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Effect of methylprednisolone injection on interleukin-4 and interferon-gamma expression following hepatitis B vaccination in mice

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Background/aim: The prevalence of hepatitis B infection is high worldwide with liver cirrhosis and hepatocellular carcinoma as important complications. Immunosuppression, especially from corticosteroids, is often cited as a cause of poor immune response and there is documented evidence of irrational administration of glucocorticoids to children and adults. Decreased expression of interleukin-4 and interferon-gamma is an indication of poor humoral and cellular immune responses, respectively. Therefore, we decided to find out if methylprednisolone injection decreases interleukin-4 and interferon-gamma expression following hepatitis B vaccination in mice.

Materials and methods: Mice were randomly divided into 2 groups. Daily intramuscular injections of methylprednisolone (15 mg/kg) were given to the test group while water for injection (0.1 mL) was given to the control group for 30 days. On day 6 all mice were given 2 µg of hepatitis B vaccine and they received a booster dose on day 27. On day 34, blood samples were collected and analyzed for interleukin-4 and interferon-gamma titers.

Results: There was positive interleukin-4 and interferon-gamma response in all groups but the differences in titers were not statistically significant.

Conclusion: At the dosages and length of exposure used in this study, methylprednisolone injection did not significantly inhibit interleukin-4 and interferon-gamma expression following immunization against hepatitis B virus in mice.

Key words: Hepatitis B vaccine, interleukin-4, interferon-gamma, immune response, methylprednisolone

1. Introduction

Hepatitis is a general term meaning inflammation of the liver and viruses are the most important causes. The causative organism of viral hepatitis B is a double-stranded DNA virus 42 nm in diameter belonging to the family *Hepadnaviridae*. It is transmitted through contact with blood or other body fluids of an infected person. Homosexuals, heterosexuals with multiple sex partners, persons who receive frequent blood transfusions, health workers who handle blood and blood products, and those who receive tattoos are important risk groups (1). It is 50 to 100 times more infectious than HIV and can survive outside the body for at least 7 days (2).

Globally, two billion people have hepatitis B infection and 600,000 of these die yearly (2). Prevalence of the disease in Nigeria falls between 11.7% and 44.7% (3).

Failure to mount immune response to hepatitis B vaccination is a common occurrence. Hepatitis B infection is a major risk factor for development of liver cirrhosis and hepatocellular carcinoma. It is found in 1/3 of cases of liver cirrhosis and 1/2 of cases of hepatocellular carcinoma (2). Chronic HBV infection accounts for 60%–80% of the world's primary liver cancers (4). It is second only to tobacco among all known human carcinogens (5).

1.1. Justification for the study

Immunization failure that exposes vaccinees to this infection is often attributed to immunosuppression associated with malnutrition, diseases like HIV/AIDS, and glucocorticoid therapy. Interestingly, irrational prescription and administration of glucocorticoids to infants and children for such ailments like bronchiolitis, asthma, skin infections, and febrile convulsions is common in poor and underdeveloped countries. Cytokines such as interleukin-4 (IL-4) and interferon-gamma (IFN-γ) are important mediators of immune response and there are no studies linking glucocorticoid therapy to low IL-4 and

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IFN-γ expression following hepatitis B vaccination, hence raising the need for this study. The choice of mice for the study was informed by the ethical issues involved in using children. Mice also have genetic similarities to humans (6).

1.2. Objectives of the study

The main objective of this study was to determine the effect of methylprednisolone injection on cytokine expression following hepatitis B immunization in mice. Specific objectives included:

- To determine the effect of methylprednisolone injection on serum IL-4 titer following hepatitis B vaccination in mice.
- To determine the effect of methylprednisolone injection on serum IFN-γ titer following hepatitis B vaccination in mice.

1.3. Hypotheses

Null hypothesis: Methylprednisolone injection does not decrease serum IL-4 and IFN-γ following hepatitis B vaccination in mice.

Alternative hypothesis: Methylprednisolone injection decreases serum IL-4 and IFN-γ following hepatitis B vaccination in mice. The null hypothesis was tested at a significance level (P-value) of 0.05.

2. Materials and methods

This study was done at the Pharmacology Laboratory, Nnamdi Azikiwe University Teaching Hospital, Nnewi and Safety Molecular Pathology Labs Ltd., Enugu, between July 2009 and June 2013. The procedures were in accordance with guidelines for the care and use of laboratory animals (7).

2.1. Sample size determination

A sample size of 40 (20 mice per group) at 95% power to detect a difference between means of 2.5 at a significant level (alpha) of 0.05 (two-tailed) was chosen using a special formula for the calculation of sample size for laboratory animals experiment (8): $N = 1 + 2C[s/d]^2$, where C = a constant (7.8) at 0.05 level of significance, s = 2.75 (standard deviation from a similar previous study) (9), and d = difference between means desired in present study.

2.2. Animal source

Forty mice (22 males, 18 females), 6–8 weeks old and weighing 28–36 g, were obtained from the animal house of the Department of Pharmacology, Nnamdi Azikiwe University. The animals were certified healthy by a veterinarian, Dr Chijioke A Ezenyeaku, of this university. Male mice were randomized into two groups of 11 mice per group. Female mice were also randomized into two groups of 9 mice per group. Each group was housed in metal cage of 60 cm × 45 cm × 30 cm. All were allowed free access to animal feed (Growers, Top Feeds, Nigeria) and clean drinking water. Left over feed and water were discarded and cages were cleaned with chlorhexidine antiseptic solution every 12 h. Artificial light by fluorescent lamp (Philips, the Netherlands; 18 W) and a light/dark cycle of 12/12 h were maintained. All mice were acclimatized for 2 weeks.

2.3. Drug source /vaccines/assay kits

Methylprednisolone injection (Methysol, Keun Wha Pharm. Co, South Korea), diazepam injection (Valium, Roche, USA), and ketamine injection (Ketalar, Popular Pharmaceuticals, Bangladesh) were procured from Pax Pharmacy, Onitsha. Hepatitis B vaccine (Euvax B, LG Life Sciences, South Korea) was procured from the Cold Chain Office, Health Department, Idemili North Local Government, Ogidi, Anambra State. IL-4 Mouse ELISA Kit (Abcam, UK) and IFN-γ Mouse ELISA Kit (Abcam, UK) were procured from Safety Molecular Pathology Laboratories Ltd., Enugu.

2.4. Experimental procedure

Each test mouse received 15 mg/kg methylprednisolone injection IM on alternate hind legs daily for 30 days (except on the 6th and 27th days). Each control mouse received 0.1 mL of water for injection intramuscularly on alternate hind legs for 30 days (except on the 6th day and 27th days). On the 6th day, all mice were given 2 μg (0.1 mL) of hepatitis B vaccine intramuscularly to the hind leg, and they received a booster dose on the 27th day. On the 34th day, blood samples were collected from all the mice for analysis.

The male mice were further randomized based on the length of exposure to methylprednisolone injections into MT_{15}: male test mice that received 15 mg/kg methylprednisolone injection daily for 15 days (n = 6), MT_{30}: male test mice that received 15 mg/kg methylprednisolone injection daily for 30 days (n = 5), and MC: male control mice that did not receive methylprednisolone injection (n = 11). MT_{15} and MT_{30} were differentiated by application of blue and red permanent marks respectively on the frontal regions. The female mice (9 test, 9 control) were similarly randomized into FT_{15}: female test mice that received 15 mg/kg methylprednisolone injection daily for 15 days (n = 5), FT_{30}: female test mice that received 15 mg/kg methylprednisolone injection for 30 days (n = 4), and FC: female control mice that did not receive methylprednisolone injection (n = 9).

The test and control mice received 2 μg (0.1 mL) of hepatitis B vaccine on the 6th day and a booster dose on the 27th day (10). An injections chart was maintained throughout the duration of the experiment. The animals were weighed before the commencement and at the end of the experiment. Deaths of animals were recorded.

2.5. Collection of blood samples

On the 34th day all mice were anesthetized, one at a time, using intramuscular ketamine and diazepam at 50 mg/

kg and 5 mg/kg, respectively (11). Blood samples were collected from the saphenous vein of each mouse using the Hoff method (12). Withdrawn blood samples were quickly transferred into plain specimen bottles and allowed to clot naturally. Thereafter, the serum was separated into another specimen bottle using a micropipette and stored at 2–8 °C until use.

2.6 Determination of immune response

Assays for serum IL-4 and IFN-γ were done using ELISA according to the manufacturer's instructions (Diagnostic Automation Inc., USA). The optical densities were converted into international units using digital software, Gen 5 (Diagnostic Automation, USA), attached to the microplate reader (Model No DAR 8000. Diagnostic Automation Inc., USA).

2.7 Statistical analysis

The mean values (±SEM) for the generated data were calculated. The D'Augostino and Pearson omnibus normality test was performed on the data. Testing of statistical significance between test and control groups of mice was done for IFN-γ and IL-4 using the Mann–Whitney test. All statistical tests were done using Graph Pad Prism 5.0 and the results were taken as statistically significant at $P < 0.05$.

3. Results

Three male mice died: 1 MT_{15} on day 7, 1 MT_{30} on day 18, and 1 MC on day 25.

There were positive IL-4 and IFN-γ titers in all groups of mice (Tables 1 and 2), but the differences in titers were not statistically significant (Tables 3 and 4). There were also no significant differences for sex or duration of methylprednisolone exposure in the groups of mice tested (Tables 3 and 4).

4. Discussion

Serum IFN-γ and IL-4 were detected in all mice groups studied. There was no statistically significant difference in serum IFN-γ and IL-4 between the methylprednisolone-exposed and control groups of mice. There were also no differences in length of exposure between the groups. This was in contrast to earlier finding in which cellular immune responses (mediated by IFN-γ) were suppressed while humoral immune responses (mediated by IL-4) were enhanced by glucocorticoids (13). Franchimont et al. demonstrated that dexamethasone blocked IL-12 (Th1)-induced STAT-4 phosphorylation without blocking IL-4 (Th2)-induced STAT6 phosphorylation in lymphocytes and suggested that this might explain the glucocorticoid-induced shift towards Th2 humoral immune response (14). In addition, Remelts et al. demonstrated that treatment with dexamethasone injection suppressed systemic cytokine response in patients with community-acquired pneumonia (15), while Mazzocca et al. demonstrated the inhibitory effect of methylprednisolone injection on serum IFN-γ levels in umbilical vein endothelial cells (16). El-Radhi et al. also demonstrated that oral prednisolone decreased serum concentration of IL-4 below the sensitivity level in asthmatic children (17).

The high doses of methylprednisolone used in this study could explain the inability of this drug to increase the serum IL-4 or decrease the serum IFN-γ titers since high doses of glucocorticoids could suppress the hypothalamic–pituitary–adrenal axis with unpredictable effects on immune function. This unpredictability is based on the fact that cytokines, which mediate immune responses, are pleiotropic (one cytokine can exert many actions) and redundant (different cytokines can exert the same action), and often influence the synthesis of other cytokines.

It is also important to note that the effect of glucocorticoids on gene expression is not very specific. Glucocorticoids decrease the expression of target genes that code for proinflammatory and immunostimulatory cytokines such as IFN-γ by a process called transrepression. The opposite effect (transactivation), which increases the expression of these genes, can also be induced by glucocorticoids. Most glucocorticoids do not distinguish between transrepression and transactivation in their mechanisms of action, making it difficult to predict what their actions on the immune system could be (18,19). Therefore, in contrast to the traditional view of glucocorticoids as immunosuppressant hormones, they are more accurately conceptualized as immunomodulatory hormones that can stimulate as well as suppress immune function, depending on the type of immune response, the immune compartment, and the cell type involved (20). This nonspecificity in action could partly explain the findings in this study.

The low levels of serum IL-4 and IFN-γ in most of the mice could be explained by the fact that mice are not natural hosts of hepatitis B virus. This notwithstanding, mice are good experimental models for hepatitis B viral studies as their hepatocytes do support hepatitis B viral replication (21).

Adjuvants are often required in order to increase immune response to hepatitis B vaccines in mice (22). In fact, Qin et al. demonstrated an increased secretion of anti-HBs, IL-12, and IFN-γ when dinucleotide adjuvants were injected together with hepatitis B surface antigen in mice (23). Although the use of adjuvants would have increased the cytokine titers in these mice, they could have equally confounded the effect of steroids on the immune response, the main focus of this study.

There were sex differences in serum IL-4 and IFN-γ levels as the female test (FT) and female control (FC)

Table 1. Mean ± SEM serum IL-4 titers (pg/mL) in the test and control groups of mice.

Male test (MT)	Male control (MC)	Female test (FT)	Female control (FC)
3.36	3.65	68.32	2.49
31.47	18.96	2.41	2.43
3.07	3.66	57.63	3.91
2.92	2.45	2.64	3.11
14.60	3.41	2.45	4.66
12.88	3.93	68.32	13.74
3.18	2.49	2.41	89.71
2.90	3.11	56.10	10.69
---	----	2.64	3.88
9.30 ± 4.56	5.21 ± 1.94	29.21 ± 10.22	14.96 ± 8.97

The test groups of mice (MT, FT) received daily intramuscular injections of 15 mg/kg methylprednisolone injections for 30 days. The control groups of mice (MC, FC) received daily intramuscular injections of 0.1 mL of sterile deionized water for 30 days. All mice groups received 2 µg (0.1 mL) of HBsAg on the 6th and 27th days.

Table 2. Mean ± SEM serum IFN-γ titers (pg/mL) in the test and control groups of mice.

Male test (MT)	Male control (MC)	Female test (FT)	Female control (FC)
3.55	6.54	34.44	2.98
32.11	23.64	12.18	2.8
8.99	6.42	27.83	4.69
2.33	2.55	3.59	3.37
5.64	5.49	6.13	5.52
2.54	5.73	25.83	16.48
3.11	4.97	4.57	68.70
7.22	2.18	4.99	10.69
----	----	6.25	4.09
8.18 ± 3.47	7.19 ± 2.42	13.98 ± 3.94	13.27 ± 6.70

The test groups of mice (MT, FT) received daily intramuscular injections of 15 mg/kg methylprednisolone injections for 30 days. The control groups of mice (MC, FC) received daily intramuscular injections of 0.1 mL of sterile deionized water for 30 days. All mice groups received 2 µg (0.1 mL) of HBsAg on the 6th and 27th days.

groups had higher average serum IL-4 and IFN-γ levels, but these differences were not statistically significant when subjected to statistical analysis.

Limitations encountered in this study include species differences between man and mice and even genetic differences between the mice groups; these could account for some of the conflicting findings. The use of monoclonal cell lines in place of animals could have eliminated these species and genetic differences. However, such a study is beyond the scope of this work in terms of personnel and laboratory facilities. The sample size of 40 mice is also a minus in terms of significance of the study to the general

Table 3. Result of statistical analyses of serum IL-4 titers (pg/mL) in various test and control groups of mice using the Mann–Whitney test.

Data analyzed	Sum of ranks in row A	Sum of ranks in row B	P-value
MT vs. MC	70	66	0.8785
MT_{15} vs. MT_{30}	18	18	1.000
FT vs. FC	87	84	0.9314
FT_{15} vs. FT_{30}	27.5	27.50	0.9155
MT vs. FT	84	87	0.5041

The test groups (MT, FT) of mice received daily intramuscular injections of 15 mg/kg methylprednisolone injections for 30 days. The control groups of mice (MC, FC) received daily intramuscular injections of 0.1 mL of sterile deionized water for 30 days. All mice groups received 2 μg (0.1 mL) of HBsAg on the 6th and 27th days. MT_{15}, MT_{30}: Male test mice exposed to methylprednisolone injection for 15 and 30 days, respectively. FT_{15}, FT_{30}: Female test mice exposed to methylprednisolone injection for 15 and 30 days, respectively.

Table 4. Result of statistical analyses of serum IFN-γ titers (pg/mL) in various test and control groups of mice using the Mann–Whitney test.

Data analyzed	Sum of ranks in row A	Sum of ranks in row B	P-value
MT vs. MC	68	68	1.000
MT15 vs. MT30	20	16	0.6857
FT vs. FC	100	71	0.2224
FT15 vs. FT30	28	17	0.5556
MT vs. FT	84	87	0.5041

The test groups (MT, FT) of mice received daily intramuscular injections of 15 mg/kg methylprednisolone injections for 30 days. The control groups of mice (MC, FC) received daily intramuscular injections of 0.1 mL of sterile deionized water for 30 days. All mice groups received 2 μg (0.1 mL) of HBsAg on the 6th and 27th days. MT_{15}, MT_{30}: Male test mice exposed to methylprednisolone injection for 15 and 30 days, respectively. FT_{15}, FT_{30}: Female test mice exposed to methylprednisolone injection for 15 and 30 days, respectively.

population. Modification of this study using cell lines in place of animals can allow a higher sample size to increase the sensitivity of the study. Further research on this topic should take the above mentioned limitations into account.

In conclusion, the results show that there were positive IL-4 and IFN-γ responses in all mice groups, but the differences in titers between the groups were not statistically significant. Therefore, it could be concluded that at the dosages and length of exposure used in this study, methylprednisolone injection did not significantly inhibit IL-4 and IFN-γ expression following hepatitis B vaccination in mice. The possible extrapolation from this could be that corticosteroids, when used in the usual clinical doses and duration of therapy, are not likely to inhibit immune response to hepatitis B and other vaccinations. This is more likely to be so since doses and duration of therapy higher than those that are usually applied in clinical practice were used in this study with no statistically significant reduction in antibody and cytokines responses.

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