0014-4754/94/070648-06$1.50 + 0.20

Efficacy of tumor cell vaccine after incorporating monophosphoryl lipid A (MPL) in tumor cell membranes containing tumor-associated ganglioside

M. H. Ravindranath*, S. M. Brazeau and D. L. Morton

Laboratory of Glycolipid Immunotherapy, John Wayne Cancer Institute, 2200 Santa Monica Blvd, Santa Monica (California 90404, USA)
Received 25 November 1993; accepted 13 January 1994

Abstract. Murine B16 melanoma expresses the ganglioside GM_3. GM_3 shed from tumor cells is immunosuppressive and promotes tumor growth[1]. Reduction or elimination of the shed GM_3 could be therapeutic, and the anti-GM_3 antibodies may reduce and clear the shed ganglioside. To test this hypothesis, mice were challenged with tumor cells, with or without inducing anti-GM_3 antibody response. Since gangliosides are poor immunogens and T-cell independent antigens, an adjuvant (monophosphoryl lipid A (MPL), a non-toxic lipid A of *Salmonella*), directed against B-cells, was employed. MPL was incorporated onto liposomes and into the surface membrane of B16 mouse melanoma cells; both are rich in GM_3. C57BL/6J mice immunized with MPL-liposomes or MPL-B16 cells responded with elevated levels of anti-GM_3 IgM. Non-immunized mice or mice immunized with B16 cells alone or ganglioside GM_3 alone (without MPL) elicited poor anti-GM_3 IgM response, confirming the GM_3's immunologic crypticity and MPL's immunopotentiating effect. MPL's immunopotentiating effect was improved by coupling it to melanoma cell membranes. C57BL/6J mice were immunized with irradiated B16 alone or MPL alone or MPL-conjugated irradiated B16. After three weekly immunizations, each mouse received a challenge dose of viable syngeneic B16. Neither MPL alone nor B16 alone had a significant effect on tumor growth or host survival; however, administration of MPL-conjugated B16 cells significantly prevented tumor growth and prolonged survival. Our results indicate that MPL-incorporated B16 cells augment the anti-GM_3 IgM response, which may reverse GM_3-induced immunosuppression by eliminating tumor-derived GM_3, and restore immunocompetence.
Key words. Tumor cell vaccine; B16 melanoma; gangliosides; GM_3; monophosphoryl lipid A (MPL); ELISA; anti-GM_3 IgM; immunization.

Solid tumors of neuroectodermal origin produce and shed large quantities of immunosuppressive sialoglycolipids or gangliosides[2]. Human cutaneous malignant melanoma expresses gangliosides GM_3, GD_3, GM_2, GD_2 and O-AcGD_3[3], whereas B16 murine melanoma expresses only GM_3[1]. The structure of GM_3 derived from B16 melanoma is similar to that of GM_3 derived from any normal tissues[1]. The simple ganglioside profile of B16 murine melanoma makes this tumor an excellent model for investigating the role of GM_3 in immunosuppression. Intraperitoneal administration of GM_3 in the B16 melanoma-bearing mice significantly augmented melanoma growth, suggesting that GM_3 shed from tumor cells may favor tumor growth[1], possibly by suppressing immune surveillance. In support of this observation[1], it was demonstrated that GM_3 preferentially suppressed the generation and activity of the cytotoxic lymphocytes in tumor-bearing mice, suggesting that melanoma-derived GM_3 in the circulation may impede antitumor functions of the immune system. Therefore, we hypothesized that reduction or elimination of tumor-derived GM_3 from the circulation may reverse immunosuppression and restore immunocompetence in tumor-bearing mice. In this regard, antiganglioside antibodies could be an ideal reagent to clear tumor-derived GM_3 from the circulation. We verified this possibility by studying the tumor growth and survival of the mice after augmenting anti-GM_3 antibodies with GM_3-rich vaccines. We observed that the immunopotential of tumor cell vaccines can be increased by conjugating the tumor cells with an adjuvant, such as MPL. Our data suggests that antigen-presentation of vaccine-associated gangliosides depends on the closer association between the ganglioside antigen and the immunopotentiating agent or adjuvant in the vaccine.

Material and methods

Mice. Strain C57BL/6J, 7- to 8-week-old male mice were obtained from Harlan Sprague Dawley Company (Indianapolis, Indiana, USA) and maintained on mouse chow and water ad libitum in the animal house facility at the University of California, Los Angeles (UCLA).

B16 Melanoma cells. B16 melanoma cells, syngeneic for C57BL/6J mice, were cultured in the laboratory in a RPMI 1640 medium containing 2 mM glutamine supplemented with 5% fetal bovine serum. An antibiotic-antimycotic mixture (penicillin, 10,000 units/ml; fungizone, 25 μg/ml; streptomycin, 10,000 μg/ml) was added to the medium. Cell viability was assessed using the trypan blue dye exclusion procedure. After 5

or 6 passages, cells were irradiated for 13 to 15 min at 7000 R at UCLA's gamma cell counter facility.

Adjuvant to augment antiganglioside antibodies. Monophosphoryl lipid A (MPL), purified from *Salmonella minnesota* R 595 was purchased from Ribi Immunochem Research (Hamilton, Montana, USA). For immunization, MPL was administered as aqueous suspensions in saline or in HBSS.

Ganglioside GM_3. N-acetyl neuraminic acid containing GM_3, purified from bovine brain and supplied by Sigma, was used in this study. Prior to experimental use, we chromatographically assessed its purity following a procedure described earlier[4]. For immunization, GM_3 is administered as an aqueous suspension prepared in either saline or in HBSS. Similarly, aqueous suspensions were prepared for MPL-GM_3.

Preparation of liposomes containing GM_3 and MPL. Small unit vesicles containing GM_3 (8 μg), MPL (8 μg) and phosphatidyl choline (PC) (8 μg) were prepared following the procedure of Wilschut[5]. In brief, GM_3, MPL and PC were dissolved in chloroform: methanol (1:1, v/v) and evaporated to dryness in small, round-bottomed flasks over a rotary evaporator. The lipid layer formed in the bottom of the flask was recovered as liposomes by adding 500 μl of warm saline (40 °C) and by intermittent vortexing and sonication for 30 min.

Incorporation of MPL to B16 cells. MPL was incorporated into the surface of B16 cells as follows: 25 μl of a suspension of irradiated B16 cells (1×10^6 cells/ml) was added to 250 μl of MPL (50 μg/ml); the mixture was shaken gently and incubated for 45 min at 24 °C. Incorporation of MPL into the cell membrane was assessed using a Polymyxin B assay[6,7]. Polymyxin B binds to membrane-conjugated MPL, thereby increasing the membrane's porosity; this increased porosity is measured by the degree of dye penetration. We used a 0.1% solution of trypan blue. Dye exclusion was initially followed at regular intervals for 1 h. Since maximum binding (50% of total cells) occurred at 30 min, we subsequently reduced the incubation time to 45 min. Incorporation of MPL into B16 cells can also be examined under a phase contrast microscope. Cells were irradiated before incorporation of MPL.

Enzyme-linked immunosorbent assay (ELISA). IgM serum antibodies were assessed using the ELISA procedure[8]. Microtiter plates (Immunolon # 1, Fischer Scientific, Pennsylvania, USA) were coated with 0.8 nmole/well of GM_3 in ethanol. Plates were dried in vacuo. The wells containing antigen were treated with phosphate buffered saline (pH, 7.4)-Tween-20 (0.1%) before the addition of diluted sera (50 μl). The titer values from the sera of saline-treated mice were used as control. The experimental values were corrected for the background (wells without antigen) and referenced against control values obtained using sera from saline-treated mice.

Immunization schedule; anti-GM_3 antibody response. 15 mice were pretreated with saline (day 0) and then immunized on day 14 (first immunization) and on or after day 28 (second immunization) with one of seven immunogens: Saline micellar MPL (8 μg), GM_3 (8 μg), GM_3-MPL (8 μg–8 μg), liposome-MPL, irradiated B16, or irradiated B16-MPL. Mice in the two B16 groups recieved 2.5×10^4 B16 cells (200 ng of GM_3) on day 14 and 5×10^4 cells (0.4 μg of GM_3) on day 28. Each immunogen was administered to 3 mice using intraperitoneal injection. The sera were collected 2 weeks after final immunization (day 42) for measuring IgM response.

Antitumor immunity. 33 mice were immunized with 100-μl injections of one of the following: Hank's balanced salt solution (HBSS, n = 9), irradiated B16 in HBSS (10^6 cells, n = 6), irradiated B16-MPL in HBSS (10^6 B16 cells with 160 μg MPL, n = 11), MPL in HBSS (160 μl, n = 4), or Bacillus Calmette Guerin (BCG, Tice strain) + HBSS (5×10^6 bacteria, n = 3). Injections were administered i.d. three times at weekly intervals. One week after the final immunization, mice were challenged by s.c. injection of B16 cells whose viability had been confirmed by dye exclusion. The challenge dose was empirically determined by measuring tumor size and days of survival following s.c. administration of 3 different doses of viable B16 cells in 16 untreated mice. Following the tumor challenge dose, the mice were observed for 60 days. The size of external tumors developing at or near the inoculation site was measured with vernier calipers every other day, beginning one week after the challenge dose. The length and width of the tumor was measured; these dimensions were multiplied and their product was divided by 2. Mice dying within 60 days of tumor challenge were examined for internal tumors in the peritoneum or various organs. Mice surviving longer than 60 days were observed for an additional month before they were sacrificed and examined for internal tumor growth.

Results

Anti-GM_3 IgM response. Mice immunized with GM_3-MPL, GM_3-liposome-MPL or GM_3-rich irradiated B16-MPL showed higher anti-GM_3 IgM antibody response than those immunized with GM_3 alone, or with irradiated B16 cells without MPL (table 1). The antibody response (as measured by absorbency of the sera at a particular dilution) in mice immunized with GM_3-MPL was found to be 2.5 times higher than in mice immunized with GM_3 alone. The level of antibody (as assessed by absorbency) in mice immunized with B16-MPL was ten times more than in those immunized with B16 alone. The results indicate: 1) the immunological crypticity of the gangliosides, as documented by significantly poor anti-GM_3 antibody response in mice immunized with B16 or GM_3 alone, and 2) the

Table 1. Anti-GM_3 IgM response in C57BL/6J mice after two immunizations[1] with various preparations of GM_3 and MPL. The absorbency values presented for treatment with each immunogen were corrected against the mean values obtained from non-immunized control mice (n = 9)

Immunogen[2]	Anti-GM_3 IgM antibody levels (ELISA absorbency)[3] 1:1000	1:2000	1:4000
MPL (8 µg/immunization/mice)	0.243 ± 0.028	0.150 ± 0.066	0.117 ± 0.106
GM_3 (8 µg/immunization/mice)	0.253 ± 0.051	0.177 ± 0.043	0.118 ± 0.077
GM_3 + MPL (8 µg–8 µg/immu/mice)	0.621 ± 0.021	0.347 ± 0.008	0.215 ± 0.046
MPL-GM_3 liposomes	0.722 ± 0.033	0.547 ± 0.064	0.318 ± 0.059
B16[4]	0.061 ± 0.007	0.000	0.000
MPL-B16[4]	0.642 ± 0.068	0.559 ± 0.114	0.379 ± 0.121

[1]Mice were pretreated with saline on day 0 and then immunized on day 14 (first immunization) and on or after day 28 (second immunization). [2]Each immunogen was administered to three mice i.p. [3]The sera were collected 2 weeks after final immunization (day 42) for measuring antibody response. [4]Mice in the two B16 groups received 2.5×10^4 cells (200 ng of GM_3) on day 14 and 5×10^4 cells (0.4 µg of GM_3) on day 28.

adjuvanticity of MPL when incorporated onto natural or artificial membranes, as evidenced by the high titers of anti-GM_3 IgM obtained after immunization with MPL-GM_3 or MPL-B16. MPL alone did not give any increase in anti-GM_3 IgM levels. It may be noted that no antiganglioside IgG antibody was produced in any of these experiments. Probably because of its pentameric nature, IgM is more effective in clearing the tumor-derived GM_3 from the vicinity of the tumor and from the circulation than the monomeric IgG. If antibodies generated after augmentation with MPL have the ability to clear the shed GM_3, then it is reasonable to anticipate reversal of immunosuppression and restoration of immunocompetence, which should be reflected in tumor regression after immunizing with MPL-incorporated membranes containing GM_3. Therefore we measured tumor growth and survival of mice challenged with tumor after they had been immunized with MPL-incorporated GM_3-expressing tumor cell membranes.

Antitumor response (restoration of immunocompetence after immunization). In order to assess the restoration of immunocompetence by membranes with incorporated MPL, we immunized mice (C57BL/6J) with irradiated B16 cells (1×10^6) with or without incorporated MPL, or with MPL alone or with HBSS as control. Development of immunocompetence was assessed by comparing tumor growth and survival in immunized and non-immunized mice after challenging with 5×10^3 syngeneic B16 cells. The challenge dose was empirically determined after s.c. administration of varying doses of living B16 cells as shown in table 2. No tumor development was observed in mice inoculated with 5×10^2 cells, suggesting the possibility of natural immunity in C57BL/6J mice against B16 melanoma cells. However, this natural immunity was ineffective when the challenge dose was increased by ten fold (5×10^3), suggesting that it was not strong enough to eliminate the melanoma cells if the cell dose was higher. The rate of tumor growth in mice inoculated with 5×10^3 cells was slower than in those given 5×10^4 cells (table 3). In order to assess the development of immunocompetence in mice treated with MPL, or MPL coupled to membranes, we selected 5×10^3 cells as the challenge dose. The figure compares the survival of mice when immunized with B16-MPL (1×10^6 cells) or with B16 alone (1×10^6). Control animals immunized with MPL or BCG alone did not show any noteworthy restoration of immunocompetence. On the other hand, 4 of the 11 mice immunized with B16-coupled to MPL (1×10^6) showed no evidence of tumor at all, and indeed survived a month after the termination of the experiment (after 2 months). Although the sample size in this experiment is small, the data pertaining to tumor growth in mice immunized with B16-MPL (table 3) further substantiate the anti-tumor effect of membrane bound MPL.

Table 3 shows the rate of tumor growth in mice immunized with various preparations. On day 30, the tumor size is minimal in mice immunized with MPL-B16 (1×10^6) compared to those immunized with B16 alone

Table 2. Survival of host and tumor growth in 7- to 8-week-old male mice C57BL/6J after s.c. administration of varying doses of live syngeneic B16 melanoma cells

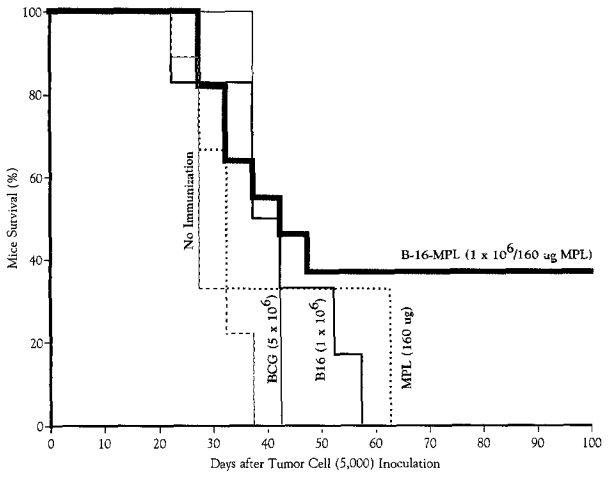
Number of cells administered[1]	Number of mice	Tumor size (mm^2) Day 17	Day 34	Days of survival
5×10^2	4	0	0	>75
5×10^3	9	0–45 (9^2)	158–350 (4^2)	24–37
5×10^4	5	72–400 (5^2)	- (0^2)	21–30

[1]The number of cells refers to viable cells; the cells were administered s.c.; [2]Number of mice still alive.

Table 3. Growth of B16 melanoma tumor in 7- to 8-week-old male mice C57BL/6J after various immunizations and challenge with syngeneic B16 melanoma cells

Immunizations	Number of mice survived		Tumor size (mm^2)
	Day 30	Day 60	Day 30 (number of mice tested)
HBSS/Saline	3/9	0/9	220–445 (9^1)
MPL	3/4	0/4	140–240 (4^1)
B16	5/6	0/6	48–290 (6^1)
B16-MPL	7/11	4^2/11	15–65 ($11^{1,2}$)

[1]Number of mice alive on day 30; [2]4 mice remained without tumor for more than 3 months. In B16-MPL immunized mice, the size of tumor is significantly smaller than in mice immunized with other immunogens.



Effect of immunization with MPL-incorporated irradiated B16 tumor cells on survival profile of syngeneic 7- to 8-week-old male mice C57BL/6J after challenge with 5×10^3 viable B16 tumor cells. B16 tumor cells were challenged after no immunization (9 mice) or immunization of BCG (5×10^6) alone (3 mice) or MPL (160 μg) alone (4 mice) or irradiated B16 cells (1×10^6) alone (6 mice) or MPL (160 μg) incorporated irradiated B16 (1×10^6) cells (11 mice). Immunization was administered i.d. weekly for 3 weeks. A week after the final immunization, live tumor cells were administered s.c. as a challenge.

(1×10^6) or MPL alone, indicating the development of immunocompetence in mice immunized with MPL-conjugated GM_3-rich tumor cells. Better survival, and retarded growth of challenge tumor cells, were observed in mice immunized with MPL-coupled B16, in contrast to mice immunized with B16 or MPL alone. This suggests the possible reversal of GM_3-induced immunosuppression and restoration of immunocompetence by membrane-bound but not free MPL, or by GM_3-rich tumor cell vaccine with MPL.

It is also evident that the development of immunocompetence depends on the challenge dose of MPL-B16. A separate experiment was done repeating the above experiment with varying doses of B16 cells (1×10^6 [with 160 μg of MPL]; 1×10^5 [with 16 μg of MPL]; 1×10^4 [with 1.6 μg of MPL]). The results were compared with those for the same doses of B16 cells without MPL. The results (table 4) showed that the days of survival decreased and the tumor growth increased with a decreasing dose of vaccine. Mice immunized with a particular dose of MPL-B16 survived better than those immunized with a corresponding dose of irradiated B16 alone.

Discussion

GM_3 is the major ganglioside of B16 melanoma cells[1]. It was observed that administration of GM_3 to tumor-bearing mice significantly augmented melanoma growth[1]. Experiments revealed that GM_3 is immunosuppressive in mice. GM_3 is also shed from the growing tumor tissues. Removing or eliminating the shed GM_3 should reverse immunosuppression and restore immunocompetence. Anti-GM_3 IgM antibodies can perform the function of clearing the shed GM_3. In this study, we attempted to augment the production of anti-GM_3 IgM by using irradiated GM_3-rich B16 cells as a vaccine. Vaccine therapy is intended to evoke the

Table 4. Differences in the dose-dependent changes in the percentage survival of mice challenged with B16 vaccine with or without incorporating MPL

Treatments	n	Days of survival								
		15	25	35	45	55	65	75	85	95
B16-MPL (1×10^6)	11	100	100	64	46	37	37	37	37	37*
B16-MPL (1×10^5)	6	100	100	50	50	33	33	0	0	0
B16-MPL (1×10^4)	6	100	100	100	67	0	0	0	0	0
B16 (1×10^6)	6	100	83	83	33	17	0	0	0	0
B16 (1×10^5)	6	100	83	83	50	0	0	0	0	0
B16 (1×10^4)	6	100	100	100	50	0	0	0	0	0
No immunization	9	100	78	22	0	0	0	0	0	0

*Mice survived without any tumor.

host's response to specific antigens on the surface of the tumor cells in the vaccine. However, if the antigens on tumor cells are immunologically cryptic, they must be unmasked to immunopotentiate the vaccine. Neuraminidase can unmask sialylated TAA (tumor-associated antigens) by removing the sialic acids[9], and this enzyme has therefore been used to pretreat autologous and allogeneic tumor cells used to immunize cancer patients[10]. Kobayashi's proposal for 'viral xenogenization' of tumor cell vaccine is yet another strategy to unmask immunologically cryptic TAA[11].

Tumor-associated gangliosides are overexpressed self-antigens in both human and animals. GM_3 is overexpressed in the murine melanoma[12], but it is still immunologically cryptic[2,3]. The same is true for GD_3, or GM_2 in human melanoma. This selective overexpression makes melanoma-related gangliosides potential target antigens for immunotherapy[2]. Previous studies, and data presented in the present study, show that gangliosides are generally immunologically cryptic. They elicit IgM but not IgG antibodies. This is not surprising because gangliosides (like other carbohydrate antigens) are incapable of T-cell recognition, owing to a failure to interact with MHC restriction elements[13,14]. Therefore, gangliosides are regarded as T-cell independent antigens. If antiganglioside antibodies were to play a significant role in reduction or elimination of gangliosides shed from tumor tissues, then the pentameric IgM would be a better candidate than the monomeric IgG.

Our finding that MPL can unmask B16-associated GM_3 is striking but not surprising because the precursors of MPL, namely lipid A and lipopolysaccharides, are potent B-cell adjuvants[15] that perform similar functions when bound to membranes[16–18]. However, unlike its precursors, MPL is non-toxic in cancer patients[19,20]. Our results support the hypothesis that MPL's immunobiological activity depends upon its mode of presentation. We found that MPL was most effective in inducing antiganglioside IgM response when conjugated to membrane liposomes or to the surface of the tumor cell; it had no significant immunopotentiating activity in aqueous preparations (micellar form). In this regard, the observations of Livingston et al.[21] are of considerable interest and relevant to this investigation. They examined the anti-GM_2 antibody responses, after immunizing mice with MPL-liposome vaccine. They incorporated both MPL and GM_2 into liposomes. When liposomes were prepared with extensive and vigorous sonication (Liposome 2a), the antibody response was much better than with those prepared by less vigorous or mild sonication (Liposome 2b). These results point out the importance of proper incorporation of MPL into ganglioside-vaccines to elicit better immune response.

Our results emphasize the importance of *incorporating* an immunopotentiating agent in a tumor cell vaccine. Currently, vaccine therapy is administered in conjunction with adjuvants such as muraminyl peptide, Freund's adjuvant, BCG, BCG-cell wall skeleton with or without trehalose dimycocholate, and *Coryne bacterium parvum*[23]. Our findings suggest that adjuvants would be more effective if incorporated in the membranes of tumor cells in the vaccine. Support for the use of a conjugated vaccine is found in early animal studies[4,23,24] demonstrating tumor suppression after injection of tumor cells mixed with BCG, but not after simultaneous injections of BCG and tumor cells at separate sites[26].

We believe that tumor cells conjugated with an immunopotentiating agent such as MPL will evoke a more powerful host response than that associated with tumor cells alone. In this study, we found that membrane-bound MPL augmented the antibody response to a cryptic ganglioside GM_3 and restored immunocompetence. We therefore propose MPL as an ideal immunopotentiating agent for active immunotherapy using tumor cell vaccines targeting ganglioside TAA. MPL is also a potentially valuable adjuvant for augmenting antiganglioside antibodies by eliminating their immunologic crypticity; this would be useful for large-scale production of therapeutic human or mouse monoclonal antiganglioside antibodies[22].

The results of this investigation could have significant relevance to the immunotherapy of human cancer. Immunotherapies of cancer can broadly be classified as adoptive, passive and active. In adoptive immunotherapy, the patient's circulating lymphocytes or tumor infiltrated lymphocytes are isolated in vitro, activated by lymphokines such as IL-2 or transduced with genes for tumor necrosis, and readministered[27,28]. This form of immunotherapy has produced several cases of dramatic and complete regression of melanoma and renal carcinoma, but the percentage of responders was very small. The reason for the lack of response in most cases has not been investigated. Our hypothesis is that the failure may be due to interaction of the activated cells with excess circulating gangliosides shed from tumor tissues.

In passive immunotherapy, patients were treated with monospecific or polyspecific antiganglioside monoclonal antibodies. Several antiganglioside antibodies (R24, 3F8 and Mab 36.1) have been tested in clinical trials[29], but complete regression was rare. It is not clear whether the antibody always reached its tumor target. Again, the failure of therapy might be due to interaction of administered antibodies with the tumor-derived gangliosides shed into the blood.

In active immunotherapy, autologous or allogeneic tumor cell vaccine is administered with a bacterial adjuvant[2,23,30]. Those patients in whom a high IgM response is elicited often survive better than those who have no or low levels of IgM antibodies[30]. IgM antibodies are often transient – the exception to the rule appears to be

antiganglioside or anticarbohydrate antibodies. We believe that in the responders, the high level of antiganglioside IgM may be clearing the shed gangliosides, whereas in the non-responders the level of the tumor-derived gangliosides may be higher, or the antiganglioside antibodies are not produced at levels adequate to reduce or eliminate the shed gangliosides. If this is true, then the use of an MPL-incorporated tumor cell vaccine would be a valuable strategy.

In conclusion, we propose that melanoma therapies should incorporate a method for reducing or eliminating tumor-derived gangliosides in the serum of tumor bearing individuals. The development of antiganglioside IgM antibodies would be a logical step, and might be useful in restoring immunocompetence lost due to the immunosuppressive activities of the shed gangliosides. Immunization using MPL with membranes containing cryptic tumor-associated gangliosides such as GM_3 may result in the augumentation of antibodies against the gangliosides; then, as a sequel to formation of antibodies, the shed gangliosides may be cleared from the circulation and immunocompetence may be restored. Our study documents that tumor cells conjugated with an immunopotentiating agent can be a better vaccine than tumor cells alone. In this regard, MPL would be an ideal immunopotentiating agent for tumor cell vaccines targeting tumor-associated gangliosides.

Acknowledgments. We wish to thank Dr. Amy Walsh and Ms. Gwen Berry for excellent editorial assistance. This work is supported by NIH grant PO1 CA12582 (DLM).

* To whom correspondence should be addressed.

1 Takahashi, K., Ono, K., Hirabayashi, Y., and Taniguchi, M., J. Immun. *140* (1988) 3244.
2 Ravindranath, M. H. and Morton, D. L., Int. Rev. Immun. *7* (1991) 303.
3 Ravindranath, M. H. and Irie, R. F., in: Malignant Melanoma: Biology, Diagnosis, and Therapy, p. 17. Ed. L. Nathanson, Kluwer Acad., Boston 1988.
4 Ravindranath, M. H., Paulson, J. C., and Irie, R. F., J. biol. Chem. *263* (1988) 2079.
5 Wilschut, J., in: Methodologie des liposomes appliquee a la pharmacologie et a la biologies cellulaire, p. 10. Eds L. D. Leserman and J. Barbet, INSERM, Paris 1982.
6 Carr, C. Jr., and Morrison, D. C., Rev. infect. Dis. *6* (1984) 497.
7 Shafer, W., and Spitznagel, J., Rev. infect. Dis. *6* (1984) 577.
8 Freudenberg, M. A., Fomsgaard, A., Mitov, I., and Galnos, C., Infection *17* (1989) 322.
9 Bekesi, J. G., Arneault, G. St., Walter, L., and Holland, J. F., J. natl Cancer Inst. *49* (1972) 107.
10 Slingluff, C. L., Vollmer, R., and Seigler, H. F., J. surg. Oncol. *39* (1988) 139.
11 Kobayashi, H., J. biol. Resp. Modif. *5* (1986) 1.
12 Hirabayashi, Y., Hamaoka, A., Matsumoto, M., Matsubara, T., Tagawa, M., Wakabayashi, S., and Taniguchi, M., J. biol. Chem. *260* (1985) 13328.
13 Hardings, C. V., Roof, R. W., Allen, P. M., and Unanue, E. R., Proc. natl Acad. Sci. USA, *88* (1991) 2740–2746.
14 Ishioka, G. Y., Lamout, A. G., Thomson, D., Bullow, N., Gaeta, F. C. A., Sette, A., and Grey, H. M., J. Immun. *148* (1992) 2446.
15 Dresser, D. W., and Philips, J. M., in: Immunopotentiation, p. 3. CIBA Foundation Symposium 18, Elsevier, Amsterdam 1973.
16 Schuster, B. G., Neidig, M., Alving, B. M., and Alving, C. R., J. Immun. *122* (1979) 900.
17 Banerji, B., Lyon, J. A., and Alving, C. R., Biochim. biophys. Acta. *689* (1982) 319.
18 Tamauchi, H., Tadakuma, T., Yasuda, T., Tsumita, T., and Saito, K., Immunology *50* (1983) 605.
19 Vosika, G. J., Barr, C., and Gilbertson, D., Cancer Immun. Immunother. *18* (1984) 107.
20 Harel, W., Shau, H., Hadley, C. G., Morgan, A. C., Reisfeld, R. A., Cheresh, D. A., and Mitchell, M. S., Cancer Res. *50* (1990) 6311.
21 Livingston, P. O., Calves, M. J., and Natoli, E. J. Jr., J. Immun. *138* (1987) 1524.
22 Irie, R. F., and Ravindranath, M. H., in: Therapeutic Monoclonal Antibodies, p. 75. Eds C. A. K. Borrebaeck and J. W. Larrick. Stockton Press, New York 1990.
23 Morton, D. L., and Ravindranath, M. H., in: Cancer Medicine, p. 967. 3rd Ed. Eds J. F. Holland, E. Frei III, R. C. Bast, D. W. Kufe, D. L. Morton and R. R. Weichselbaum. Lea & Febiger, Philadelphia 1993.
24 Bartlett, G. L., and Zbar, B., J. natl Cancer Inst. *48* (1972) 1709.
25 Tanaka, T., and Takunaga, T., Gann *62* (1971) 433.
26 Bast, R. C. Jr., Zbar, B., Borsos, T., and Rapp, H. J., N. Engl. J. Med. *290* (1974) 1413.
27 Rosenberg, S. A., Lotze, M. T., and Yang, J. C., Ann. Surg. *210* (1989) 474.
28 Rosenberg, S. A., Packard, B. S., and Aebersold, P. M., N. Engl. J. Med. *319* (1988) 1676.
29 Vadhan-Raj, S., Cordon-Cardo, C., Carswell, E., Mintzer, D., Dantis, L., Duteau, C., Templeton, M. A., Oettgen, H. F., Old, L. J., and Houghton, A. N., J. clin. Oncol. *6* (1988) 1636.
30 Morton, D. L., Foshag, L. J., Hoon, D. S. B., Nizze, J. A., Wanek, L. A., Chang, C., Davtyan, D. G., Gupta, R. K., Elashoff, R., and Irie, R. F., Ann. Surg. *216* (1992) 463.