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Demonstration of Immunogenicity with the Poorly Immunogenic B16 Melanoma[1]

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ABSTRACT

Animal tumors of spontaneous origin have consistently been found to be less immunogenic than are tumors induced by chemical carcinogens or oncogenic viruses. Spontaneous tumors rarely demonstrate significant immunogenicity in classical transplantation rejection tests. This study demonstrates a novel approach to the question of tumor immunogenicity using viable tumor cells in diffusion chambers. Classical transplantation rejection was induced to the poorly immunogenic, spontaneous B16 murine melanoma by implantation of diffusion chambers containing viable B16 melanoma tumor cells. Implantation of B16 chambers i.p. for at least 4 weeks induced specific, long-term resistance to subsequent live B16 tumor cell challenge. In contrast, immunization with irradiated tumor cells for the same time interval resulted in a delay of tumor growth but had no effect on survival. These studies demonstrate the possibility that a significant antitumor immune response against the B16 melanoma may be induced without the presence and negative regulatory influences of a progressively growing B16 tumor. In addition, the diffusion chamber sensitization technique may detect the antigenicity of some tumors which are not detectable by classical transplantation rejection tests. Thus, the diffusion chamber technique provides another avenue for testing the immunogenicity of tumors, especially those of spontaneous origin.

INTRODUCTION

In 1976, Hewitt *et al.* (9) summarized their attempts to demonstrate the existence of tumor-specific antigens on 27 mouse tumors of spontaneous origin using standard transplantation techniques. With 7 randomly selected tumors, prior immunization of recipients with homologous lethally irradiated cells failed to provide protection against subsequent live tumor cell challenge. This lack of immunogenicity was attributed to a lack of tumor-specific antigens and has been noted by other investigators (8–11, 20). The apparent lack of tumor-specific antigens on tumors of spontaneous origin has resulted in a call for reappraisal of the prospects for specific immunotherapy of human cancer (1).

The B16 murine melanoma is a rapidly growing, metastatic tumor of spontaneous origin (7). It has been used in a wide variety of studies on tumor metastasis and has generally been characterized as poorly or nonimmunogenic (6, 12). Studies by Bystryn (6) and Poskitt *et al.* (14) have demonstrated that the B16 melanoma sheds soluble TAA.[3] The TAA shed by the B16 melanoma tumor could induce a humoral response in the syngeneic host; however, immunization by the TAA shed by cultured B16 melanoma monolayers gave only a delay in growth following challenge with viable B16 tumor cells.

The diffusion chamber was introduced by Prehn *et al.* in 1954. The filter of the diffusion chamber is impermeable to cells but allows free passage of body fluids and macromolecules (15, 17). The chamber allows the culturing of viable tumor cells within the peritoneal cavity of an animal without the biological consequences of a growing, invasive tumor (15, 17, 18). The primary objective of these studies was to determine if B16 melanoma grown in diffusion chambers could induce specific sensitization of the host that results in transplantation rejection of a secondary minimum lethal B16 tumor cell challenge. This study demonstrates that transplantation resistance can be induced by viable B16 melanoma cells growing in diffusion chambers despite the fact that this tumor is poorly immunogenic in classical transplantation rejection tests.

MATERIALS AND METHODS

Eight-week-old male C57BL/6J mice were obtained from West Seneca Laboratories (West Seneca, N. Y.) and were fed a standard diet of pellets and tap water *ad libitum*.

Tumors. B16 murine melanoma cells were established in tissue culture in this laboratory and were originally obtained from The Jackson Laboratory (Bar Harbor, Maine). This tumor is of spontaneous origin and does not regress spontaneously (7).

MCA-38 cells were established in tissue culture in this laboratory by Tan *et al.* (19). The original tumor line was obtained by chemical induction using 1,2-dimethylhydrazine.

Tumor Cell Suspensions. Tumor cell suspensions were obtained by exposure of tumor cell monolayers in culture to a solution of 0.25% trypsin and 0.25% EDTA. The suspension was washed and resuspended in Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.). Each cell suspension was counted by means of a Neubauer hemocytometer and adjusted to the desired tumor cell concentration. Viability of suspensions was assessed by exclusion of 0.2% trypan blue. Cell suspensions with viability exceeding 99% were used.

Diffusion Chambers. Diffusion chambers were assembled as previously described (2). Briefly, two 1.25-cm-diameter, 0.22-μm-pore size Millipore filters (Millipore Corp., Bedford, Mass.) were glued to either side of a 1.25-cm-diameter Lucite ring. Assembled chambers were sterilized by dry heat (320°, 2 hr) prior to inoculation with tumor cells or media. B16 or MCA-38 diffusion chambers were inoculated with 1×10^5 (0.1 ml) of the appropriate cells through a porthole in the Lucite ring. The porthole was then sealed with paraffin wax. Diffusion chambers were implanted and removed by midline laparotomy of ether-anesthetized animals. After *in vivo* incubation of the loaded chambers was complete, the contents of the chambers were tested for viability by trypan blue exclusion and for tumorigenicity by bioassay.

Classical Immunization. Spent medium from monolayers of B16 cells was harvested by the protocol of Poskitt *et al.* (14). Intact B16 cells were arrested in their growth by exposure to 10,000 rads of irradiation (G. E.

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[3] The abbreviations used are: TAA, tumor-associated antigens; MCA-38, murine colon adenocarcinoma tumor.

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Maxitron 250). Groups of 10 mice received 1.0 ml of spent tissue culture media or 10^6 irradiated cells once a week for 4 weeks.

Tumor Challenges. All tumor challenges were done 5 days after removal of the diffusion chambers or 7 days after classical immunization. Mice received 5×10^3 B16 melanoma cells s.c. for B16 challenges and 1×10^5 MCA-38 cells for MCA-38 challenges. These doses were selected from dose-response studies as the minimum threshold doses to give reproducible 100% tumor takes in normal animals. All challenges were given in the left medial thigh allowing weekly observation of tumor appearance and growth as well as survival.

Experimental Design. In the first experiment, groups of 8 to 12 mice received diffusion chambers containing 1×10^5 viable B16 melanoma tumor cells or media alone as a control. The chambers were removed from the animals at 1, 2, 4, or 8 weeks. Five days after the chambers were removed, the mice received a challenge of 5×10^3 B16 cells. Challenged mice and controls were monitored weekly.

Next, the effect of conditioned media in diffusion chambers was examined. Media from confluent B16 melanoma cultures were placed into chambers which in turn were placed into mice. The chambers were removed at 4 or 8 weeks, and the mice were challenged.

The viability and tumorigenic potential of the B16 cells within the diffusion chambers following i.p. implantation were examined. The contents of 15 randomly selected chambers were tested upon removal of the chamber.

The longevity of the induced immune response was studied in animals shown to be immune after 8-week B16 chamber immunization. Animals remaining tumor free 25 weeks after the initial B16 challenge were rechallenged with 5×10^3 B16 cells and monitored for tumor appearance, growth, and survival. The specificity of the induced immune response was tested by immunizing mice with either B16 or MCA-38 in diffusion chambers and then cross-challenging the immunized mice. Chambers containing media alone were used as controls for nonspecific immunostimulation. Classical immunization protocols consisted of immunizing mice with spent tissue culture media from B16 cells as well as irradiated tumor cells.

Statistical Analysis. Survival data for the different chamber exposure times were analyzed by Student's *t* test. Tumor incidence data were analyzed by χ^2 analysis. Statistical significance between experimental and control results was assessed to the 95% confidence level.

RESULTS

In the first experiment, the efficacy of immunization by diffusion chambers containing viable B16 melanoma tumor cells was investigated. Normal 8-week-old male C57BL/6J mice received i.p. diffusion chambers containing 10^5 B16 cells. Control animals received sterile media in chambers. The chambers were removed at 1, 2, 4, or 8 weeks, and the mice were challenged with 5×10^3 B16 cells 5 days later. Any mice showing signs of chamber leakage at the time of removal were excluded from the study. Chart 1 illustrates the effect of B16 chamber exposure time as a function of percentage of survival. Implantation of B16 chambers for 1 or 2 weeks had no effect upon challenge tumor growth and lethality. Four- or 8-week implantation, however, gave a dramatic increase in survival (87 and 75% respectively; $p > 0.005$). Implantation of chambers containing sterile media had no effect upon the challenge tumor. This indicates that implantation of the chamber itself does not provide a significant nonspecific immunostimulus. It was also discovered that conditioned media from confluent B16 monolayers had no effect upon tumor growth when placed in diffusion chambers. This outcome may be a dose-related phenomenon.

To establish the viability and tumorigenic potential of cells within the chambers following i.p. implantation, 15 chambers were selected at random at the time of removal for further study. The chambers contained a gray gelatinous mass with darkly pigmented spots scattered throughout. Cell viability exceeded 90% by trypan blue exclusion, even after 8-week implantation. In addition, *in vivo* implantation of chamber contents resulted in tumors in 14 of 15 of the s.c. transplants.

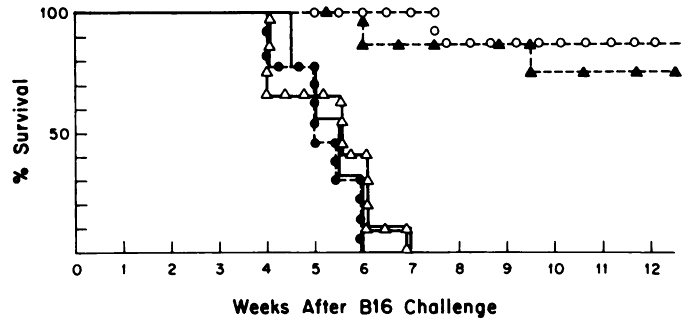

Chart 1. Survival of B16 diffusion chamber-immunized mice following challenge with 5×10^3 viable B16 tumor cells. ——, chamber control (media); △, B16 chamber, 1 week; ●, B16 chamber, 2 weeks; ○, B16 chamber, 4 weeks; ▲, B16 chamber, 8 weeks.

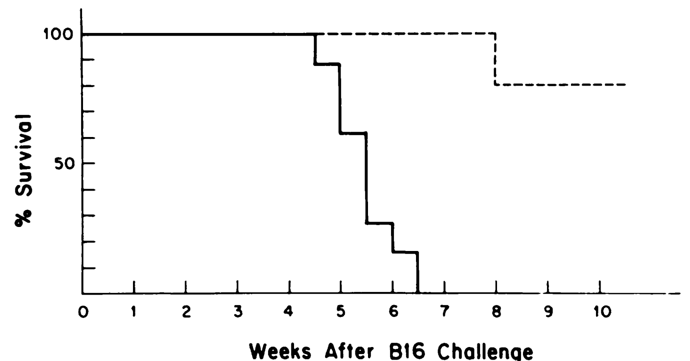

Chart 2. Longevity of diffusion chamber-induced immunity: resistance to second challenge 25 weeks after initial B16 challenge. ——, second challenge control; ----, B16-immunized mice, second challenge.

The longevity of the induced immune response was examined in animals shown to be immune after 8 weeks of B16 chamber implantation. Animals remaining tumor free 25 weeks after the initial B16 challenge were rechallenged with 5×10^3 B16 cells. As shown on Chart 2, 80% of the immune animals rejected the second challenge successfully, whereas control animals died within 6 to 7 weeks. Those animals in the immunized group which succumbed to the challenge developed tumors later and survived longer than did the control animals ($p > 0.005$).

The specificity of the antitumor response induced by B16 diffusion chambers was studied with the aid of the non-cross-reactive, syngeneic, chemically induced colon tumor MCA-38. Two groups of mice were immunized with B16 diffusion chambers, and 2 groups received MCA-38 chambers. After 8 weeks, the chambers were removed; 5 days later, the mice were challenged. One group of B16 chamber-immunized mice received 5×10^3 B16, as did one group of MCA-38 chamber-immunized mice. The other B16 and MCA-38 chamber-immunized groups were challenged with 1×10^5 MCA-38 s.c. Table 1 illustrates the specificity of diffusion chamber immunization. B16 chamber immunization was protective against B16 challenge but not against MCA-38 challenge. Likewise, MCA-38 chambers were not protective against B16 and even proved ineffective against

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Table 1
Specificity of B16 diffusion chamber-induced immunity

Diffusion chamber immunization	Tumor challenge	Tumor incidence
Media	B16	7/7
B16	B16	0/7
MCA-38	B16	10/12
Media	MCA-38	7/7
B16	MCA-38	7/7
MCA-38	MCA-38	13/14

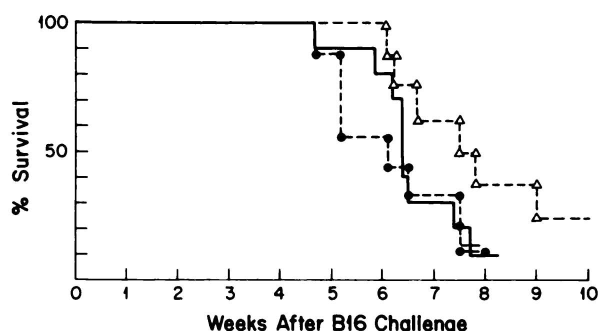

Chart 3. Survival of mice immunized with spent media from B16 cells or irradiated B16 cells following challenge with 5×10^3 viable B16 tumor cells. ——, media control; ●, spent B16 media; △, irradiated B16 cells.

itself, despite the fact that MCA-38 has been shown to be immunogenic by classical transplantation rejection tests (17). This result is discussed below since it may be a function of the tumor itself rather than a reflection upon the technique.

As an additional control, groups of mice were immunized once a week for 4 weeks with 10^6 irradiated B16 cells or spent culture medium from B16 cells. The control group received tissue culture media. The results in Chart 3 indicate that immunization with spent culture medium from B16 cells had no effect upon challenge tumor growth and lethality. However, immunization with irradiated tumor cells resulted in a delay of tumor growth ($p < 0.05$) but had no effect upon survival.

DISCUSSION

Tumors of spontaneous origin are considered by some investigators (1, 8, 9, 11) to be more appropriate models for the experimental study of cancer than are tumors induced by chemical carcinogens or oncogenic viruses because of their ability to invade and metastasize. With few exceptions, most tumors induced by chemical carcinogens or oncogenic viruses do not spontaneously metastasize. In addition, tumors induced by chemical carcinogens or oncogenic viruses are immunogenic (10). The lack of immunogenicity of spontaneous tumors has been attributed to the poor expression, or lack, of tumor-specific antigens. These findings have raised serious questions about tumor immunology as a discipline and specific immunotherapy as a potential treatment modality (1, 3, 16).

In the present study, C57BL/6J mice were sensitized by placing B16 melanoma cells into diffusion chambers and inserting the chambers i.p. After a period of 4 weeks, 87% of these mice were capable of rejecting a threshold challenge dose which gave 100% tumor takes in normal C57BL/6J mice. Tumor rejection was not observed in animals bearing B16 chambers for less than 4 weeks. The presence of tumor antigens is further supported by studies that have demonstrated the release of large quantities of macromolecules including TAA into spent media of cultured B16 melanoma cells. Indeed, these TAA have been shown to elicit a humoral response in both rabbits and the syngeneic C57BL/6J host (6, 14).

In this study, sensitization appeared to be immunologically specific inasmuch as mice sensitized to the B16 melanoma did not alter the growth of a MCA-38 challenge tumor. Sensitization of C57BL/6J mice to the MCA-38 tumor by the diffusion chamber technique did not provide protection against challenge with either MCA-38 tumor or B16 tumor cells. Previous studies have demonstrated that the MCA-38 tumor is immunogenic by classical transplantation rejection tests (12).[4] They retain most of their surface antigens, or they release small amounts of surface antigen at a slow rate (10). In contrast, poorly immunogenic rat breast tumors shed their surface antigens. Thus, the host may not become sensitized with an immunogenic tumor placed in a diffusion chamber unless the *in vivo* period of sensitization is increased to provide an adequate antigen dose.

The B16 tumor has been evaluated by classical transplantation rejection techniques and has been defined as weakly immunogenic (6, 12). B16 tumor bearers can cause a delay in the growth of a secondary tumor challenge 10 days after implantation of 10^5 cells. The delay in tumor growth of the secondary challenge was not evident 15 days after implantation of 10^5 tumor cells (16). However, these previous experiments do not take into account the longer time interval of sensitization in the diffusion chamber experiments. In this study, groups of mice were immunized by classical procedures for the same time interval as that used in diffusion chamber implantation. Successive immunizations with B16 cells arrested in their growth by irradiation but not with spent culture medium from B16 cells resulted in a delay of tumor growth but had no effect upon survival of mice. Thus, the clear-cut immunoprotection observed with diffusion chamber implantation was not simply due to the extended time interval. Studies on the mechanism of immunoprotection are currently in progress.

The results of this study are important from several perspectives. (*a*) They provide a novel way to demonstrate the immunogenicity of a tumor that has been classified as poorly immunogenic. Recently, attempts have been made to increase the immunogenicity of tumor cells by "tumor xenogenization" (4, 5). However, tumor xenogenization is dependent upon antigenicity of the tumor in the first place. (*b*) The diffusion chamber technique may be useful in demonstrating the antigenicity of tumors classified as nonantigenic and therefore useful to tumor xenogenization protocols. (*c*) The diffusion chamber technique may also provide an additional avenue of immunotherapy.

Studies are presently being undertaken to determine the potential of diffusion chamber technology in the prevention of recurrence and appearance of metastasis following resection of a B16 primary tumor.

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