Cell Mediated Immune Response in Miniature Sinclair Swine Bearing Cutaneous Melanomas

D.H. Jones and M.S. Amoss, Jr.*

ABSTRACT

Miniature Sinclair swine bearing cutaneous melanomas and nonmelanoma bearing littermate controls were tested for lymphocyte blastogenic response in a microculture assay. Lymphocytes were assayed for ^{3}H-thymidine incorporation following stimulation with concanavalin A, phytohemagglutinin or pokeweed mitogen. Treatment with all mitogens revealed a significantly ($p<0.05$) reduced proliferative capacity in melanoma swine as compared to littermate controls. These results lend further support for the use of miniature Sinclair swine as a research model for human melanoma.

RÉSUMÉ

Cette expérience consistait à vérifier, à l'aide d'une microtechnique de culture cellulaire, la réponse blastogénique des lymphocytes de porcs nains Sinclair, atteints de mélanomes cutanés, ainsi que celle de congénères témoins. Après avoir stimulé la blastogénèse des lymphocytes avec de la concanavaline A, de la phytohémagglutine ou du mitogène phytolaque, on détermina l'incorporation de ^{3}H-thymidine à ces lymphocytes. L'utilisation des trois mitogènes précités révéla une réduction appréciable ($p<0,05$) de la capacité proliférative des lymphocytes des porcs atteints de mélanomes cutanés, comparativement aux témoins. Ces résultats justifient davantage l'utilisation de porcs nains Sinclair, à titre de modèle de recherche sur le mélanome humain.

Over the last several years, there has been a steadily rising incidence of all primary cutaneous malignancies, including malignant melanomas (15). The use of appropriate animal models of human disease syndromes is important in the study of the biology of the disease process and the development of new treatment regimens. Melanomas have been reported in swine as early as 1926 (7). The majority of the early reports described the incidence of malignant melanomas in young pigs of the Duroc-Jersey breed (13). Several recent publications (9, 10, 20) have reported and described melanomas in a breed of miniature swine developed at the University of Missouri, Sinclair Comparative Medicine Research Farms. In 1974 it was suggested that Sinclair swine were an appropriate animal model for the study of melanoma (16). Cutaneous melanomas develop spontaneously both pre- and postnatally. In these swine spontaneous regression of cutaneous tumors is quite high and often results in complete reduction of the tumor mass leaving only an area of localized depigmentation and fibrosis (18). It is estimated that 10-20% of the malignant melanomas in man spontaneously regress (17).

In swine, cutaneous invasion of malignant melanomas into the deep reticular dermis or into the panniculus is often associated with a high incidence of metastasis. Like man, the organs most often affected by metastasis are the lymph nodes, lungs and liver (18). Recently, swine melanoma cells have been adapted to prolonged growth in culture. It was found that these cells appeared morphologically similar to human cultured B-16 melanoma cells. Doubling times and frequency of DOPA positive cells seen in swine melanoma culture were very similar to that found in B-16 melanoma cell cultures (2).

Numerous studies of malignant melanoma in man (3, 4, 6, 8, 11, 12, 14, 19, 21, 22) have reported a decreased responsiveness of peripheral blood lymphocytes to mitogen stimulation. The purpose of the present study was to determine whether porcine lymphocytes obtained from swine bearing cutaneous melanomas would respond to mitogenic challenge in a manner similar to nonmelanoma bearing littermate controls.

Twenty-four miniature swine two to three months of age bearing cutaneous melanomas (Clark stage equivalent two to three) and 13 nonmelanoma bearing littermate controls were obtained from the Texas A&M University miniature swine herd.

Twelve mL syringes containing 1 mL of preservative-free heparin (1000 units/mL) in RPMI-1640 as an anticoagulant were used to obtain sterile cardiac blood sam-

*Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, Texas A&M University, College Station, Texas 77843.

Submitted September 15, 1981.

ples. The lymphocytes were purified by a modification of the method of Böyum (5). Six mL of whole blood was carefully layered onto 5 mL of sterile Ficoll-Hypaque solution (sp. gr. 1.080) in 17 x 100 mm plastic tubes with caps. The tubes were centrifuged at 1200 x g at 20°C for 45 minutes. The lymphocyte band at the plasma Ficoll-Hypaque interface was aspirated with a sterile Pasteur pipette and transferred to 12 x 7 mm plastic tubes with caps containing 3 mL of RPMI-1640 and sodium heparin. The tubes were thoroughly mixed and centrifuged for ten minutes at 400 x g at 20°C. The supernatant was discarded and 3 mL of RPMI-1640 containing antibiotics (penicillin 200 units/mL and streptomycin 200 μg/mL) were added to the cell button and mixed thoroughly. The tubes were again centrifuged at 400 x g at 20°C. The supernatant was discarded and 3 mL of complete RPMI-1640 which contained 10% v/v of fetal calf serum, 2 mM L-glutamine, 25 mM Hepes (pH 7.4), and antibiotics (as above) were added per tube. The cells were resuspended and centrifuged again for ten minutes at 400 x g at 20°C and the top 2 mL of supernatant discarded. The cell pellet was resuspended in the remaining 1 mL of fluid and the cells counted using a Coulter counter. The cell suspension was adjusted to a density of 5 x 10^6 cells/mL using complete RPMI-1640.

One-hundred μL aliquots containing 5 x 10^5 cells were dispensed into round bottom microtiter culture plates. Control cell cultures received an additional 100 μL for RPMI while experimental cultures received an equivalent volume containing the appropriate mitogen. Concanavalin A[1] (Con A), pokeweed mitogen[2] (PWM) and phytohemogglutinin[3] (PHA) were used to stimulate lymphocyte blastogenesis. The mitogens were reconstituted according to the manufacturer's specifications and working dilutions of Con A (2.0 μg/mL, 4.0 μg/mL), PWM (1:128, 1:64) and PHA (1:100) were used. Nine replicate control cultures were plated for each animal. Triplicate cultures were plated for each dose level of mitogen. Cultures were incubated at 37°C and 98% humidity for 72 hours and then pulsed with 50 μL of RPMI containing 1.0 μCi of tritiated thymidine (specific activity 6.7 Ci/mM) for 18 hours. Cultures were terminated by cooling to 4°C. All cultures were harvested by collection of cells onto glass fiber filters using a multiple automated sample harvester. The filter papers were dried and the individual discs (containing cell contents) punched out and placed in scintillation vials containing 3 mL of scintillation fluid. Samples were counted for five minutes in a liquid scintillation spectrometer.

Stimulation indices were determined by dividing the mean counts per minute from triplicate stimulated cultures by the mean counts per minute for nine control cultures. Final values are reported as the Stimulation Index ± standard error of the mean (S.E.M.). A Student's t-test was calculated using the means to determine differences at the $p<0.05$ significance level.

Blastogenic stimulation of peripheral blood lymphocytes obtained from swine bearing cutaneous melanomas resulted in significantly lower ($p<0.05$) ^{3}H-thymidine incorporation into cellular DNA as compared to littermate controls (Table I). Diminished responsiveness of peripheral T-lymphocytes to mitogenic stimulation has also been reported in human patients with malignant melanoma.

Lymphocytes obtained from both tumor bearing and littermate control swine responded to increased doses of mitogen with higher stimulation indices. Although lymphocytes obtained from melanoma bearing swine responded to increased quantities of mitogen in a dose response manner, the level reached was always much lower than that of lymphocytes obtained from nontumor bearing controls. Berkelhammer *et al* (1) have reported that swine bearing regressing malanomas exhibited substantially greater *in vitro* lymphocyte reactivity against allogenic swine melanoma target cells than did swine bearing melanomas. The results of the present study lend further support for the use of miniature Sinclair swine as a research model for human melanoma.

ACKNOWLEDGMENTS

This research was supported in

TABLE I. Tritiated Thymidine Incorporation into Porcine Peripheral Blood Lymphocytes Following Mitogen Stimulation

	Stimulation Index[a, b, c]				
	Con A (2 μg/mL)	Con A (4 μg/mL)	PWM (1:128)	PWM (1:64)	PHA (1:100)
Littermate Control Swine (N = 13)	26.04 ± 8.50[d]	49.80 ± 10.90[e]	108 ± 16.91[f]	117.72 ± 15.77[g]	70.2 ± 17.39[h]
Melanoma Swine (N = 24)	6.65 ± 2.12[d]	15.17 ± 2.6[e]	23.99 ± 4.31[f]	54.85 ± 7.76[g]	33.75 ± 8.79[h]

[a]Mean Stimulation Index ± S.E.M.
[b]Based on triplicate cultures per mitogen, per animal
[c]Values with the same superscript are significantly different at $p<0.05$

[1]Difco Laboratories, Detroit, Michigan.
[2]Grand Island Biological Company, Grand Island, New York.
[3]Difco Laboratories, Detroit, Michigan.

part by NIH-Biomedical Science Grant 5 S07 RR07090-14 and the Texas Agricultural Experiment Station, Project No. 15618-6376, Texas A&M University System. Technical Article No. 16456.

REFERENCES

1. **BERKELHAMMER, J., B.M. ENSIGN, C.J. HECKER, G.D. SMITH** and **R.R. HOOK.** Correlation of *in vitro* lymphocyte reactivity with *in vivo* swine melanoma growth and regression. AACR Abstracts. 318. 1981.
2. **BERKELHAMMER, J., S.H. CAINES, D.L. DEXTER, E.H. ADELSTEIN, R.W. OXENHANDLER** and **R.R. HOOK.** Adaptation of Sinclair swine melanoma cells to long-term growth *in vitro*. Cancer Res. 39: 2960-2963. 1979.
3. **BERNENGO, M.G., G. CAPELLA, M. PERRUCIO** and **G. ZINA.** Relationship between T and B lymphocyte values and prognosis in malignant melanomas. Br. J. Dermat. 98: 655-662. 1978.
4. **BICKHARDT, R.** and **J. KUNZE.** Beiträge zur immunologishen reaktions-bereitschatt beim malignen melanon. Z. Hautkr. 49: 315-333. 1972.
5. **BÖYUM, Y.** Isolation of mononuclear cells and granulocytes. Scand. J. clin. lab. Invest. 21: 1-108. 1968.
6. **CATALONA, W.M.D., W.F. SAMPLE** and **P.B. CHRETIEN.** Lymphocytes reactivity in cancer patients: Correlation with tumor histology and clinical stage. Cancer 31: 65-71. 1973.
7. **CAYLOR, H.D.** and **C.F. SCHOLTTHAUER.** Melano-epitheliomas of swine: transplantation and cultural experiments. Archs Path. 2: 343-351. 1926.
8. **DE GAST, G.C., H.S. KOOPS, H.A. HUIGES, J. OLDHOFF** and **H.O. NIEWEG.** Humoral and cell mediated immune response in patients with malignant melanoma. Cancer 36: 1289-1297. 1975.
9. **FLATT, R.E., C.C. MIDDLETON, M.E. TUMBLESON** and **C. PEREZ-MESA.** Pathogenesis of benign cutaneous melanomas in miniature swine. J. Am. vet. med. Ass. 153: 936-941. 1968.
10. **FLATT, R.E., L.R. NELSON** and **C.C. MIDDLETON.** Melanotic lesions in the internal organs of miniature swine. Archs Path. 93: 71-75. 1972.
11. **GOLUB, S.H., T.X. O'CONNELL** and **D.I. MORTON.** Correlation of *in vivo* and *in vitro* assays of immunocompetence in cancer patients. Cancer Res. 34: 1833-1837. 1974.
12. **GROSS, N.G.** and **A.C. EDDIE-QUARTEY.** Monitoring of immunologic status of patients receiving BCG therapy for malignant disease. Cancer 37: 2183-2193. 1976.
13. **HJERPE, C.A.** and **G.H. THEILEN.** Malignant melanomas in porcin littermates. J. Am. vet. med. Ass. 144: 1129-1131. 1964.
14. **KLEIN, R., G.R.F. KRÜGER** and **K. SESTERHENN.** T-lymphocyten im peripheren blut. Ein untersuchung an 113 patienten mit malignem melanom. Z. Hautkr. 53: 62-66. 1978.
15. **LEE, J.A.H.** The trend of mortality from primary malignant tumors of skin. J. invest. Dermat. 59: 445-448. 1973.
16. **MILLIKAN, L.E., J.L. BOYLON, R.R. HOOK** and **P.J. MANNING.** Melanoma in Sinclair swine: A new animal model. J. invest. Dermat. 62: 20-30. 1974.
17. **NATHANSON, I.** Biological aspects of human malignant melanoma. Cancer 20: 650-655. 1967.
18. **OXENHANDLER, R.W., E.H. ADELSTEIN, J.P. HAIGH, R.R. HOOK** and **W.H. CLARK.** Malignant melanoma in the Sinclair miniature swine. Am. J. Path. 96: 707-714. 1979.
19. **PRITCHARD, D.J., R.E. RITTS, W.F. TAYLOR** and **G.C. MILLER.** A perspective study of immune responsiveness in human melanoma. Cancer 41: 2165-2173. 1978.
20. **STRAFUSS, A.C., A.R. DOMMERT, M.E. TUMBLESON** and **C.C. MIDDLETON.** Cutaneous melanoma in miniature swine. Lab. Anim. Care 18: 165-169. 1968.
21. **THATCHER, N.M.K., N. PALMER** and **G.D. GASUMA.** Lymphocyte function and response to chemoimmune-therapy in patients with metastitic melanoma. Br. J. Cancer 36: 751-762. 1977.
22. **ZIEGLER, J.L., M.G. LEWIS, J.M.S. LUYOMBYA** and **J.W.M. KIRYABURIE.** Immunological studies in patients with malignant melanoma in Uganda. Br. J. Cancer 23: 729-734. 1969.