SPANDIDOS PUBLICATIONS

BIOMEDICAL REPORTS 10: 183-188, 2019

Anti-*Toxoplasma gondii* antibodies attach to mouse cancer cell lines but not normal mouse lymphocytes

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Received January 24, 2018; Accepted January 4, 2019

DOI: 10.3892/br.2019.1186

Abstract. *Toxoplasma gondii* (*T. gondii*) is prevalent intracellular parasite and a cause of worldwide infection in the human population. An inhibitory effect of this parasite on cancer growth has been demonstrated in cell culture and animal models. To determine whether the anticancer activities of *T. gondii* are associated with host immune response, in the current study the reactivity of anti-*T. gondii* antiserum with the surface of cancer cell lines was investigated. Anti-*T. gondii* antibodies were raised in rabbit and the reaction of this antiserum in comparison with other anti-parasite antisera (anti-*T. vaginalis*, anti-hydatid cyst fluid, anti-protoscolices antigens) with mouse melanoma or breast cancer cells lines was investigated using flow cytometry. Anti-*T. gondii* antiserum reacted markedly with the surface of mouse melanoma and breast cancer cells, and less so with the normal mouse spleen lymphocytes. Meanwhile, the other anti-parasite antisera did not react strongly with the surface of cancer cells compared with normal mouse spleen lymphocytes. In summary, it has been demonstrated herein that anti-*T. gondii* antiserum may selectively react with the surface of mouse cancer cells but not with normal mouse spleen lymphocytes. Therefore, further study on anti-*Toxoplasma* antibodies may be useful for directing the application of selective drug delivery in cancer treatment.

Introduction

Toxoplasma gondii (*T. gondii*) is a protozoan parasite that infects approximately 1/3 of the human population worldwide (1). Anticancer activities of this parasite have been demonstrated in previous studies (2-4). Notably, it has been observed that non-replicating *T. gondii* reverses tumor associated immunosuppression (5). Antitumor effects of *T. gondii* antigens in a murine sarcoma 180 tumor model have also been demonstrated (4). In another study, Balb/c inbred mice were injected with *T. gondii* antigens and then challenged with WEHI-164 fibrosarcoma cells, and significant inhibition of tumor growth was observed (6). In other work Choo *et al* (2) injected mice with *T. gondii* antigens and observed that malignant glioma growth was inhibited significantly in comparison with a control group. Kim *et al* (3) challenged two groups of mice with Lewis lung carcinoma, and infected one with *T. gondii* as an experimental group. They observed that survival rate was significantly increased in the parasite-injected mice. Additionally, angiogenesis in the experimental group was notably inhibited (3). In other work, it has been demonstrated that intradermal injection of *T. gondii* stimulated a potent antitumor immune response *in vivo* (7). In this regard it has also been shown that *T. gondii* is able to generate therapeutic antitumor immunity against ovarian cancer (8). Besides *T. gondii*, anticancer activities of other parasites including *Trypanpsoma cruzi* (9), malaria parasite (10) and hydatid cyst [larval stage of *Echinococcus granulosus* (*E. granulosus*)] (11-13) have also been reported. The mechanisms underlying the anticancer effects of parasites are not well defined. However, it has been suggested that the immune response raised by the parasites may nonspecifically affect the growth of cancer. For instance, it has been demonstrated that *Trypanosoma cruzi* (*T. cruzi*) is able to elicit a protective immune response against colon and mammary cancers (9). The existence of antibody against *T. cruzi* in mouse was associated with inhibition of tumor growth *in vivo* (14). In another study, it was demonstrated that cell-mediated immunity had a role in antitumor activity of *T. cruzi* (15). In this regard, the presence of common antigens between cancers and certain parasites has been reported (16-18). To identify some of the mechanisms of the anticancer activities of parasites, in the present work a reaction panel of anti-parasite antisera with the surface of mouse melanoma and breast cancer cell lines was investigated.

Materials and methods

Antigen preparation. T. gondii purified tachyzoites were purchased from the Pasture Institute, Tehran, Iran. The

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Key words: antibodies, breast cancer, cell surface, melanoma, *Toxoplasma gondii*

tachyzoites were sonicated in PBS, centrifuged for 2 min at 600 x g at room temperature, and the supernatant containing *T. gondii* antigen was maintained at -20˚C. *Trichomonas vaginalis* (*T. vaginalis*) parasites maintained in liquid nitrogen from the Department of Parasitology, Isfahan University of Medical Sciences (Isfahan, Iran) were cultured in TYIS medium (produced in-house) and the antigen prepared as described in our previous work (19). Briefly, the parasites were harvested from culture medium, washed three times and sonicated, and *T. vaginalis* crude antigen was kept at -20˚C. Sheep lungs or livers infected with hydatid cysts were obtained from slaughtered sheep in Fasaran slaughter house in Isfahan, Iran. Hydatid cyst fluid was then aspirated and examined under a light microscope. Fluid exhibiting the presence of protoscolices was then centrifuged for 2 min at 600 x g and the supernatant containing hydatid cyst fluid antigen was stored at -20˚C. The packed protoscolices were also sonicated in PBS, centrifuged for 2 min at 600 x g and the supernatant stored as protoscolices antigen.

Cell culture. Mouse melanoma (B16F10) and breast (4T1) cancer cell lines were purchased from the Pasture Institute. The cells were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with a humidity of 70% and 5.0% CO_2 as previously mentioned (20). Normal lymphocytes were isolated from normal mouse spleens and prepared for further experimentation.

Animal experiments. Rabbits and mice were used in the current study, which were purchased from the Pasture Institute. A total of 6 female Balb/c, inbred, 2-month-old mice were used to prepare spleen cells. For this purpose, the mice were euthanized by intraperitoneal injection of 250 mg/kg body weight pentobarbital (Sigma, 3636 under sterile conditions and their spleens removed. Subsequently, the spleen cells were extracted, counted and their viability checked by using trypan blue staining. To prepare cells, spleens were removed from scarified mice and transferred to a Petri Dish containing Isotonic saline. Spleens were then minced using a Scalpel blade and the mixture was passed through a four-layered gauze to remove large debris. Isotonic saline containing cells were then washed twice with isotonic saline and centrifuged at 600 x g for 2 min at room temperature. Subsequently, cells were further suspended in isotonic saline. To stain cells with Trypan blue (Merck, 50 μl prepared cells was mixed with 50 μl Trypan blue stain at room temperature. Following 15 min, one drop of mixed cells with trypan blue was applied to a Neubauer slide (HBG, Germany) and counted using light microscope (magnification, x400).

Four month-old male rabbits were used to raise antibodies against parasites antigens. The animals were maintained in an appropriate animal research facility and mice kept in groups of six per cage (rabbits one/cage) and fed with clean food and water. The rabbits were housed at 20-25˚C with a humidity of 70-80% with a 12 h light/dark cycle. The animal research protocols of the present study were approved by Isfahan University of Medical Sciences Ethics Committee with approval number IR.mui.REC.1394.824.

Preparation of rabbit antisera. Volumes of 1 ml containing 2 mg of the different parasite antigens and sonicated breast and melanoma cancer cells were emulsified in the same volume (1 ml for each antigen) of Freund's adjuvant (Sigma-Aldrich; Merck KGaA) at room temperature and each antigen injected subcutaneously into a male 4-month-old, white New Zealand rabbit (n=5 in total). Injections were repeated fortnightly. In all rabbits, complete Freund's adjuvant was used for the first injections and incomplete Freund's adjuvant for the boosters. Following the third booster, 1 ml blood samples from the ear vein of each rabbit were collected. To prepare sera, the blood samples were centrifuged at 3,000 x g for 5 min at room temperature. The sera were then checked for the presence of specific antibodies using home-made ELISA tests as described in our previous study (20). In those rabbits with a high titer (optical density >1.5) of antibodies, the last booster was injected into each rabbit and then blood samples were collected and the sera kept at -20˚C until use. For the injection of rabbits or bleeding no anesthesia method was used. However, following final bleeding the rabbits were euthanized by intravenous injection of 150 mg/kg pentobarbital.

For ELISA, 96 wells plates were coated with the antigens (*T. gondii*, *T. vaginalis* or hydatid cyst protoscolices crude antigens) diluted 1:20 with carbonate buffer. Following overnight incubation at 37˚C, the plates were blocked with 1% bovine albumin (Merck KGaA) and then washed with sodium chloride buffer containing 0.05% Tween-20, and then diluted (1:100) antisera were added and incubated at 37˚C for 1 h. The plates were then washed and a secondary antibody (A6154; Sigma-Aldrich was added and incubated at 37˚C for 1 h. Finally, following washing, the plates were incubated with the chromogenic substrate and the optical density of wells was read at 450 nm using an ELISA reader (Kbiokit-ELx800).

Reaction of antisera with the cancer cell lines. Mouse cancer cells were harvested from culture medium and normal lymphocytes obtained from normal mouse spleens. All the cells were washed with PBS and incubated for 1hour at 37˚C with different antisera (1:100) which raised in rabbits against different mentioned antigens, namely anti-*T. gondii*, anti-*T. vaginalis*, anti-hydatid cyst fluid, anti-protoscolices antigens, anti-melanoma cells, anti-breast cancer cells or normal rabbit serum primary antibodies. After 1 h, the cells were washed with sodium chloride buffer containing 0.05% Tween-20, and secondary antibodies conjugated with fluorescein isothiocyanate (Donkey anti-rabbit IgG BioLegend, Inc., San Diego, CA, USA; 406403; 1:1,000) were applied in the dark at 4˚C for 1 h. Following the last washing of the test tubes, cells were analyzed using a flow cytometer. BD Cell Quest Pro software version 6 (BD Biosciences, San Jose, CA, USA) was used for analysis.

Statistical analysis. The experiments were repeated three times and data are presented as the mean ± standard deviation. A linear mixed model test was used for statistical analysis. In comparison of means, $P<0.05$ was considered to indicate significance.

Results

Reaction of anti-Toxoplasma antibodies with surface of B16F10 melanoma cells. The reaction of antiserum against

Table I. Results of flow cytometry analysis (M2 cell percentage of total cells) indicating the level of reaction of different antisera with the surface of mouse melanoma cancer cells (B16F10) and mouse normal spleen cells.

	Anti-melanoma (B16F10) antiserum	Anti-*Toxoplasma gondii* antiserum	Normal rabbit serum	Background fluorescence
B16F10	10.28±0.49	11.14±1.48[a]	1.91±0.31	1.11
Spleen cell	-	9.32±0.21	7.79±0.79	3.18

[a]P<0.05 B16F10 cells vs. spleen cells.

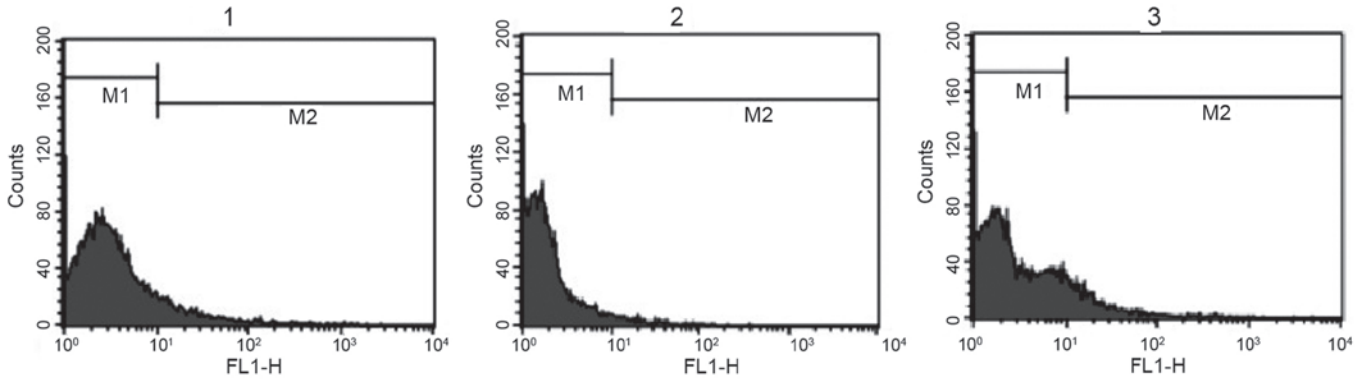

Figure 1. Reaction of antisera against mouse melanoma cancer cells, normal rabbit serum and *Toxoplasma gondii* with the surface of live melanoma cancer cells. M1 and M2 indicate cells that were not reactive or reactive with the treated antiserum, respectively.

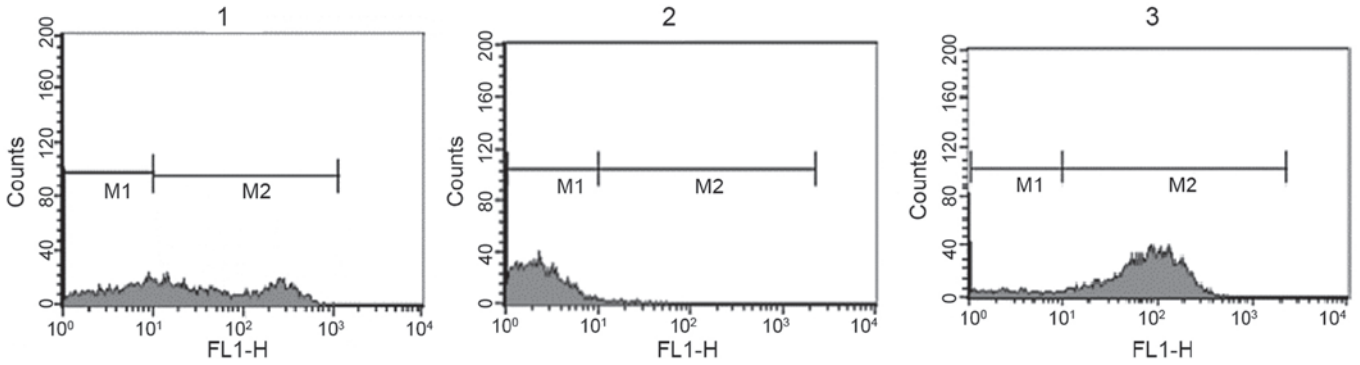

Figure 2. Reaction of antisera against breast cancer cells, normal rabbit serum and *Toxoplasma gondii* with the surface of live breast cancer cells. M1 and M2 indicate cells that were not reactive or reactive with the treated antiserum, respectively.

T. gondii and normal rabbit serum with the surface of mouse melanoma cells was investigated. Anti-*T. gondii* antiserum exhibited a marked reaction with melanoma cells, while the reaction of normal rabbit serum was weak. In this experiment, anti-melanoma cell antiserum was used as a positive control and normal rabbit serum as a negative control (Fig. 1 and Table I). Analysis of experimental repeats indicated that the difference in M2 (reactive) cells between the anti-*T. gondii* antiserum and normal rabbit serum groups was statistically significant (P<0.05).

Reaction of anti-Toxoplasma antibodies with surface of 4T1 breast cancer cells. The reaction of antisera against *T. gondii* and normal rabbit serum with the surface of mouse breast cancer cells was investigated. Again, *T. gondii* antiserum exhibited a strong reaction with breast cancer cells while the reaction of normal rabbit serum was weak. Anti-breast cancer cell antisera were used as a positive control and normal rabbit serum as a negative control (Fig. 2 and Table II). When the experiment was repeated, the difference in M2 cells between the anti-*T. gondii* antiserum and normal rabbit serum groups was statistically significant (P<0.05).

Reaction of anti-Toxoplasma antibodies with surface of mouse normal spleen lymphocytes. The reaction of anti-*T. gondii* antiserum and normal rabbit serum with the surface of mouse normal spleen lymphocytes was investigated. The results revealed that the reaction of anti-*Toxoplasma* antibodies with melanoma cells was greater than the reaction with mouse normal lymphocytes. However, the reaction of normal rabbit

Table II. Results of flow cytometry analysis (M2 cell percentage of total cells) indicating the level of reaction of different antisera with the surface of mouse breast cancer cells (4T1) and mouse normal spleen cells.

	Anti-4T1 antiserum	Anti-*Toxoplasma gondii* antiserum	Normal rabbit serum	Background fluorescence
4T1	62.14±7.97	81.77±22.2[a]	2.06±0.69	0.17
Spleen cells	-	7.79±0.79	9.32±0.21	3.18

[a]P<0.05 vs. spleen cells.

Table III. Results of flow cytometry analysis (M2 cell percentage of total cells) indicating reaction of antisera against Trichomonas vaginalis and hydatid cyst crude protoscolices antigen with the surface of breast cancer cells, melanoma cancer cells or normal mouse spleen lymphocytes.

	Anti-*Trichomonas vaginalis* antiserum	Anti-hydatid cyst protoscolices antiserum	Background florescence intensity
Melanoma cancer cells	3.74±0.74	2.11±0.35	1.11±0.14
Breast cancer cells	5.75±1.23	3.64±0.82	0.17±0.09
Normal mouse spleen lymphocytes	11.23±2.39	9.43±1.97	3.18±0.68

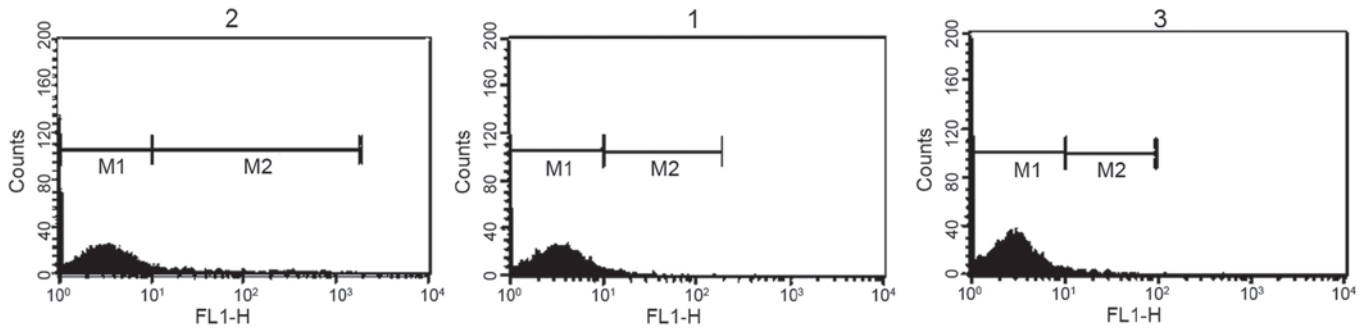

Figure 3. Reaction of normal rabbit serum and antisera against *Toxoplasma gondii* with the surface of normal mouse spleen lymphocytes. Background fluorescence has been shown as a control. M1 and M2 indicate cells that were not reactive or reactive with the treated antiserum, respectively.

serum with mouse normal lymphocytes was greater than the reaction with melanoma cells (Fig. 3 and Tables I and II).

Reaction of anti-T. vaginalis and anti-protoscolices antibodies with surface of melanoma, breast cancer or mouse normal spleen lymphocytes. To determine if antisera against different parasites exhibited a reaction similar to that of anti-*T. gondii* antiserum, the reactions of antisera against *T. vaginalis* and hydatid cyst crude protoscolices antigen with the surface of breast cancer cells, melanoma cells and normal mouse spleen lymphocytes were investigated. The results revealed no statistically significant differences in the reactions of the sera with cancer cells compared with spleen lymphocytes (Table III).

Discussion

Results of the present work indicated that anti-*T. gondii* antiserum selectively reacts with the surface of murine melanoma and breast cancer cells and not with normal mouse spleen lymphocytes. Additionally, the results revealed that this selective reactivity did not occur with other anti-parasitic antisera including anti-*T. vaginalis* or anti-hydatid cyst protoscolices antigen. This is a notable finding as through selective attachment of anti-*T. gondii* antibodies to cancer cells, these antibodies may by valuable carriers of cancer immunotherapeutics. In this regard, Salanti *et al* (21) demonstrated that a glycosaminoglycan protein of Plasmodium falciparum selectively attached to cancer cells, and they proposed that this protein may be a viable candidate for cancer immunotherapy.

Reaction of anti-*Toxoplasma* antibodies with the surface of breast and melanoma cancer cells implies that there are shared epitopes on the surface of cancer cells and *Toxoplasma* antigens. In this context, existence of common antigens between certain parasites and cancers has been documented (16,17). The majority of these antigens are specific mucin glycoproteins, which have important roles in the metastasis of cancer cells (22) and in the interaction of parasites and their hosts. For instance, expression of tumor-associated antigen in adult and larval stages of *E. granulosus* has been observed. Additionally,

cross-reaction of the sera of cancer patients with 40 and 27 kDa bands of hydatid cyst antigens has been demonstrated (18,23).

The effect elicited by cross-reaction of antibodies raised against *Toxoplasma* with epitopes on the surface of cancer cells remained undetermined in the present study. However, the attachment of the antibody to the cancer cells surface may exert some lethal effects on the cancer cells. For instance, it has been shown that *Trypanosoma cruzi* antigens stimulated protective immunity against colon and mammary cancers (9). Alternatively, this cross-reaction may render the cancer cells vulnerable to the complement system. In this regard it has been reported that complexes of antigen-antibody are able to activate the complement system (24).

In previous years there has been considerable focus on enhancing antibody activity through conjugation with cytotoxic drugs (25-28). Thus, with further work it may be possible to conjugate humanized purified anti-*Toxoplasma* monoclonal antibodies to a cytotoxic drug to improve its suitability for site-selective drug delivery. Such anti-*Toxoplasma* monoclonal antibodies may have the advantage of being effective against different cancers. It has been demonstrated that drug targeting may improve the efficacy of therapy and reduce side effects associated with drugs (29). Immunohistochemistry is now recommended to investigate the reaction of anti-*Toxoplasma* antibodies with human breast cancer tissues.

One of the main problems in the treatment of cancer is that most of the drugs used to target cancer cells are also cytotoxic to normal tissues. Attempts have been made to overcome this problem by coupling anticancer drugs to antibodies which have some degree of specificity for cancer antigens. In this regard there are numerous patents regarding use of antibodies for drug delivery. For instance, in a patented method by Sahin *et al* (30), monoclonal antibodies against claudin-18 were used for the treatment of different cancers. Patil *et al* (31) also conjugated a polymalic acid platform to a monoclonal antibody to enable specific drug delivery to treat different cancers. Furthermore, conjugation of an antibody with a cytotoxin or an enzyme for the selective treatment of cancers has been proposed (32).

Through further study it may be possible to conjugate humanized purified anti-*Toxoplasma* monoclonal antibodies to a drug for selective treatment of certain cancers. Tumor-associated antigens (TAAs) are expressed by tumor cells and can be recognized by the immune system. Most known TAAs have been found to be expressed by melanoma cancer cells, and few TAAs have been recognized in other tumors (33). TTAs exhibit poor immunogenicity, which results in lack of a sufficient immune response to control cancer growth (34,35). It has been demonstrated that vaccination with TAA peptides generally fails to induce a specific immune response in mice (36). Immunization with parasite-derived antigens, which have shared epitopes with TTAs, may overcome this disadvantage and induce a high degree of protective immunity. In this regard, it has been observed that for virally induced tumors, prophylactic vaccination with synthetic peptides was effective in animal models (37,38), whereas for non-virally induced tumors this vaccination was less effective (39,40). Thus, induction of a strong immune response may be one of the critical advantages of immunotherapy with parasitic antigens.

In conclusion, in the present study it was indicated that anti-*Toxoplasma* antiserum selectively reacts with the surface of mouse cancer cells but not with normal mouse spleen lymphocytes. With further work these selectively binding antibodies may be a useful tool in cancer immunotherapy.

Acknowledgements

The present study was prepared from thesis by a PhD student (Mrs Mahshid Shakibapour) and an MSc student (Miss Fereshteh Mohamadi) from Isfahan University of Medical Sciences.

Funding

The present work was supported by a grant (grant no. 3953009) from Isfahan University of Medical Sciences (Isfahan, Iran).

Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

FM and MS performed the experiments. SS performed the experiments and wrote the first draft of the manuscript. AA consulted immunohistochemical procedures. ST assisted with experiments. HD supervised experiments and prepared final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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