Effect of Purified 1-Hydroxyphenazine Pigment on B rosette formation against Secondary hydatidosis

Zaman A. A. Ibrahim

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

Abstract

The effect of purified 1-hydroxyphenazine pigment which was generated from *Pseudomonas aeruginosa* on specific immune response B cells inside the body of white BALB/C mice against experimental secondary hydatidosis and the infectivity of protoscoleces was studied.

In comparison with negative control mice groups (P.B.S.) the results showed that the higher purified concentrations (100) μmole/ml of this pigment had suppressive effect on this specific immune response B cells(B-Rosette formation) and this effect was highly significant after (6) weeks from challenge dose with protoscoleces intraperitoneally (I.P) against this pigment, and this effect reflects the protoscoleces infectivity which increased due to suppression of B rosette formation while the mitogen Phytohaemagglutinin (PHA) showed a significant stimulation of this specific humoral response which leads to decrement in protoscoleces infectivity in comparison with higher pigment concentrations .

KEYWORDS: 1-hydroxyphenazine.B cell. Rosette formation. Hydatid. Cyst.

تأثير ألصباغ 1- هيدروكسي فينازين النقيه في ألتشكل الزهري البائي ضد ألخمج التجريبي بالاكياس العداريه

د. زمان عبد ألصاحب عبد أبراهيم / فرع العلوم ألأساسيه- كلية ألتمريض_جامعة بغداد- العراق

الملخص

تمت دراسة تأثير الصباغ 1-هيدروكسي فينازين ألمعزوله والنقيه من Pseudomonas aeruginosa في التشكل الزهري البائي داخل جسم الفئران البيض BALB/C ضد الخمج التجريبي بالاكياس العدارية ومدى تأثيرها على خمجية الروؤيسات البدائيه (infectivity of protoscolices) .

اظهرت النتائج مقارنة مع مجموعة السيطرة (P.B.S) بان التراكيز العاليه لهذه الصباغ (100) مايكرومول/مل لها تأثيرا مثبطا على ألتشكل الزهري للخلايا ألبائيه، وان هذا التأثير قد ازداد بصوره معنوية بعد مرور ستة اسابيع من ألخمج التجريبي بجرعة التحدي بالروؤيسات البدائية وان هذا التأثير يعكس مدى خمجية(Infectivity) هذه الرؤيسات والتي ازدادت لتثبيط فعالية التشكل ألزهري للخلايا أللمفاويه ألبائيه ، فيما أظهر ألمشطر أللانوعي (PHA) تحفيزآ في ألأستجابه ألمناعيه ألخلطيه ألمتخصصه والتي أدت الى نقصان في خمجية ألرؤيسات ألبدائيه مقارنة مع تراكيز الصباغ ألعاليه.

كلمات مفتاحيه-1-هيدروكسي فينازين. خلايا بائيه. تشكل زهري.عدري.كيس.

1- Introduction

Echinococcosis or hydatidosis is the most serious world wide human zoonotic disease caused by larval stage hydatid cyst of the dog tapeworm *Echinococcus granulosus* (1) ,which is widespread in Mediterranean region (2).

Despite inducing host cellular and humoral immune response this parasite is highly successful parasite that develops progress and ultimately causes chronic disease (3). This parasite secretes some antigens that are thought to be responsible for immunomodulatory activities promoting its survival within a mammalian host (4). These parasites have extraordinary abilities to control host immune rejection mechanisms and defending themselves from host human attack (5).

1-1.*Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic human pathogen of immunocompromised individuals. It is typically infects the pulmonary tract causing both pulmonary damage and high mortality rates in patients with cystic fibrosis and other forms of bronchiectasis (6).

Pseudomonas aeruginosa produces a number of virulence factors. The most common products secreted by this bacterium are phenazine pigment exotoxins such as pyocyanine and its metabolite 1-hydroxyphenazine (7), or secretes a variety of pigments, including, fluorescein (yellow-green and fluorescent, now also known as pyoverdin), and pyorubin (red-brown) (8).

Some studies had shown the ability of *P. aeruginosa* to produce phenazines is critical for killing parasites (9), fungi (10) and induction of neutrophil apoptosis (11).

In addition to the phenazine pigment, 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 (12), or maybe toxins like lipopolysaccharide (13), fluorescin (14) or mucoid substances like alginate (15).These products have biological effects on host cells that may contribute to some inflammatory states like apoptosis in respiratory epithelial cells (16), or immunological effects on some of the specific immune response cells and its products like T lymphocytes (17) or B lymphocytes (18), and interleukins (19), while others may affect some of the innate immune response elements like macrophages (20) and complement (21).

1-2. B lymphocytes.

In mammals B lymphocytes are specific immune cells that are developed 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) and in these secondary organs they start to produce circulating antibodies when it stimulated with either mitogens or T-independent antigenic stimulation (22).

These specific humoral immune cells during early maturation undergoes several immunoglobulin gene rearrangements that establish B-cell specific receptors before it travels to the secondary lymphoid organs and the blood in which 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 (23).

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 (24). This test is considered one of the antibody-dependant humoral measurements (25).

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.

2- Materials and Methods.

2.1. Source of protoscolices

All hydatid cysts were collected from patients resident in some of Baghdad hospitals (Iraq), and protoscoleces were isolated aseptically from cysts according to the method of (26). The number was adjusted to 2000 protoscoleces/1ml of sterile phosphate buffered saline (PBS; pH = 7.2) and their viabilities were determined according to the method adopted by (27) using eosin stain.

2-2. Design of experiments:

The inbred males (Females excluded) BALB/C mice groups were prepared to be injected as follow:

Four groups were inoculated intraperitoneally (I.P) with four purified concentrations of 1-hydroxyphenazine (25, 50, 75, 100) µmole/1ml (28). After seven days they were given the same concentrations as a booster dose of the pigment, and after same period they were infected (I.P) with 2000 protoscolices/1mL (P.B.S) as a challenge dose .The fifth group was inoculated (I.P) with 1mL of sterile (PBS) and used as negative control group. The sixth group was inoculated (I.P) with (100µgm/ml) non-specific mitogen Phytohaemagglutinin (PHA) and challenge dose with same

number of protoscolices and used as positive control. After (2, 4, 6) weeks B-lymphocytes were separated according to [29] method and mixed with Erythrocyte-Antibody-Complement (EAC) according to [30] 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 mice were killed and dissected under dissecting microscope and the infectivity of protoscoleces was investigated and recording cysts number and their diameters using vernier micrometer.

2-3. Statistical Analysis:

The suitable statistical methods were used in order to analyze and assess the results; they include the followings (31):

2-3-1 Descriptive statistics:

Summary statistic of the readings distribution (mean, SD, SEM, minimum & maximum).

2-3-2 – Inferential statistics:

These were used to accept or reject the statistical hypotheses, they include the followings:

Analysis of variation ANOVA (f-test).

Least significant difference LSD (f-test).

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).

2-3-3-Computer & programs:

All the statistical analysis was done by using Pentium-4 computer through the SPSS program (version-10) and Excel application.

3- RESULTS:

After two weeks of mice groups exposure to protoscoleces as a challenge dose, 1-hydroxyphenazine caused decrement in B- rosetting formation and this decrement 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 showed no significant difference (P>0.05) in B-rosetting formation in comparison with negative and positive control groups.(Table-1), and this decrement is continue 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 PBS (Table-1).

Pigment concentration (75)µmole/ml showed high significant decrement P<0.01 in B- rosetting formation after 6 weeks of the challenge dose(11.25±3.653) in comparison with

negative control group PBS and the positive control groups PHA, while the mice groups exposed to (100))µmole/ml showed highly Significant decrement(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 protoscoleces according to cyst growth and development (numbers and diameters) in comparison with PHA which show significantly decrease the infectivity of protoscoleces, but this decrement of 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 protoscoleces 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)	22.4 ± 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 protoscoleces infection.

Pigment concentrations µmole/ml	Cysts numbers			Cysts diameters(mm)		
	Mean ± S.D			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).

4-Discussion.

The ubiquitous host range of *Echinococcus* Metacestode exemplifies the extraordinary ability of these parasites to control host immune rejection mechanism (32).

From all above, the results showed that the higher concentrations of 1- hydroxyphenazine reduce rosettings phenomenon, while PHA is a good phytomitogenic which able to stimulates and proliferates T lymphocytes. These T cells secret cytokines in turns activated B lymphocytes (33). The protoscolices with PHA both are a good non-specifically mitogenic for unprimed T and B lymphocytes *in vitro.* (34).

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 but, generally, (35) found that the concentration (12.5)µmole/ml of phenazine derivative pyocyanine had suppressive effect on interleukin-2(IL_2) production, which play very important role in proliferation and differentiation of B-lymphocytes , and this effects increased proportionally with pigment concentrations.

These results agree with (28,36,37),which they said that all phenazine derivatives had suppressive effect on B-rosette formation and this effect depend on concentration that used in that experiment because the higher concentrations affect the CD16 which considered as B-cell surface receptor for EAC complex which in turns reduce the percentage of B-rosetting.

B-rosette formation is one of specific humoral immune responses which depend on B lymphocyte. These

cells have both FC-Receptor and surface immunoglobulins receptors (Sig), 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 [30].

This study agree with [18] 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 of (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 [38] and till now numerous questions regarding this mechanism remain unanswered [39]. In summary, our 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. *P. 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[40] .

Acknowledgements:

Sincerely deep thanks to all surgeons and staff members in all Baghdad–city hospitals for their great help to obtain intact hydatid cyst after surgical operations with our best heart wishes for them.

Great thanks to Ghasan mudafer in the Teaching Laboratories Directorate/Ministry of Health for his help in doing the statistical analysis of this research results.

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