Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 2, 2021, Pages. 3314 – 3327
Received 20 January 2021; Accepted 08 February 2021.

Post vaccination Unresponsiveness to Recombinant Hepatitis B Vaccine in Healthy children in Basrah: Association with IL-18& INF-gamma Serum Level

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ABSTRACT

Background: The association of IL18 with the development of antibodies tohepatitis B virus surface antigen (anti-HBs) in vaccinated children and the role of IL18 and INF-g in promoting the responses to HBV vaccine. We studied whether these cytokines concentrations contribute to anti-HBs development (titer≥ 10 IU/L) in relation to age and duration from the last vaccine dose administration.

Methods: In 400 vaccinated children who completed the standard series of HBV vaccine quantitatively estimating the levels of anti-HBsAg, IL18 and INF-g by specific ELISA kits. The associationsbetween the INF-g and IL18 levels and the risk of impaired anti-HBs development were estimated bycomputing the odds ratios and their 95% confidence intervals .

Results: The rate of responses among vaccines was 77% leaving high rate of non-responders (23%) who are susceptible to infection despite the total vaccination coverage. These non-responders distribution was negatively correlated with age and duration from the last vaccine dose. The pattern of responses to HBsAg vaccine significantly correlated with serum concentration of IL18 and INF-g,(p = 0.001) .Combination of IL18 levels with increased quantity of INF-g (p = 0.05), (p = 0.046) was associated with an increased chance for the development of anti-HBs in vaccinated children.

Conclusion: Development of anti-HBs is associated with quantity of IL18 and INF-γ that correlated negatively with age and duration of vaccination which is associated with overtime loss of protective antibody.
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Keywords: Antibodies to surface antigen of hepatitis B virus, INF-g, HBV vaccine, IL18

Introduction

The WHO strategy for an effective control of HBV infection and its complications is mass vaccination of neonates and children within the framework of Expanded Program on Immunization (EPI)[1]. Vaccination with the major surface antigen of the virus (HBsAg) induces protective antibodyresponse in the majority of vaccine recipients.A serum level of at least 10 mIU/ml of anti- HBs antibodies reached after vaccination has been proposed to be the lowest limit for protection[2]. However, approximately 5-10% of immunized individuals are enabled to develop the minimum protective anti-Hbs titers of 10 mIU/ml after completion of primary vaccine series remaining at risk for HB infection [3,4].

The group of non-responders patients could represent an important reservoir of HBV susceptible persons that will persist ashealthy carriers, contributing significantly to the spread

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of the disease[5]. Thus, the problem ofunresponsiveness to HBV vaccine constitutes a critical public healthmatter worldwide [6]. To date, the underlying mechanisms responsible for non-response phenomenon against HbsAg in vaccinated healthy subjects are poorly understood [7,8]. The process of anti-HBs production is T cell-dependent and requires T helper (Th)-cell activation [9].Altered Th1 immune response has been thought toaccount for non-responsiveness to HBV vaccine [10,11]. It is well recognized that IL-18 strongly enhances thesecretion of interferon-γ (IFN-γ) which, in turn, further skews theimmune response toward a Th1 phenotype [12].

IL-18 is known to lead the production of other proinflammatory cytokines ,it works with IL-12 to induce cell-mediated immunity [13], where IL-12 can stimulate the production of IL18 and have synergies with IL-18 in the activation of NK cells and cytotoxic T lymphocyte (CTL) cells.

Several studies were conducted to examine the duration of protection after immunization with recombinant HBV vaccines, but these were related to seroconversion in selected clusters of vaccine recipients [14,15]. In Iraq ,the national prevalence rate of HBs Ag was 1.6% and correlated positively with age. The prevalence rate of anti-HBs antibodies was 17%[15].

This study aim is to investigate the association of IL-18 and INF-γ serum concentration with pattern and magnitude of responses to HBV vaccination in Basrah governorate.

Materials And Method

A Total of 400 blood samples collected from apparently healthy children (191 female and 209 male) with age between 2-13 years old who completed the primary series of HBV vaccination (hepB1,hepB2,hepB3). These blood samples were collected from 10 Primary Health Care Centers (PHC) in Basrah province, including: 1-Al Seef, 2-Al Jamhoria, 3-Al Reesala, 4-Al thager, 5-Al shafi, 6-Al Hartha, 7-Shatt Al Arab, 8-Al Rahmma, 9-Bahila, and 10-Abu Al Khaseeb. They were collected through the period from December 2018 to August 2019.All children were substantially healthy, with no acute or chronic diseases and a history of immune illnesses, any immune compromising conditions (such as autoimmune diseases) and blood transfusion or were excluded. Informed consent of children parents were taken and the protocol was approved by medical college ethical committee.

Data collection.

Data collection for children about health condition were obtained through special questionnaire form. They covers questions related to name, age, sex, residence, immunization

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history against hepatitis B and the number of doses taken, previous infection, surgical intervention, dental surgery, genetic diseases and blood transfusion, underlying diseases, obesity. One questionnaire form for each individual and information was taken from the parents of children.

Inclusion Criteria

The inclusion criteria was apparently healthy children who completed the primary series of HBV vaccination . Age of children was from 2-13 years-old, with no any underlying diseases or obesity.

Sample collection and Processing

Five mlVenious blood samples were drawn from the vein, by using a disposable butterfly blood collection needle and After centrifugation, serum of each sample was transferred by using clean pipette into sterile Eppendorf tube andkept frozen at -20C◦ until the time of testing**.**These samples were used forHBsAgtesting and quantitative estimation of HBsAg, anti- HBsAb, IL18 and INF-γ concentration.

HBsAb Detection.

Qualitative determination of HBs antibody by an enzyme linked immunosorbent assay(ELISA) to determine antibodies for hepatitis B virus surface antigen (Anti-HBs) in human serum for evaluating levels of antibody response to HBsAg-vaccine, this test was done by using AccuDiag™HBsAb (Quantitative) ELISA Kit (COD. 1710-12 USA) according to the manufacturer's instructions.HBsAg was estimated negative for all samples by using the HBsAg Combo Rabid test, (catalog # :R0042C CTK Company UK).

Detection of IL-18 and INF- γ.

Quantitative determination of IL-18 and INF-gamma concentrations in serum was done by usingHuman Interleukin 18(IL-18) ELISA Kit (Elabscience®, Catalog No : E-EL-H0253. USA) and Quantitative determination of IFN-γ concentrations in serum "or plasma" done by using Human IFN-γ(Interferon Gamma) ELISA Kit (Elabscience®, Catalog No : E-EL-H0108. USA)according to the manufacturer's instructions.

The Sandwich-ELISA principle is used in this ELISA kits. The micro ELISA plate provided in this kit was pre-coated with a Human IL-18-specific antibody. Standards or samples are

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added to the wells of the Micro ELISA plate. Following the kit manufacture's steps .The optical density (OD) is spectrophotometrically measured at a wavelength of 450 nm(± 2 nm). The OD value is proportional to the Human (IL-18, INF-γ andanti-HBsAb) concentration. Human (IL-18 and INF-γ) concentration in the samples calculated by comparing the samples OD with the standard curve constructed from the standards provided by the kit manufacturer. Similarly , the procedure steps was followed for detection of IL18, INF-γand anti-HBs antibodies using their specific ELISA kits.

Statistical Analysis

Statistical Package for Social Science (SPSS) version 20 was used for statistical analysis of the data. To evaluate the difference between the study classes, Chi-square (X2) and Fisher's Exact tests were used.Student's t-test was used for comparing the means. The level of significance was set at $P < 0.05$.

Result

The overall serologic positivityof HBsAb in the study population is presented in Table - 1.Where the responders children to HB vaccine (HBsAb>10 mIU/ml) were 77% while non-responders children (HBsAb<10 mIU/ml) were 23% from the total of 400 children with an age range between 2-13 years.

Table (1): The overall HBsAbseropositivity among vaccinees in Basrah Governorate

Type of responses	No (%)	Nature of participant
Responders	308(77)	HBsAb more than 10m IU/ml
Non-responders	92 (23)	HBsAb less than 10mIU/ml
Total	400 (100)	children with age between 2-13

The percentage of responders and non- responders to HB vaccine in each PHC areas is presented in table (2).The highest seropositivity among vaccinated children was reported in Shatt Al Arab, Al Reesala and Abu Al Khaseeb (93.3%, 83.3% and 83.3%) respectively. But lower percentage of responders recorded in Al Hartha health center. While the highest percentage of non-responders registered in Al Hartha health center (34.5%) and lower percentage was in Shatt Al Arab (6.7%).

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Table (2): Distribution of responder and non-responders according to the PHC areas.

Residency	The health centers	No. (%)	Responders (%)	Non-responders (%)
Urban	**1-Al Seef**	45	34 (75)	11 (24.4)
	2-Al Jamhoria	55	43(78.2)	12 (21.8)
	3-Al Reesala	36	30 (83.3)	6 (16.7)
	Subtotal	136	107(78.68)	29(21.32)
Rural	**4-Al Thager**	30	22 (73.3)	8 (26.7)
	5-Al Shafi	31	23 (74.2)	8 (25.8)
	6-Al Hartha	55	36 (65.5)	19 (34.5)
	7-Shatt Al Arab	30	28 (93.3)	2 (6.7)
	8-Al Rahmma	36	27 (75)	9 (25)
	9-Bahila	28	20 (71.4)	8 (28.6)
	10-Abu Al Khaseeb	54	45 (83.3)	9 (16.7)
	Subtotal	264	201(76.14)	63(23.86)
	Total	400	308 (77)	92 (23)

Table (3) showsthe distribution of responding to HB vaccine in relation to gender. The responders was 46.8 % in female and 53.2% in male, while non-responder was 51.1% and 48.9% in female and male respectively.The difference between responders and non-responders was not statistically significant (P=0.4).

The seropositivity was 78.7% among children living in urban areas and 76. 1% among children living in rural areas, while 21.3% of nonresponders children in urban area and 23.9% in rural area. This difference is statistically not significant (P>0.05).(Table-3).

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Table (3): The distribution of HBsAb responses among vaccines in relation to gender.

Gender	No. (%)	Responders (%)	Non-responders (%)
Female	191 (47.8%)	144(46.8%)	47 (51.1%)
Male	209 (52.3)	164(53.2%)	45(48.9%)
Total	400	308 (77 %)	92 (23%)
			X^2 = 0.53 P. value = 0.4
Residence			
Urban	107 (78.7%)	29 (21.3%)	136(34%)
Rural	201 (76.1%)	63 (23.9%)	264(66%)
Total	308(77%)	92(23%)	400
X2 = 0.32 P. value = 0.56			

The distribution of the study population in relation to age and responses to HBV vaccinationis presentedin table -5.For comparison purpose, the studied populations were divided into 3 groups in relation to their age; 2-5 years , 6-9 years and 10-13 years. The highest percentage of responders to HBV vaccination was observed at age (2-5) years which was 91.25%, then decreased gradually until it reach 79.84% in the age group (6-9) years, and the lower percentage at age 10-13 years was 54.3%, while lower percentage of non-responders was at age (2-5) years (8.75%) and increased in age group 6-9 (20.16%) then reach to highest percentage in age group (10-13) years which was 45.7%.There is a significant difference between various age groups (P=0.000) which reflect the impact of vaccine duration. Therefore seropositivity to HBV vaccine is negatively correlated with age and duration from the administration of the last dose of vaccine. Accordingly , anti-HBV antibody loss by 5 years interval post vaccination showed gradual increase in the percentages of waning immunity (Table-4) which reach 12.42% by 6-9 years and 37.45% by 10-13 years post vaccination.

Table (4): The distribution of the study population in relation to age and responses to HBV vaccination

Age groups	No. (%)	Responders n(%)	Non-responders n(%)	P. value	Ab loss by 5y
(2-5)	**160 (40%)**	146 (91.25%)	14 (8.75%)	0.000	8.75%
(6-9)	**124 (31%)**	99 (79.84%)	25 (20.16%)	0.000	12.42%

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(10-13)	**116 (29%)**	63 (54.3%)	53 (45.7%)	0.42	37.45%
Total	**400**	308 (77%)	92 (23%)	0.000	

The concentration level of IL18 and INF- gamma in non-responders and responders children was estimated by using an Enzyme Linked Immuno Sorbent Assay(ELISA) technique .Table (5), demonstrated that the mean concentration of IL18 showed marked increase in the responders children (18.67±8.07 pg/ml) than the non-responders group (10.27 ± 5.17 pg / ml) , this difference was statistically significant (P value = 0.000) . IL-18 levels among all participants ranged from 5.27 – 26.67 pg/ml.

The mean value of IFN-γ concentration was significantly higher in withresponders group (23.48± 10.87 pg/ml) as compared with the non-responders (14.31±7.74 pg/ml) as illustrated in Table (5) , (P value < 0.05) following the same trend of IL-18 levels, (INF-g levels in all participants ranged from 7.43 -33.48 pg/ml).

Table (5):Serum Concentration Level of IL18 and IFN-γ in the Responders and Non-responders Groups.

Groups Mediators	**Responders (n=308)**	**Non-responders (n=92)**	P. value
IL18 Mean ± SD	18.67 ± 8.07	10.27 ± 5.17	0.000
	IL18 range : 5.27 – 26.67 pg/ml		
IFN-γ Mean ± SD	23.48 ± 10.87	14.31 ± 7.74	0.000
	INF-g range : 7.43 – 33.48 pg/ml		

Table (6), shows the quantitative grading of responses among responders in relation to the levels of IL18 and INF-g and titers of anti-HBs considering the high responders those posses IL18 greater than the mean value of IL18 concentration (14.47pg/ ml) and the mean for INF-γ (18.9 pg/ml), among those with anti-HBs antibody greater than 100mIU/ml . The low responders, those with anti-HBs Ab titer > 10 mIU/ml and <100mIU/ml who have cytokines (IL18 and INF-γ) below the mean concentration values.

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Table (6): The Distribution of the Study Population in Relation to the Mean Value of IL18 and IFN-γ among responders .

Mediators	Levels	Responders (n=308) (%)		Non-responders (n=92 : 23%)
		High responders (n=163: 41%)	Low responders (n=145:36%)	
IL18 (mean value 14.47pg/ml)	Lower than M.V.	44 (27%)	42 (29%)	73 (79.3%)
	Higher than M.V.	119 (73%)	103 (71%)	19 (20.7%)
X^2 = 78.35df = 2P. value = 0.000				
IFN-γ (mean value 18.9pg/ml)	Lower than M.V.	39 (23.9%)	37(25.5%)	76 (82.6%)
	Higher than M.V.	124(76.1%)	108(74.5%)	16 (17.4%)
X^2 = 100.99 df = 2			P. value = 0.000	

*** High responder: >100 mIU/ml, Low responders: > 10 mIU/ml <100mIU/ml**

The study population with high responding accounted for 41% (>100mIU/ml) and those with low response to HBV vaccine was 36% (>10 mIU/ml and <100mIU/ml). Individuals with low IL18/anti-HBs represent 29% and those with low IL18 and high anti HBs antibody(>100 mIU/ml) represent 27%. Although there were high IL18 levels , still some of them showed lower responding (<100mIU/ml). However, 20.7% of non-responders showed high IL18 concentration (greater than the mean value). These differences were statistically significant (P<0.001). The pattern of responses of INF-g in association with antibody development following the same trend of IL18 in which there were 17.4% of non-responders showed high concentration of INF-g (> INF-g mean value) Table (6).

The concentration of IL18 in female and male among responders and non-responders children was presented in table (7). Level of IL18 in the serum of female responders elevated more than female non-responders, which was17.51pg/ml and10.49 pg/ml.This difference was statistically significant(P< 0.05). Also IL18 level in male of responders children was high compared with male of non-responders children, which were 19.88 pg/ml and 10.04 pg/ml in male of responders and non-responder respectively. The differences between male in the non-responders and responders children was statistically significant P = 0.001).

The concentration of IFN-γ in female and male among responders and non-responders groups presented in the table -7. Level of IFN-γ in femaleresponders higher than non-responders

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female, which was 22.23pg/ml responder, and 13.21pg/ml non-responders female.This difference was statistically significant (P=0.009). Also IFN-γ level in the male of responders group was high as compared with male of non-responders group, which were 24.79 pg/ml and 15.47 pg/ml respectively. The differences between male in responders and non-responders groups was statistically significant (P=0.001) .

Table (7):Serum Concentration level of IL18 and IFN-γ in the responders and non-responders according to gender.

Gender	Female		Male	
Immune Markers	**Responders (n=144)**	**Non-responders (n=47)**	**Responders (n=164)**	**Non-responders (n=45)**
IL18 Mean ± SD	17.51 ± 8.71	10.49 ± 5.77	19.88 ± 9.89	10.04 ± 6.26
P. value	**0.01**		**0.001**	
IFN-γ Mean ± SD	22.23 ± 10.34	13.21 ± 7.92	24.79 ± 11.65	15.47 ± 8.03
P. value	**0.009**		**0.001**	

The association of serum levels of IL18 and INF- γ with age of the study groups summarized in table (8)which demonstrated that there were highly significance differences in the quantity of both IL18 and INF- γ in relation to age groups in the studypopulation. There is a negative relation between the mean concentration of IL18 and IFN-γas the age increased, the concentration of these immune markers decreased gradually with time among responders children in which the high levels of IL18 and INF-g observed at early age group (2-5 years), This difference was statistically significant (P value <0.05 for both) .

Table (8):Serum Concentration level of IL18 and IFN-γ in the responders and non-responders according to age groups

Age groups		**IL18 Mean ± SD**	**IFN-γ Mean ± SD**
2-5	**Responders (n=146)**	25.53 ± 11.93	32.42 ± 12.58
	Non-responders (n=14)	13.1 ± 7.31	19.61 ± 9.35
P. value		0.004	0.01
6-9	**Responders (n=99)**	18.43 ± 10.46	22.69 ± 12.2
	Non-responders (n=25)	12.38 ± 6.14	14.82 ± 6.16

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P. value		0.04	0.01
10-13	**Responders (n=63)**	16.74 ± 6.87	23.54 ± 10.44
	Non-responders (n=53)	6.76 ± 3.44	11.01 ± 5.94
P. value		0.000	0.000

Discussion

The development of an effective vaccine and successful vaccination is a major achievement of modern medicine and has the potential to eradicate this infection from humankind.The vaccine is highly immunogenic, and dramatically reduces morbidity and mortality related to hepatitis B [16].

The WHO strategy for effective control of HBV infection and its complications is mass vaccination of neonates and children within the framework of Expanded Program on Immunization (EPI) which introduced in Iraq since 1993 [17].The national prevalence rate of HBs Ag was 1.6% and correlated positively with age. The prevalence rate of anti-HBs antibodies was 17%. The prevalence rate of anti-HBs antibodies in <10 years of Iraqi children is only 32.2%, during the period between 2010-2012 which raise the issue of incomplete coverage of hepatitis B vaccine during the years preceding that study [15].

Vaccination with the major surface antigen of the virus (HBs Ag) induces protective antibody response in the majority of vaccine recipients exceeding 95 – 100% [18,19].This study give a recent insight of HBV vaccination since all participants completed the series of standard vaccine doses and despite this high rates of vaccine coverage, the non-responders in this study represent high proportion of vaccines (23%) where the world wide non-responding rates range is 5-10%[3].The responding rate found in this study closer to that reported from Baghdad (77.2%) by Al-Thwani*etal* 2008[20] and less than that reported in Diyala (83.6%) by Hasan and Mustafa (2017)[21] and Basrah (82.5%) by Mansour and Hasony(2009)[22]. These minor differences may reflect the use of different testing methods and the sample size beside the early vaccine administration.However, this immunity gaps of anti-HBs antibody in the community of non-responders individuals could represent an important reservoir of HBV susceptible persons that will persist as healthy carriers, contributing significantly to the spread of the disease [23,24]. Thus, the problem of unresponsiveness to HBV vaccine constitutes a critical public health matter worldwide [25]. Although there were no significant

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differences in the seroconversion post HBV vaccination between urban and rural areas , but there is a variation in the distribution of non responders at various PHC sectors in Basrah,where the non-responder rate in Harthaarea,north of the city represent the highest (34.5%) which is almost similar to that reported by Atalla*et al*(2013)[15]. However, Lee *et al*(2017)[26] reported a higher rates of non-responders(26.2%). The high rates of non-responders in this study may be attributed to the inadequate vaccination schedule and late introduction of HBV vaccine in the immunization program in Iraq and there is no school based immunization schedule similar to that implemented in other countries [27,28] or due to the inadequate logistic measures stressed on the vaccination program that reflected on the outcome of the vaccination program.

The antibody titer after vaccine administration ranges from <10m IU/mL (non-responder) to > 10m IU/mL upto>10000m IU/mL (responders) [28,29] . At least three doses are necessary for a minimally acceptable immune response (anti-HBs antibody titer ≥10m IU/mL)[30].Between 8–42% of people with protective antibody following vaccination lost it within 5 years[31], which is almost agreed with this study findings. However, the time taken for the disappearance of antibody shows wide variation [32]. Although the possibility of developing HBsAg among subjects who respond to an HBV vaccine is almost nil, risks persist for those not developing anti- HBs antibody (anti-HBsAg) conversion with the decline of anti-HBsAg titer. However, it was demonstrated that after a usual three-dose HBV vaccination, approximately 90% (range: 74–100%) of the subjects who received the vaccine remained protected for ≥30 years regardless of the anti-HBs antibody titer [24], but this trend not observed in this study as the post HBV vaccination correlate negatively with age and duration from the last vaccine dose administration.

Looking into the effect of IL18 and INF-γ serum concentrations on the pattern of responses to HBV vaccine .These mediators are an important cytokine of Th1 response [33], that has an important role in the immune response develops against hepatitis B vaccination . In a study performed by Jafarzadeh*et al.*,(2003) reported the inadequate IFN-γ and the related IL18 production in peripheral blood mononuclear cells against the hepatitis B vaccine in vitroin infants with hepatitis B vaccine unresponsiveness [34], and inadequate IFN-γ production was found to be associated with hepatitis B vaccine unresponsiveness [35]. These cytokines in this study was implicated in the regulation of responses to HBV vaccine as IL18 and IFN-γ levels in the subjects who were responsive greater than that among unresponsive to hepatitis B vaccination which is in agreement with the above reports.

In conclusion, this study demonstrated diminishedproduction of the cytokines IFN-γ, and IL-18 in PB from healthynon-responders to HB vaccine, suggesting insufficient orlack of Th1 and Th2 responses. This could be becauseof a defect in either the primary HBsAg-specific T-cellrepertoire or antigen presentation[33]. whichstrongly suggests the contribution ofIL-18 cytokine levels in children which may lead to the dysfunctionof antigen-presenting cells in unresponsiveness to hepatitis Bvaccine.

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