

Use of *Mycobacterium vaccae* (SRL172) as an Immunomodulating Agent in Association with Murine Melanoma Model B16-F10

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Summary

Because of immunotherapeutic effects of *Mycobacterium vaccae* (*M.vaccae*) by inducing Th1 responses, we tried a heat-killed suspension of *M.vaccae* in mice models (C57BL/6J), which had already been challenged by tumor cell lines B16-F10. The Immunization consisted of subcutaneous injection of irradiated B16-F10 cells, as an antigen, plus *M.vaccae* into the tail base at two inguinal sites of mice, which were injected by live wild type B16-F10 cells. Survival and tumor growth was recorded every three days for a period of three months. Increase in survival was observed whilst decrease in tumor growth was recorded. The result indicates that the heat-killed suspension of the bacterium is a powerful immunological adjuvant.

Key words: melanoma cell, immunomodulating agent, *Mycobacterium vaccae*, vaccine

Introduction

Many animal tumor models are used to improve and develop protocols for combating cancer. In melanoma there are different cell clones with different immunological and metastatic properties. The B16 melanoma originally arose, spontaneously from C57BL/6J mice and many B16 clones with different grade of stringency have been derived from the original tumor cell line. B16-F10 is a very metastatic and aggressive clone with lung tropism but with a poor immunogenicity (Fidler 1975). K1735-M2 is a clone derived from K1735 melanoma, which is less aggressive than B16-F10. There are very few tumor associated fully characterized in the B16 melanoma. One of them is B700 antigen, which is expressed on human melanoma cells and related to serum

albumin (Gersten & Hearing 1992). Some of the melanoma antigens including B700 are shared by B16-F10 and K1735 (Deleo *et al*1991).

Considering all advantages of using heat-killed suspension of *M.vaccae* such as its ability to modulate the immune response against tumor cell lines shifting Th2 responses to Th1 and showing no side effects (Baban 1998), the suspension could be used in most of animal melanoma models both easily and safely.

In this study the efficacy of using the bacterium as an immunomodulating agent by mixing it with murine melanoma model B16-F10 was evaluated.

Materials and Methods

Vaccine preparation. B16-F10 cells were harvested with 0.02% Ethylene Diamine Teta-acetic Acid (EDTA) in phosphate buffer saline (PBS) washed three times with PBS, resuspended in it, and subjected to 7000 rads of gamma-irradiation.

Adjuvant. *M.vaccae* (NCTC 11659) was provided by Stanford Rook Ltd, London, England and cultivated on Sauton's medium solidified with 1.5% agar. The bacterial growth was scraped off the medium at the end of the logarithmic phase of growth and was suspended in M/15 borate buffered saline (pH8.0) at a concentration of 10mg wet weight/ml. This corresponds to 10^7 bacilli in 0.1ml. The suspension was autoclaved at 121°C for 15 min to ensure sterility and was subsequently stored in the dark at 4°C.

Immunization. 6 groups of C57BL/6J black mice each of 10 were received different vaccine protocols as shown in table 1.

Table 1. Immunization schedules

Group	Immunization protocol	Priming*
A (control)	5×10^6 irradiated B16-F10 cells	-
B (control)	5×10^6 irradiated B16-F10 cells	+
C	5×10^6 irradiated B16-F10 cells plus 10^7 <i>M.vaccae</i>	-
D	5×10^6 irradiated B16-F10 cells. plus 10^7 <i>M.vaccae</i>	+
E	5×10^6 irradiated B16-F10 cells plus 10^9 <i>M.vaccae</i>	-
F	5×10^6 irradiated B16-F10 cells plus 10^9 <i>M.vaccae</i>	+

*Some of the groups were primed with *M.vaccae* by feeding for a month starting on day 4 (minus means before challenging day or starting day) by injecting 5×10^8 *M.vaccae* bacilli into the water bottle of the animal cage twice weekly, and then giving plain water for a week before immunization.

Immunization consisted of subcutaneous injections into the tail base at two inguinal sites on day 10 of 5×10^6 irradiated B16-F10 cells with or without *M. vaccae*. On day 0, all groups of mice were challenged on the left flank by subcutaneous injection of 5×10^6 live wild type B16-F10 cells.

Survival and tumor growth were recorded every three days for a period of three months and subcutaneous diameter was measured using calipers. Mice were killed when tumor diameter exceeded 15mm or when the tumor was ulcerated.

Results

Group F showed delayed tumor growth when compared to the control group A ($P=0.003$). Total protection was also conferred and was long lived (3 months) and sustained in 40% of the mice of group F (Figure 1a). Compared to the control group

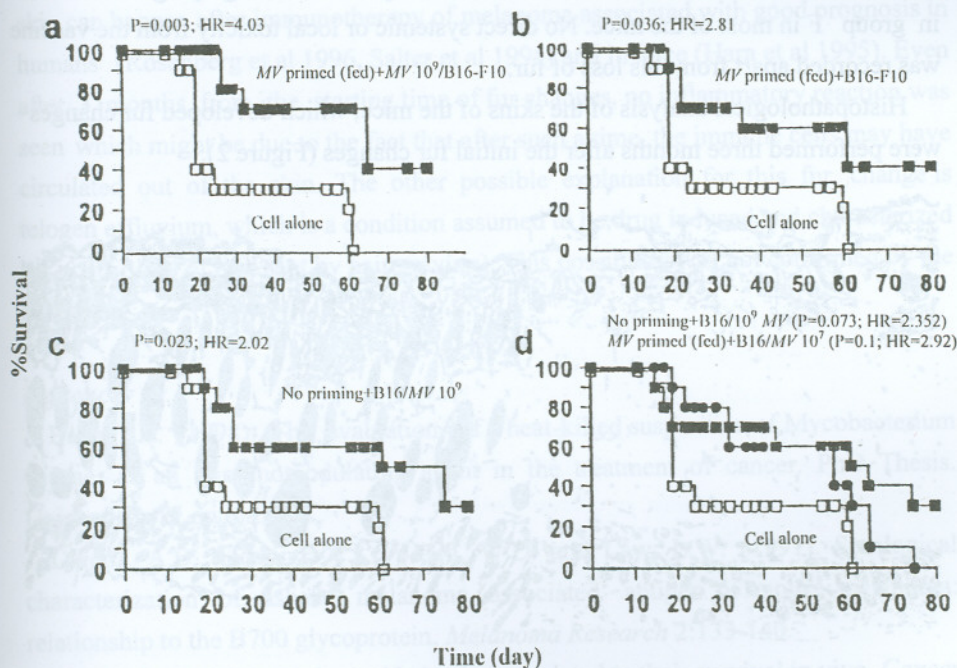


Figure 1. The survival of the mice in the different immunized groups is presented below: panel a: group A versus group F; panel b: group A versus group B; panel c: group A versus group E; panel d: group A versus groups C and D. The value between the different survival curves and the survival curve of group A is shown for each figure. HR means Hazard Risk.

A, the survival curves of group B (Figure 1b) and E (Figure 1c) were significantly prolonged ($P=0.023$ and $P=0.036$, respectively). The increased survival of the groups of mice which receive a tumor vaccine regimen containing 10^7 *M.vaccae* bacilli (group C and D) did not reach statistical significance difference compared to the survival curve of group A ($P=0.073$ and $P=0.1$, respectively, Figure 1d).

Discussion

After the live tumor challenge (around day 5 to 10) all the mice in group F developed fur changes characterized by fur graying and fur loss. At the time of live tumor challenge, a limited area of the left flank of each mouse was shaved. The mice from group F did not regain their fur in the shaved area whereas in the other groups the fur of the mice grew again. The fur loss extended to the other parts of the body, especially the back and the other flank. Localized patches of fur graying were noticed in group F in most of the mice. No direct systemic or local toxicity from the vaccine was recorded apart from this loss of fur.

Histopathological analysis of the skins of the mice, which developed fur changes were performed three months after the initial fur changes (Figure 2).

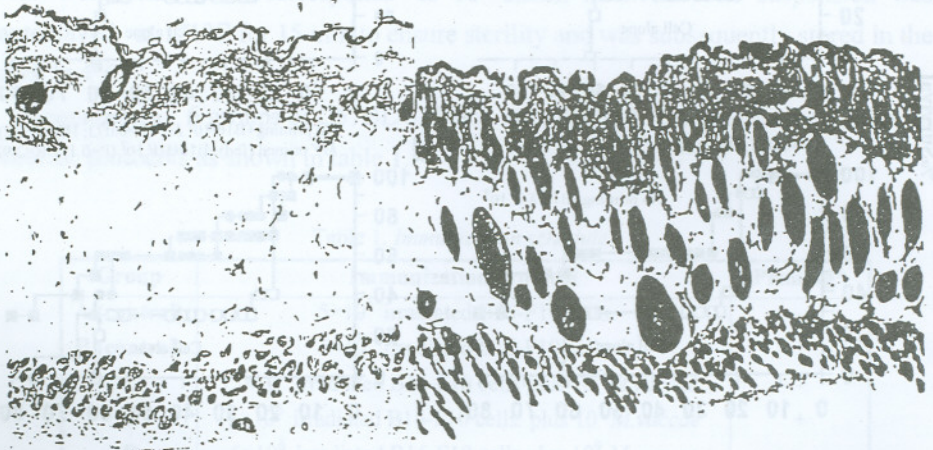


Figure 2. Section of the skin of a long-term survivor from groups F (left) and C (right)

The main purpose of the experiment was to follow to survival and therefore we decided not to kill the mice for histopathological reason until the completion of

surviving curves. No inflammatory reaction was detected at this time.

Our results show that heat killed *M.vaccae* is effective in the B16-F10 model when combined with irradiate cells. Taking into account the aggressiveness and the poor immunogenicity of the B16-F10 model, this implies that *M.vaccae* when mixed with an antigen is a very powerful immunological adjuvant. Causing no local or systemic toxicity in mice and only minor side effects in man is an important factor in favors of *M.vaccae* in comparison with other adjuvant such as incomplete Freund adjuvant. Moreover considering makes it as a superior product even to BCG.

There is no clear explanation for the fur changes in protected mice in our prevention experiment. Cross reaction between the melanoma cells in the vaccine and the hair follicles is one possibility since melanoma cells share antigens with the normal melanocytes of the hair follicles e.g. tyrosinase (and gp75) gp, 100 and MelanA/Mart-1 antigen. It has been shown also that color changes of hair/fur and skin can happen after immunotherapy of melanoma associated with good prognosis in humans (Rosenberg et al 1996, Salter et al 1995) and in mice (Hara et al 1995). Even after 3 months from the starting time of fur changes, no inflammatory reaction was seen which might be due to the fact that after such a time, the immune cells may have circulated out of the skin. The other possible explanation for this fur change is telogen effluvium, which is a condition assumed to be drug induced and characterized by acute loss of hair (not by hair graying). This possibility was not confirmed by the biopsies.

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