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Induction of oral tolerance towards hepatitis B envelope antigens in a murine model

Israel Gotsman [a], Roy Beinart [a], Ruslana Alper [a], Elazar Rabbani [b], Dean Engelhardt [b], Yaron Ilan [a,*]

[a] *Department of Medicine, Liver Unit, Hadassah University Hospital, PO Box 12000, IL91120 Jerusalem, Israel*
[b] *ENZO, Biochem, New York, USA*

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Abstract

Background: Hepatitis B virus (HBV) is a non-cytopathic virus, and the hepatocellular injury that occurs as a consequence of HBV infection is mediated by the host antiviral immune response. Subjects with natural tolerance to HBV have minimal or no liver injury despite chronic viremia. We have shown that immune tolerance towards viruses can be induced by oral administration of viral proteins. *Aims*: To test whether oral induction of tolerance can be induced towards HBV antigens, and whether oral tolerance induction downregulates preexisting anti-HBV immune response. *Methods*: Oral tolerance was induced via feeding of five low oral doses of HBV proteins (HBsAg + preS1 + preS2, BioHepB). This was followed by two inoculations with the BioHepB vaccine. Humoral immune tolerance was evaluated by measuring serum levels of anti-HBs antibody titers at monthly intervals. To determine if oral tolerance induction downregulates pre-existing anti-HBs immunity, mice were inoculated twice with the BioHepB vaccine, followed by feeding of BioHepB-HBV proteins. *Results*: Feeding of HBV proteins markedly inhibited production of anti-HBs antibodies in naive mice. Anti-HBs titers were 45 versus 135 mIU/ml, in tolerized versus non-tolerized controls ($P < 0.005$). Moreover, oral tolerance induction effectively down-regulated pre-existing immunity and reduced the anti-HBs titers in previously immunized mice to 112 versus 223 mIU/ml, in tolerized compared with non-tolerized controls ($P < 0.01$). *Conclusions*: Induction of oral tolerance towards HBV proteins downregulates the antiviral humoral immune response in naive mice, and in the presence of preexisting anti-HBV immunity. This approach should be further investigated as a method for alleviation of antiviral-mediated liver injury in chronic HBV hepatitis.

Keywords: Hepatitis B virus treatment; Oral tolerance; Immune response; Chronic liver disease

1. Introduction

Chronic Hepatitis B virus (HBV) infection is a serious health problem worldwide (Alter et al., 1990; Margolis et al., 1991). Results of experimen-

* Corresponding author. Tel.: +972-2-6777337; fax: +972-2-6420338.

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tal infection in cultured hepatocytes, and the presence of asymptomatic carriers of the virus indicate that the virus itself is non-cytopathic (Ganem, 1982; Blum et al., 1991; Chisari and Ferrari, 1995). The host immune response clears the virus in the majority of cases. However, in some cases, the immune response fails to clear the virus, and continues to be directed at the infected hepatocytes, leading to chronic liver disease (Alberti et al., 1986; Liaw et al., 1988; Blum et al., 1991; De Jongh et al., 1992; Chisari and Ferrari, 1995). Current treatment strategies, which include antiviral therapy and attempts to augment the antiviral immune response have been largely disappointing (Hoofnagle and Di Bisceglie, 1997).

In the context of the current understanding of the immunological mechanisms involved in HBV-mediated liver injury, it is likely that chronic HBV-associated inflammation of the liver can be alleviated by tolerance induction towards HBV antigens. Recently others and we have shown that it is possible to induce tolerance towards viruses thereby downregulating cellular and humoral antiviral immune responses (Ilan et al., 1996a, 1997a,b,c; Takahashi et al., 1996). Out of the methods used for tolerance induction, oral tolerance emerged as the most efficient (Ilan et al., 1997c). Oral tolerance is the induction of immunological hyporesponsiveness towards specific antigens or, through a bystander effect, towards other antigens present at the site, via secretion of immunosuppressive cytokines (Weiner, 1994). Enteral exposure to high doses of antigen induces tolerance by clonal inactivation of antigen-specific T-cells, while the feeding of a low antigen dose leads to induction of regulatory cell secreting factors that suppress generation of antigen-specific effector cells (Weiner, 1994, 1997). Induction of oral tolerance by feeding of adenoviral structural proteins induces immune tolerance towards these viruses. Moreover, oral tolerance effectively downregulates the preexisting anti-adenovirus immune response (Ilan et al., 1998a).

The aim of the present study was to evaluate whether it is possible to induce humoral immune tolerance towards hepatitis B antigens, and to evaluate whether oral tolerance induction is an effective method for downregulating pre-existing anti-HBV immune response. In the present study we demonstrated for the first time that oral administration of low doses of HBsAg + PreS1 + PreS2 induced peripheral humoral immune tolerance and downregulated pre-existing anti-HBV immune response.

2. Materials and methods

2.1. Animals

Normal inbred, female BALB/c mice (25–30 g), were obtained from the Animal Core of the Hadassah-Hebrew University Medical School. Mice were maintained on standard laboratory chow and kept in 12 h light/dark cycles. All animal experiments were carried out according to the guidelines of the Hebrew-University-Hadassah Institutional Committee for Care and Use of Laboratory Animals, and with the Committee's approval.

2.2. Induction of anti-HBV immune response

BioHepB recombinant hepatitis B vaccine (Bio-Technology General LTD, Israel), which contains three surface antigens of the hepatitis B virus: HBsAg, PreS1 and preS2, was used for induction of anti-HBV immune response (Shouval et al., 1994). This vaccine was chosen as it has been shown previously to improve immunogenicity compared to other vaccines, and induces a high level of seroconversion and high antibody titer (Shouval et al., 1994). We tested the optimal vaccine dose required for induction of an effective antiviral immune response. Three groups of mice consisting of ten animals each were studied. Mice in each group were injected intraperitoneally (i.p.) with one of three doses of the BioHepB vaccine: 0.2, 0.4 or 0.8 mcg. Mice were followed for serum anti-HBs antibody titers 30 days after vaccine inoculations.

2.3. Induction of oral tolerance towards HBV antigens

Recombinantly prepared HBsAg + PreS1 + PreS2 antigens (BioHepB, BioTechnology Gen-

Table 1
Experimental and control groups

Group	Antigen fed	Days of feeding	Days of BioHepB vaccination	Days of anti-HBs evaluation
A	None	None	1, 30	30, 60
B	HBsAg+pre S1+pre S2	−10–−1	1, 30	30, 60
C	BSA	−10–−1	1, 30	30, 60
D	HBsAg+pre S1+pre S2	1–10	1, 30	30, 60
E	BSA	1–10	1, 30	30, 60
F	HBsAg+pre S1+pre S2	30–40	1, 30	30, 60
G	BSA	30–40	1, 30	30, 60
H	HBsAg+pre S1+pre S2	1–10	None	30, 60

eral LTD, Israel) were used as target antigens for induction of peripheral immune tolerance in the present study. Peripheral tolerance was induced through oral administration of low-dose HBV antigens (BioHepB), 1 mcg/feeding, using a feeding-atraumatic needle, on alternate days for 10 days (a total of five doses). Control groups received similar doses of bovine serum albumin (BSA).

2.4. Experimental groups

Eight groups of mice consisting of ten animals each were studied (Table 1). Experimental groups A–G were inoculated twice i.p. with the BioHepB vaccine (0.04 mcg) at 1-month intervals. Mice in control group A were vaccinated on days 1 and 30 without oral feedings, and were followed for serum anti-HBs antibody titers 30 days after each injection on days 30 and 60. To determine the effect of oral tolerance induction on the humoral anti-HBV immune response in naive animals, mice in-groups B and C were fed, 10 days prior to vaccination, with HBV envelope proteins (BioHepB) or bovine serum albumin (BSA), respectively. This was followed by two inoculations i.p. with the BioHepB vaccine on days 1 and 30. Mice were followed for serum anti-HBs titers 30 days following each immunization, on days 30 and 60. To determine the effect of oral tolerance induction on pre-existing anti-HBV immune response, mice in-groups D and E were inoculated i.p. with the BioHepB vaccine followed by oral administration of HBV envelope proteins (BioHepB) to mice in experimental group D, or BSA to mice in control group E, beginning on vaccination day. Mice in both groups were re-immunized with the BioHepB vaccine on day 30, and anti-HBs antibody titers were measured 30 days following the second injection on day 60 (Fig. 1). For further determination of the effect of oral tolerance induction on anti-HBV immunity, mice in-groups F and G were inoculated twice with the BioHepB vaccine on days 1 and 30, followed by oral admin-

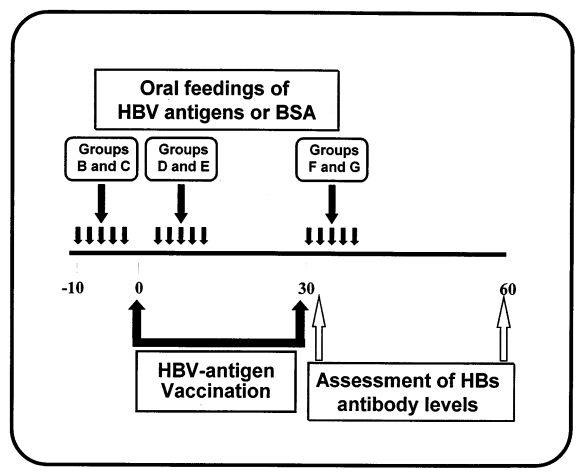

Fig. 1. The experimental protocol. Oral tolerance was induced via feeding of five low oral doses of HBV proteins. This was followed by two inoculations with the BioHepB vaccine. Humoral immune tolerance was evaluated by measuring serum levels of anti-HBs antibody titers at monthly intervals (A). To determine if oral tolerization downregulates pre-existing anti-HBs immunity, mice were inoculated twice with the BioHepB vaccine, followed by feeding of BioHepB-HBV proteins as outlined in (B) and (C).

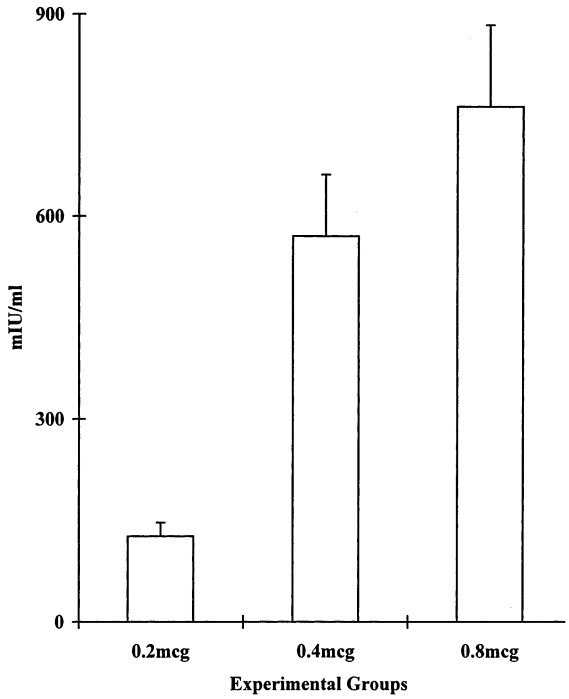

Fig. 2. Anti-HBsAg antibody serum levels 30 days following i.p. inoculation with three different doses of the recombinant BioHepB vaccine. A significant difference between the dose of 0.2 mcg compared with 0.4 and 0.8 mcg ($P < 0.05$ and $P < 0.03$, respectively) was seen. No significant difference between 0.4 and 0.8 mcg doses was observed ($P = 0.20$).

istration of HBV envelope protein (BioHepB) to mice in experimental group F, or of BSA to mice in control group G. Mice were followed for anti-HBs titers before oral tolerance induction on day 30, and following tolerance induction on day 60 (Fig. 1). To determine the effect of oral administration of HBV envelope proteins (BioHepB) on anti-HBs titers, mice in group H received five oral doses of HBV antigens without vaccination. Mice were followed 30 days later for development of anti-HBs serum levels.

2.5. Assessment of anti-HBs humoral immune response

Antibodies to HBsAg were detected by a commercial solid phase radioimmunoassay (RIA, Ausab, Abbott Laboratories, North Chicago, IL). A World Health Organization reference serum was used for quantitative analysis of anti-HBs by RIA, utilizing the Hollinger formula and expressed in mIU/ml. Mice in all experimental and control groups were followed for anti-HBs antibody titers 30 days following each inoculation of the BioHepB vaccine, throughout the study.

2.6. Statistical analysis

Results were analyzed using the standard Student's *t*-test.

3. Results

3.1. Induction of anti HBV immune response

Three groups of mice were followed for anti-HBs antibody titers post-inoculation with three different doses of the BioHepB vaccine. A significant difference between a dose of 0.2 mcg compared with 0.4 and 0.8 mcg was observed. Vaccination with 0.2 mcg induced anti-HBs titers of 126.5 ± 72.8 mIU/ml compared with 570.5 ± 358.5 and 762.0 ± 423.1 mIU/ml, following inoculation with 0.4 and 0.8 mcg, respectively ($P < 0.05$ and $P < 0.03$ for 0.4 and 0.8 versus 0.2, respectively, and $P = 0.2$ for the difference between 0.4 and 0.8 mcg; Fig. 2). Consequently, a vaccination dose of 0.4 mcg, administered i.p. was used in the study.

3.2. Evaluation of the effect of oral administration of HBV-envelope proteins on anti-HBs titers

Oral administration of HBV-envelope proteins without immunization had no effect on anti-HBs antibody titers. Mice in Group H that were fed HBV envelope proteins had undetectable anti-HBs serum levels 30 days after vaccination.

3.3. Evaluation of the effects of tolerance induction on anti-viral humoral immune response

Induction of anti-HBV peripheral immune tolerance was evaluated by measuring anti-HBsAg antibody production at monthly intervals in mice

fed prior to HBV vaccination. Administration of HBV proteins prior to BioHepB vaccination markedly downregulated the antiviral humoral immune response. Thirty days following the first inoculation of the vaccine, serum anti-HBs antibody levels were 385 ± 154 versus 709 ± 290 and 777 ± 346 mIU/ml) in tolerized, HBV-envelope proteins fed (group B), compared with non-tolerized non-fed controls (group A), and BSA-fed (group C), controls, respectively ($P < 0.013$, Fig. 3A). Moreover, 30 days following the second inoculation with the BioHepB vaccine, anti-HBs antibody titers were $66\,495 \pm 44\,007$ versus $95\,257 \pm 58\,320$ and $123\,607 \pm 76\,130$ mIU/ml, in tolerized versus non-tolerized controls (groups A and C, $P < 0.05$, Fig. 3B).

3.4. Evaluation of the effect of oral tolerance induction on preexisting anti-HBV immune response

The effect of tolerance induction towards HBV proteins on pre-existing anti-viral immune response was evaluated by measuring anti-HBs antibody titers in anti-HBV immunized mice orally treated with BioHepB antigens or BSA. The feeding of HBV antigens immediately after exposure to HBV vaccination, reduced anti-HBs titers to $70\,572 \pm 32\,030$ mIU/ml in tolerized (group D) compared with $95\,257 \pm 58\,320$ and $115\,198 \pm 40\,715$ mIU/ml in non-tolerized controls (groups A and E), 60 days following BioHepB inoculation ($P < 0.05$ compared to control groups A and E, Fig. 4).

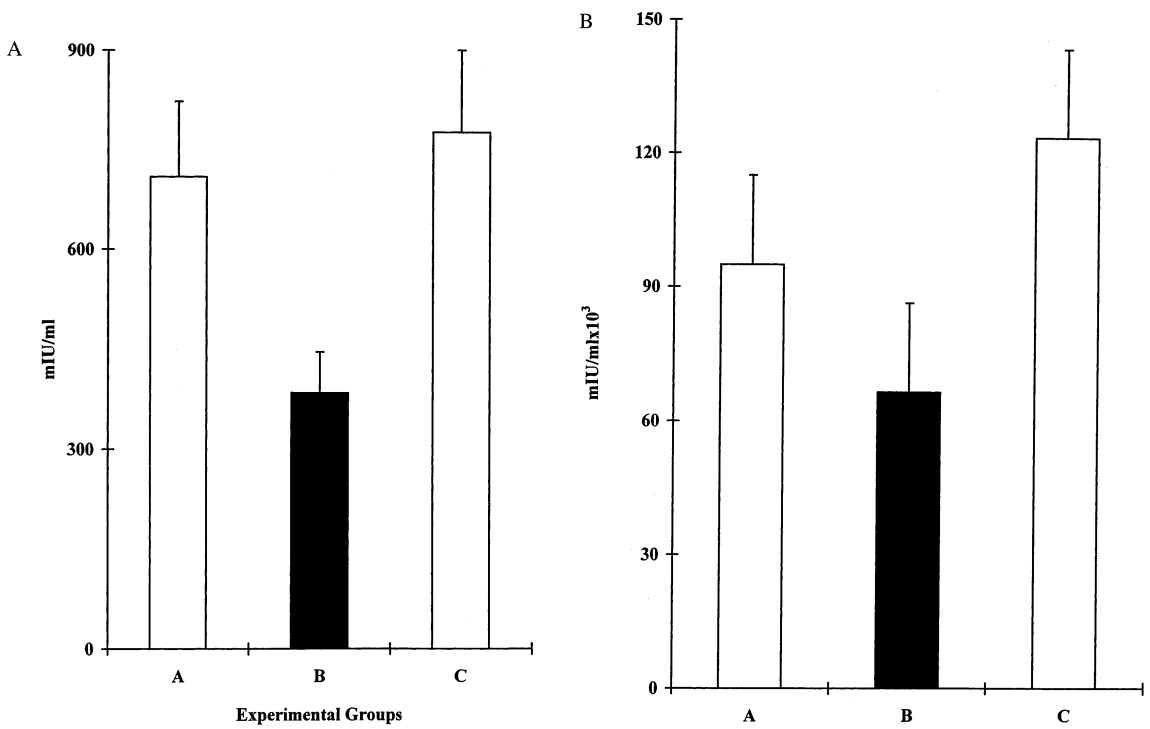

Fig. 3. The effect of oral administration of HBV proteins on tolerance induction towards viral antigens: Mice were fed with HBV envelope proteins followed by two inoculation of the BioHepB vaccine on days 10 and 40. Anti-HBs antibody levels were measured 30 days following each injection, on days 40 (A) and 70 (B). Marked reduction in anti-HBs antibody titers was observed in tolerized HBV-envelope-proteins fed mice (group B, black bar) versus non-tolerized non-fed controls and BSA-fed controls (groups A and C, respectively, white bars) on day 40 (Fig. 3A, $P < 0.013$), and on day 70 (Fig. 3B, $P < 0.05$)

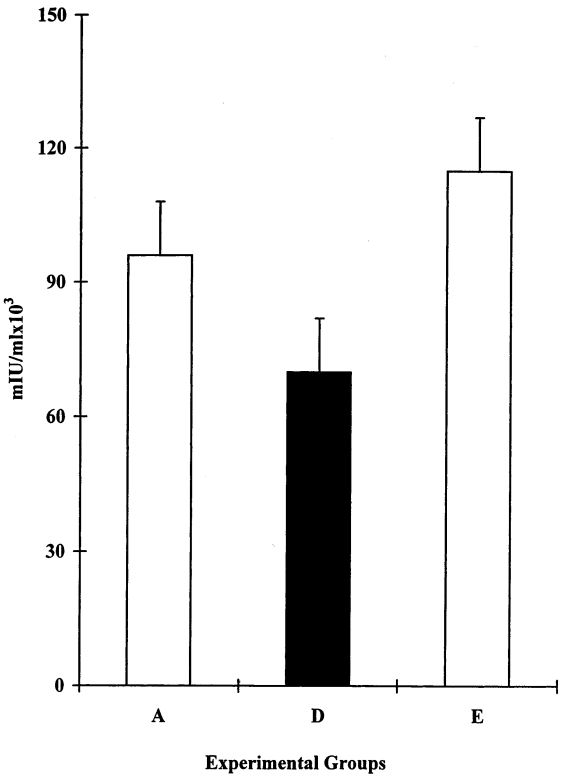

Fig. 4. The effect of oral tolerance induction towards hepatitis B virus in mice with preexisting immunity towards HBV: Mice were immunized with the BioHepB vaccine followed by oral tolerance induction. A second inoculation of the vaccine was administered on day 30, and serum antibody levels were measured 30 days later on day 60. A reduction in anti-HBs serum levels was observed in tolerized HBV-envelope proteins fed mice (group D, black bar) versus non-tolerized BSA-fed controls (group E, white bar) on day 60 ($P < 0.05$).

Moreover, feeding of HBV antigens 30 days following two immunizations with the vaccine, significantly reduced anti-HBs antibody titers. At 30 days following the first immunization, prior to oral feedings of HBV antigens, anti-HBs antibody titers were similarly elevated in groups A, F, and G. There were no significant differences in HBs antibody titers between the groups (Fig. 5A). However, 30 days following the second immunization, anti-HBs antibody titers were significantly reduced in mice fed with HBV antigens. HBs antibody serum levels were $57\,354 \pm 37\,673$ versus $95\,257 \pm 58\,320$ and $98\,195 \pm 57\,256$ mIU/ml, in tolerized, HBV-envelope proteins fed, versus non-tolerized non-fed controls (group A), and BSA-fed controls (group G), respectively as measured on day 60 ($P < 0.01$, Fig. 5B).

4. Discussion

Oral administration of low dose HBV-envelope proteins downregulated the humoral immune response towards viral epitopes in naive animals thus inducing partial peripheral immune tolerance. Moreover, oral administration of HBV envelope proteins effectively down-regulated the preexisting anti-HBV immune response, and significantly inhibited anti-HBs antibody production in mice vaccinated with HBV antigens.

It was previously shown that induction of peripheral tolerance towards specific viral antigens abrogates both humoral and cellular components of the antiviral immune response (Ilan et al., 1997c). Virus injection during the newborn period, intrathymic injection of the whole virus, viral proteins or virus-infected hepatocytes, and transient immunosuppression with FK506 around the time of viral injection, were all found effective in tolerance induction towards viruses in naive animals (Ilan et al., 1996a, 1997a,b; Takahashi et al., 1996). These tolerization methods work by producing clonal anergy, clonal ignorance, or generation of T lymphocytes that down-regulate antigen- specific immune response (Ilan et al., 1996b).

Oral tolerance is a recognized procedure for induction of antigen-specific immune tolerance. It has been possible to induce tolerance to a variety of antigens by feeding animals or humans with the specific antigens or related proteins (Weiner, 1997; Strobel and Mowat, 1998). This method has been used to prevent or alleviate several autoimmune disorders, including collagen-induced arthritis, experimental colitis, graft-versus-host disease, and experimental allergic encephalomyelitis in animals, and for the treatment of rheumatoid arthritis and multiple sclerosis in humans (Miller et al., 1992; Trentham et al., 1993; Weiner et al., 1993; Chen and Weiner, 1996; Von Herrath et al., 1996; Ilan et al., 1998b,c; Trentham, 1998; Trop et al., 1999). We have previously shown that oral tolerance towards adenoviral antigens effec-

tively prevents the antiviral immune response (Ilan et al., 1997c). This approach of tolerization was the most effective and least toxic of all methods described above. Thus it has the greatest potential for clinical application. Adoptive transfer of tolerance by transplantation of immune cells from orally tolerized donors to sublethally irradiated recipients, supported the existence of suppressor cells in this setting (Ilan et al., 1996b). The results of the present study suggest that administration of low doses of HBV envelope proteins can induce tolerance towards HBV epitopes. Feeding of preS1 + preS2 + HBsAg recombinant complex of the HBV-enveloped proteins into naive animals downregulated the anti-HBV immune response upon vaccination (groups B and C).

Oral administration of several antigens to an animal already sensitized to the antigen may, in some cases, downregulate the antigen-specific immune response, rather than boost it (Weiner, 1997). The feeding of antigenic proteins in some chronic autoimmune disease models ameliorated pre-existing disease (Miller et al., 1992; Weiner et al., 1993; Chen and Weiner, 1996; Von Herrath et al., 1996; Ilan et al., 1998c; Trop et al., 1999). We have recently demonstrated that oral tolerance can prevent the secondary immune response in the presence of preexisting anti-adenovirus immunity in animals (Ilan et al., 1998a). Enteral administration of adenoviral structural proteins into preimmunized rats down-regulates both humoral and cellular antiviral immune response. In the present study we have demonstrated that oral administration of HBV proteins to previously immunized mice, induces humoral tolerance towards HBV envelope peptides (experimental groups D and E). Moreover, tolerance induction downregulated the secondary antiviral humoral immune response. Induction of anti-HBV immune tolerance, following two inoculations of the vaccine, effectively inhibited anti-HBs antibody production (experimental groups F and G).

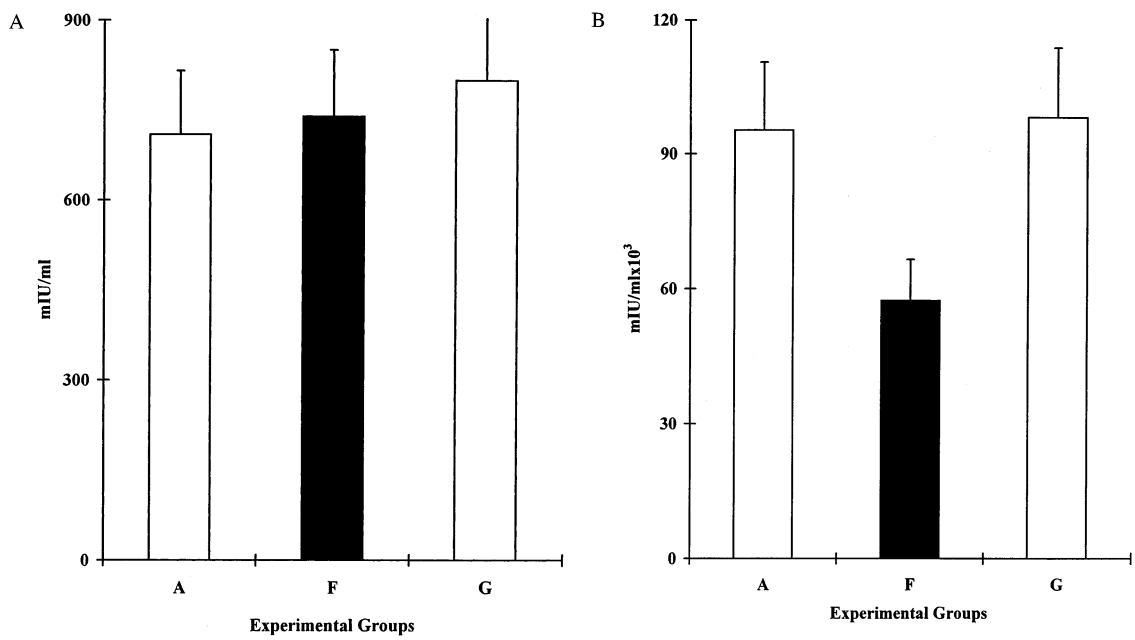

Fig. 5. The effect of oral tolerance induction on preexisting secondary anti-HBV immune response: Mice were inoculated twice with the BioHepB vaccine on days 1 and 30, followed by oral tolerance induction. There was no difference in anti-HBs serum levels measured at 30 days prior to oral feedings between groups A, F and G. However, a significant reduction of anti-HBs serum levels was observed in tolerized HBV proteins-fed mice (group F, black bar) versus non-tolerized BSA-fed controls (group G, white bar) on day 60 ($P < 0.01$).

Hepatitis B virus infection accounts for 10% of chronic liver disease in the Western World (Alter and Margolis, 1990). The antiviral immune response accounts for both viral clearance and the severity of hepatitis (Ganem, 1982; Blum et al., 1991; Chisari and Ferrari, 1995). Following acute HBV infection, the majority of adult patients develop an effective antiviral immune response that leads to viral clearance and long-lasting antiviral immunity (Blum et al., 1991; Chisari and Ferrari, 1995). HBV clearance during acute hepatitis is associated with a strong polyclonal cytotoxic T lymphocyte (CTL) response against viral envelope, nucleocapsid and polymerase proteins that persists for decades after clinical recovery (Guidotti et al., 1994; Rehermann et al., 1996). In contrast, patients who fail to mount an effective immune response to the virus are at risk of developing chronic infection. The host antiviral immune response, rather than viral factors, is critical for the pathologic consequences of the infection (Chisari and Ferrari, 1995).

The majority of patients with HBV infection who do not clear the virus become 'healthy' carriers (Koziel, 1996; Tang et al., 1998; Marco et al., 1999). Currently 5% of the world's population are carriers of HBV (Ganem, 1982; Alter et al., 1990; Blum et al., 1991; Chisari and Ferrari, 1995). These naturally tolerized people appear to be immunologically tolerant to the virus, are asymptomatic and do not exhibit evidence of hepatic dysfunction despite evidence of intrahepatic viral load (Kurose et al., 1997). The current understanding of the absence of hepatic pathology in healthy carriers is that the virus, by itself, is not injurious to liver cells (Ganem, 1982; Chisari and Ferrari, 1995). At the cellular level, studies in transgenic mice have shown that some HBV viral antigens (e.g. HBeAg in neonatal period) may act as tolerogens, leading to induction of an antigen-specific suppressor T-cell population (Milich et al., 1987, 1995). Several lines of evidence support the notion that natural or acquired tolerance towards HBV can prevent or markedly alleviate the severity of hepatitis. Neonates, in whom HBV-specific tolerance occurs because of deletion of HBV-recognizing specific T-cell clones, clear the virus very slowly (Bozkaya et al., 1997; Kurose et al., 1997). Following perinatal infection, 95% of subjects become chronic carriers. Most perinatally infected subjects do not develop HBV-mediated liver damage (Kurose et al., 1997). This group represents the largest population of healthy HBV carriers (Ganem, 1982; Chisari and Ferrari, 1995). Similarly, immunosuppressed hosts (e.g. chemotherapy-treated patients) tend to have prolonged viral persistence after HBV infection, but have a milder acute liver injury (Ganem, 1982; Hasegawa et al., 1994; Gish et al., 1995). Treatment with Campath-1, an anti-T cell agent, was reported to alleviate viral hepatitis (Nagler et al., 1996). These examples indicate that naturally developed, or acquired, anti-HBV tolerance, are associated with milder liver disease or none at all (Ganem, 1982; Chisari and Ferrari, 1995).

Current strategies to treat chronic HBV hepatitis include antiviral medications or attempts to augment the antiviral immune response (Hoofnagle et al., 1988; Ilan et al., 1993a,b; Dienstag et al., 1995; Hoofnagle and Di Bisceglie, 1997). However, complete viral eradication and long-term benefit are uncommon. Patients chronically infected with HBV cannot mount an effective antiviral immune response while exhibiting immune-mediated hepatitis. In contrast, subjects who are tolerized to the virus become carriers but do not develop chronic liver disease. Induction of specific tolerance towards viral-antigens could allow long-term alleviation of the disease, leaving the general immunological defense of the recipient intact.

In conclusion, feeding of HBV envelope proteins induced peripheral humoral immune tolerance towards viral antigens. Hepatocellular damage following HBV infection is mediated by the host anti-HBV immune response, rather than virus-induced cytopathy. Therefore, induction of antiviral tolerance may downregulate the inflammatory immune response and alleviate hepatitis. Thus it could be envisaged to treat chronic HBV patients by inducing specific immune tolerance to HBV, rather than focusing solely on viral suppression. This method has the potential to be used in clinical practice to tolerize patients against viral antigens and modify their clinical course from chronic active hepatitis to a benign carrier state. It

opens up the possibility for understanding the immune target antigens involved in the pathogenesis of HBV-related disorders.

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