In vivo antitumor effect of proteoglycan fraction from Agaricus brasiliensis does not depend on the production of antitumor antibodies

O efeito antitumoral in vivo da fração proteoglicana de Agaricus brasiliensis não depende da produção de anticorpos antitumorais

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**Lindsey Castoldi**  
Doutora  
Institution: Instituto de Ciências da Saúde  
Adress: Universidade Federal de Mato Grosso, Avenida Alexandre Ferronato 1200, Cidade Jardim, Sinop, Mato Grosso, Brasil  
E-mail: lindsey.castoldi@ufmt.br

**Andrée Vanessa Pinto Domeneghini**  
Doutora  
Institution: Centro Universitário Central Paulista  
Adress: Rua Miguel Petroni, 5111, Lote Habitacional São Carlos 1, São Carlos, São Paulo, Brasil  
E-mail: patologiaunicep@yahoo.com.br

**Ramon Kaneno**  
Pós-doutorado  
Institution: Universidade Estadual Paulista  
Adress: Rua Professor Dr. Antônio Celso Wagner Zanin, 250, Distrito de Rubião Junior, Botucatu, São Paulo, Brasil  
E-mail: rskaneno@yahoo.com.br

**ABSTRACT**
Polysaccharides isolated from the edible mushroom Agaricus brasiliensis were previously shown to have antitumor, antiproliferative, and immunomodulatory activity. Here, we evaluated the in vivo effect of the acid-treated fraction from A. brasiliensis (ATF) on the subcutaneous growth of Ehrlich tumor cells (EHR) and on the production of tumor-specific antibodies. Mice (n=10) were inoculated with 2 × 10^6 EHR and injected subcutaneously in the tumor inoculum region with 0.1 mL ATF or saline. Control (tumor-free) group received ATF or saline. Treatments were carried out for 7, 14, or 30 days, with three consecutive doses and an interval of 4 days between the first and last doses, being repeated until the end of each experimental period. Histopathological analysis shows the infiltration of mononuclear and polymorphonuclear cells into the tumor site of all tumor-bearing mice. Tumor stimulated the influx of polymorphonuclear cells in the early stages, especially at 7 days, while the influx of mononuclear cells was higher in the final stages, at 14 and 30 days in all animals, independently of the treatment with ATF. Treatment of animals for 30 days reduced the tumor weight in 30% but we did not find a correlation with the antitumor antibody production since both treated and untreated mice were able to produce them.
Keywords: acid-treated fraction, agaricus blazei murrill, agaricus brasiliensis, ehrlich tumor, antibodies.

RESUMO
Polissacarídeos isolados do cogumelo comestível Agaricus brasiliensis demonstram ter atividade antitumoral, antiproliferativa e imunomoduladora. O objetivo deste trabalho foi avaliar a ação da fração ácido tratada do A. brasiliensis (ATF) sobre o desenvolvimento subcutâneo (sc) do tumor de Ehrlich (EHR) e na produção de anticorpos antitumorais. Os animais (n=10) foram inoculados (sc) com 2 x 10^6 células de EHR e tratados (0.1 mL, sc) na região do inóculo tumoral com ATF ou salina. Animais dos grupos controles (livres do tumor) foram tratados com ATP ou salina. Os tratamentos foram realizados durante 7, 14 ou 30 dias, com 3 doses consecutivas com intervalo de 4 dias entre a primeira e última dose, sendo repetido até o final de cada período experimental. A análise histopatológica do tumor demonstrou que a presença de células mononucleares e polimorfonucleares foi similar entre os grupos durante todo o experimento. O desenvolvimento tumoral estimulou o influxo de células polimorfonucleares nas fases iniciais, principalmente aos 7 dias, enquanto o influxo de células mononucleares foi maior nas fases finais, aos 14 e 30 dias. Apesar do tratamento durante 30 dias resultar na redução do peso tumoral em 30%, não houve diferença entre os grupos ATF/EHR e EHR quanto a produção dos anticorpos anti-EHR, pois tanto os camundongos tratados quanto os não tratados foram capazes de produzi-los.

Palavras-chave: fração ácido-tratada, agaricus blazei murrill, agaricus brasiliensis, tumor de ehrlich, anticorpos.

1 INTRODUCTION
Cancer is the second main cause of death worldwide just after coronarian diseases.¹ Despite the variety of drugs available to treat cancer, tumor cells are able to develop drug resistance.² In addition, side effects of chemo and radiotherapy lead many patients to search for alternative methods to help the anticancer fight.² The use of natural products as a complementary treatment to modern medicine for different types of cancers has increased in recent years, mainly due to their immuno-stimulating activity and few side effects.²⁻⁴ Edible mushrooms are among the most popular nutraceutical food used to fight cancer, due to their putative antiviral, antibiotic, anti-inflammatory, immunomodulatory and antitumor activities.⁴⁻⁵ Mushrooms have low calorie but are a rich source of proteins, lipids, carbohydrates, fibers, minerals, and vitamins (mainly B2, C, D).⁵⁻⁶

Agaricus brasiliensis Wasser et al. or A. blazei Murill.⁷ is one of the most consumed and studied mushrooms, especially concerning its antitumor activity.² This species was discovered in Brazil and began to be studied in Japan in 1969.⁸⁻¹¹ Polysaccharides and proteoglycans isolated from A. blazei have antitumor,
antiproliferative, anti-mutagenic, and immunomodulatory activities. In 1998, Ebina and Fujimiya developed an acid-treated soluble oxalate fraction (ATF) from \textit{A. blazei}. The ATF consists mainly of \((1\rightarrow4)\)-\(\alpha\)-D-glucan and \((1\rightarrow6)\)-\(\beta\)-D-glucan and has direct antitumor action on tumor cells, and indirect antitumor action through increased immune response mediated by natural killer (NK) cells, specific cytotoxic cells, and macrophages.

Based on these studies we have previously investigated the activity of ATF in the Ehrlich tumor model. This tumor is a murine spontaneous mammary carcinoma, maintained though successive animal passages in its ascitic form. These cells can be subcutaneously (s.c.) implanted in mice of any being not affected by MHC compatibility, and not spontaneously regressing. In this model of study, we observed that the fraction inhibited tumor growth, reduced the production of interleukin (IL)-10, and modulated tumor-induced immunosuppression. In another study, we also found that ATF is able to stimulated the fungicidal activity of mouse peritoneal macrophages, increasing their levels of hydrogen peroxide, and the expression of the mannose receptor. In human monocytes, the increased activity was associated with the expression of toll-like receptor (TLR) 4 and TLR2, and with the production of tumor necrosis factor-alpha (TNF-\(\alpha\)) and IL-1\(\beta\).

The effects of ATF on the components of the immune system is well documented, but its effect on the production of antibodies has not been investigated. Although antibodies may be less important in mediating effective antitumor immune responses, individuals with tumors produce antibodies against tumor-specific or tumor-associated antigens whose effector activity is associated with complement activation or cytotoxicity mediated by macrophage and NK cells, which express receptors for the Fc portion of IgG. In this study we assessed the presence of antitumor serum antibodies in peripheral blood of animals bearing s.c. Ehrlich tumor, and the leukocyte infiltration at the tumor site, following the \textit{in situ} treatment with ATF of \textit{A. brasiliensis}.

2 MATERIAL AND METHODS

2.1 EXPERIMENTAL DESIGN

Male BALB/c mice (n=10) were subcutaneously (s.c.) inoculated on the right flank with \(2 \times 10^6\) Ehrlich tumor cells after trichotomy. Animals were treated with three consecutive doses of 0.1 mL of \textit{A. brasiliensis} ATF (EHR/ATF) or buffered saline (EHR) was inoculated at the tumor inoculum site, with an interval of 4 days between the first and
last dose during 7, 14, or 30 days. Tumor-free mice used as control group were treated subcutaneously with extract (ATF) or saline solution (Control). After this period, the animals were euthanized with an overdose of sodium pentobarbital (Sigma-Aldrich, Saint Louis, MO, USA), followed by cardiac puncture. Serum was recovered and examined for anti-Ehrlich antibodies by indirect immunofluorescence. Each tumor mass was removed for histopathological analysis.

2.2 ANIMALS

The experiment was carried out using 45-day-old, male, BALB/c isogenic strain mice obtained from the Centro de Bioterismo of Universidade Estadual de Campinas (UNICAMP), São Paulo, Brazil. The animals were kept in the animal house of the Department of Pathology, School of Medicine of Botucatu, Universidade Estadual Paulista (UNESP), in polypropylene boxes, at a constant temperature of 22°C with ad libitum access to water and feed (Nuvital, Campinas, São Paulo, Brazil) throughout the study. All procedures involving animals followed the international standards for handling and using experimental animals and were approved by the Ethics Committee on Animal Experimentation of the School of Medicine of Botucatu, UNESP (protocol no. 287).

2.3 EHRLICH TUMOR

Ehrlich tumor was maintained in its ascitic form in Swiss mice through successive and periodic passages in another animal. For the study, ascitic cells were recovered, washed with phosphate buffered salt solution (PBS), and their viability was assessed based on their ability to exclude trypan blue dye. We considered a viability of at least 70% to prepare a cell suspension in PBS, adjusted to $1 \times 10^7$ cells mL$^{-1}$ to be s.c. inoculated (0.1 mL/mouse in the EHR and EHR/ATF groups).

2.4 ATF EXTRACT FROM A. BRASILIENSIS

Approximately 1,000g of dried and powdered fruiting bodies of A. brasiliensis Wasser et al., provided by Department of Plant Production, School of Agronomic Sciences of Botucatu, UNESP, were extracted three times with 9 L of 80% ethanol by boiling for 22 h. The insoluble residue was recovered by centrifugation at 5,000 × g for 10 min and extracted another three times with 9 L of distilled water by boiling for 22 h each time. The final insoluble residue was recovered by centrifugation (5,000 × g, 10 min) and extracted twice in 9 L of 5% ammonium oxalate at 100 °C for 10 h. The resultant
ammonium oxalate soluble extract was filtered, dialyzed, and concentrated. This material was then treated with 1M HCl for 24 h at room temperature, followed by the pH adjustment 7.0 by adding 1M NaOH, and remotion of particulate material by centrifugation. The supernatant, here referred as ATF, was sterilized by filtration through a 0.22 µm pore size membrane (Millipore, Billerica, MA, USA) and furtherly lyophilized.12-13, 16 This ATF was reconstituted with saline solution at the concentration of 5 mg mL⁻¹ to be inoculated into the animals.

2.5 HISTOPATHOLOGICAL ANALYSIS

The tumor inoculum site was excised and placed in vials containing 10% buffered formalin. After 24 h, the material was washed in water and kept in 70% alcohol until the time of cutting and embedding in paraffin. The sections were stained with hematoxylin and eosin for histopathological analysis. The tumor mass was assessed for the presence of mononuclear or polymorphonuclear cells. Each section was scored from 0 to 3, to indicate the absence of cells (0), weak cell infiltration (1), moderate cell infiltration (2), or intense cell infiltration (3). Mice from the non-tumor groups (ATF or Control) were assessed for treatment stress. We also looked giant tumor cells, mitotic figures, and necrotic areas.

2.6 ANALYSIS OF ANTI-EHRlich ANTIBODIES BY INDIRECT IMMUNOFLUORESCENCE

Cryopreserved Ehrlich tumor cells thawed in a water bath at 37°C and quickly resuspended in 10 mL of complete culture medium [RPMI 1640 culture medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Nutricell, Campinas, São Paulo, Brazil), 200 mM L-glutamine, 25 mM HEPES, 40 UI gentamicin, and 2 g L⁻¹ sodium bicarbonate]. Cells were washed twice by centrifugation at 1,500 rpm for 10 min and cell viability was assessed by trypan blue exclusion test. Considering the minimum viability of 70%, cells were incubated at 37°C, under 5% CO₂ for 24 h. Subsequently, cells were washed and suspended in 1% albumin solution (Sigma-Aldrich) at 4 × 10⁶ cells mL⁻¹. About 50 µL this cell suspension was put on glass slides and kept at room temperature for 5 min, when the liquid excess was aspirated. Glass slides were dried at room temperature and the cell layer was fixed with cold acetone and allowed to dry. Each slide
was individually wrapped in a tissue paper and aluminum foil, and stored at – 20ºC until use.

For the analysis of anti-Ehrlich antibodies, sera of studied mice were diluted at 1:20 and individually put on previously prepared fixed Ehrlich cells, and incubated in a humid chamber at 37 ºC, under 5% CO₂ for 30 min. Glass slides were then washed with PBS for 10 min and added with FITC-labeled anti-mouse IgG antibody (BD Biosciences, San Jose, CA, USA, diluted at 1:80). After a 60 min incubation at room temperature inside a dark box, slides were washed with PBS for 5 min, dyed with a 0.01% Evan’s blue dye solution and mounted with buffered 50% under a glass coverslip for observation by fluorescence microscopy. A pool of serum obtained from High Antibody Responder Mice (Biozzi mice)²¹ immunized with Ehrlich tumor cells was used as positive control, while a pool of serum from normal animals was used as negative control. Data were expressed as the reciprocal of the dilution.

2.7 STATISTICAL ANALYSES

The groups were compared at each time of assessment using the non-parametric Kruskal-Wallis test. The effect of treatment time in each group was analyzed by Friedman's non-parametric test. The groups with tumors, which were treated with ATF extract were compared with those untreated for 7, 14, or 30 days using the Mann-Withey test. All tests were performed considering a 5% significance level.

3 RESULTS

3.1 HISTOPATHOLOGICAL ANALYSIS

Table 1 shows the median and semi-amplitude scores of the mononuclear cell analysis, according to the treatment group and period. The presence of mononuclear cells was similar between EHR and EHR/ATF groups throughout the experiment, but at 14 and 30 days tumor development induced an increased influx of mononuclear cells to the inoculum site.

Table 2 shows that similarly to mononuclear cells, the intensity of the polymorphonuclear infiltration was similar between EHR and EHR/ATF groups throughout the experiment. However, the higher influx of polymorphonuclear cells was induced in the early stages of tumor development (7 days of treatment).

Although treatment with ATF extract for 30 days resulted in a 30% reduction in tumor weight, there was no difference between the EHR/ATF and EHR groups regarding
the presence of mitotic figures, giant neoplastic cells, or areas of necrosis (data not shown).

Table 1: Median and semi-amplitude of the scores of the analysis of mononuclear cell infiltrate, according to group and time of sacrifice

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TIME (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>CONTROL</td>
<td>2.0 ± 1.0 a*Aα</td>
</tr>
<tr>
<td>ATF</td>
<td>2.0 ± 1.0 aA**α</td>
</tr>
<tr>
<td>EHR</td>
<td>3.0 ± 1.0 aAα</td>
</tr>
<tr>
<td>EHR/ATF</td>
<td>3.0 ± 1.0 aAα</td>
</tr>
</tbody>
</table>

Histopathological analysis was performed on hematoxylin and eosin-stained sections of solid Ehrlich tumor from animals treated with Agaricus brasiliensis extract for 7, 14, or 30 days. The cell infiltrate received scores from 0 to 3, with 0 representing the absence of cells, 1 weak cell infiltrate, 2 moderate cell infiltrate, and 3 intense cell infiltrate.

* Lowercase letters indicate comparison of the treatment effect within the same time.
* Capital letters indicate comparison of groups with tumors within the same time.
# Greek letters indicate time comparison within the same group

Non-parametric analysis of variance technique for the three-factor model (treatment effect, tumor, and time), complemented with the respective multiple comparison tests, \( P < 0.05 \).

Table 2: Median and semi-amplitude scores for the polymorphonuclear infiltrate cell infiltrate analysis, according to group and time of sacrifice

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TIME (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>CONTROL</td>
<td>0.0 ± 0.0 a*Aα</td>
</tr>
<tr>
<td>ATF</td>
<td>0.0 ± 0.0 aA**α</td>
</tr>
<tr>
<td>EHR</td>
<td>3.0 ± 0.5 aBβ</td>
</tr>
<tr>
<td>EHR/ATF</td>
<td>3.0 ± 1.5 aBβ</td>
</tr>
</tbody>
</table>

Histopathological analysis was performed on HE-stained sections of solid Ehrlich tumor from animals treated with Agaricus brasiliensis extract for 7, 14, or 30 days. The cell infiltrate received scores from 0 to 3, with 0 representing the absence of cells, 1 weak cell infiltrate, 2 moderate cell infiltrate, and 3 intense cell infiltrate.

* Lowercase letters indicate comparison of the treatment effect within the same time.
* Capital letters indicate comparison of groups with tumors within the same time.
# Greek letters indicate time comparison within the same group
Non-parametric analysis of variance technique for the three-factor model (treatment effect, tumor, and time), complemented with the respective multiple comparison tests, \( P < 0.05 \).

3.2 CIRCULATING ANTITUMOR ANTIBODIES

Table 3 shows the median and total semi-amplitude of antitumor antibody titers assessed by indirect immunofluorescence. The production of antitumor antibodies was detectable after 14 days of treatment and was more evident at 30 days. Treatment with ATF did not change the production of anti-Ehrlich antibodies in tumor-bearing mice.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TIME (days)</th>
<th>TIME TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>CONTROL</td>
<td>0 ± 0 a*A**</td>
<td>0 ± 0 aA</td>
</tr>
<tr>
<td>ATF</td>
<td>0 ± 20 aA</td>
<td>0 ± 10 aA</td>
</tr>
<tr>
<td>EHR</td>
<td>0 ± 0 aA</td>
<td>0 ± 20 aB</td>
</tr>
<tr>
<td>EHR/ATF</td>
<td>0 ± 20 aA</td>
<td>0 ± 20 aB</td>
</tr>
</tbody>
</table>

The presence of anti-Ehrlich antibodies in the animals' serum was evaluated by indirect immunofluorescence reaction on slides pre-fixed with Ehrlich tumor cells, and the data are presented as a reciprocal of the dilution.

* Lowercase letters indicate comparison of groups within the same time
* Capital letters indicate time comparison within the same group

Non-parametric analysis of variance technique for the three-factor model (treatment effect, tumor, and period), complemented with the respective multiple comparison tests, \( P < 0.05 \).

4 DISCUSSION

In this study we investigated the effect of the ATF, extracted from the edible mushroom *A. brasiliensis* on the leukocyte infiltration, and the production of antibodies by mice s.c. implanted with Ehrlich cells. Treatment of tumor-bearing mice with ATF was able to control the growth of Ehrlich solid tumor, reducing the tumor mass by 30%.

Ehrlich tumor cells are considered highly immunogenic\(^{15,22}\) and can promote a strong humoral response, especially against the mucin-1 protein\(^{23,24}\) and tumor cell
surface carbohydrates.\textsuperscript{22,23,25} Ehrlich ascitic tumor cells also have gangliosides on their surface.\textsuperscript{26} These include GM3, GM2, GM1, GD3, GD1a, and GT1b.\textsuperscript{26} Normal animals immunized with attenuated Ehrlich cells, GT1b, or anti-GT1b antibodies are protected against the growth of this carcinoma due to the production of IgM antibodies.\textsuperscript{22,23,25,27}

Differently, we only investigated the generation of IgG, since it represents a long-term immunity.\textsuperscript{20,28} We found that both the animals treated with ATF and those receiving just saline solution, were able to produce anti-Ehrlich IgG, showing that such antibodies have no relevant role in the resistance against the development of Ehrlich tumor cells. This data contrast with our previous studies, in which we observed an inverse correlation between mice survival and humoral response (anti-Ehrlich IgG) following the treatment with aqueous extracts of different varieties of the mushroom \textit{Lentinula edodes}.\textsuperscript{29} Although the administration of dichloromethane or methanol extracts of \textit{A. blazei} stimulated antibody production, it inhibited NK cell and lymphoproliferative activity of animals bearing Ehrlich tumors.\textsuperscript{30} Fantuzzi et al.\textsuperscript{31} demonstrated that the aqueous extract of \textit{A. brasiliensis} did not change the secretion of secretory IgA in animals treated with it. In a previous study, Kakajima et al.\textsuperscript{4} observed that the treatment of normal animals with hot water extract of \textit{A. blazei} increased the production of antibodies against sheep red blood cells, possibly due to the differentiation of B lymphocytes induced by IL-6 and IL-1\textsuperscript{2} produced by activated T lymphocytes and macrophages. The collective findings support the idea that the glucans and proteoglycans present in \textit{A. brasiliensis} ATF extract differentially affect immune effector cells\textsuperscript{2}, they modulate the production of pro- and anti-inflammatory cytokines\textsuperscript{16,17} and consequently, the balance of cellular and humoral responses.\textsuperscript{29}

We observed that both groups of tumor-bearing mice induced the infiltration of polymorphonuclear and mononuclear cells into tumor-site, without a relevant influence of ATF inoculated \textit{in situ}. Ehrlich carcinoma has escape mechanisms that suppress the host's immune system by reducing the responsiveness of T lymphocytes and progressively decreasing the activity of splenic NK cells.\textsuperscript{32} Transforming growth factor-beta 1 (TGF-\beta 1) and a range of non-functional receptors that allow tumor cells to escape the potent inhibitory effects of TGF-\beta on their own growth have been identified at tumor implantation sites.\textsuperscript{32,33} Conversely, this cytokine can inhibit the function of TCD4\textsuperscript{+} cells and prevent the removal of the tumor by the immune system.\textsuperscript{33} This is evident by the drastic reduction of TCD4\textsuperscript{+} cells in the spleen of the animals in the initial days of tumor
Furthermore, macrophages may contribute to these suppressive effects, as they can also secrete immunosuppressive cytokines, such as TGF-β and IL-10.

Thus, we conclude that the ATF extract of *A. brasiliensis* did not change the production of anti-Ehrlich IgG despite modulating cell influx to the tumor site. Future studies should identify tumor antigens and the mechanisms involved in the modulation of the humoral response promoted by the compounds in the fraction.

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