Author's response to reviews

Title: Immunity to melanin and to tyrosinase in melanoma patients, and in people with vitiligo

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Author's response to reviews: see over
To the Editor of BMC Complementary and Alternative Medicine
Professor Tom Rowles

Dear professor Rowles,

Firstly we want to express our gratitude to you, and to the reviewers of our manuscript. Our replies to your constructive comments are included in the amended version of the manuscript and we think that they enhanced its quality.

Kind regards,

Zorica Juranic et al.,

The answers on the comments:

Reviewer: An M.T. Van Nuffel
Reviewer's report:
The article deals with the question whether diet e.g. mushrooms that carry the antigen tyrosinase and melanin, could contribute to the adaptive immune response in order to avoid melanoma relapse after resection of the primary tumor in patients without metastasis. Therefore, in the first instance they look whether and which type of humoral immunity is present in melanoma patients and compare therefore against vitiligo patients and healthy donors.

Major compulsory revisions
1) The authors mention in their manuscript that human and mushroom tyrosinase share some immunogenic epitopes. However, they do not indicate the exact percentage of homology between both proteins. Please include this in the manuscript.

The answer is put in the section Methods, and in References:

Mushroom tyrosinase, purchased from Sigma Aldrich, has been reported to have 16.32 % sequence identity and 41.08 % sequence similarity with human tyrosinase.

Furthermore, they do not show whether the immunoglobulins found were directed against the shared epitopes or against epitopes specific for mushroom tyrosinase. I believe this is crucial information to estimate the protective potential of the present immunoglobulins against malignant melanoma. They could for instance screen with synthetic human tyrosinase as they did for melanin to assure that the presence of the immunoglobulins can contribute to the control of malignant melanoma.
Reply:

In our study we determined the levels of anti-tyrosinase IgG, IgM and IgA autoantibodies, (specific for mushroom tyrosinase) as clearly stated in the manuscript text. Furthermore, data concerning the detection of anti-tyrosinase antibodies, significant for melanoma and vitiligo, are previously published and cited in the text. Obviously, detected antibodies could bind antigenic determinants of human and mushroom tyrosinase. (Moreover it was reported that mice immunized with mushroom tyrosinase generated a high titer of anti-tyrosinase antibodies and after the inoculation of melanoma cells developed a lower number of lung metastases compared with an unvaccinated control group (Fishman et al., ref. (23)).)

The answer is put into the text of the manuscript.

2) The authors claim in the last sentence of the results section that the PBMC of healthy individuals proliferate better upon stimulation with melanin compared to the PBMC of melanoma patients. To me this is not visible in figure 4, panel A. Only in combination with PHA a slight increased proliferation is observed, which is likely due to the PHA. Therefore, this statement should be changed.

Reply:

Stimulation of PBMC for proliferation by synthetic melanin and PHA although present in patients with melanoma, was more pronounced in healthy people. (It is changed in the text.)

Minor essential revisions
1) Tyrosinase is a membrane associated glycoprotein that is directed towards the melanosomes as it is a key enzyme in the melanin synthesis. To which extend does it becomes expressed at the surface to allow opsonization by immunoglobulins? Also for melanin. What is its surface expression? Please mention this in the manuscript.

Reply:

-Two forms of tyrosinase exist: intracellular membrane bound form (consisting of inner, transmembrane and cytoplasmic domain), and soluble form. It is proposed that membrane soluble forms could serve as an antigen. Tyrosinase is detected in the serum as well. In addition, melanocytes possess phagocytic activity and express MHC II molecules, therefore can present antigens derived from tyrosinase and melanin. (Baharav et al., 1996).

-Melanin is an intracellular pigment, but its role as an antigen in immune control of melanoma is proved in vivo. It is important to note that anti-melanin IgM antibodies labeled with (188) Re are reported to be successful in radioimmunotherapy of experimental metastatic melanoma.

The answers are included in the text.

2) If eating mushrooms could lead to humoral immunity against tyrosinase and melanin, it is important that the mushroom consumption habits of the patients in the three groups were the same in order to compare the presence and intensity of this humoral immunity against these antigens. Is it checked that this habit is comparable in the three groups: the melanoma patients, the vitiligo patients and the healthy donors?

Please comment and include this in the manuscript

**Reply:**

This suggestion will be useful for further investigation. In this preliminary analysis, we did not examine mentioned characteristics of tested subjects.

3) The authors mention that IgA would have a blocking effect, while IgM would be important to control the disease. Can they explain more in the discussion section why that could be? Are they sure that this is a direct effect of this isotype of immunoglobulin?

**Reply:**

Enhanced levels of anti-tyrosinase and anti-melanin IgA antibodies are found in 14 out of 62 and in 12 out of 62 patients with melanoma, respectively. Mentioned elevated IgA levels were more frequent in patients with metastatic disease. On the contrary, the presence of elevated IgA antibodies was less frequent in healthy persons. Moreover, presence of enhanced levels of IgA antibodies was not observed in patients with vitiligo. These results and results from previous work by Juranic et al. (reference 24) may indicate that in melanoma patients the IgA express its blocking action. Even in the presence of enhanced percentage of CD89 (FcαR) expressed by granulocytes, monocytes, macrophages, T lymphocytes and NK cells, it seems that there is no IgA-dependent cellular cytotoxicity as the metastatic disease exists. These results point to the anti-melanin IgA, and anti-tyrosinase IgA blocking function.

One of the crucial properties of IgM antibody is its ability to activate classical complement pathway. Regarding suggested protective role of IgM antibodies, decreased levels of anti-tyrosinase IgM antibodies are observed in 28 out of 62 melanoma patients and in 24 out of 36 patients with metastatic disease. Decreased levels of anti-melanin IgM antibodies are found in 11 out of 62 melanoma patients (and in 7 out of 35 patients with metastatic disease). Moreover, significantly lower levels of anti-tyrosinase IgM and anti-melanin IgM antibodies are detected in
sera of melanoma patients compared to healthy controls. It is also important to note that anti-melanin IgM antibodies labeled with (188) Re are reported to be successful in radioimmunotherapy of experimental metastatic melanoma.


(The answer is put into the text.)

4) Throughout the figures, it might for some readers be difficult to see the difference between the red and bright red squares used to represent the data of the melanoma patients respectively with and without metastatic disease. Maybe it is an option to use open and filled squares?

It is done.

5) PBMC is written fully for the first time in the material and methods section, while it is already used in the abstract and results section. Please write the full name the first time it is used. Also ADCC and MTT are only provided as an abbreviation. Please write this in full as well the first time the abbreviation is used.

These corrections are in the amended version of the manuscript.

Discretionary Revision

1) Is a minimal amount of mushrooms necessary to eat before humoral immune responses occur? The effect of diet consisting of approximately 20g of edible mushrooms in one usual meal consumed twice weekly is planed to be determined.

Does the kind of mushrooms plays a role (e.g. brown mushrooms probably contain more tyrosinase and melanin)?

May be, it shall be determined in the next study.
Reviewer: Masaru Toriyama

Reviewer's report:
Discretionary Revisions
1. Abstract did not summarize the current study.
The aim of the paper is to determine the intensity of humoral immunity to melanoma-associated antigens. But conclusion said that humoral immunity to melanoma-associated antigens found even in healthy people showed that these antigens were immunogenic. Furthermore, the conclusion in abstract is inconsistent with that in main part of the paper. I cannot catch what the authors will elucidate.

Reply:
The article deals with the question whether consummation of edible mushrooms that carry the antigen tyrosinase and melanin, could contribute to the adaptive anti-melanoma immune response. If the answer is yes, this could be considered as the aim for another new study—whose results could give the answers—is it possible by mushroom consumption to prevent melanoma, or avoid melanoma relapse after resection of the primary tumor, in patients without metastasis. Therefore, in the first instance they look whether and which type of humoral immunity is present in melanoma patients and compare it in vitiligo patients and healthy donors.

2. The study is very preparative.
If the authors would propose the method of diet for melanoma patients, they should test whether the proposal is correct or incorrect. I think the proposal is very important, but the results described in this paper have poor relevance to the proposal. There are few scientific inference between humoral immunity and the proposed diet method.

Reply
The data obtained in this work suggest that proposal that consuming mushrooms could induce humoral immunity to tyrosinase and to melanin.
Yes, there are reports on the antitumor action of mushrooms. In scientific literature we have not found data indicating that mushroom consumption could induce serum the special IgA, IgM and IgG anti-melanoma, or anti-tyrosinase immunity.

3. This study is focused on nowhere.
I understand that melanoma is very serious disease. Then studies on melanoma were done very well. There are many target points to investigate such as development, growth, malignant transformation, infiltration, metastasis and so on. If the authors will show the humoral immunity and melanoma scientifically, they should develop the studies from one or two of the view points above mentioned.

Reply
We have published several scientific articles dealing with melanoma control by chemical agents, or by immune mediated actions. We also think that food could be very important source of
additional control of malignant diseases, by immune mediated and pharmacological-antitumor action as well.