABSTRACT

The effect of allogeneic cell therapy on tumor growth was studied in a murine model of mammary carcinoma (4T1) as an experimental model of solid tumors in humans. i.v. inoculation of 4T1 (H-2d) cells into syngeneic mice [BALB/c or (BALB/c×C57BL/6)F1] (F1) carrying the H-2d histocompatible antigens results in tumor colonies in the lungs that finally cause the death of all of the mice. Sublethally irradiated F1 mice were inoculated with 4T1 cells to simulate minimal residual disease and with immunocompetent splenocytes derived from naive donors of F1 (syngeneic), BALB/c (syngeneic to the tumor but semiallogeneic to the host), or C57BL/6 (allogeneic to the tumor and semiallogeneic to the host) mice. The survival of F1 tumor-bearing mice that were treated with allogeneic C57BL/6 splenocytes was significantly prolonged (P < 0.02) compared with hosts given F1 or BALB/c-derived splenocytes that are syngeneic to 4T1 tumor cells. Adoptive transfer of lung cells that were isolated from F1 primary mice inoculated with 4T1 cells and syngeneic BALB/c or F1 splenocytes led to local tumor growth and death in secondary recipients. In contrast, only 1 of 22 secondary recipients developed tumors when inoculated with lung cells derived from F1 mice given allogeneic C57BL/6 splenocytes. All of the 21 secondary hosts survived disease-free for a follow-up time of >200 days. These results indicate that immunocompetent cells allogeneic to the mammary carcinoma cells were able to inhibit tumor development in the primary hosts and to prevent tumor growth in the adoptive recipients, which suggests that allogeneic cell therapy may be an efficient antitumor tool to eradicate minimal residual disease in human solid tumors.

INTRODUCTION

Disease recurrence of malignant hematopoietic cells and certain solid tumors is the most frequent complication after conventional and even after high-dose chemoradiotherapy combined with autologous stem cell transplantation (1, 2). In patients with acute and chronic leukemia as well as in patients with other malignancies of the hematopoietic system, eradication of residual disease or reversing relapse can be accomplished by infusion of allogeneic immunocompetent cells that induce the GVL effect, as has been shown at our center and can be accomplished by infusion of allogeneic immunocompetent splenocytes derived from naive donors of H-2d, syngeneic to 4T1 tumor-bearing mice. The more recent observation suggesting that alloBMT may induce measurable responses against metastatic breast cancer (14).

MATERIALS AND METHODS

Mice. BALB, C57, and F1 mice aged 10–12 weeks, were purchased from Harlan Sprague Dawley and maintained in the animal facility of the Hadassah University Hospital, with food and water ad libitum, in full compliance with all of the regulations for protection of animal rights.

Tumor. 4T1 is a BALB-derived tumor cell line (H-2d) established from a cell subpopulation isolated from a single, spontaneously arising mammary tumor of a BALB/cfC3H mouse where the "f" stands for fostering (15). 4T1 cells were maintained by passage in vitro in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, and 1% nonessential amino acids. Preparation of cells for injection included harvesting by 0.25% trypsin in 0.05% EDTA, washing the cells in DMEM culture medium, and resuspension in RPMI 1640 containing 10% fetal bovine serum.

For adoptive transfer experiments, lungs were removed from these F1 mice on day 10 after tumor inoculation and, after teasing into single cell suspensions, were injected i.v. in mice in a volume of 0.1 ml or 0.25 ml, respectively. All of the tissue culture media and reagents were purchased from Biological Industries (Beit Ha’emek, Israel). Cells were kept at 37°C in a humidified 5% CO2/air incubator.

Measurement of Primary Tumor Growth in Vivo. Local i.d. tumors were measured once a week in two perpendicular dimensions with a Taxol-(r) pitemeter caliper. Tumor size in cm3 was calculated by the formula (a × b2)/2, where b is the smaller dimension of the tumor.

Experimental Design of Cell Therapy. F1 mice were conditioned by nonlethal TBI (4 Gy) or lethal TBI (11 Gy), using a 6-MeV linear accelerator at a dose rate of 1.9 Gy/min. Sublethally irradiated F1 recipient mice were inoculated i.v. 24 h later with 2 × 104 4T1 cells and 4 h afterward with 15 × 106 splenocytes derived from F1, BALB, or C57 mice. F1 mice conditioned with lethal TBI (11 Gy) were reconstituted 24 h later with 15 × 106 syngeneic F1 BM cells given together with the splenocytes. Twenty-four hours after BM reconstitution and splenocytes inoculation, mice were inoculated i.v. with 0.25 ml of 2 × 104 4T1 tumor cells. In some experiments, as detailed in the "Results," mice were inoculated i.d. with 0.1 ml of 2 × 104 4T1 tumor cells. BM cells were prepared by flushing RPMI 1640 through the shafts of the femora of the donors with a 25-gauge needle followed by centrifugation at 230 × g and resuspension in Hanks’ medium for i.v. injection. Splenocytes were prepared by teasing spleens into single cell suspensions, centrifugation at 230 × g, and resuspension in Hanks' medium for an i.v. injection of 0.25 ml. For adoptive transfer experiments, lungs were removed from these F1 mice on day 12 after tumor inoculation and, after teasing into single cell suspensions, were injected i.d. in a volume of 0.1 ml into naive secondary BALB hosts.

Statistical Analysis. The statistical significance of tumor size was evaluated by the standard two-tailed, unpaired, Student t test. The Kaplan-Meier method was used to calculate the probability of survival as a function of time after tumor inoculation or after adoptive transfer of lung cells into naive.
RESULTS

Induction of i.d. and Lung Tumors

Injection of 4T1 (H-2d) cells into syngeneic hosts carrying the H-2d histocompatible antigens resulted in local tumor growth after i.d. inoculation or in tumor colonies in the lung after i.v. injection. Tumor colonies were isolated from the lungs of mice injected i.v. with 4T1 tumor cells and adoptively transferred i.d. into naive syngeneic recipients leading to local tumor growth and the death of the secondary hosts from pulmonary metastases. Adoptive transfer of 4T1 from pulmonary colonies was time dependent and resulted in tumor development in the secondary hosts only if allowing for a sufficient time in the primary hosts before isolation of tumor cells for adoptive transfer into the secondary recipients (Fig. 1). Lung cells isolated 7 days after i.v. inoculation with $2 \times 10^4$ 4T1 cells did not result in tumor development in the secondary hosts for a follow-up period of >42 days whereas the same number of lung cells isolated after 14 and 22 days led to marked local tumor growth (0.819 and 1.763 cm$^3$, respectively) as measured in naive secondary BALB recipients on day 28 after adoptive cell transfer (Fig. 1). The consistent kinetics for adoptive transfer of lung metastases was used to design an experimental system to test the effect of alloCT on metastatic and local tumor cell growth as described below. Adoptive transfer of lung tumor colonies to secondary recipients was used to determine the presence or elimination of minimal residual disease after alloCT.

The Effect of alloCT on i.d. Tumor Growth

F1(H-2d/b) mice were exposed to nonlethal whole body irradiation (4GY) and on the next day were inoculated i.d. with $2 \times 10^4$ 4T1 cells to simulate minimal residual disease. On the day of tumor inoculation, all of the 22 mice were assigned to 4 experimental groups, each given i.v. $15 \times 10^6$ splenocytes derived from F1(H-2d/b), BALB(H-2d), or C57(H-2b) mice. A control group of mice did not receive splenocytes. The effect of allogeneic splenocytes on tumor growth was evaluated by serial tumor measurements as shown in Table 1. Tumor size was significantly lower in mice receiving C57 cells fully allogeneic to the 4T1 tumor cells compared with tumor sizes measured in mice inoculated with BALB- or F1-derived splenocytes syngeneic to the 4T1 tumor cells ($P = 0.04$ and 0.03 for day 28 and 36, respectively). Tumor size measurements in the control group of mice which did not receive splenocytes were similar to numbers measured in experimental groups given syngeneic splenocytes. Reduced tumor size did not affect the survival of C57-inoculated F1 mice (median = 45 days) which followed a pattern similar to that of BALB-treated F1 mice (median = 47 days). In these two experimental groups, BALB→F1 and C57→F1, splenocytes were semiallogeneic to F1 hosts and may have caused GVHD; however, survival of these mice was not statistically different ($P > 0.1$) from survival of untreated controls (median = 56 days) or F1 mice inoculated with F1 splenocytes (median = 68 days). The same experimental design used for mice that received syngeneic and allogeneic splenocytes i.d. into the tumor area, did not affect tumor size (data not shown).

The Effect of alloCT on Growth of Lung Metastases Induced by i.v. Inoculation of 4T1 Tumor Cells

The fact that alloCT with donor lymphocytes that was given i.v. effectively reduced i.d. tumor growth (Table 1) led us to test the response to i.v. alloCT on lung tumors induced by i.v. inoculation of 4T1 cells. Inoculation of allogeneic splenocytes into recipients exposed to nonlethal or lethal irradiation induces a GVH disease that may cause the death of tumor-bearing recipients. Because our final goal was to detect evidence of GVT effects, we decided to bypass lethal GVHD by designing adoptive transfer experiments in which lung tumor cells isolated from primary hosts were inoculated i.d. into secondary syngeneic recipients; therefore, local tumor growth could be monitored by serial measurements and death in these secondary hosts would be related to tumors only.

A. alloCT after Sublethal Irradiation. F1 mice were exposed to nonlethal irradiation (4GY) and 24 h later were inoculated with $2 \times 10^4$ 4T1 cells and $15 \times 10^6$ splenocytes derived from either syngeneic F1, mice (group 1); BALB donors (group 2), semiallogeneic to the host but syngeneic to the tumor; or C57 donors (group 3), semiallogeneic to the host and allogeneic to the tumor. Twelve days later, lungs were removed and $1.5 \times 10^6$ cells were adoptively transferred i.d. into naive BALB mice. In each experimental group, five mice of the original F1 hosts were kept to determine survival of the primary tumor recipients. Fig. 2 presents the probability of survival of these primary F1 hosts shown by Kaplan-Meier curves. The probability of survival at day 60 was 0%, 7%, and 50% in F1 mice treated with splenocytes derived from F1, BALB, and C57 mice, respectively. Survival time of mice treated with C57-derived splenocytes fully allogeneic to the 4T1 tumor cells was significantly longer than in untreated mice.

### Table 1: The effect of syngeneic and allogeneic splenocytes on i.d. tumor growth and the survival of sublethal irradiated (BALBXC57) F1 mice

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$^a$ Tumor size presented as the mean ± SE of 5–6 mice in the experimental groups.

$^b$ Tumor size was significantly smaller in F1 mice treated with spleen cells derived from C57 mice ($P = 0.04$ and 0.03 for day 28 and 36, respectively) than in F1 mice treated with spleen cells derived from BALB or F1 mice or from untreated mice.

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Fig. 1. Tumor sizes measured in BALB recipients on day 28 after adoptive transfer of $1.5 \times 10^6$ lung cells derived from (BALBXC57) F1 mice that were inoculated i.v. with $2 \times 10^4$ 4T1 tumor cells. Adoptive transfer was carried out on days 7, 14, and 22 after tumor inoculation (5 mice/time point).

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Probability of survival on day 200, calculated from Kaplan-Meier curves, was 0%, 21%, and 95.5% in adoptive recipients inoculated with 15 x 10^6 splenocytes, syngeneic (BALB (H-2d) and F, (H-2d/h)) or allogeneic [C57(H-2h)] to the tumor cells. Experimental groups treated with syngeneic or allogeneic splenocytes consisted of 10-14 mice pooled from three experiments.

Adoptive transfer experiments were carried out under the same conditions as described for mice exposed to sublethal irradiation (section A). i.d. tumor sizes in the secondary recipients are listed in Fig. 5. A marked antitumor effect of alloCT was noted when using C57 splenocytes against metastatic disease in mice bearing 4T1 tumor cells whenever fully allogeneic immunocompetent cells are used for immunotherapy. Disease-free survival for the C57→F, experimental group was significantly prolonged (P = 0.0001) compared with that of secondary recipients inoculated with lung cells derived from F,→F, recipients (group 1) or BALB→F, recipients (group 2).

B. alloCT after Lethal TBI and Syngeneic Reconstitution. F, mice exposed to 110 Gy and reconstituted with syngeneic F,-derived BM cells were inoculated with 15 x 10^6 splenocytes derived from 1) F, mice, 2) BALB mice, or 3) C57 mice; 24 h later, they were inoculated i.v. with 2 x 10^4 4T1 tumor cells to simulate minimal residual disease after autologous BM transplantation. Nine F, mice given F, splenocytes died of lung metastases within a median of 29 days (range, 24–52) after tumor inoculation as did the control group of 11 naive F, mice inoculated with tumor cells only (median, 31 days; range, 24–52 days). F, mice that were reconstituted with F,-derived BM after lethal TBI and given BALB splenocytes or C57 splenocytes developed GVHD, which was severe in 10 BALB-treated F, mice leading to their death without lung metastases within a median of 10 days (range, 7–11 days,) and moderate GVHD in 7 C57-treated F, mice, which died with very small lung metastases within a median of 29 days (range, 17–70). In parallel, control groups of F, mice that were exposed to lethal TBI, given syngeneic F, BM cells, and treated with splenocytes derived from F, or C57 mice but not with tumor cells were monitored for GVHD-related death. Mice inoculated with BALB or C57 splenocytes developed severe or moderate GVHD, respectively, and died within a median of 12 and 22 days, respectively, whereas mice that were given F, splenocytes without subsequent tumor cells survived for over 140 days. These results indicate that mice of experimental groups 2 and 3 died of GVHD, whereas mice in group 1 died because of tumor development.
inoculated with 4T1 tumor cells only (group 4). All of the mice with measurable tumors died within days 39 and 57, whereas secondary hosts inoculated with lung cells derived from C57-treated mice were alive and tumor free for more than 200 days (Fig. 6). Survival of the secondary recipient inoculated with F1-derived lung cells given C57 splenocytes (group 3) was significantly different (P = 0.0001) from survival of secondary recipients inoculated with lung cells derived from F1 mice not treated (group 4) or treated with F1 (group 1) or BALB (group 2) splenocytes, as calculated by the log-rank test. Probability of survival at day 52, as calculated by Kaplan-Meier curves was 12.5, 14, 100, and 22% in groups 1, 2, 3 and 4, respectively.

DISCUSSION

alloCT was used to treat a murine mammary carcinoma as a potential new modality for metastatic epithelial tumors. The series of experiments presented in this study demonstrate the induction of effective antitumor responses in mice inoculated with immunocompetent H-2b-lymphocytes, allogeneic to H-2b-mammary carcinoma cells. In contrast, H-2b lymphocytes, which were semiallogeneic to the tumor-bearing host (H-2b) but syngeneic to the tumor cells, or immunocompetent H-2b-lymphocytes, which were syngeneic to both the tumor-bearing host and the tumor cells, were completely ineffective in eradicating metastatic tumor cells. Under the experimental conditions used, the adoptive transfer of lung cells obtained from mice inoculated with only 4T1 cells resulted in consistent metastatic disease and death in all of the secondary recipients. The prevention of tumor growth in secondary recipients of lung cells that were obtained from mice treated with lymphocytes allogeneic to the tumor cells indicated eradication of metastatic disease in response to alloCT.

The fact that local i.d. tumor was only partially reduced in size after i.v. infusion with allogeneic immunocompetent cells and not in response to i.d. inoculation emphasizes the importance of the traffic of antitumor effector cells through the blood vessels. Lymphocyte migration to the tumor through the blood vessels may be associated with better homing and adhesion of effector cells to the target cells. After both sublethal and lethal TBI, alloCT induced marked GVT effects. Despite the strong GVH reaction that was induced by BALB splenocytes after lethal TBI, no antitumor response could be induced. It seems that in our murine model, the GVH reaction was not sufficient to induce a GVT effect. Our results (a) emphasize the importance of using immunocompetent cells that are fully mismatched to the tumor cells; and (b) suggest that the curative GVT effect is not merely a by-product of a GVH reaction directed against host alloantigens but rather is caused by recognition of tumor antigens presented in the context of alloantigens and being presented to MHC-restricted T lymphocytes. A direct identification of GVT effector cells will help in designing an efficient therapy in tumor-bearing hosts and may be safer for application in cancer patients.

GVT effects against solid tumors were documented previously in our laboratory in (NZB X NZW) F1 female mice developing spontaneous sarcoma (18); 24% of (NZB X NZW) F1 recipients developed spontaneous sarcoma at 1 year of age, whereas no tumor was observed in BALB/c->NZB X NZW F1 chimeras with no clinical manifestations of GVHD. Similarly, our recent publication (14), with the same 4T1 metastatic mammary carcinoma model, indicated statistically significant reduced primary tumor cell growth in C57->(BALB X C57) F1 and DBA2->BALB chimeras in comparison with untreated controls, again pointing to the existence of GVT effects. Preliminary clinical experience at our center suggests that GVT effects may also be induced by allogeneic peripheral blood lymphocytes derived from histocompatible siblings in patients with metastatic breast cancer after autoBMT (19) or alloBMT (11). Transient elimination of metastatic liver disease in one patient and prolongation of disease-free survival in five additional patients was documented in patients undergoing alloCT with donor lymphocyte infusion after autoBMT (19). Prolonged alloCT effects may be achieved in recipients after alloBMT when host-versus-graft tolerance, induced by infusion of donor stem cells, may enable durable GVT effects in stable chimera (11).

The data presented here, together with our work published recently (14), suggest that GVT effects that are mediated by allogeneic lymphocytes could be used as a new modality in patients who are fully resistant to available chemotherapy. Although GVL effects (20–22) and GVT effects (11, 14)—independent of GVHD—were previously demonstrated both in experimental models and in humans, in clinical practice, one should always be aware of the relationship between beneficial GVT effects and detrimentalGVHD. Induction of GVL or GVT effects by alloCT carries the risk of uncontrolled GVHD (3, 4).

One way to overcome the development of uncontrolled GVHD is donor lymphocyte infusion in graded increments (4). We have shown
previously that by increasing the time interval between alloBMT and alloCT, a greater resistance to GVHD develops (21, 23). This phenomenon, also confirmed by others (24), suggests that alloCT may be induced later after alloBMT, preferably in an outpatient setting, while controlling for GVHD by infusion of graded increments of donor lymphocytes with the option to discontinue therapy as soon as the threshold of GVHD is reached. In the future, better control of GVHD, while exploiting the GVL and GVT potential of donor lymphocytes, may be accomplished by introducing the thymidine kinase suicide gene into donor lymphocytes before stem cell infusion, thus providing the option to eradicate donor lymphocytes if uncontrolled GVHD develops (25).

An immunological approach such as alloCT may be a practical way of controlling residual disease in patients with metastatic breast cancer or other metastatic solid tumors who are at high risk to relapse or with evidence of disease fully resistant to all of the other available modalities. Our experimental system could be useful for testing GVT effects mediated by allogeneic lymphocytes leading to the more effective and safer cure of metastatic diseases.

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REFERENCES


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