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Upregulation of MHC class II antigen on dendritic cells from hepatitis B virus transgenic mice by interferon-γ: abrogation of immune response defect to a T-cell-dependent antigen

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SUMMARY

The experiments presented here were performed to see whether the level of expression of major histocompatibility complex (MHC) class II antigen (Ia antigen) on dendritic cells, one of the most critical antigen presenting cells (APC), influences the humoral immune response in hepatitis B virus (HBV) transgenic mice. We have reported that transgenic mice had a low responsiveness in specific antibody production to keyhole limpet haemocyanin (KLH), a T-cell dependent, HBV-unrelated antigen compared with the age, sex, and major histocompatibility-matched normal mice,[1] due to a significantly lower T-cell stimulatory capacity of transgenic mice-derived dendritic cells, possibly as a result of significantly lower level of Ia antigen.[1] Immunohistochemical staining has shown that treatment of transgenic mice with mouse recombinant interferon-γ (IFN-γ), daily for six consecutive days resulted in an increased expression of Ia antigen on splenic dendritic cells. Again, flow cytometric analyses have further confirmed the significant increase in the expression of Ia antigen on dendritic cells, isolated from transgenic mice treated with IFN-γ compared with the same from the untreated or phosphate-buffered saline (PBS)-treated transgenic mice. Transgenic mice immunized with two optimum doses of KLH (5 μg/mouse) could not produce anti-KLH antibodies in sera, but injecting transgenic mice with the same doses of KLH together with IFN-γ resulted in the production of anti-KLH antibodies in sera. Again, KLH-primed normal mice-derived T/B lymphocytes produced anti-KLH antibody, when cultured with dendritic cells from IFN-γ-treated transgenic mice expressing a higher level of Ia antigen, but not with the same from PBS-treated or untreated transgenic mice. Treatment of transgenic mice with IFN-γ resulted in a reduced level of hepatitis B virus (HBV) DNA in liver and in sera. These experiments have shown that the level of expression of Ia antigen on dendritic cells is a critical factor for its APC capability and its modulation of IFN-γ may be used for immune therapy in HBV carriers.

INTRODUCTION

The high rate of incidence and prevalence of hepatitis B virus (HBV) infection in the world, the susceptibility of HBV carriers to develop hepatocellular carcinoma, the absence of effective and economical therapeutic intervention for HBV carriers and the existence of a considerable number of non-responders to HBV vaccination among general populations demand a better and complete understanding about the immunological mechanisms in HBV carriers.[2] It has been reported that both cellular and humoral immune responses to HBV-related antigens are defective in human HBV carriers[3–6] and in HBV-transgenic mice.[7] HBV carriers can not produce specific antibody to hepatitis B surface antigen (HBsAg) *in vivo* and most of these studies *in vitro* have documented the abnormalities in function of regulatory and helper T cells, and the inability of B cells to produce specific antibody in response to HBsAg.[2–6] On the other hand, some investigators could not detect any substantial defects in the function of T and B lymphocytes in HBV carriers.[8,9] Under such diverse reports about the immune response of HBV carriers on HBV-regulated antigens, it was interesting to elucidate the immune response of HBV carriers to HBV-unrelated antigens to have further insight into the exact immune status of HBV carriers. Recently, we have used transgenic mice[10] produced by microinjecting the full genome of HBV into the fertilized eggs of C57BL/6 mice, expressing HBV-related antigens and mRNAs in sera and tissues as an animal model of HBV carrier state.[1] When normal C57BL/6

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Abbreviations: APC, antigen-presenting cells; HBV, hepatitis B virus; KLH, keyhole limpet haemocyanin; HBsAg; hepatitis B surface antigen; HBeAg, hepatitis B e antigen; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; OD, optical density; SD, standard deviation; PCR, polymerase chain reaction.

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mice were immunized with an optimum dose of keyhole limpet haemocyanin (KLH), all mice produced anti-KLH antibody both *in vivo* and *in vitro*. On the other hand, only a few of the transgenic mice responded very poorly *in vivo* and most of the transgenic mice could not produce anti-KLH antibody *in vitro*, being primed with the same dose of KLH.[1] Further experiments have revealed that the T-cell stimulatory capacity of transgenic mice-derived antigen-presenting dendritic cells was low compared with the same from the normal mice, probably due to a low expression of Ia antigen on dendritic cells; we have postulated that the low expression of Ia antigen on dendritic cells in transgenic mice would be one of the main factors for low, but not non-responsiveness of transgenic mice in antibody response to KLH. A part of our hypothesis was found to be valid as anti-KLH antibody production was detected in culture supernatant when T/B cells from KLH-primed transgenic mice and dendritic cells from unprimed normal mice, expressing much higher levels of Ia antigen, were cultured in the presence of exogenous KLH.[1] IFN-γ (interferon-γ) has been reported to be a potent stimulator of Ia antigen both *in vivo* and *in vitro*[11–13] and have also been reported to modulate the expression of other cytokines and production of immunoglobulins.[14] Again, IFN-γ has been reported to inhibit the expression of all HBV-related antigens in primary hepatocyte cultures from HBV carriers.[15] In human HBV carriers, IFN-γ was shown to have less potent antiviral activity compared with α-IFN, although the immunomodulatory role of IFN-γ in HBV infection has been reported.[4,16] However, the exact mechanism related to its immunomodulatory capability is still not clear. Treating transgenic mice with IFN-γ in our short-term protocol of six days, we found an increased expression of Ia antigen on dendritic cells, an increased level of interleukin 2 (IL-2) in sera, a decreased signal for HBV DNA in liver by *in situ* hybridization and a decreased level of HBV DNA in sera by polymerase chain reaction (PCR) and all of these factors have probably contributed to the better antigen presenting cell (APC) capability of dendritic cells for specific immune response to KLH both *in vivo* and *in vitro* resulting in abrogation of immune response defect of transgenic mice to a T-cell dependent antigen.

MATERIALS AND METHODS

Animals

Transgenic mice (1.2HB-BS10) were produced by microinjecting a partial tandem duplication of the complete HBV genome into fertilized eggs of C57BL/6 mice and these transgenic mice produced HBsAg, HBeAg and HBV DNA in sera. These transgenic mice also expressed HBV-specific mRNAs in tissues such as liver, kidney and testes.[10] Transgenic mice and normal C57BL/6 mice (Charles River Inc, Nagoya, Japan) were housed separately and bred in our animal facility and used for immunization at 6–8 weeks old.

Reagents

The antigen tested was KLH (Polyscience Inc, Warrington, PA). Collagenase was purchased from Sigma (St Louis, MO). Cytotoxicity medium containing 0·3% bovine plasma albumin and 25 mM HEPES in RPMI-1640 and lymphocyte-M, a separation medium containing Ficoll 400 and sodium diatrizoate, density 1·0875 ± 0·0005 were obtained from Cedarlane Lab. Ltd (Hornby, Ontario, Canada).

Antibodies

The antibodies used in this experiment have been described in detail in a previous report.[1] In short, monoclonal antibodies (mAb) to Thy-1.2 (clone 5a-8), and Lyt-1.2 (clone CG16) (Cedarline), 33D1 (TIB227), from American Type Culture Collection (ATCC), and CD45R (clone RA-3-3A1/6.1, TIB 146; ATCC) were used to deplete T cells, dendritic cells and B cells, respectively with low-tox complement (C) (Cedarline). Hamster anti-mouse CD11c (N418), a dendritic cell-specific antibody,[17] fluorescein isothiocyanate (FITC) conjugated anti-hamster immunoglobulin G (IgG) (Cappel, Malvern, PA), and FITC-conjugated anti-mouse Ia (clone M5/114) (Boehringer Mannheim Biochemica, Mannheim, Germany) were used for staining. Peroxidase-conjugated affinity purified anti-mouse IgM and IgG from E.Y. Laboratories (San Mateo, CA) were used in enzyme-linked immunosorbent assay (ELISA). Biotinated anti-mouse Ia and N418 were used as primary antibodies and FITC-conjugated streptoavidin (Biomeda, Foster City, CA) and rhodanine-conjugated goat anti-hamster Ab (E.Y. Laboratories) were used as second antibodies in immunostaining for Ia antigen and N418, respectively.

Immunization with KLH and treatment with IFN-γ

Groups of mice were injected with either KLH, or IFN-γ or both. When mice were injected with KLH only, they received two injections of KLH, i.p. in PBS at an interval of two weeks according to our previous protocol.[1] Mice were injected with IFN-γ for six consecutive days at a daily dose of 5×10^4 U, i.p. according to the already described protocol with some modifications.[12] When the mice received both KLH and IFN-γ, the first injections were started at the same time. Mice were bled before and various time points after immunization. In most of the experiments, mice were primed with 5 μg of KLH, i.p. in PBS and boosted once after two weeks. A group of transgenic mice receiving a daily injection of PBS for six days and some mice receiving no treatment were used as controls.

Cell preparations

Various cell populations were prepared from mouse spleen according to the methods described previously with some modifications.[1,18] Briefly, lymphocytes containing T and B cells (T/B cells) were obtained by passing spleen cells through Sephadex G-10 columns. In order to deplete dendritic cells from T/B cells, these were treated with 33D1 + C in some experiments. The isolation of dendritic cells have been described fully in a previous communication.[1] In short, the single cell suspensions of spleen were centrifuged on dense BPA column ($p = 1.082$) and the cells at the interface were collected and adhered onto a plastic surface, which were then recovered and depleted of the Fc receptor bearing cells by rosetting with antibody-coated sheep erythrocytes. In some experiments, dendritic cells were treated with a mixture of anti-Thy-1.2, anti-Lyt-1.2, anti-CD45R plus C to eliminate contaminating T and B cells.

Cell culture for antibody production

This was done according to the already described methods with slight modifications.[19] Spleen cells (5×10^6) from KLH-primed mice were cultured in 1·0 ml of RPMI-1640 medium supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol and

20 μg/ml of gentamycin sulphate in the presence of 0·5 μg/ml of KLH in 24-well culture plates for 10 days. In some experiments, T/B cells (5×10^6) in combination with dendritic cells (10^5) were used instead of bulk spleen cells. At the end of culture, the supernatants were collected, centrifuged and filtered. One half of the aliquots of all samples were stored at 4° until assay, usually within 2–3 days and the other halves were stored at −20° to assay again after the whole experiments.

Detection of specific antibody response

KLH-specific antibody production was determined by ELISA according to the already described method.[1,20] In short, binding capacities of peroxidase-conjugated goat anti-mouse IgM and IgG were assayed as enzymatic reaction of hydrolysis of a substrate, orthophenylenediamine, by measuring the optical density (OD) value at 492 nm with an ELISA reader (SJeia Auto Reader, Model ER-8000, Sanko Junyaku Co. Ltd, Tokyo, Japan). Pooled sera from unprimed mice were taken as negative controls, and standard sera rich in anti-KLH IgG antibody and anti-KLH IgM antibody were used as positive controls. In the KLH-specific antibody assay, the cut-off value for a positive response was the mean ± 3 standard deviation (SD) of the OD value for the negative control. Since the OD values at 1 : 128 000 and 1 : 32 000 dilutions of anti-KLH IgG-rich and anti-KLH IgM rich standard sera were almost equal to the respective cut-off value, their OD value was designated as 1·0 DtgU and 1·0 DtmU, respectively. Levels of anti-KLH antibody in the specimen were estimated based on the standard curve plotting OD values at different dilutions of anti-KLH antibody-rich standard sera. In the present experiments, we have shown anti-KLH IgG, as in most cases we measured the antibody from secondary immune response.

Estimation of HBV-related antigens and serum IL-2

HBsAg and HBeAg in sera were estimated by the reverse passive haemoagglutination method and by the ELISA method using a commercial kit (Mycell, Tokyo Institute of Immunology, Tokyo, Japan; ELISA kit, Mizuho Medi, Shionogi, Osaka, Japan). Serum IL-2 levels were estimated by an ELISA method utilizing the commercial ELISA kit (IL-2 estimation kit, Genzyme, Cambridge, MA).

In situ *hybridization of HBV DNA in liver*

In situ hybridization for HBV DNA was done using the *in situ* hybridization kit utilizing non-radioactive digoxigenin labelled DNA probe (Kreatech Biochemistry, Amsterdam, the Netherlands). In short, paraffin-embedded liver specimens were cut in 4–6 μm sections, heat fixed at 56° for 16 hr, deparaffinized in xylene, digested in pepsin in 0·01 N HCl (1 : 80), prehybridized in prehybridization mixture and then a digoxigenin labelled HBV DNA probe was applied to the specimen, and denatured at 90° and finally the staining for positive samples was detected by incubating with alkaline phosphatase labelled anti-digoxigenin, and subsequently with nitroblue tetrazolium and 5-bromo-4-chloro-indolyl-phosphate. Positive signals for HBV DNA were detected as black granules in the liver. Controls were stained with a negative control probe supplied with the kit, and we also stained liver specimens from patients with hepatitis C virus for HBV DNA. No black granules resembling HBV DNA were detected in controls (data not shown).

Detection of HBV DNA by PCR in transgenic mice

Total DNA was isolated from sera by an already published method with some modifications.[21] In short, sera were collected cautiously from mice and were digested with proteinase K for two hours at 56° and total DNA was extracted by a standard protocol with phenol/chloroform/isoamylalcohol (25 : 24 : 1), and precipitated with ethanol. The pellets were resuspended in TE-buffer (10 mM Tris HCl, pH 8·0, 1 mM ethylenediamine tetraacetic acid (EDTA), pH 8·0) and either used immediately or frozen at −20°. The amount of total DNA was measured and suspended at 50·0 ng, 5·0 ng, 0·5 ng, 0·05 ng, 0·005 ng, 0·0005 ng, and 0·00005 ng/μl by diluting the samples serially with TE buffer. HBV DNA sequence in the serum sample was amplified by PCR with synthetic oligonucleotides spanning X/precore/core region of HBV DNA. For 1·0 μl of total DNA, we used 25 pmol sense primer (nt 1725–1747, AAGGACTGGGAGGAGTTGGGGGA), 25 pmol anti-sense primer (nt 1966–1946, GAGAGAAAAAACGGAAGACTG), 2·5 mmol dATP, dCTP, dGTP, and dTTP each, 5 μl 10 × PCR buffer (100 mM Tris HCl, pH 8·3, 500 mM KCl) and 2.5 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) in 46 μl total volume. After a 5 min denaturing step at 94°, we ran 35 cycles, each lasting 60 seconds at 94°, 90 seconds at 50°, and 150 seconds at 72°. After the last cycle, there was an additional 7 minutes at 72° to complete elongation. The PCR product was electrophoretically visualized in an ethidium-bromide stained 3% agarose gel. The presence of a band 242 bp long was considered a positive result.

Cytofluorometry

This was done according to a previously described procedure with slight modifications.[1,18] Briefly, cells were stained with an optimum dilution of FITC-conjugated specific antibody directly or stained with an optimum dilution of primary antibody followed by FITC-conjugated antibody, washed and suspended in PBS containing 1% bovine serum albumin and 0·02% sodium azide and finally $FITC^+$ cells were counted in a flow cytometer (EPICS profile, Coulter Corp, Hialeah, FL). Cells unstained and stained with FITC-conjugated second antibody alone served as a control.

Immunostaining

Tissues from spleen were snapped frozen in M-1 embedding matrix. 5 μm cryostat sections were fixed in acetone and stained with primary antibodies containing biotinated anti-mouse Ia and anti-mouse CD11c (N418) simultaneously for one hour at room temperature in humid atmosphere. FITC-conjugated streptoavidin and rhodanine-conjugated anti-hamster antibodies were used as second antibodies for staining Ia antigen and anti-mouse CD11c (N418), respectively. Fluorescence images were taken using an Axioskop microscope equipped with the appropriate excitation–emission filters for rhodanine or fluorescein (Zeiss, Oberkochen, Germany). Photographs were taken at the same site of the specimen using filter for fluorescein and rhodanine simultaneously to characterize cells positive for both N418 and Ia antigen. The individual images of fluorescein and rhodanine were green and red, respectively. The cells positive for both antigens stained yellow and represented the expression of Ia antigen on dendritic cells.

Table 1. Defects of antigen-presenting activity of dendritic cells from HBV-transgenic mice

Cells prepared from*			
Whole spleen (5×10^6)	T/B cells (5×10^6)	Dendritic cells (10^5)	Anti-KLH IgG Ab† DTgU/200 μl
A. Culture containing autologous cells			
Normal mice			8·7 ± 1·3
Transgenic mice			0·6 ± 0·1
	Normal mice	Normal mice	9·8 ± 2·6
	Transgenic mice	Transgenic mice	0·7 ± 0·1
B. Co-culture experiments			
	Transgenic mice	Normal mice	5·0 ± 0·9
	Normal mice	Transgenic mice	1·2 ± 0·3

* Normal mice and HBV-transgenic mice, were immunized i.p. twice with 5 μg of KLH in PBS at an interval of two weeks and used for experiments five months after last immunization. T/B cells; G-10-non-adherent cells, dendritic cells were obtained from spleen adherent cells by detaching with EDTA followed by depleting Fc-receptor positive cells with EA-rosette formation.[1] Percentage of anti-CD11c positive cells was estimated to be 75–90% and was added to the culture at 10^5/well. Numbers in parentheses indicate the numbers of cells/ml.

† Culture supernatant from each well were collected 10 days after the onset of culture of the cells indicated in the presence of 0·5 μg of KLH/ml. Anti-KLH IgG antibody in the culture supernatant were determined by ELISA method. Levels of anti-KLH IgG were expressed as DTgU/200 μl of supernatant. Each value represents the mean ± SD of the duplicate assay of triplicate cultures of five experiments. Values more than 1·0 were considered as positive.

RESULTS

Defect of antigen-presenting capacity of dendritic cells in HBV-transgenic mice

Similar to our previous experiments,[1] normal mice responded by producing anti-KLH antibody of both IgM and IgG classes in a dose-dependent manner after being immunized with graded doses of KLH in PBS. It was found that two injections with 5 μg of KLH in PBS was sufficient for anti-KLH antibody production by all normal mice *in vivo*. On the other hand, most of the transgenic mice could not produce anti-KLH antibody in sera being primed with 5 μg of KLH (data not shown). When the whole spleen cells from normal mice injected with 5 μg of KLH were cultured for 10 days in the presence of exogenous KLH, anti-KLH antibody was detected in all culture supernatants, even one year after secondary immunization, whereas transgenic mice primed twice with 5 μg of KLH could not produce the KLH-specific antibody *in vitro*, as shown in Table 1 (part A). Similar findings have been seen when autologous T/B cells and dendritic cells were cultured together. On the other hand, in the co-culture experiments, T/B cells from the KLH-primed transgenic mice produced anti-KLH antibody when cultured with dendritic cells from normal mice, but not with the same from the transgenic mice (Table 1, part B). Thus our findings on the defect of transgenic mice in humoral immune response to KLH and the role of dendritic cells in this regard was reconfirmed.[1]

Upregulation of Ia antigen on dendritic cells from transgenic mice by IFN-γ

The present experiments were designed to see whether the Ia antigen expression on dendritic cells from transgenic mice could be upregulated by IFN-γ and in case of an upregulation whether this would be reflected in better functional capability or not. We injected transgenic mice with IFN-γ, daily for six consecutive days and the mice were sacrificed and spleen were snapped frozen and the cryostat sections were stained for Ia antigen and anti-CD11a (N418), a dendritic cell-specific marker[17] to see the staining pattern of Ia antigen on splenic dendritic cells. Cells positive for both antigens appeared yellow and we found an increase in the intensity and frequency of double positive cells in the spleen from transgenic mice after treatment with IFN-γ (Fig. 1a) compared with spleen from transgenic mice receiving no treatment (Fig. 1b). Thus evidence suggesting the upregulation of Ia antigen on splenic dendritic cells in transgenic mice due to treatment with IFN-γ was found.

Next, we isolated dendritic cells from transgenic mice before and after injecting IFN-γ and the expression of Ia antigen were checked by flow cytometry. The mean fluorescence intensity of Ia antigen of dendritic cells from transgenic mice without and with treatment with IFN-γ was 103·4 ± 36·8 and 309·2 ± 65·8, respectively (mean ± SD of five experiments). Fig. 2 shows the representative staining pattern of Ia antigen on dendritic cells isolated from transgenic mice with or without treatment with IFN-γ. A significant increase of Ia antigen expression in fluorescence intensity on transgenic mice-derived dendritic cells due to injection with IFN-γ was seen.

Role of IFN-γ for anti-KLH antibody production *in vivo*

Next, it was seen whether IFN-γ has any role in specific antibody production to KLH in transgenic mice. Table 2 shows

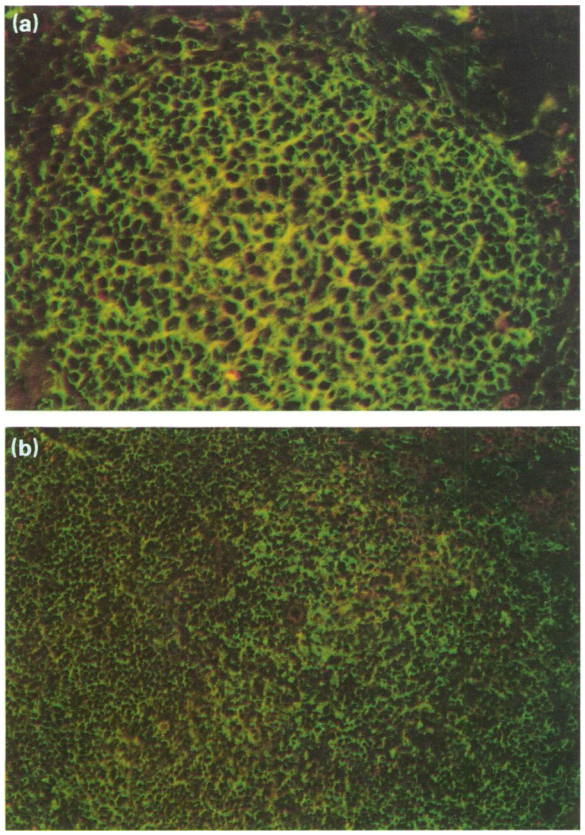

Figure 1. Expression of Ia antigen on dendritic cells from transgenic mice with (a) and without treatment (b) with IFN-γ. Transgenic mice were injected with IFN-γ for six days and sacrificed. The spleen sections were treated with anti-Ia antigen and anti-mouse CD11c (N418), a dendritic cell-specific marker[17] and subsequently stained with FITC- and rhodanine-conjugated second antibodies. Cells positive for both Ia and N418 were stained yellow and represented the Ia antigen expression on dendritic cells. An increase in the intensity and number of cells positive for both the antigens were seen in spleen treated with IFN-γ (magnification factor: Fig. 1a × 320; Fig. 1b × 160).

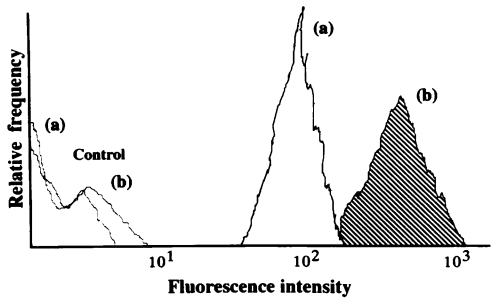

Figure 2. Increased expression of Ia antigen on dendritic cells from HBV-transgenic mice after treatment with IFN-γ. Spleen dendritic cells were isolated from transgenic mice without (a) and after treatment (b) with IFN-γ for six days and stained with FITC-conjugated anti-Ia mAb in a direct method. Controls indicate fluorescence intensity of dendritic cells stained with a subclass matched-FITC-conjugated second antibody alone. Each of a representative staining pattern was shown here. In most cases more than 90% of each population was stained with hamster anti-mouse CD11c mAb (N418) and FITC-conjugated goat anti-hamster antibody (data not shown).

the production of anti-KLH antibody by transgenic mice injected with KLH and IFN-γ or PBS. Transgenic mice injected with two injections of 5 μg of KLH or KLH and PBS at an interval of two weeks could not produce anti-KLH antibody in sera, but when transgenic mice were injected with KLH together with IFN-γ significant amounts of anti-KLH antibody was produced in sera. Although these findings have shown a role of IFN-γ in upregulation of the expression of Ia antigen on dendritic cells from transgenic mice and in overcoming the immune response defect of transgenic mice in specific antibody production to KLH *in vivo*, we did a series of experiments *in vitro* to characterize the cell/s responsible for these, especially to clarify the role of dendritic cells in this regard.

Improved antigen-presenting capacity of dendritic cells treated with IFN-γ

In order to achieve this, we established a mice model for *in vitro* specific antibody production by immunizing normal mice with two injections of 5 μg of KLH in PBS, i.p. The kinetics of anti-KLH antibody production in these mice *in vitro* revealed that spontaneous production (without the addition of exogenous KLH in culture) of KLH-specific antibody was seen up to four months after last immunization. However, spleen cells from KLH-primed normal mice produced anti-KLH antibody five months after last immunization when an adequate amount of exogenous KLH were added to the culture. Thus immunizing normal mice with two doses of KLH (5 μg/mouse) and observing them for five months after last immunization, an animal model for *in vitro* specific antibody production was established in which the role of T and B lymphocytes, as well as APC could be studied. The cultures containing different cell populations as indicated in Table 3 were done for 10 days in the presence of exogenous KLH and anti-KLH antibody in the culture supernatant was estimated by ELISA. It was found that in most cases, dendritic cells from PBS-treated transgenic mice could not provide adequate help for anti-KLH antibody production to T/B cells from KLH-primed normal mice, but the normal mice-derived dendritic cells and dendritic cells from transgenic mice treated with IFN-γ expressing an increased level of Ia antigen provided substantial help for anti-KLH

Table 2. Role of IFN-γ in anti-KLH antibody production by transgenic mice *in vivo*

Mice	Treatment with	Anti-KLH IgG antibody DTgU/μl
Transgenic mice	no treatment	0·7 ± 0·5
Transgenic mice	PBS	0·8 ± 0·5
Transgenic mice	IFN-γ	5·3 ± 0·8

Transgenic mice were primed twice with 5 μg of KLH in PBS and injected with PBS or IFN-γ for six days starting from the first day of injection with KLH or kept without injecting anything as described in Materials and Methods. Anti-KLH antibodies in sera were estimated by an ELISA, two weeks after last immunization. Anti-KLH IgG antibody has been shown as DTgU/μl and values 1·0 or more were considered as positive. Mean and SD of five experiments have been presented.

Table 3. Improved antigen-presenting cell activity of dendritic cells from γ-IFN-treated transgenic mice

T/B cells (5×10^6)	Dendritic cells (10^5)	Anti-KLH IgG antibody DTgU/200 μl
Normal mice	Transgenic mice–untreated	0·9 ± 0·5
Normal mice	Transgenic mice–PBS	1·2 ± 0·7
Normal mice	Transgenic mice–γ-IFN	5·7 ± 1·2
Normal mice	Normal mice–PBS	9·5 ± 1·6

Normal mice were primed with 5 μg of KLH in PBS, twice at an interval of two weeks and T/B cells were isolated as G-10 non-adherent cells, five months after last immunization. Unprimed transgenic mice were injected with PBS, γ-IFN or left untreated. Unprimed normal mice were treated with PBS only. Dendritic cells from these unprimed mice were isolated and the fluorescence intensity of Ia antigen was checked by flow cytometry (Fig. 1a). The different cell populations were cultured for 10 days and anti-KLH IgG antibodies were estimated in the supernatant, which has been tabulated as DTgU/200 μl, 1·0 or more of which were considered as positive. Mean and SD of five experiments have been tabulated.

antibody production to KLH-primed normal mice-derived T/B cells (Table 3). Thus it became evident that the upregulation of Ia antigen on dendritic cells from transgenic mice by IFN-γ was reflected in better APC function for specific humoral immune response to KLH.

IFN-γ treatment induces IL-2 production in transgenic mice

Table 4 shows the level of IL-2 in sera in normal and transgenic mice treated with KLH alone or KLH with IFN-γ. Both normal and transgenic mice did not show detectable amounts of IL-2 in resting state. When normal mice were injected with KLH, there was production of a significant amount of IL-2 in sera, but transgenic mice being injected with KLH did not show any detectable amount of IL-2 in sera. On the other hand, injecting transgenic mice with KLH together with IFN-γ resulted in the production of detectable amounts of IL-2 in sera.

Table 4. Induction of IL-2 production by IFN-γ in transgenic mice

Sera collected from	Serum IL-2 level pg/ml
Normal mice	undetectable
Normal mice injected with 2 doses of KLH	20·1 ± 4·6
Transgenic mice	undetectable
Transgenic mice injected with 2 doses of KLH	undetectable
Transgenic mice injected with IFN-γ	19·2 ± 1·8
Transgenic mice injected with IFN-γ and KLH	21·4 ± 2·2

Normal and transgenic mice were either injected with two injections of KLH or treated with two injections of KLH and six daily injections of IFN-γ or received no treatment as described in Materials and Methods. Sera were collected 24 hours after last injection with KLH or IFN-γ. IL-2 levels in sera were estimated by an ELISA method using the IL-2 estimation kit. Mean ± SD of five experiments are shown here.

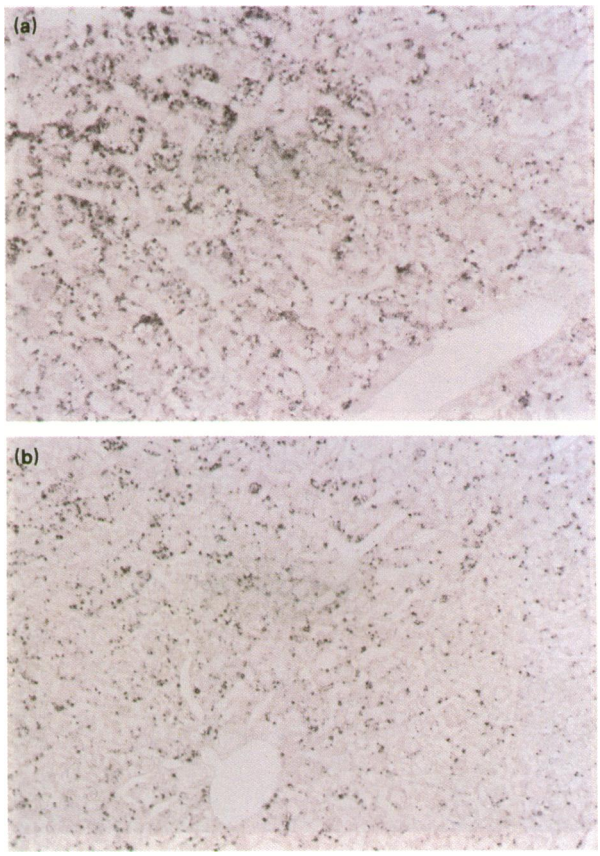

Figure 3. *In situ* hybridization for HBV DNA in liver from transgenic mice was done before (a) and after (b) treatment with IFN-γ as described in Materials and Methods. HBV DNA were seen as black granules in the liver using the deoxigenin labelled HBV DNA probe. A decrease in the signals for HBV DNA has been found in mice being treated with IFN-γ compared with the same before treatment (magnification factor ×320).

Anti-viral capability of IFN-γ in HBV transgenic mice

The titres of HBsAg or HBeAg in sera in transgenic mice did not change significantly after six days treatment with IFN-γ. *In situ* hybridization for HBV DNA showed that there was a decrease in the signal for HBV DNA in liver after treatment with IFN-γ compared with the same before treatment (Fig. 3). In untreated transgenic mice, there were some aggregations of black granules in some parts of the specimen, which was not seen in transgenic mice after treatment with IFN-γ (Fig. 3). In order to further study the role of IFN-γ treatment on the level of expression of HBV DNA in sera, a semi-quantitative estimation of HBV DNA in sera from transgenic mice was done before and after treating with IFN-γ. Total DNA from sera of both untreated transgenic mice and transgenic mice treated with IFN-γ were extracted and the amount of total DNA was measured and adjusted to different concentrations ranging from 50 ng/μl to 0·00005 ng/μl by diluting 10-fold serially. HBV DNA sequence in the sera were amplified by PCR using HBV-specific probes and the presence of HBV DNA was seen in different concentrations of total serum DNA extracted from untreated transgenic mice or transgenic mice treated with IFN-γ. Fig. 4 shows the data from eight HBV-transgenic mice

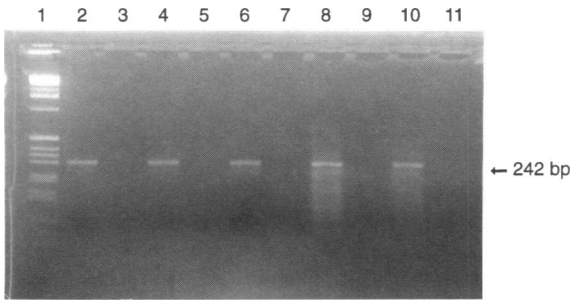

Figure 4. HBV DNA sequence in sera after amplifying by PCR in HBV-transgenic mice before and after treatment with IFN-γ. The details of PCR for HBV DNA have been described in Materials and Methods. Total DNA was isolated from HBV transgenic mice before and after treatment with IFN-γ. The amount of DNA was measured and adjusted to different concentrations ranging between 50 ng/μl and 0·00005 ng/μl. Lane 1 represents molecular marker and a band 242 bp in length indicates the presence of HBV DNA. 0·005 ng of total DNA from untreated HBV transgenic mice were amplified by PCR and were allowed to run in lanes 2, 4, 6 and 8. Total DNA of 0·5 ng from sera of transgenic mice treated with IFN-γ were amplified by PCR and allowed to run on lanes 3, 5, 7 and 9. Known positive and negative controls were allowed to run in lanes 10 and 11, respectively.

(four receiving no treatment and four receiving IFN-γ for six days) regarding the presence of HBV DNA in sera. All four HBV-transgenic mice receiving no treatment showed specific bands indicating the presence of HBV DNA when 0·005 ng of DNA from sera was amplified by PCR. On the other hand, we could not detect any HBV DNA sequence in sera using 100 times more DNA (0·5 ng) from HBV-transgenic mice sera treated with IFN-γ for six days and amplified by PCR (Fig. 4). Thus, we had evidence of a lower level of HBV DNA in sera in transgenic mice treated with IFN-γ for six days compared with transgenic mice receiving no treatment.

DISCUSSION

The inability of HBV-infected individuals to elicit an adequate immune response to HBV is considered to be responsible for the acquisition of chronic HBV carriership and this has been attributed to immunological tolerance to HBV-related antigens, although HBV carriers have shown evidence of defects in immune responses not only to HBV-related antigens but also to other general immunological parameters.[2–5] Transgenic mice produced by genetic engineering and expressing HBsAg, HBeAg and HBV DNA in sera and relevant mRNAs in different tissues have served as an animal model of HBV carrier state, and we have reported that transgenic mice are not only unresponsive to HBV-related antigen, but they responded poorly to KLH, a HBV-unrelated, T-cell dependent antigen.[1] We have further characterized the cells responsible for this and have reported that the antigen-presenting dendritic cells from transgenic mice expressed significantly lower levels of Ia antigen compared with normal mice and had low T-cell stimulatory capacity in allogeneic mixed leucocyte reaction and oxidative mitogenesis model. It was also shown that the immune response defects of transgenic mice could be overcome *in vitro* by using dendritic cells from normal mice, expressing considerably higher levels of Ia antigen.[1] Thus, we have speculated that the low expression of Ia antigen on transgenic mice-derived dendritic cells would be one of the critical factors for the immune response defect in transgenic mice.

In order to justify our hypothesis, we have undertaken the present experiments to upregulate the expression of Ia antigen on dendritic cells from transgenic mice and to see its functional significance. IFN-γ is an immunomodulatory agent and upregulates the expression of Ia antigen both in humans and mice.[11–13,22,23] Several other divergent roles of IFN-γ in immune response, such as restoration of antigen-presenting capacity,[24] augmentation of antibody response,[25] and abrogation of immune unresponsiveness[26] have been reported. Particularly, it has been reported that in HBV infection, IFN-γ has been shown to down-regulate HBV replication both *in situ*[16] and in primary hepatocyte cultures.[15]

Treating transgenic mice with IFN-γ and staining the spleen for Ia antigen and N418, a dendritic cell-specific marker, we got indirect evidence of a possible role of IFN-γ on the expression of Ia antigen on dendritic cells from transgenic mice. In order to have more direct evidence, we isolated dendritic cells from spleens of transgenic mice and Ia antigen expression on dendritic cells was monitored by immunofluorescence. We found that there was a significant increase in the expression of Ia antigen in fluorescence intensity on dendritic cells from transgenic mice after treating with IFN-γ compared with dendritic cells isolated from transgenic mice treated with PBS only (Fig. 1). The IFN-γ treated transgenic mice-derived dendritic cells expressing higher level of Ia antigen acted as an efficient APC for providing help to the T/B cells from KLH-primed normal mice to produce anti-KLH antibody, but the PBS-treated dendritic cells from transgenic mice or untreated dendritic cells from transgenic mice was almost as inefficient as APC for specific antibody production *in vitro* (Table 3).

The exact mechanism underlying the improved functional capacity of dendritic cells treated with IFN-γ is not exactly known. Guidotti *et al.*, using another line of HBV–transgenic mice have shown that HBV gene expression is inhibited in transgemic mice by a non cytolytic mechanism and is mediated by IFN-γ, and TNF-α.[27] We have checked the levels of HBV DNA in transgenic mice before and after treating with IFN-γ. Although, we could not detect any significant change in the titre of HBsAg and HBeAg in sera, *in situ* hybridization for HBV–DNA in liver tissue have shown a decreased intensity for the signal of HBV DNA after treatment with IFN-γ (Fig. 3). In addition to this, semiquantitative measurement of HBV DNA by PCR has shown that the HBV DNA levels were found to be decreased considerably in sera from transgenic mice after treatment with IFN-γ (Fig. 4). We treated transgenic mice for only six days with IFN-γ, and this may not be sufficient to exhibit the reduced level of HBV-related antigens, but the influence of reduced viral replication on increased Ia expression on dendritic cells could be one of the main factors to overcome the immune response defect.

In order to explore the immunomodulatory role of IFN-γ in transgenic mice, we estimated the serum IL-2 levels in mice before and after treating with IFN-γ. Both normal and transgenic mice did not show any detectable levels of IL-2 in sera at the resting state. Immunizing normal mice with KLH in PBS showed significant levels of IL-2 in sera, but IL-2 was not detectable in sera in transgenic mice after immunizing with KLH. On the other hand, when transgenic mice were

immunized with KLH and treated with IFN-γ for six days, detectable levels of IL-2 were detected in sera of most of the transgenic mice. Recently, it has been shown that IL-2 and IL-4 are needed for the development of IL-4 producing cells[28] and it has been reported that dendritic cells are responsible for the initial priming of T cells and able to cause optimum expansion of antigen-specific T cells for the production of lymphokines characteristic of both Th1 and Th2 subsets, i.e. IFN-γ and IL-4.[29] We postulate that increased expression of Ia antigen on dendritic cells due to treatment with IFN-γ resulted in better antigen presentation, which in turn resulted in the production of IL-2 by activated T cells and contributed to the ablation of immune response defect of transgenic mice to KLH.

These experiments have shown that by modulating the expression of Ia antigen on dendritic cells with IFN-γ, the immune response defect can be overcome, although the status of different costimulatory molecules in transgenic mice remains to be clarified in the future.

It has been reported that the non-responders to HBV vaccination have a very specific failure in antigen presentation,[30] although the nature of the failure has not been characterized fully. Using HBV-unrelated antigens, we have shown that tolerance to HBsAg is not the hall-mark of immunological abnormality in HBV carriers.[1] Rather, the defect of antigen-presenting capacity of dendritic cells in HBV infection[1] and defect of antigen-presentation in non-responder of HBV vaccination[30] are also critical factors in this regard. The present communication has also shown a possible way to circumvent this defect, which has a tremendous clinical importance for therapy of chronic human HBV carriers and for the non-responders to HBV vaccine recipients.

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