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BRIEF REPORT

Immunosuppressive effect of purified pyocyanine pigment on T-lymphocytes viability against experimental infection with hydatid cyst protoscolices

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ABSTRACT

Background: The effect of pyocyanine pigment, which was isolated and purified from *Pseudomonas aeruginosa*, on specific lymphocytes viability inside the body of white male Balb/c mice against the experimental secondary hydatidosis and the infectivity of protoscolices was studied in comparison with negative control mice groups, phosphate buffered saline (PBS) and positive control group (immunoferon).

Materials and methods: Four groups of male Balb/c mice were intraperitoneally (IP) inoculated with four purified concentrations of pyocyanine (25, 50, 75, 100µm/ml). Seven days later, they were given the same concentrations as a booster dose of the pigment, then 7 days later they were intraperitoneally infected with 2000 protoscoleces /mL (PBS) as a challenge dose. The fifth group was intraperitoneally inoculated with 1ml of sterile PBS and used as a negative control group, while the sixth group was intraperitoneally inoculated with 100µmg/ml immunoferon and received the challenge dose of 2000 protoscoleces/ml PBS and served as the positive control group.

Results: The concentrations of 50, 75 and 100µm/ml of this pigment had suppressive effect on these specific immune response cells. This effect was statistically significant ($p<0.01$) after six weeks from the challenge dose with intraperitoneal protoscolices infection. This effect revealed that the protoscolices infectivity increased due to suppression viability of T lymphocytes, while the immunoferon showed a significant stimulation of these specific cellular cells, which decrease the protoscolices infectivity in comparison with higher pigment concentrations.

Conclusion: Pyocyanine is a toxic pigment causing suppression of T-cells activity, especially at higher concentrations which allow protoscolices development and growth.

Keywords: *Pseudomonas aeruginosa, Pyocyanine, T cell, Protoscolices.*
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INTRODUCTION

Cystic echinococoosis (CE) is a widespread chronic endemic helminthic disease caused by infection with metacestodes (larval stage) of the tapeworm *Echinococcus granulosus* (1).

Ultimate growth of the cyst depends on its location inside the body of the host, therefore, in some organs are unable to expand freely, whereas in others, modest growth results in serious impairments to the function of vital structures or

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even in death (2). Hydatid cyst secretes major immunodominant antigens which are thought to be responsible for immunomodulatory activities promoting its survival within a mammalian host (3). These activities have extraordinary abilities to control host immune rejection mechanisms through alteration or suppression of the functional lymphocytes by secreting substances from the cyst wall which interfere locally with immunocompetent cells which facilitate long term survival of the parasite (4).

Pseudomonas aeruginosa is an opportunistic pathogen in human, causing many adverse effects like direct tissue damage with a greater cytotoxic potential urinary tract infection (5) and pneumonia (6). Many virulence factors, excreted by this bacterium, affect the immune system during the course of infection causing both acute and chronic diseases. These factors are either toxins like lipopolysaccharides (LPS) which suppresses host immune response causing persistent infection (7), or sometimes toxic pigment pyocyanine (8) and its derivative, 1-hydroxyphenazine (9). The majority of these factors have biological effects on host cells which may contribute to some inflammatory states like epithelial cell apoptosis (10), while other products may affect some of the natural (innate) immune response elements like macrophage (11) and complement (12), or immunological effects on some of the specific immune response cells like T lymphocytes (13).

T lymphocytes are specific immune cells, which are differentiated primarily in the thymus, and considered as the central control and development of the immune responses (14). They play very important role against parasitic infections especially with *Echinococcus granulosus* whether this role is pathogenic or protective immune response (15).

The aim of this study is to find out the effect of phenazine pigment (pyocyanine) on both viability of T lymphocytes and cyst protoscolices infectivity in vivo.

MATERIALS and METHODS

Six groups of white male Balb/c mice, each containing 8-9 mice, aged 10-12 weeks and weighted 21-22gm, were used for experimental infection. They were housed in plastic cages under convenient conditions room temperature 22-25°C with food and water supply under our care and expense.

All hydatid cysts were collected from resident patients in some of Baghdad hospitals, Iraq. Protoscolices were isolated from cysts aseptically (16). Their numbers were adjusted to 2000 protoscolices/ml of sterile phosphate buffer saline (PBS, locally prepared) with pH 7.2. Their viabilities (viability must be more than 98%) were tested (17) using eosin stain (BDH, England).

The inbred male Balb/c mice groups were prepared to be injected as follow:

- Four groups were intraperitoneally injected four purified concentrations of Pyocyanine (25, 50, 75 and 100μm/ml). Seven days later, they were given the same concentrations as a booster dose of the pigment, and after the same period, they were intraperitoneally infected with 2000 protoscolices/ml PBS as a challenge dose.
- The fifth group was intraperitoneally injected 1ml of sterile PBS and used as the negative control group.
- The sixth group was intraperitoneally injected 100μgm/ml immunoferon (Arabian company for drugs, Jordan) and received challenge dose with the same number of protoscolices and served as the positive control group.

After 2, 4, and 6 weeks, T lymphocytes were isolated and their viabilities were tested by dye exclusion using 0.2% trypan blue stain (BDH, England) (18). One hundred cells were counted and percentage of lymphocytes viability was calculated by haemocytometer (Neubaur, England) under a compound microscope (Olympus, Japan).

Finally, 25 weeks later, all mice were killed and dissected under a dissecting microscope. The infectivity of protoscolices was investigated by recording cyst numbers and their diameters were determined by vernier micrometer (Memmert, Germany).

RESULTS

Two weeks following the exposure to protoscolices as a challenge dose, pyocyanine caused highly significant decrease in lymphocyte viability ($p<0.01$), especially among mice which were exposed to concentrations of 50, 75 and 100µm/ml of pigment (60.25±2.52, 44.50±5.89, and 31.50±4.34, respectively), while the least concentration (25µm) showed no significant difference (87.50±4.56) in comparison with the negative (88.75±2.86) and positive control group (90.25±2.33) (table 1).

Table 1. Effect of purified pyocyanine on lymphocyte viability 2, 4 and 6 weeks following the infection with 2000 protoscolices/ml PBS as a challenge dose

	Lymphocytes viability		
	2nd week	**4th week**	**6th week**
Pigment concentrations(µm/ml)			
25	87.50±4.56	84.75±3.25	77.25±1.550
50	60.25±2.52	51.00±2.23	45.25±2.43
75	44.50±5.89	30.25±4.31	21.00±1.56
100	31.50±4.34	22.50±3.66	15.50±1.34
Negative control	88.75±2.86*	89.75±2.60	88.25±1.44
Immunoferon	90.25± 2.33	90.75±2.22	89.50±3.16

* Data are shown in mean ±standard deviation

This significant decrease was continued during the 4th week of exposure to challenge dose with protoscolices which exposed to 75 and 100 µm/ml in comparison with the negative (89.75±2.60) and positive control group (90.75±2.22) ($p<0.01$, $p<0.05$, respectively) (table 1). Similarly, pigment concentrations of 75 and 100µm/ml demonstrated significant decrease ($p<0.01$) 6 weeks following the challenge dose (21.00±1.56 and 15.50±1.34, respectively) in comparison with the negative and positive control groups (88.25±1.44 and 89.50±3.16, respectively) (table 1). This decrease in the lymphocyte viability causes statistically significant ($p<0.01$) increment in protoscolices infectivity (both cyst numbers and diameters) in comparison with immunoferon as presented in table 2. Therefore, higher concentrations of pyocyanine had suppressive effects on the viability of lymphocytes, while immunoferon was able to stimulate and proliferate T cells.

Table 2. Effect of purified pyocyanine pigment on cysts numbers and diameters 25 weeks following the protoscolices infection

	Number of cysts	**Diameter of cysts (mm)**
Pigment concentrations (µm/ml)		
25	2.25±1.46*	1.42±0.46
50	8.35±3.69	1.33±0.58
75	11.63±4.25	1.63±0.42
100	17.13±4.50	3.11±1.63
Negative control	-	-
Immunoferon	2.50±1.71	0.65±0.26

* Data are shown in mean ±standard deviation

DISCUSSION

Like many helminthic parasites, hydatid cysts develop sophisticated mechanisms for avoiding the cytotoxic effects of the immune response (19).

To our knowledge, no previous study has been conducted to investigate the effect of this pigment (Pyocyanine), which was isolated and purified from *Pseudomonas aeruginosa*, on T lymphocytes as immunomodulators against parasites and in particular against experimental hydatidosis, however, it is generally accepted that phenazine pigment causes firstly local suppression of T lymphocytes proliferation which may interfere with cellular immune responses, secondly this pigment inhibits the production of one of the essential lymphokines, interleukin-2 (IL-2) and its receptor

on T cell membranes (20). Nevertheless, it has been indicated that *P. aeruginosa* phenazine pigment causes inhibition of human lymphocytes proliferation *in vitro* in the presence of killed *P. aeruginosa* and purified pyocyanine is a strongly inhibitor for lymphocyte proliferation more than the crude pigment (21).

Some authors indicated that a number of virulence toxic factors secreted by this pathogen, especially phenazine pigments and its derivatives, have biological effects on host cells or they have pathophysiological effects on host tissue that impact the host immune response (22,23). Additionally, this pigment had a toxic effect on T lymphocytes activity and functions.

This pigment like all phenazine pigments generated by *P. aeruginose*, may affects on interleukin-2 (IL-2) production from T helper cells which are responsible for T lymphocytes activation (24), while inoculation of mice with alive protoscolices manifested high marker (IL-4, IL-5, and IL-10) which are responsible for cyst progression and establishment (19). In addition to the toxicity of this pigment, the juvenile hydatid cyst fluids are able to suppress or destroy lymphocytes in direct contact with cyst (25). However, being infected with higher dose of protoscolices may result in non-specific suppression of T cell activity (26).

In conclusion, pyocyanine is a toxic pigment (dose dependent) causing decrease of T cells viabilities, especially at higher concentrations, which let protoscolices to develop and growth and accordingly *P. aeruginosa* may pave the way for the infection with the hydatid cyst. Finally, the mechanism of phenazine pigment action is not well known (9) and till now, numerous questions regarding this mechanism remain unanswered (27).

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REFERENCES

1. Rigano R, Buttari B, Profumo E, Ortona E, Delunardo F, Margutti P, et al. Echinococcus granulosus antigen B impairs human dendritic cell differentiation and polarizes immature dendritic cell maturation towards Th2 cell response. Infect Immun 2007;75(4):1667-78.

2. John DT, Petri WA, editors. Markell and Voge's. Medical Parasitology. 9th ed. Philadelphia: Saunders; 2006.

3. Mamuti W, Sako Y, Nakao M, Xiao N, Nakaya K, Ishikawa Y, et al. Recent advances in characterization of Echinococcus antigen B. Parasitol Int 2006;55:57-62.

4. Annen JM, Köhler P, Eckert J. Cytotoxicity of Echinococcus granulosus cyst fluid in vitro. Zeit Parasitenkd 1981;65:79-88.

5. Buonanno AP, Damweber, BJ. Review of urinary tract infection. US Pharm 2006;31(6):26-36.

6. Sadikot RT, Blackwell TS, Christman JW, Prince AS. Pathogen-host interaction in Pseudomonas aeruginosa pneumonia. Am J Respir Crit Care Med 2005;171(11): 1209-23.

7. Cryz SJ, JR, Pitt TL, Furer E, Germanier R. Role of lipopolysaccharide in virulence of Pseudomonas aeruginosa. Infect Immun 1984;44:508-13.

8. Price-Whelan A, Dietrich LE, Newman DK. Pyocyanine alters redox homeostasis and carbon flux through central metabolic pathways in Pseudomonas aeruginosa PA14. J Bacteriol 2007;189(17):6372-81.

9. Muller M, Sorrell TC. Inhibition of the human platelet cyclooxygenase response by the naturally occurring phenazine derivative 1-hydroxyphenazine. Prostagl 1995;50(5-6):301-11.

10. Rajan S, Cacalano G, Bryan R, Ratner AJ, Sontich CU, Van-Heerckeren A, et al. Pseudomonas aeruginosa induction of apoptosis in respiratory epithelial cells: analysis of the effects of cystic fibrosis transmembrane conductance regulator dysfunction and bacterial virulence factors. Am J Respir Cell Mol Biol 2000;23: 304-12.

11. Wilson R, Dowling RB, Jackson AD. The biology of bacterial colonization and invasion of the respiratory mucosa. Eur Respir J 1996;9:1523-30.

12. Kindt TJ, Goldsby RA, Osborne BA, editors. KUBY: Immunology. 6th ed, New York, W.H. Freeman company; 2007.

13. Epelman S, Neely GG, Ma LL, Gjomarkaj M, Pace E, Melis M, et al. Distinct fates of monocytes and T cells directly activated by Pseudomonas aeruginosa exoenzymes S. J Leukoc Bio 2002;71:458-68.

14. Male D, Brostoff J, Roth DB, Roitt I, editors. Immunology. 7th ed, New York, Mosby Inc. 2006.

15. Romagnani S. Induction of Th1 and Th2 responses: a key role for the 'natural' immune response? Immunol Tod 1992;13:379-81.

16. Smyth JD. In vitro culture of Echinococcus spp. In: Proceedings of 13th International Congress of Hydatidology. Madrid, 1985; p.84-9.

17. AL-Qaoud KM, Abdel-Hafez SK. Humoral and cytokine responses during protection of mice against secondary hydatidosis caused by Echinococcus granulosus. Parasitol Res 2005;98:54-60.

18. Hudson L, Hay FC, editors. Practical immunology. 3rd ed. Oxford: Blackwell Scientific Publications, 1989.

19. AL-Qaoud KM, Abdel-Hafez SK. The induction of T helper type-1 response by cytokine gene transfection protects mice against secondary hydatidosis. Parasitol Res 2008;102(6):1151-5.

20. Nutman J, Berger M, Chase PA, Dearborn DG, Miller KM, Waller RL, et a1. Studies on the mechanism of T cell inhibition by Pseudomonas aeruginosa phenazine pigment pyocyanine. J Immunol 1987; 138(10):3481-87.

21. Sorensen RU, Klinger JD, Cash HA, Chase PA, Dearborn DG. In vitro inhibition of lymphocyte proliferation by Pseudomonas aeruginosa phenazine pigments. Infect Immun 1983;41(1):321-30.

22. Leidal KG, Munson KL, Denning GM. Small molecular weight secretory factors from Pseudomonas aeruginosa have opposite effects on IL-8 and RANTES expression by human airway epithelial cells. Am J Respir cell Mol Biol 2001;25(2):186-95.

23. Buret A, Cripps AW. The immunoinvasive activities of Pseudomonas aeruginosa; relevance for cystic fibrosis. Am Rev Respir Dis 1993;148:793-5.

24. Ulmer AJ, Pryjma J, Tarnok Z, Ernst M, Flad HD. Inhibitory and stimulatory effects of Pseudomonas aeruginosa pyocyanine on human T or B lymphocytes and human monocytes. Infect Immun 1990;58(3):808-15.

25. Wattal C. Evaluation of human cellular immune function in Echinococcosis. Ind J Med Res Sec A: Infect Dis 1990; 91:214-17.

26. Allan D, Jenkins P, Connor RJ, Dixon JB. A study of immunoregulation of Balb/c mice by Echinococcus granulosus equines during prolonged infection. Parasit Immunol 1981;3(2):137-42.

27. Look DC, Stoll LL, Romig SA, Humlicek A, Britigan BE, Denning GM. Pyocyanine and its precursor phenazine-1-carboxylic acid increase IL-8 and intercellular adhesion molecule-1 expression in human airway epithelial cells by oxidant-dependent mechanisms. J Immunol 2005;175:4017-23.