10.5005/jp-journals-10018-1074
ORIGINAL ARTICLE

The Adoptive Transfer of HBsAg-specific Splenocytes from Balb/c Congenic Donors into HBsAg Transgenic Mice is not associated to Histopathological Damage

Yadira Lobaina, Daymir García, Aracelys Blanco, Dunia Hernández, Heidy Trujillo, Freya Freyre, Nelson Merino, Jose Suárez, Julio Ancízar, Leticia Martínez, Julio Cesar Aguilar

ABSTRACT

Background: Hepatitis B virus (HBV) infections remain as a major health problem. It has been estimated that about 370 million people are chronically infected worldwide. Chronic infection also increases the risk of liver diseases such as cirrhosis and hepatocellular carcinoma. Current antiviral therapies fail to control viral replication in the long term. Viral persistence has been associated with a defect in the development of HBV-specific cellular immunity. The limitations of the current available therapies underline the need for alternative therapies. The development of a HBV therapeutic vaccine still remains as a feasible option of treatment despite several drawbacks. Recently, approaches like adoptive T-cell therapy have been evaluated.

Materials and methods: The adoptive transfer was done intraperitoneally using Balb/c mice as donors and HBsAg-transgenic mice as receptors. The HBsAg concentration, specific IgG response and biochemical parameters was evaluated in transgenic mice sera by ELISA. The histopathological analysis of the main organs was carried out.

Results and discussion: In the present work we demonstrated that the adoptive transfer of HBV-specific cellular immunity did no cause histopathological damage. The potent immune response transferred was developed in non-Tg Balb/c mice after multiple simultaneous intranasal and subcutaneous immunizations with NASVAC, a novel HBV therapeutic vaccine candidate. The results indicated that the transference of immunity is safe and also capable of transiently reducing the HBsAg concentration in the sera of transgenic mice. These data support the safety of the therapeutic vaccination using NASVAC and also are in line with the usefulness of the T-cell therapy for chronic hepatitis B.

Keywords: Adoptive transfer, Donor lymphocytes, Liver injury, Therapeutic vaccine, Hepatitis B virus.

How to cite this article: Lobaina Y, García D, Blanco A, Hernández D, Trujillo H, Freyre F, Merino N, Suárez J, Ancízar J, Martínez L, Aguilar JC. The Adoptive Transfer of HBsAg-specific Splenocytes from Balb/c Congenic Donors into HBsAg Transgenic Mice is not associated to Histopathological Damage. Euroasian J Hepato-Gastroenterol 2013;3(2):97-102.

Source of support: Nil

Conflict of interest: None

INTRODUCTION

Chronic infection with hepatitis B virus (HBV) is an important medical problem, with a reservoir of more than 350 million chronically infected carriers worldwide. Cirrhosis and hepatocellular carcinoma associated with chronic HBV infection are among the most important human death causes in high prevalence regions.

Currently, several drugs are recommended for treatment of patients with chronic hepatitis B. These drugs can be divided into two main groups, immunomodulatory drugs like interferon and antiviral drugs including lamivudine, adefovir, entecavir, tenofovir and telbivudine.[1,2] In general these drugs are expensive, have a limited efficacy, rarely produce a sustained response and their prolonged use is associated to important adverse events.[3]

The immunotherapy based in the development of vaccine candidates constitutes one of the most promissory strategies for future treatment of chronic hepatitis B. The effectiveness of these candidates will depend on the generation of a potent humoral and cellular immune response capable to overcome the tolerance against the viral antigens established in patients.[4,5] Other strategies include the adoptive transfer of T-cell immunity; this novel approach has demonstrated their feasibility in some viral chronic infections like citomegalovirus, Epstein-Barr virus and HBV. Studies on adoptive T-cell transfer for the treatment of chronic viral infections are currently extended using either *in vitro* expanded, unmodified or receptor-modified, retargeted T cells.[6]

In the case of the hepatitis B chronic infection previous reports have shown that the passively transferred immunity from a naturally immune donor is effective in the viral clearance.[7] Such works indicated the relevancy of the cytotoxic T-cell response in the resolution of the acute and chronic infection.[8] However, a concern in the development of therapeutic vaccines and adoptive T-cell strategies is the potential immunopathological damage related with the generation or adoptive transfer of a potent cellular response against the viral antigens expressed on liver and other organs as a consequence of the chronic infection.

It was reported in experiments with transgenic mice that the deposition of immune complex antigen-antibody specific against HBsAg could cause damage at liver and kidney levels.[9] Based on all the above evidences, the present work explore with more details safety aspects related with the nasal/parenteral administration of the therapeutic vaccine candidate NASVAC. With that purpose we simulated the environment of a chronic patient that develops a potent immune response against the surface and nucleocapsid antigens of HBV after the administration of said vaccine

candidate. As a model of this scenario we employed HBsAg transgenic mice that received a passive intraperitoneal transfer of immune cells (splenocytes coming from immune congenic Balb/c mice that were immunized by nasal and parenteral routes with three dose of the NASVAC candidate).

MATERIALS AND METHODS

Animals

Balb/c mice (H-2d haplotype, CENPALAB) as donors, and HBsAg (+) transgenic mice (Balb/c genetic background, CIGB) as receptors[10,11] were employed in this study.

Generation of Anti-HBsAg Immunity on Donor Mice

An immunization schedule employing Balb/c mice, female, from 8 to 12 weeks was conducted. Mice were simultaneously inoculated by intranasal and subcutaneous routes with a vaccine candidate called Nasvac that contains the surface and nucleocapsid antigens of HBV.[12] Doses were administered on days 0 and 14; an additional booster dose was administered before the transfer protocol.

The evaluation of the humoral immune response generated by this treatment was carried out by ELISA, measuring the HBsAg-specific IgG and IgG subclasses after each dose. With the aim of studying the cellular immune response, 10 days after the first dose, an ELISPOT assay was performed to measure the specific secretion of gamma interferon by spleen $CD8^+$ T lymphocytes. The results of these evaluations were taken to select the group immunized with Nasvac that generated the higher cellular response.[12] Based on that, animals from the group immunized by IN/SC route with NASVAC were selected as donors of splenocytes for the adoptive transfer experiments. Additionally, animals from the same schedule, immunized with PBS 1×, were selected as donors of splenocytes for control of nonimmune transference.

Obtention of Immune Splenocytes

Fifteen days after receiving the booster dose the donor animals and the animals from the control group were sacrificed and the spleens were processed. Total spleen cells were purified as previously reported.[13] Vials containing 30×10^6 cells in 100 µl of PBS 1× were obtained for individual transfer. Two of this vials received a restimulation previous to the transfer. The restimulation treatment was carried out using 500 µl of Nasvac formulation and 500 µl of supplemented RPMI culture medium, and the cells were incubated 1 hour at 37°C and 5% CO_2, with agitation of 15 minutes. This process was performed in 15 ml corning tubes. After the treatment the two restimulated samples were washed with PBS 1× and resuspended independently in 100 µl of PBS 1× for the adoptive transfer.

Adoptive Transfer of Immunity

As receptors, we employed transgenic mice of both sexes that expressed HBsAg[10,11] with 16 to 20 weeks of age. The mice were randomly assigned to the different groups of treatments. Before the transfer, we carried out a blood extraction to check the basal level of HBsAg in the sera of the transgenic mice. Two days after, we administered intraperitoneally (IP) 30 to 50×10^6 splenocytes in a volumen of 100 µl of PBS 1× per mice.

Blood extractions were carried out by the retro-orbital plexus, weekly, during 5 weeks. In the week 8th post-transfer, the animals were sized, weighed, bleeded and sacrificed. The main organs were weighed and preserved in phormol for histological analysis. A portion of the blood extracted at that point was employed in the determination of blood biochemical parameters such as liver transaminases (ALT), alkaline phosphatase (ALP) and creatinine (CRT).

Serum HBsAg Quantification in Transgenic Mice

An inhouse direct sandwich ELISA qualified at CIGB was employed for serum HBsAg quantification. Briefly, the solid phase was coated using 10 µg/ml CB HepB4 monoclonal antibody (CIGB-Sancti Spiritus, Cuba) at 50°C during 20 minutes, after sensitization the plates were washed 4-fold with a 0.05% Tween 20 water solution and several dilutions of the serum samples were dispensed and incubated during 1 hour at 50°C. A standard curve of HBsAg (CIGB, Cuba) generated by serial dilutions and a pool of nonimmune mice sera as negative control was dispensed. Afterwards, the wells were incubated with the CB Hep-1 peroxidase conjugate during 30 minutes at the same temperature. Finally, the plates were washed 8-fold and the substrate solution based on O-phenylenediamine and hydrogen peroxide was applied. After 15 minutes, the reaction was stopped with 2N sulfuric acid solution and the plates were read using an optical density detector (Multiskan Sensident, Merck, USA) at 492 nm wavelength.

HBsAg or HBcAg-specific IgG Determination in Sera

The ELISA used to evaluate HBsAg or HBcAg-specific IgG has been previously described.[13] Briefly, the solid phase was sensitized with HBsAg or HBcAg at 5µg/ml of each protein, then the plates were blocked using 2% skim milk and after washing the sera samples applied at different dilutions, then it was incubated with the anti-mouse IgG peroxidase

conjugate (SIGMA, USA). Finally, the plates were washed 5-fold and the O-phenylenediamine/hydrogen peroxide substrate solution was applied. After 15 minutes the reaction was stopped with 2N sulfuric acid solution and the plates were read at 492 nm wavelength with a Multiskan Sensident (Merck, USA) reader.

RESULTS AND DISCUSSION

HBsAg Concentration in Sera

The results presented here are representative of two similar adoptive transfer experiments. The majority of mice that received immune splenocytes showed a significant decrease of HBsAg concentration since the first day post-transfer (Graph 1). Statistical differences were found between time 0 and the 1st and 2nd weeks post-transfer ($p < 0.05$). Between the 3rd and 4th weeks forward the HBsAg concentrations began to increase, indicating that the control established by the transferred immunity was vanishing. Since, this point and until the week 8th statistical differences were not found in the antigenemia when compared to time 0.

In the case of the mice that received splenocytes with HBs-specific immunity a marked decrease in the HBsAg concentration in sera was detected, more notably between days 7th and 28th. However, for the mice that received placebo-splenocytes or PBS 1× (Graph 2), although some fluctuations in the serum HBsAg concentration was detected, no statistic differences were found when compared to time 0. As shown in Graph 2, HBsAg values obtained for this group of animals are always above 5 µg/ml.

The present results indicate that efficiently decrease of circulating HBsAg can be achieved by adoptive transfer of immunity in the transgenic mice model. The antigenemia control established by the transferred immune response was effective for 1 to 2 weeks after a single splenocyte transfer.

Other similar works based in adoptive transfer of immunity reported in literature[14] show higher levels of HBsAg control (in the order of several months), however in this cases the transgenic mice used as receptors had lower basal serum HBsAg levels.

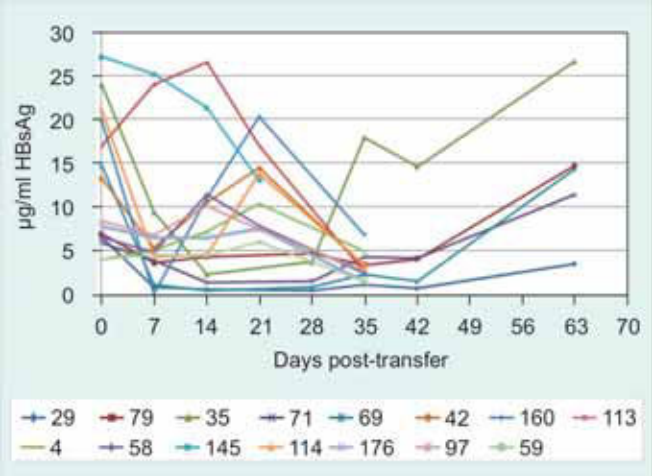
HBsAg-specific Serum IgG Response

The majority of mice that received splenocytes with anti-HBsAg immunity showed a specific response of IgG (Graph 3A). In the case of animals with HBs-specific IgG response the titers were high (>10^4) and began to decrease between the 2nd and 3rd week, this could be related with the antigenemia increase found at this time. On the other hand, the groups that received placebo-splenocytes or saline solution did not showed HBs-specific antibody titers (Graph 3B).

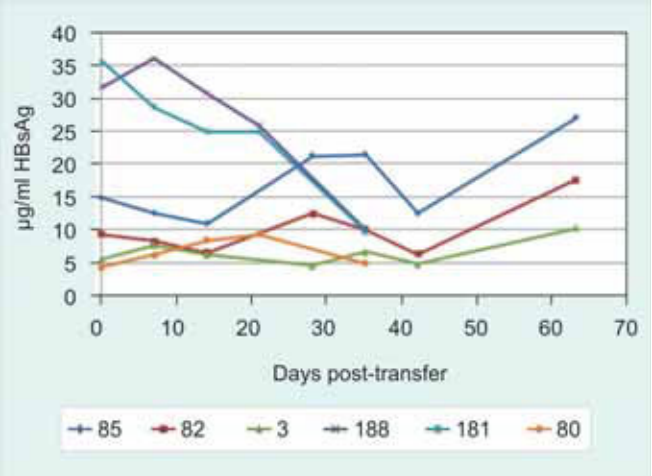
HBsAg-specific antibodies titers increased after the adoptive transfer until the 2nd week post-transfer, these results correspond with the results from Schirmbeck et al[14] demonstrating that the HBsAg expressed constitutively in the transgenic mice constitutes a restimulation for the specific antibody response. They also reported a potent and sustained antibody response that persisted by 5 months after the transfer. In our model, as commented above, transgenic mice expresses higher basal levels of HBsAg in sera compared to the reports from literature. Even in this scenario, during the 2 months our study lasted, the specific IgG titers remained detectable and above 1:1,000.

HBcAg-specific IgG Response in Sera

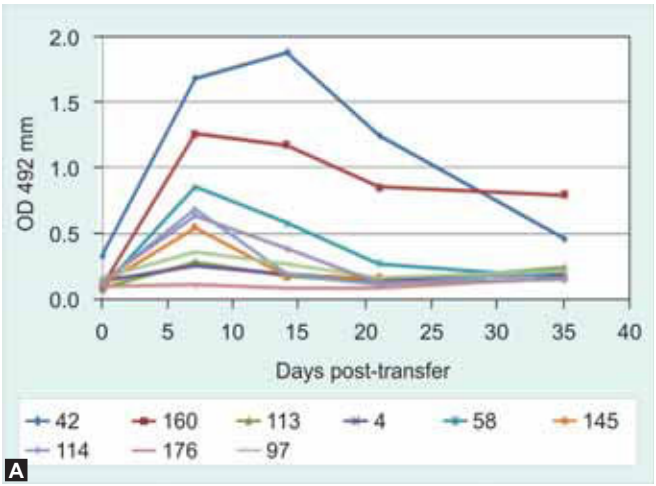
Taking into account that the transferred immune splenocytes come from Balb/c mice with a potent immune response against HBsAg and HBcAg, we decided to evaluate if the anti-HBcAg response remains in the sera of receptor transgenic mice.

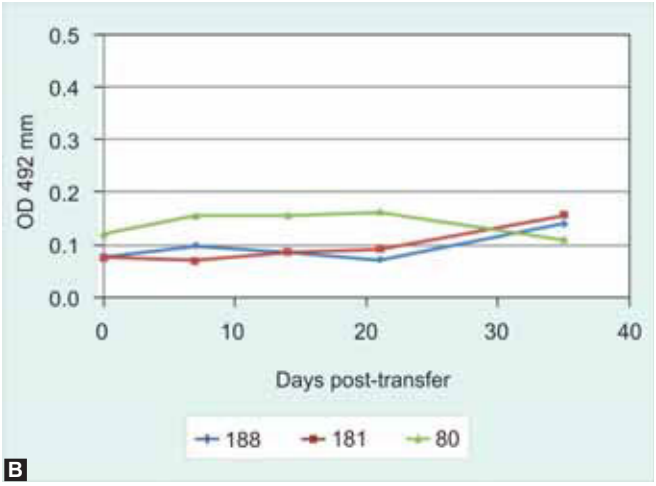


Graph 1: Antigenemia (concentration of HBsAg in µg/ml) in sera at different evaluation point for immune-transferred mice

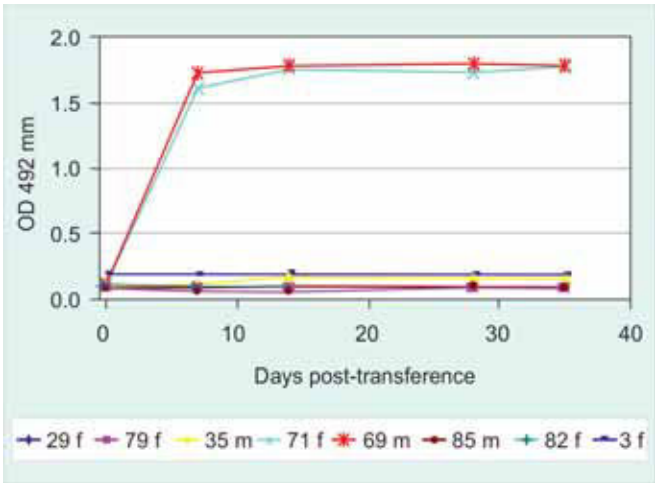


Graph 2: Antigenemia (concentration of HBsAg in µg/ml) in sera at different evaluation point for placebo-transferred mice





Graphs 3A and B: HBsAg-specific IgG response in sera, a representative sample of the evaluated mice, (A) immune-transferred mice and (B) placebo-transferred



Graph 4: HBcAg-specific IgG response in sera by ELISA

As we shown in Graph 4, only mice that received splenocyte previously stimulated *in vitro* with Nasvac formulation, mice 69 and 71, maintained a high HBcAg-specific IgG response. Animals that received splenocytes with HBcAg-specific immune response but without restimulation *in vitro* before transfer (mice 29, 35 and 79) did not show specific response in sera, behaving similar to mice that received placebo-splenocytes or saline solution.

This result constitutes the unique difference regarding immune response found between mice that received splenocytes restimulated *in vitro* previous to transfer and the group of animals that received immune splenocytes without *in vitro* restimulation and support the issue of HBsAg restimulation *in vivo* with the HBsAg from recipient as it was only after *in vitro* HBcAg restimulation that the B cells were capable of inducing IgG response able to be detected in recipient mice.

Blood Biochemical Parameters

At week 8th post-transfer blood samples were taken and a portion of the blood was employed for the determination of hemochemistry parameters, such as alanine aminotransferase, ALP and CRT (Table 1). These specific enzymes are commonly used as marker of liver and kidney damage. We known that the transgenic mice employed express the HBsAg constitutively in the liver, kidney and other organs,[11] that's why constitutes a concern the potential damage induced by the presence of a potent specific immune response.

Table 1: Biochemical parameters in blood at the 8 week post-transfer (ALT: alanine aminotransferase, ALP: alkaline phosphatase). Normal values for humans: ALT: 7 to 33 UI/l, CRT: 0.2 to 1.3 mg/dl, ALP: 30 to 120 U/l. Normal values for nontransgenic mice: ALT: 28 to 69 UI/l, CRT: 0.1 to 0.3 mg/dl, ALP: 144 to 240 U/l

Mice	*ALT (UI/l)*	*ALP (UI/l)*	*Creatinine (mg/dl)*
29	19.833	198	0.665
35	26.833	224.4	0.87
79	27.416	195.799	0.614
69	23.333	321.199	0.614
71	34.416	189.199	0.665
42	14	139.7	0.487
160	9.33	130.9	0.536
113	1.75	nd	0.487
4	25.08	67.1	0.341
58	nd	nd	0.438
145	dead	dead	dead
114	17.5	306.9	0.73
176	1.75	6.6	0.799
97	1.75	nd	nd
59	19.25	170.5	0.73
Controls			
3	21	167.199	0.717
82	29.166	201.3	0.717
85	35	233.199	0.665
188	8.16	nd	nd
181	8.75	nd	nd
80	25.08	219.9	0.63

The values obtained for all mice in the study (treated and placebo mice) are included in the normal range reported

(for humans, ALT: from 7 to 33 UI/l, CRT: from 0.2 to 1.3 mg/dl). However, the ALP values obtained for the majority of mice are above the normality range (for humans from 30 to 120 U/l).

The statistical comparison between treated (mice that received anti-HBsAg immune splenocytes transfer) and not treated (mice that received placebo-splenocytes or saline solution transfer) did not yield significant differences for the evaluated parameters. In summary, the results of the biochemical parameters under study evidenced that the two most probable organs that could be damaged as a consequence of the immune response did not show any sign of damage. In this sense, the obtained results support the safe use of NASVAC in therapeutic vaccination.

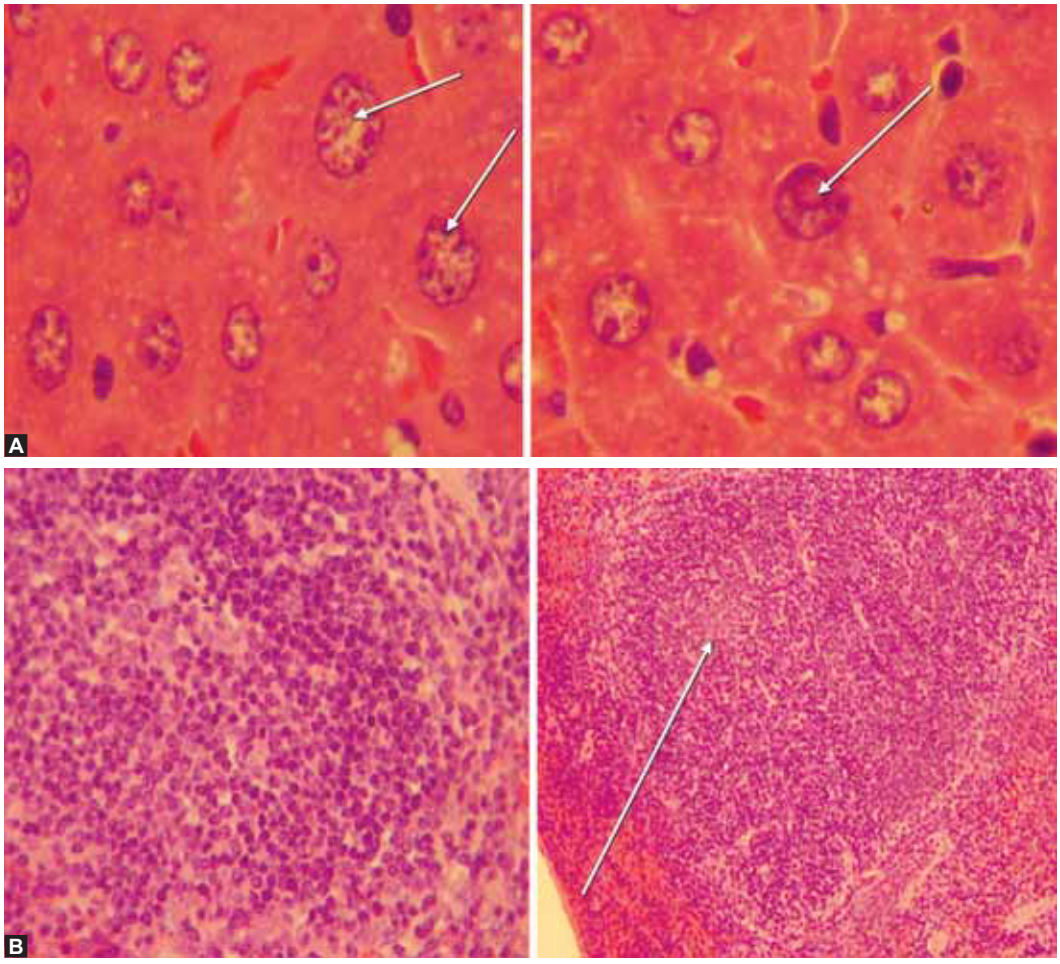
Histological Analysis

At week 8th post-transfer, mice were sacrificed and the main organs were extracted and weighed. Neither differences regarding animal weigh nor the weight of the main organs between treated and not treated mice were detected. However, between sexes there are differences in the weighs of the male mice, which is normal in these animals.

The main organs were extracted and included in paraformaldehyde for histological analysis. As results of this work, no alterations of the organs of the transgenic mice were observed (Figs 1A and B) in association to the adoptive transferred immunity. In addition, no damage was observed in the liver or kidneys, the organs most likely to be damaged. Other organs where the presence of HBsAg has been demonstrated[11] were not damaged either. We did not find histological differences between treated and not treated mice.

CONCLUSION

Taking into account the absence of damage after adoptive transfer of immunocytes we conclude that the adoptive transfer of immunity induced by the therapeutic vaccine candidate comprises HBsAg and HBcAg antigens that is safe and decrease in a transient manner the levels of circulating HBsAg in sera. In addition, these results indirectly demonstrated the safety of the immunity induced by the nasal/parenteral administration of the NASVAC vaccine candidate in a model of immunotolerance subversion.



Figs 1A and B: Representative photograph of the histopathology analysis of the organs (A) liver, (B) spleen. The arrows pointed out different findings without clinical relevance

REFERENCES

1. Hilleman MR. Overview of the pathogenesis, prophylaxis and therapeusis of viral hepatitis B, with focus on reduction to practical applications. Vaccine 2001 Feb;19(15-16):1837-1848.
2. Nash K. Telbivudine in the treatment of chronic hepatitis B. Adv Ther 2009 Feb;26(12):155-169.
3. Lok ASF. The maze of treatments for hepatitis B. N Engl J Med 2005 Jun;352(26):2743-2746.
4. Bertoletti A, Ferrari C, Fiaccadori F. Role of the cell-mediated immune response in the pathogenesis of hepatitis B virus infection: possible immune-therapeutic strategies. Acta Biomed Ateneo Parmense 1996;67(3-4):87-93.
5. Webster G, Bertoletti A. Quantity and quality of virus-specific CD8 cell response: relevance to the design of a therapeutic vaccine for chronic HBV infection. Mol Immunol 2001 Dec;38(6):467-473.
6. Bohne F, Protzer U. Adoptive T-cell therapy as a therapeutic option for chronic hepatitis B. J Viral Hepat 2007 Nov;14 (Suppl 1): 45-50.
7. Lau GK, Suri D, Liang R, Rigopoulou EI, Thomas MG, Mullerova I, et al. Resolution of chronic hepatitis B and anti-HBs seroconversion in humans by adoptive transfer of immunity to hepatitis B core antigen. Gastroenterology 2002 Mar;122(3): 614-624.
8. Boni C, Penna A, Ogg GS, Bertoletti A, Pilli M, Cavallo C, Cavalli A, Urbani S, Boehme R, Panebianco R, et al. Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B: new perspectives for immune therapy. Hepatology 2001 Apr;33(4):963-971.
9. Zi XY, Yao YC, Zhu HY, Xiong J, Wu XJ, Zhang N, Ba Y, Li WL, Wang XM, Li JX, et al. Long-term persistence of hepatitis B surface antigen and antibody induced by DNA-mediated immunization results in liver and kidney lesions in mice. Eur J Immunol 2006 Apr;36(4):875-886.
10. Castro FO, Pérez A, Aguilar A, de la Riva G, Martínez R, de la Fuente J, Herrera L. Expresión del gen del antigeno de superficie del virus de la hepatitis B en ratones transgénicos. Interferón y Biotecnología 1989;6(3):251-257.
11. García E, González VE, González-Griego A, Salgado A, Fernández A, Pezzella M, Alerm A. Correlación del ADN y del antigeno de superficie del virus de hepatitis B en tejidos de animales transgénicos. Rev Cubana Invest Biomed 2000;19(1): 8-9.
12. Lobaina Y, Trujillo H, Garcia D, Gambe A, Chacon Y, Blanco A, Aguilar JC. The effect of the parenteral route of administration on the immune response to simultaneous nasal and parenteral immunizations using a new HBV therapeutic vaccine candidate. Viral Immunol 2010;23(5):521-529.
13. Aguilar JC, Lobaina Y, Muzio V, García D, Pentón E, Iglesias E, Pichardo D, Urquiza D, Rodríguez D, Silva D, et al. Development of a nasal vaccine for chronic hepatitis B infection that uses the ability of hepatitis B core antigen to stimulate a strong Th1 response against hepatitis B surface antigen. Immunol Cell Biol 2004 Oct;82(5):539-546.
14. Schirmbeck R, Wild J, Stober D, Blum HE, Chisari F, Geissler M, Reimann J. Ongoing murine T1 or T2 immune response to the hepatitis B surface antigen are excluded from the liver that expresses transgene-encoded hepatitis B surface antigen. J Immunol 2000 Apr;164(8):4235-4243.

ABOUT THE AUTHORS

Yadira Lobaina (Corresponding Author)

Department of Hepatitis B, Biomedical Research Unit, Center for Genetic Engineering and Biotechnology, Havana, Cuba, PO Box 6162 Fax: (53-7)2714764, e-mail: yadira.lobaina@cigb.edu.cu

Daymir García

Department of Hepatitis B, Biomedical Research Unit, Center for Genetic Engineering and Biotechnology, Havana, Cuba

Aracelys Blanco

Department of Animal Facility, Biomedical Research Unit, Center for Genetic Engineering and Biotechnology, Havana, Cuba

Dunia Hernández

Department of Hepatitis B, Biomedical Research Unit, Center for Genetic Engineering and Biotechnology, Havana, Cuba

Heidy Trujillo

Department of Hepatitis B, Biomedical Research Unit, Center for Genetic Engineering and Biotechnology, Havana, Cuba

Freya Freyre

Department of Hepatitis B, Biomedical Research Unit, Center for Genetic Engineering and Biotechnology, Havana, Cuba

Nelson Merino

Department of Animal Facility, Biomedical Research Unit, Center for Genetic Engineering and Biotechnology, Havana, Cuba

Jose Suárez

Department of Animal Facility, Biomedical Research Unit, Center for Genetic Engineering and Biotechnology, Havana, Cuba

Julio Ancízar

Department of Animal Facility, Biomedical Research Unit, Center for Genetic Engineering and Biotechnology, Havana, Cuba

Leticia Martínez

Department of Animal Facility, Biomedical Research Unit, Center for Genetic Engineering and Biotechnology, Havana, Cuba

Julio Cesar Aguilar

Department of Hepatitis B, Biomedical Research Unit, Center for Genetic Engineering and Biotechnology, Havana, Cuba