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RESEARCH ARTICLE

Expressions of IFN-γ and IL-2 in Biliary Atresia Mice Model After Exposure with Rhesus Rotavirus (RRV)

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Abstract

Background: Biliary atresia (BA) is a cholangiopathic obstructive disorder with unclear etiology and pathogenesis . It was thought that BA is originated from bile duct epithelium viral infection, followed by immunological processes and ended with biliary cirrhosis.

Objective: To determine the effect of induction and duration of illness after rhesus rotavirus (RRV) exposure to changes IFN-γ and IL-2 in mice model of BA.

Methods: Fourty-eight babies from 20 pregnant Balb/c mice were randomized into 2 groups that received placebo or 1.5 $x10^6$ PFU RRV intraperitoneally within less than a day after birth. Each group was terminated on day 3, 7, 14 and 21, further examined the expression of IFN-γ and IL-2 with flowcytometer. Statistical analysis using Mann Whitney and Kruskal Wallis .

Result: Differences in the expression of IFN-γ and IL-2 day 3, 7, 14 and 21. IFN-γ day 3 control 1.72 (0.05) vs trial groups 2.66 (0.05) p=0.020, day 7 0.86 (0.07) vs 1.64 (0.05) p=0.011, day 14 2.66 (0.08) vs 97.22 (1.27) p=0.006, day 21 10.14 (0.84) vs 29.45 (1.05) p=0.011. Each of them differ significantly with control p<0.001 and trial groups p=0.002. Expression of IL-2 day 3 1.15 (0.04) vs 2.73 (0.09) p=0.021, day 7 1.66 (0.17) vs 2.93 (0.09) p=0.011, day 14 2.97 (0.20) vs 5.31 (1.19) p=0.006, day 21 5.41 (0.86) vs 29.43 (1.04) p=0.011. Each of these differ significantly with control p<0.001 and trial groups p=0.002.

Conclusions: Induction and duration of illness after RRV exposure influence the expression of IFN-γ and IL-2 in the mice model of biliary atresia.

INTRODUCTION

Biliary atresia (BA) is a rare neonatal disease which characterized with chronic obctructive biliary system, and usually manifest in the first months of life. It affects approximately 1 in 5000–15000 live births worldwide. Ascending obstruction of the biliary tree may cause severe cholestasis and rapidly resulting biliary cirrhosis.[1,2]

The etiology of BA remains uncertain. Some evidences suggested the involvement of primary perinatal hepatobiliary viral infection followed by secondary damage of biliary tracts which mediated by immunological process.[3,4,5] Additionally, both human and murine studies of BA had demonstrated elevated levels of pro-inflammatory cytokines including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-10, and IL-18.[6] The existence of IFN-γ over-expression demonstrated that Th1-related cytokines is a critical point in the

pathogenesis of BA. The IFN-γ, through CD4+ T-cells may also play an important role in the pathogenesis of BA.[7] It is likely that the pro-inflammatory cytokines such as IL-2, IL-12, TNF-α and other soluble mediators such as iNOS, work synergistically to stimulate BA.[3]

Activated effector T-cells produce cytokines that can directly damage epithelial cells or indirectly cause damage them through stimulation of other immune cells. T-cells within the liver of BA patients have been shown to secrete Th1 cellular cytokines such as IFN-γ, IL-2, and TNF-α. It was an unique phenomenon to BA and not found in other neonatal cholestatic diseases.[8] Study of Bezerra *et al.* (2002), using gene expression microarray techniques observed that there were upregulation of proinflammatory cytokine genes including IFN-γ and osteopontin, and down-regulation of Ig genes in liver biopsy of BA cases. It suggested that there was inhibition of Th2 pathway.[9]

Interleukin-2 (IL-2) is a peptide produced solely by activated type 1 T lymphocytes (Th1 and Tc1) and is the major T-cell growth factor. It also increases immunoglobulin synthesis and induces cytolytic activity by NK cells and lymphokine-activated killer cells. The present study demonstrate that IL-2 mRNA expression significantly increase within the liver in BA compare with other cholestatic disorders.[10] Little is known about the etiopathogenesis of biliary atresia; consequently, there has been slow progress in developing improved therapies or preventative strategies during the past decade. The purpose of this study is to investigate the influence of induction and duration of illness after rhesus rotavirus (RRV) exposure to changes in the expression of IFN-γ and IL-2 in mice models of BA.

MATERIALS AND METHODS

Biliary atresia mice model

Twenty pregnant BALB/c mice were devided into two groups those were ten mice in study group and the other ten mice as control group respectively. The mice were kept sepparately in sepparate cages and a virus-free environment at the Laboratory of Molecular Biology the Faculty of Medicine Brawijaya University Malang Indonesia. They had free access to chow and water. Each delivered babies of mice from mice in the study group were given a single intraperitoneal (i.p.) injection of as much as 0.05 mL containing of 1.5 X 106 pfu/mL of RRV strain MMU 18006 (ATCC Virginia # VR 1739), while babies from the control group were injected with 0.05 mL of balanced salt solution (BSS). All of the injections were performed not more than 24 hours after birth. Infected babies of mice died within the first 2 days after birth, or were not fed by their mothers, were not included for further analysis. The babies of mice were weighted on the day of delivery and then were sacrificed by cervical dislocation on day 3, 7, 14 and 21 after birth. Liver and biliary tissues were removed by standard surgical procedure for further processes The protocol of this study had been approved by the Ethical Committe of Health Research Faculty of Medicine Brawijaya University Malang Indonesia (Reg #.. 361/EC/KEPK-83/11/2012).

Flowcytometry analysis

Tissue was homogenized and red cells lysed with ACK buffer. Liver immune cells were enriched by Percoll gradient (40/60). Single-cell suspensions were incubated with Fc-block and ready for stained. Mouse IFN-γ and IL-2 staining kits was used according to the manufacturer's instructions (eBioscience, San Diego, CA). Cells were visualized with FACS Caliber flow cytometer (Becton-Dickinson, Mountain View, CA), FlowJo (Tree Star, Inc., Ashland, OR) software used for analysis. Flowcytometric analysis was done at Biomedical Laboratory the Brawijaya University Malang Indonesia.

Statistical analysis

Data were analyzed statistically using IBM SPSS 20 software on personal computer. Descriptive analysis was used to describe the characteristics of the study subject. Analysis of the average for numerical data used independent sample t-test, Mann Whitney test, and Kruskal-Wallis. Data were analyzed using 95% confidence level (α=0.05).

RESULT

There were totally 48 newborn mice/ babies elligible in this study, consised of two groups, those were 24 infected by RRV 1.5 x 10^6 PFU intraperitoneally less than 24 hours after birth as study group and the others 24 injected with buffered saline as control group.

The expression of IFN-γ

Expression of IFN-γ of the RRV group on the day 3, 7, 14, and 21 were quantitatively higher than the control group ($p<0.05$) (Table 1). It can also be seen that the expression of IFN-γ in the liver tissues gradually increased, both in control and treatment group (Table 1 and Figure 5).

Table 1. The expression of IFN-γ

Variable	Day	Control group Median (interquartil)	Treatment group Median (interquartil)	*p** between groups per variables
IFN-γ	3	1.72 (0.05)	2.66 (0.05)	0.020*
	7	0.86 (0.07)	1.64 (0.05)	0.011*
	14	2.66 (0.08)	97.22 (1.27)	0.006*
	21	10.14 (0.84)	29.45 (1.05)	0.011*
	*p**	<0.001**	0.002**	<0.001**

*Significant differences by Mann Whitney test at α=0.05
** Significant differences by Kruskal Wallis test at α=0.05

The following chart showed the expression of IFN-γ (median) from time to time in the control group compared with RRV group:

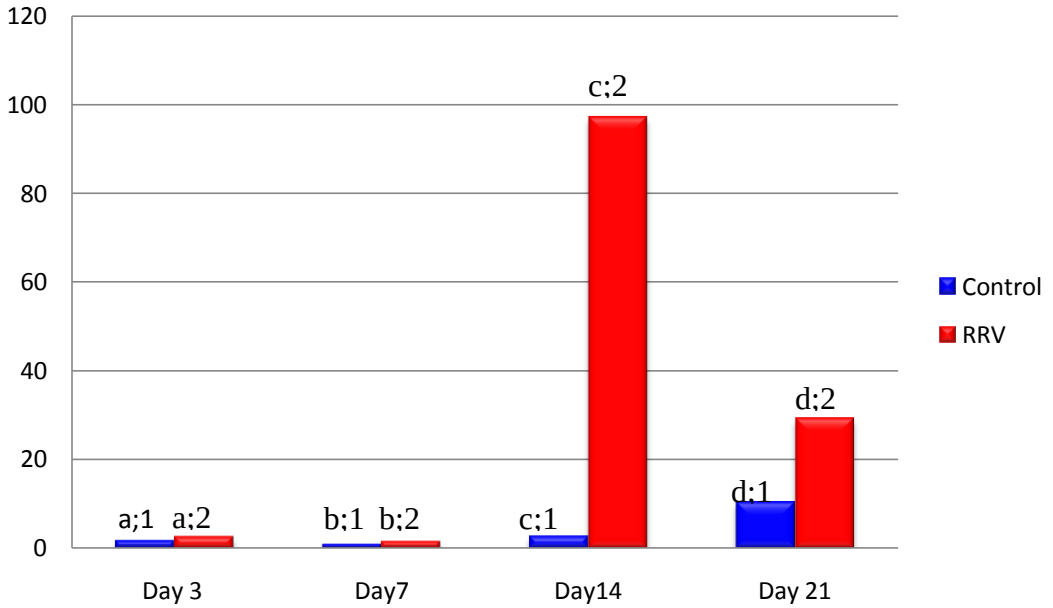

Figure 5. Effect of duration of illness after RRV exposure to the expression of IFN-γ in the liver tissues of RRV mice group and control groups.
Letter notification: differences between group
Number notification: differences among group

Induction of RRV in the RRV group caused the increase of expression of IFN-γ especially after day 7 of RRV induction and reached the peak on day 14, then decreased on day 21 but it was still higher than the control group.

The expression of IL-2

Expression of IL-2 of the RRV group on the day 3, 7, 14, and 21 were quantitatively higher than the control group ($p<0.05$) (Table 2). It can also be seen that the expression of IFN-γ in the liver tissues gradually increased, both in control and treatment group (Table2 and Figure 6).

Table 2. The expression of IL-2

Variable	Day	Control group Median (interquartil)	RRV group Median (interquartil)	*p** between groups per variables
IL-2	3	1.15 (0.04)	2.73 (0.09)	0.021*
	7	1.66 (0.17)	2.93 (0.09)	0.011*
	14	2.97 (0.20)	5.31 (1.19)	0.006*
	21	5.41 (0.86)	29.43 (1.04)	0.011*
	p	<0.001**	0.002**	<0.001**

* Significant differences by Mann Whitney test at α=0.05
** Significant differences by Kruskal Wallis test at α=0.05

The following chart showed the expression of IL-2 (median) from time to time in the control group compared with RRV group:

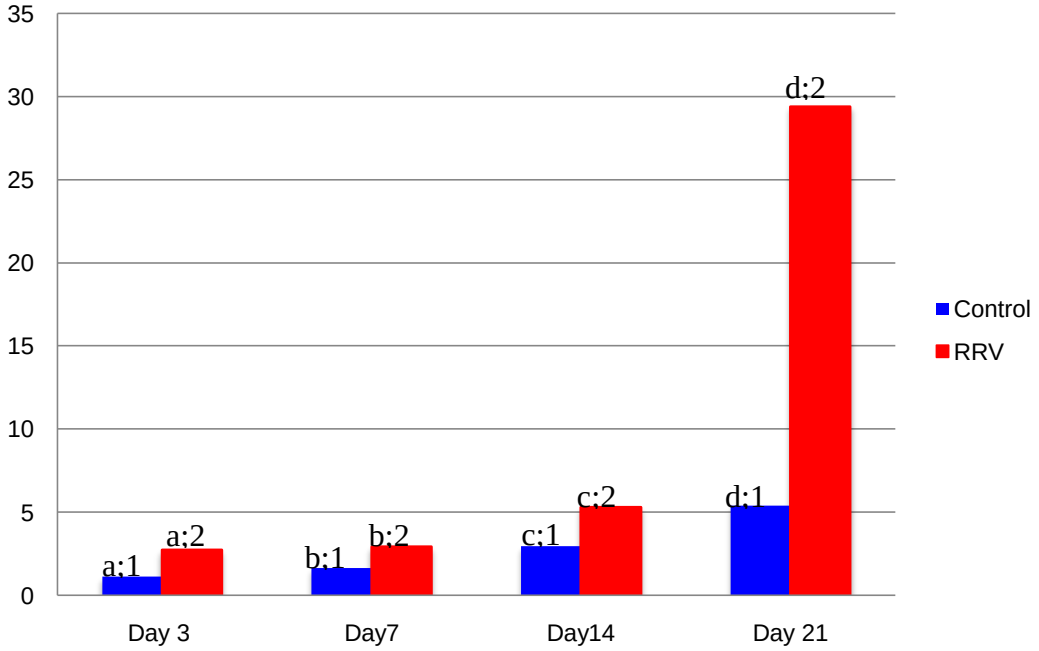

Figure 6. Effect of duration of illness after RRV exposure to the expression of IL-2 in the liver tissues of RRV mice group and control groups.
Letter notification: differences between group
Number notification: differences among group

The expression of IL-2 in babies mice liver tissue were signicantly increase started from day 3 and reached the peak on day 21 after induction of RRV(P<0.05)

DISCUSSION

In this study, the mortality rate of mice in the RRV group occurs more frequently, especially before day 7 and between day 14 and 21. Mortality is caused the babies mice look sick and eaten by its parent in accordance with its nature. Baby mice that sick is most likely due to the induction of RRV as evidenced by the death of the RRV group more than the control. These results are similar with Allen[11] and Bessho[12] who obtain more than 80% of mice that have undergone RRV-induced biliary atresia on the 14 day of termination. Petersen[13] in his research got the lethality number reaches 100% in neonatal mice intervention after day 21 post-induction.

Hypothesis that pro-inflammatory cytokines important for the pathogenesis of BA has been tested on mice models induced RRV.[12] Bile duct damage initiated by viral infection which is followed by the release of antigens

"self" that has changed and activate auto-reactive T-cells and specific bile ducts, which causes chronic fibroschlerosis injury to the bile duct.[3] In this study, the cellular adaptive immune response that occurs is reflected in the presence of expression of IFN-γ and IL-2.

Expression of IFN-γ in this study increased and peaked at around day 14, then decreased after day 21. But the expression of IFN-γ on day 21 compared with the control group was still significantly higher. Expression of IFN-γ by biliary tract cells decreased after day 21, probably due to the original biliary tract cells has undergone fibrosis[14,15] so that cells had been replaced by fibroblasts.[16]

Study of Shivakumar find that the pivotal role of lymphocytes and IFN-γ in duct obstruction become evident in mice lacking IFN-γ. Without IFN-γ, the sequential switch to a lymphocyte-based hepatic inflammation do not occur, duct obstruction completely prevent, and extrahepatic bile ducts maintain the lumenal continuity with the duodenum. In according with a central role for IFN-γ in duct obstruction, administration of recombinant IFN-γ following RRV infection resulted in recurrence of biliary atresia in IFN-γ-deficient mice.[17]

Other study find that base on the hepatic overexpression of IFN-γ in liver of infants at the time of diagnosis, newborn mices which are inoculated with RRV carrying an inactivating mutation in the IFN-γ gene. In these mices, cholestasis induced by RRV is transient, extrahepatic bile ducts are free of obstruction, and survival improve substantially.[12]

The expression of IL-2 increased after RRV induction and the increase with the time sequence begins after day 7 after RRV induction.

Longitudinal study of soluble inflammatory mediators on 21 infants with BA when portoenterostomi Kasai and 6 months after, report that adhesion molecules and pro-/anti-inflammatory cytokines are involved in the pathogenesis of BA. In the early of study, there is no significant difference in cytokine levels between BA and control, but IL-2 and IL-10 are significantly higher than other cholestatic disease controls. In the first 6 months after portoenterostomi, all plasma cytokine and adhesion molecule levels increase significantly, except IL-10. It is concluded that the process of BA is progressive inflammation and involve both Th and macrophage immune responses, that can not be fixed only with portoenterostomi.[18]

Shinkai et al, demonstrate that IL-2 mRNA expression is significantly increased within the liver in BA compare with other cholestatic disorders. Although their methods can not identify the cellular source of IL-2 production, they presume that type 1 cytotoxic lymphocytes (Tc1) are increased in the portal tracts of liver with BA, which result in bile duct destruction. IL-2 may act as a driving force in portal inflammation by Tc1.[10]

This study has several limitations, which are not carried out the clinical judgment of the mice from time to time because of the difficulty in distinguishing the clinical state of the mice, as well as a laboratory marker is not done to compare the histopathological findings and the immune response occurs. This study used flowcytometry to quantitatively measure the amount of expression of IFN-γ and IL-2 with the extra hepatic bile duct samples and liver tissues of mice, so it can not be distinguished whether the expression is derived from the biliary tract or liver tissue. To distinguish which cells expressing IFN-γ and IL-2, immunohistochemical examination should be done.

The results of this study provide additional evidence of the truth of the hypothesis that the induction of RRV resulted in changes the expression of IFN-γ and IL-2 in the pathogenesis of BA, thus opening discourse to do further studies for new strategies in the medical management of BA. Progressive increase expression of IFN-γ and IL-2 beginning on day 7 with a peak at day 14 (IFN-γ) shows that the possibility of a good time for medical intervention performed around day 7 and before day 14, because after day 14, the occurrence of BA already irreversible.

CONCLUSION

The research approves that induction and duration of illness after rhesus rotavirus exposure effect on the expression of IFN-γ and IL-2 in mice models of biliary atresia. The peak of IFN-γ expression is on day 14, while in the same time IL-2 expression starts to sharply increase.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this article.

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