Study of Some Parameters to Evaluate Immunization against Leishmaniasis in Golden hamsters

دراسة بعض المؤشرات لتقييم التمنيع ضد الخمج بداء اللشمانيات في الهامستر الذهبي

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Abstract

Incubation period, smears, impression and parasite culture for each liver, spleen, foot and blood were observed along five times follow up (15,30, 45, 60, 75, 90) days after challenge infection with virulent *Leishmania tropica* isolate in four groups of golden hamsters, inoculated with (one, two, three) doses of different antigens as following: Group (1) inoculated with autoclaved killed *Leishmania tropica*, Group (2) inoculated with BCG vaccine alone while Group (3) Inoculated with mixed vaccines (autoclaved killed *Leishmania* + BCG) and (4) control animals inoculated with phosphate buffer saline. Group 3, was considered as the best vaccine in this study because animal inoculated with this vaccine showed the following results compared with other antigens: - No lesion appeared along 90 days of following up.- Negative foot impression and culture up to 15 and 30 days of infection respectively, - Negative spleen and liver impression up to 30 days of infection when comparing with other groups.

المستخلص

اختبر في هذه الدراسة كل من فترة الحضانة ، المسحات ، الطبعات ومزارع الطفيلي لكل من الكبد ، الطحال، وساده القدم والدم بأعتماد ست فترات للمتابعة (90 ، 75، 60 ، 45 ، 30 ،15) يوما بعد الخمج بعزلة ذات فوعة لطفيلي اللشمانيا الجلدية وذلك في اربعة مجاميع من حيوانات الهامستر الذهبي التي حقنت بجرعة وجرعتين وثلاث جرع من المستضدات المختلفة وكما يلي: المجموعة الأولى حقنت بعالق طفيلي اللشمانيا المقتولة بحرارة المؤصده ، المجموعة الثانية حقنت بلقاح الـ BCG ، المجموعة الثالثة حقنت بخليط اللقاحين السابقين اما المجموعة الرابعة فقد حقنت بمحلول دارئ الفوسفات الملحي واعتبرت كمجموعة سيطرة . وقد كان خليط اللقاحين هو الأفضل في هذه الدراسة في تمنيعه لحيوان الهامستر ضد خمج لاحق باللشمانيا الجلدية ، وذلك لأن الحيوانات التي حقنت به اظهرت النتائج الآتية: لم تظهر الآفة الجلدية على مدى 90 يوم من خمج التحدي مقارنة بالمجاميع الأخرى . النتائج كانت سالبة لكل من طبعات و مزارع القدم المخمجه لغاية (15 ، 30) يوم على التوالي من خمج التحدي بالمقارنة مع نتائج المجاميع الأخرى . النتائج كانت سالبة لطبعات كل من الطحال والكبد ولغاية 30 يوم من خمج التحدي مقارنة مع نتائج المجاميع الأخرى .

Introduction

Leishmaniasis is a group of diseases caused by over 20 known species of pathogenic protozoan parasites of the genus *Leishmania* with divers clinical features ranging from self-limiting cutanoues leishmaniasis to visceral disease[1,2]. The various species of *Leishmania* are transmitted by sand flies, amastigotes, librated from host cells in the insect's gut, transform into promastigotes, which multiply there and finally introduced into a new hosts when sandfly again feed [3].

The importance of *Leishmania* as a human pathogen has stimulated a large number of researches deal with immunization against Leishmaniasis especially in experimental animals [4,5,6]. Evaluation the potency of vaccines against Leishmaniasis is not so easy, investigators examined several parameters to measure im

provoked by antigens used in immunization, such as the delayed type of hypersensitivity test (skin test), lymphocyte transformation, interferon gamma production [6,7,8] and following up of experimentally infected animal after immunization [9]. In this paper we addressed the use of incubation period, smears, impression and parasite culture for each liver, spleen, foot and blood to evaluate the success immunization against experimental infection of *Leishmania tropica* using three different antigens with (one, two, three) doses for each antigens.

Materials and Methods

Isolation of the Leishmania parasite

Leishmania tropica was isolated from a lesion in the left arm of a 21 years-old male at Baghdad Teaching Hospital/ Baghdad according to [10].

Media

1. **Semi –Solid medium:** This medium was prepared according to [11] and was used for parasite isolation from man and reclaim from the infected animal tissue.
2. **Biphasic Medium:** This media was prepared according to the method of [12] and used for parasite cultivation in order to prepare vaccine antigens and for preparing injecting dose.

Autoclaved Killed Leishmania (AKL) Vaccine preparation

Method of [13] was used in preparing *Leishmania tropica* vaccine with little modification [14] as following:

1. Promastigote of *Leishmania tropica* was cultivated in Biphasic Medium at 26^{o}C instated of RPMI-1640.
2. Promastigotes were harvested and concentrated using centrifuge at 3200 rpm.
3. The promastigotes were washed five times with phosphate buffer saline PBS, and counted using Haemocytometer to get the final concentration of immunization dose which was 1×10^{7} parasites/ 0.2 ml.

The promastigotes were separated and transferred into several autoclavable containers and was put in autoclave at 121^{o}C for 15 minutes, and then the containers were kept at 4^{o}C.

BCG Vaccine

BCG vaccine was obtained from "The National Centre for Drug Control and Researches/ Baghdad/ Iraq", the vaccine was made by " Japan BCG Laboratory" each vial contained 0.5 mg of lyophilized vaccine and each 1 ml of vaccine contained 8.26 $\times 10^{6}$ cells of *Mycobaterium bovis*.

Animals

Sixty male of Golden hamsters (*Mesocricetus auratus*) aged (8-10) weeks were obtained from "The National Centre for Drug Control and Researches/ Baghdad/ Iraq". Animals were separated into four groups each group contained 15 animal which inoculated as following:

Group (1): inoculated with Autoclaved killed *Leishmania* (AKL) per 0.2 ml.

Group (2): inoculated with 1.4×10^{6} cell per 0.2 ml [15].

Group (3): inoculated with mixed inoculums of both (1×10^{7} AKL/0.2 ml) and (1.4×10^{6} unit of BCG/ 0.2 ml).

Group (4): was considered as control group. Animals in this group were inoculated with 0.2 ml phosphate buffer saline.

(One, two, three) doses for each of the previous antigen were used with an interval of 15 days between each dose and the other.

All previous animals were inoculated intradermally in the left hind footpad using 1 ml sterile syringe for each animal. After 15 days of immunization, all animals were inoculated with (challenge dose) 5×10^7 promastigotes of virulent *Leishmania tropica* isolates / 0.2 ml .

Dissections

All animals of the four groups were dissected along five times of follow up (30, 45, 60, 75, 90) days after challenge infection, the follow up of the of *L.tropica* infection was done using different parameters:

1. Incubation period from infection date to the appearance of lesion and/or lesions [10].
2. Smears, Impression and cultivation of (liver, spleen, foot, blood) after challenge infected dose [14].

Results

Lesions appearance results

After challenge infection with virulent *Leishmania tropica*, no lesion was noticed along 90 days of following up in animals immunized with different dose of mixed vaccine (AKL+BCG), while it appears in animals immunized with single dose of (AKL) and in animals inoculated with (one, two, three) doses of (BCG) at (30, 45, 75) days of infection respectively, in control groups lesions appeared during the first 2 weeks after infection Figure (1).

Impressions, Smears and cultures results

Foot

Results of foot impressions were negative up to 15 days of infection in animals immunized with different dose of (AKL) and animals immunized with different dose of mixed vaccine (AKL+BCG). While the results of cultivation were negative up to 30 days after infection in previously mentioned animals.

Foot impression and cultivation results were positive during the first week of infection in control groups and during the first 2 weeks in animal immunized with different dose of BCG Table (1).

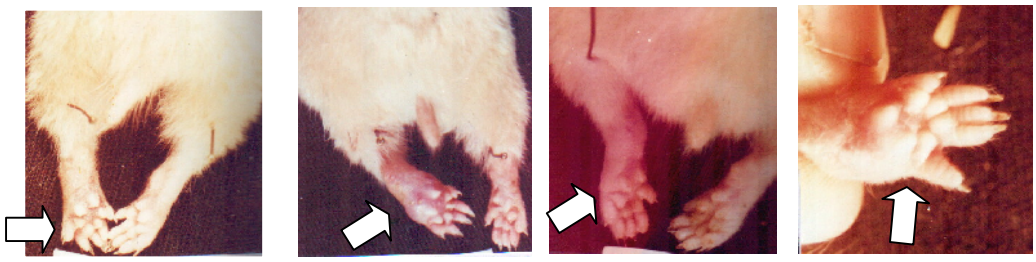
Spleen and Liver

Results of spleen and liver impression were negative up to (15, 30) days of infection respectively in animal immunized with different dose of each (AKL) and (AKL+BCG).While liver and spleen cultivation results were negative in the above mentioned animals along 90 days of follow up.

Impression and cultivation of both liver and spleen were positive after 15 days of infection in control group and animal immunized with one or three dose of (BCG), while impressions were positive at 30 days in two dose (BCG) immunized animal Table (2, 3).

Blood

Both smears and cultivation of blood was negative up to 90 days of infection for all groups of animal used in this study.



A B C D

Figure (1): comparison of food pad of different animal groups
A: animal inoculated with AKL
B: animal inoculated with BCG
C: animal inoculated with AKL + BCG
D: animal inoculated with PBS

Table (1): Impressions and parasite culture of skin after challenge infection

No of dose	Days of infection/ days	SKIN AKL		BCG		AKL + BCG		PBS	
		I	C	I	C	I	C	I	C
	7	-	-	-	-	-	-	+	+
1	*15*	-	-	+	+	-	-	+	+
	30	+	-	+	+	-	-	+	+
	45	+	+	+	+	+	+	+	+
	60	+	+	+	+	+	+	+	+
	75	+	+	+	+	+	+	+	+
	90	+	+	+	+	+	+	+	+
2	*7*	-	-	-	-	-	-	+	+
	15	-	-	+	-	-	-	+	+
	30	+	-	+	+	+	-	+	+
	45	+	+	+	+	+	+	+	+
	60	+	+	+	+	+	+	+	+
	75	+	+	+	+	+	+	+	+
	90	+	+	+	+	+	+	+	+
	7	-	-	-	-	-	-	+	+
3	*15*	-	-	+	+	-	-	+	+
	30	+	-	+	+	+	-	+	+
	45	+	+	+	+	+	+	+	+
	60	+	+	+	+	+	+	+	+
	75	+	+	+	+	+	+	+	+
	90	+	+	+	+	+	+	+	+

I:Impression **C:Culture**

AKL :Autoclaved killed *Leishmania* **PBS: phosphate buffer saline**

Table (2): Impressions and parasite culture of spleen after challenge infection

No of dose	Days of infection/days	SPLEEN AKL I	AKL C	BCG I	BCG C	AKL + BCG I	AKL + BCG C	PBS I	PBS C
	7	-	-	-	-	-	-	-	-
1	*15*	-	-	+	+	-	-	+	+
	30	+	-	+	+	-	-	+	+
	45	+	-	+	+	+	-	+	+
	60	+	+	+	+	+	-	+	+
	75	+	-	+	+	+	-	+	+
	90	+	-	+	+	+	-	+	+
2	*7*	-	-	-	-	-	-	-	-
	15	-	-	-	+	-	-	+	+
	30	+	-	+	+	-	-	+	+
	45	+	-	-	+	+	-	+	+
	60	+	-	+	+	+	-	+	+
	75	+	-	+	+	+	-	+	+
	90	+	-	+	+	-	+	+	+
	7	-	-	-	-	-	-	-	-
3	*15*	-	-	+	+	-	-	+	+
	30	+	-	+	+	-	-	+	+
	45	+	-	+	+	+	-	+	+
	60	+	-	+	+	+	-	+	+
	75	+	-	+	+	+	-	+	+
	90	+	-	+	+	-	+	+	+

I:Impression **C:Culture**

AKL :Autoclaved killed *Leishmania* **PBS: phosphate buffer saline**

Table (3): Impressions and parasite culture of liver after challenge infection

No of dose	Days of infection/days	LIVER AKL I	AKL C	BCG I	BCG C	AKL + BCG I	AKL + BCG C	PBS I	PBS C
	7	-	-	-	-	-	-	-	-
1	*15*	-	-	+	+	-	-	+	+
	30	-	-	+	+	-	-	+	+
	45	+	+	+	+	+	-	+	+
	60	+	-	+	+	+	-	+	+
	75	+	-	+	+	+	-	+	+
	90	+	-	+	+	+	-	+	+
2	*7*	-	-	-	-	-	-	-	-
	15	-	-	-	+	-	-	+	+
	30	-	-	+	+	-	-	+	+
	45	+	-	+	+	+	-	+	+
	60	+	-	+	+	+	-	+	+
	75	+	-	+	+	+	-	+	+
	90	+	*	+	+	+	*	+	+
	7	-	*	-	-	-	-	-	-
3	*15*	-	-	-	+	-	-	+	*
	30	-	-	+	+	-	-	+	+
	45	+	-	+	+	+	-	+	+
	60	+	-	+	+	+	-	+	+
	75	+	-	+	+	+	-	+	+
	90	+	-	+	+	+	-	+	+

I:Impression **C:Culture**

AKL :Autoclaved killed *Leishmania* **PBS: phosphate buffer saline**

***: Contamination**

Discussion

Golden hamsters were used in this study because some investigators proved that these animals were the suitable host for experimental *L.tropica* infection [16], in other hands gender and age of the hosts are also play an important role in the immune response provoked by *Leishmania* [17], so we used only male aged (8-10) weeks.

Promastigotes (infective stage of *Leishmania spp*.) was used in vaccine preparation, which were harvested in stationary phase, because promastigote in stationary phase can be considered to be more virulent and immunogenic than promastigotes in log phase [18], as well as the second and third boosting dose of these antigens were administrated due to the recommendation of [19] who recommended to use more than one dose for killed vaccine. After challenge infection with virulence *L. tropica*, no lesion appeared along 90 days of following up in animal immunized with different dose of mixed vaccine (AKL+BCG), while it appeared in animals immunized with single dose of (AKL) and in animals inoculated with one, two and three doses of (BCG) at (30,45,75) days of infection respectively, control groups lesions appeared during the first two weeks after infection. These results confirmed that mixed vaccine (AKL+BCG) may provoke strong immune response represented by T-helper (1) stimulation which produce different interleukins such as interferon gamma an macrophage activation factors which may play very important role in killing or reduction the parasites in skin [20, 21] .

Results of spleen and liver impression were negative up to (15, 30) days of infection respectively in animal immunized with different dose of each (AKL) and (AKL+BCG). While liver and spleen cultivation results were negative in the above mentioned animals along 90 days of follow up. These results may correlate with the high levels of lymphocytes and macrophages which induce different immunological factors that killed the parasites especially in spleen which have large amount of macrophages [21], these factors can reduce or prevent the transforming of amastigotes to promastigotes in culture, this is may be the reason of the negative cultivation results in both liver and spleen belonging to the animals immunized with different doses of each (AKL) and (AKL+BCG).

Both smears and cultivation of blood was negative up to 90 days of infection for all groups of animal used in this study. These results agreed with [22], who showed the difficulty of getting positive culture or smear from blood especially when blood was taken from heart directly.

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