Immunosuppression of B-rosette formation by I-Hydroxyphenazine pigment against experimental hydatidosis

Zaman A. A. Ibrahim

Abstract:

Background: Hydatidosis is a widespread chronic zoonotic disease caused by helminthic larval stage of tapeworm *Echinococcus granulosus*. It has a serious medical and public health problems because it is asymptomatic disease and appears after the cyst grows and well developed in the local area. So, recently many substances are used to activate and modulate the immune system of the host in order to control the cyst growth and development.

Objective: To study the effect of this phenazine pigment 1-Hydroxyphenazine (1-HP), which is generated by *Pseudomonas aeruginosa*, on one of the specific immune B-cell- reactions against experimental hydatidosis *in vivo* and the possible effect on the infectivity of the protoscolices?

Methods: Four groups of male balb/c mice were used for experimental hydatidosis against intraperitoneally (I.P.) inoculated four purified concentrations (25, 50, 75 and 100µm/ml) of 1-HP in comparison with sterile P.B.S. used as a negative control group and the sixth group was inoculated with 100µg/ml Phytohaemagglutinin (P.H.A.) and received the challenge dose of 2000 protoscolices/ml P.B.S. and used as a positive control group.

Results: The results showed that the higher purified concentration 75, 100 µmole/ml of this pigment had suppressive effect on this specific immune response B-cells (B-Rosette formation). This effect was highly significant in comparison with the positive and with the negative control groups.

Conclusion: 1-HP is a dose dependant toxic pigment causing suppression of B-cells activity, especially at higher concentration which allows protoscolices to develop and grow.

Keywords: 1-hydroxyphenazine, B cell, Rosette formation, Hydatid, Cyst

Introduction

Hydatidosis or hydatid disease is caused by larval stage of the dog tapeworm *Echinococcus granulosus* which is an important zoonotic cestode genus and has a worldwide distribution [1, 2].

Despite inducing a strong host cellular and humoral immune response, this parasite is a highly successful parasite that develops, progress, and ultimately causes chronic disease [3]. Hydatid cysts secrete some antigens that are thought to be responsible for immunomodulatory activities of this parasite promoting its survival within a mammalian host [4].

This parasite in human is potential dangerous disease [5] and it has mechanisms that they are used to evade the host immune response and defending themselves from host human attack [6].

Pseudomonas aeruginosa is an opportunistic human pathogen of immunocompromised individuals. It is commonly associated with chronic, progressive lung disease in individual with cystic fibrosis [7]. It produces a number of virulence factors. The most common of these factors which secreted by this bacterium are phenazine pigment exotoxins such as pyocyanine and its metabolite 1-hydroxyphenazine [8]. Some studies had shown that, some of these products have ability for killing parasites [9] and fungi [10].

In addition to the phenazine pigments, this pathogen generates other virulence factors that affect the immune system during infection causing both acute and chronic diseases, these factors are either enzymes like elastase [11] or may be toxins like lipopolysaccharide [12], fluorescin [13] or mucoid substances like alginate [14]. These products have biological effects on host cells that may contribute to some inflammatory states like apoptosis in respiratory epithelial cells [15]. In addition to that, it have immunological effects on the specific immune response cells and its products like T-lymphocytes or

B- Lymphocytes [16] and interleukins [17]. While others may affect some of the innate immune response elements like macrophages [18] and complement [19].

In mammals B-Lymphocytes are specific immune cells that are produced primarily in the bone marrow and fetal liver. They mature there before proceedings via circulation to the secondary lymphoid organ like lymph node, spleen and mucosal- associated lymphoid tissue (MALT). In these secondary organs they start to produce circulating antibodies when it stimulated with either mitogens or T-independent antigenic stimulation [20]. These specific humoral immune cells during early maturation undergo several immunoglobulin gene rearrangements that establish B-cell specific receptors before it travels to the secondary lymphoid organs. In the blood, they interacts with antigen that triggers cell division and formation firstly plasma cell which produce large amount of specific antibody, which bind to that specific antigen, and secondly memory cell which responsible for anamnestic response [21].

One of these receptor is C3 receptor which enable B-cell to bind with erythrocyte coated with antibody and complement (EAC) forming rosette

shape and this EAC rosette complex formation is due presence of receptor on B-cell for C3 and such rosette do not formed by T- cells [22]. This test is considered one of the antibody-dependant humoral measurements [23].

The aim of this study is to investigate the effect of this phenazine pigment (1-hydroxyphenazine) produced by *P. aeruginosa* on one of the specific immune cell- reaction against experimental hyadatidosis in vivo and the possible effect on the infectivity of the protoscolices.

Materials & Methods:

Source of protoscolices.

Six groups of white male balb/c mice (each group with 12-14mice; age 5-6 weeks; weight 23-25gm) were used for experimental infection. All hydatid cysts were collected from patients resident in some of Baghdad hospitals (Iraq). The protoscolices were isolated aseptically from cysts according to the method of [24]. The number was adjusted to 2000 protoscolices/ml of sterile phosphate buffered saline (P.B.S.; pH = 7.2) and their viabilities were determined according to the method adopted by [25] using eosin stain.

Design of experiments: The inbred males (females excluded) balb/c mice groups were prepared to be injected as follow:

Four mice groups were inoculated with four purified concentrations of 1-hydroxyphenazine (25, 50, 75, and 100) µmole/1ml [26]. After seven days they were given the same concentrations as a booster dose of the pigment. Then after the same period they were infected (I.P.) with 2000 protoscolices/ ml (P.B.S.) as a challenge dose .The fifth group was inoculated (I.P.) with ml of sterile (P.B.S.) and used as a negative control group. The sixth group was inoculated (I.P.) with (100µg/ml) non-specific mitogen Phytohaemagglutinin (P.H.A.) and challenge dose with same number of protoscolices and used as a positive control. After 2, 4, 6 weeks B-lymphocytes were separated. according to method and mixed with Erythrocyte-Antibody-Complement [27] (EAC) according to [28] method. Thin films of this mixture on very clean slides were done after (4 hour) incubation for B-rosette. All the films were fixed with 70% alcohol and stained with Wright-Giemsa stain. They were examined microscopically and 200 lymphocytes were counted. B-rosette forming cells (At least 3-5 SRBC bound to B Lymphocytes) were considered positive & counted. After 25 weeks all alive mice were killed and dissected under dissecting microscope and the infectivity of protoscolices was investigated by recording cysts number and their diameters were determined by using vernier micrometer.

Statistical Analysis:

The suitable statistical methods were used in order to analyze and assess the results; they include ANOVA and LSD [29].

Note: The comparison of significant (P-value) in any test were: S= Significant difference ($P<0.05$).

HS= Highly Significant difference ($P<0.01$).NS= Non Significant difference ($P>0.05$).

Results:

After two weeks of mice groups exposure to protoscolices as a challenge dose,

1-hydroxyphenazine caused decrease in B-rosetting formation and this decrease was highly significant ($P<0.01$) specially among mice groups which exposed to high concentrations (100) µmole/ml of pigment which were (11.2±1.461) for B-rosettings, while low concentration (50, 25) µmole show no significant difference ($P>0.05$) in B-rosetting formation in comparison with the negative and positive control groups.(Table-1). This decrease was continued highly significant($P<0.01$) in B-rosetting formation for mice groups which were exposed to (100)µmole/ml (9.50±1.472) after 4 weeks in comparison with negative control group P.B.S. (**Table-1**). Pigment concentration (75µmole/ml) showed high significant decrease $P<0.01$ in B- rosetting formation after 6 weeks from the challenge dose inoculation (11.25±3.653) in comparison with negative control group P.B.S. and the positive control groups P.H.A., while the mice groups which exposed to 100 µmole/ml showed highly Significant decrease ($P<0.01$) in

B-rosettings formation (5.3±2.855) in comparison with both negative and positive control groups (Table-1). The results reflect the infectivity of protoscolices according to cyst growth and development (numbers and diameters) in comparison with P.H.A. which show significantly decrease the infectivity of protoscolices, but this decrease of the infectivity was sometimes less or not significant($P>0.05$) between some concentrations with respect to the cysts diameters**(Table-2).**

Table-1: Effect of purified 1- hydroxyphenazine on B-rosettings in *vivo* after 2, 4 and 6 weeks from protoscolices infection.

Pigment concentrations μmole/ml	B- Rosettings After 2 weeks		After 4 weeks		After 6 weeks	
	Mean ± S.D.		Mean ± S.D.		Mean ± S.D.	
P.B.S. (- control)	2.42 ± 0.337		22.7±0.683		20.6±2.439	
P.H.A.(+ control)	26.2 ± 1.883		26.4±5.635		24.2 ±4.918	
25	22.0 ±2.160	$	22.2±3.090	$	22.0 ±4.243	$
50	21.6 ±1.566	$	22.0 ±6.218	$	11.4 ±1,377	*
75	21.8 ±4.062	#	11.2 ±5.472	*	11.25 ±3.653	*
100	11.2 ±1.461	*	9.50±1.472	*	5.30 ±2.855	*

* HS= P<0.01 # S = P< 0.05 $ NS = P>0.05.

Table-2: Effect of purified 1- hydroxyphenazine pigment on cysts numbers and diameters after 25 weeks from protoscolices infection.

Pigment concentrations μmole/ml	Cysts numbers Mean ± S.D		Cysts diameters(mm) Mean ± S.D	
P.H.A.(+control)	1.66 ± 0.3633		1.838 ± 0.8222	
25	3.55 ± 0.4743	$	1.888 ± 0.6745	$
50	7.35 ± 1.9971	#	2.813 ± 1.2135	$
75	14.63 ± 7.3268	*	2.875 ± 5.4600	$
100	19.13 ± 0.8662	*	3.131 ± 0.9482	#

* HS= P<0.01 # S = P< 0.05 $ NS = P>0.05

One way ANOVA of cyst number showed P=0.00 highly significant (P<0.01.)

One way ANOVA of cyst diameter showed P=0.136 Non-Significant (P>0.05).

Discussion:

The ubiquitous host range of *Echinococcus* metacestode exemplifies the extraordinary ability of these parasites to control host immune rejection mechanism [30]. The results showed that the higher concentrations of 1- hydroxyphenazine reduce rosetting phenomenon, while P.H.A. is a good phytomitogenic which able to stimulates and proliferates T lymphocytes. These T cells secret cytokines in turns activated B lymphocytes [31]. The protoscolices with P.H.A. both are good non-specifically mitogenic for unprimed T and B lymphocytes *in vitro* [32]. No studies were found about the effect of this pigment (1-hydroxyphenazine) which isolated and purified from *Pseudomonas aeruginosa* on B-lymphocytes rosetting formation as immunomodulators against parasites especially against secondary experimental hydatidosis. It was found that pyocanine which is a phenazine pigment produced by P. *aeruginosa* has dual dose-dependent stimulatory as well as inhibitory effects on immune response in vitro, and it is toxic when added at a high concentration [16].

B-rosette formation is one of specific humoral immune responses which depend on B-lymphocyte [22]. These cells have both FC-Receptor and surface immunoglobulins receptors, so, the increment of the antigen concentration and time of exposure may reduce the ability of these cells to bind with EAC complex to form B-rosette shape due to the saturation of (CD16) surface receptors of these cells which is considered as receptor for EAC complex [28]. This study agree with [33] who said that the higher concentrations of phenazine pigment has ability to suppress the B-cell differentiation to antibody forming cells due to the suppression(IL-2) receptor on B-cell which is important in B-cell proliferation and differentiation.

Finally, the mechanism of phenazine pigment was not well known [34] and till now numerous questions regarding this mechanism remain unanswered [7].

In conclusion, the results demonstrate that the *P. aeruginosa* pigment, 1-PH, induces suppression B-rosetting phenomenon (especially at higher concentrations) against experimental hydatidosis in mice which is associated with a significant increase in the virulence of the protoscoleces in mice.

Pseudomonas aeruginosa may pave the way for the infection with the hydatid cysts. Alternatively, the existing hydatid infection may become more aggressive in patients colonized with some strains of this bacterium which secretes phenazine pigment.

Further studies are needed to understand the mechanism by which the pigment suppresses the immune response *in vivo*, and really many researches now carried on to see the effects of low concentrations of purified phenazine pigments which produced by this pathogen and may be modulates the immune response against experimental hydatidosis [35].

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Department of Basic Sciences, College of Nursing, University of Baghdad, Iraq