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Characterization of T-cell tolerance to hepatitis B virus (HBV) antigen in transgenic mice

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SUMMARY

We made three different lines of hepatitis B virus (HBV) transgenic mice which express different amounts of hepatitis B e antigen (HBeAg) and/or hepatitis B core antigen (HBcAg) to analyse the cellular mechanisms of HBcAg specific T-cell tolerance. BS10 (official designation, 1.2HB-BS10) transgenic mice, which contain the whole HBV genome, express relatively high amounts of HBeAg in the serum and HBcAg in the liver. SPC mice, which contain hepatitis B virus core and precore gene, express small amounts of HBeAg in the serum but not HBcAg in the liver. SC33 mice, which contain only hepatitis B core gene, do not express HBeAg in the serum but express HBcAg in the liver. BS10 mice showed a very low anti-HBc antibody response after primary and secondary immunizations with recombinant HBcAg compared to transgenic host C57BL/6 (B6) mice. SPC mice showed an almost equal level of anti-HBc antibody response compared to B6 mice. SC33 mice contained anti-HBc antibody even before immunization and showed high titres of anti-HBc antibody response after immunization with HBcAg. Analysis of cellular site(s) of low responsiveness of BS10 mice revealed that proliferating and helper T cells are specifically tolerant to HBcAg. B cells and antigen-presenting cells in BS10 mice were not defective. SC33, SPC and BS10 mice differ a little in their developmental expression of HBc/HBeAg. Our results suggest critical roles of the nature (circulating versus non-circulating) as well as the time of expression of self-antigens in T-cell tolerance.

INTRODUCTION

The immune system acquired the ability to respond to and battle against myriads of environmental foreign antigens by preparing diverse lymphocyte clones during development. In spite of enormous diversity created by random assortment of VDJ or VJ segments in the T-cell receptor genes (reviewed in ref. 1), clones which react against self-component are deleted and/or inactivated to avoid anti-self immune responses.[2] This is a state called self-tolerance. The mechanism of self-tolerance is one of the central questions in modern immunology and is under intensive investigation in many laboratories. A recent technological advance, to create transgenic mice by injecting foreign genes into fertilized eggs, has offered one of the ideal experimental strategies to investigate the mechanism of self-tolerance against circulating and/or tissue-specific antigens since such introduced gene products exist from the very early developmental stage. These studies provided supportive evidence for central clonal deletion[3,4] as well as peripheral inactivation[5,6] of self-reactive T-lymphocyte clones.

Recently, Araki *et al.*[7,8] made C57BL/6 transgenic mice which contain the whole hepatitis B virus genome to analyse the mechanism of the pathogenesis of immune-mediated viral hepatitis. Preliminary experiments revealed that this strain of HBV transgenic mice showed reduced T-cell proliferative response to hepatitis B core antigen (HBcAg), suggesting the acquisition of T-cell tolerance to HBcAg. This line of HBV transgenic mice seems to offer a unique situation for the effects of circulating versus non-circulating self-antigens on the T-cell tolerance induction since it expresses both circulating hepatitis B e (HBe) and non-circulating hepatitis B core (HBc) transgene products. Although HBe and HBc antigens are serologically distinct,[9,10] the primary amino acid sequences show significant identity and are highly cross-reactive at the helper T-cell level[11] and the dominant T-cell sites in B10 ($H\text{-}2^b$) mice were reported to reside in p120–140 peptide which is shared between HBe and HBcAg.[12] To analyse the effects of circulating and non-circulating antigens on tolerance induction at the T-cell level, we made two additional lines of HBV transgenic mice (SPC and SC33) with different transgene constructs. SPC transgenic mice, which contain HBV core and precore gene, express circulating hepatitis B e antigen (HBeAg) in the serum but little HBcAg in the liver. SC33 transgenic mice, which contain only the HBc gene but no precore gene, do not express circulating HBeAg but contain HBcAg in the liver. In these two lines of transgenic mice, we used serum amyloid P (SAP) component gene promoter to

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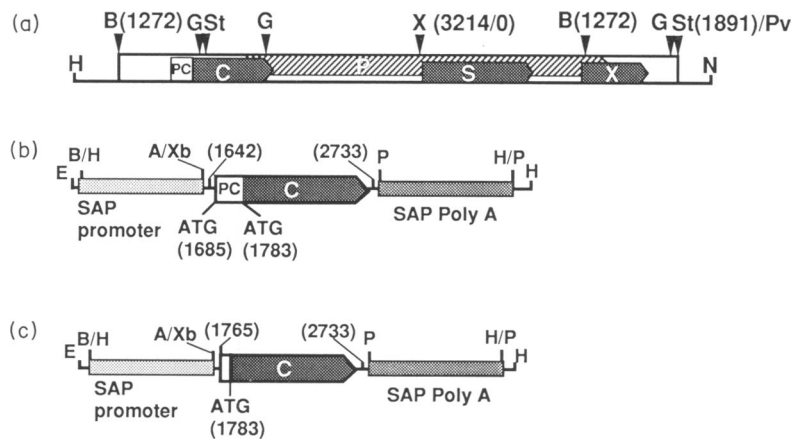

Figure 1. DNA constructs used for production of transgenic mice. (a) The HBV genome was derived from the plasmid pBRHBadr4.[15] The 1.2HB-BS10 (BS10) DNA[7] carries one full length of HBV (*Bam*HI fragment) plus a 619 base pair (bp) overlapping region (*Bam*HI/*Stu*I fragment). The coding region of HBsAg, HBcAg, HBVx, precore and polymerase are displayed with hatched arrows marked S, C, and X, an open box marked PC, and a striped arrow marked P, respectively. (b, c) The SAP promoter (*Hin*dIII/*Avr*II fragment, ~0·6 kb) and the SAP poly A signal (*Pst*I/*Hin*dIII fragment 0·9 kb) were derived from a genomic DNA clone of the human SAP gene.[16] The *Avr*II site and the *Pst*I site are located 14 bp upstream from the start codon and 40 bp upstream from the poly A signal, respectively. The SPC (b) and SC33 (c) were constructed by inserting the HBV DNA fragments (1642-2733) and (1783-2733) between the SAP promoter and poly A signal, respectively. The former contains the complete precore (PC) plus core open reading frame (ORF) (b), and the latter contains only core ORF (c). A, *Avr*II, B, *Bam*HI; E, *Eco*RI; G, *Bgl*II, H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; St, *Stu*I; X, *Xho*I; Xb, *Xba*I. The nucleotide numbers marked along the HBV DNA start at the unique *Xho*I site.

express viral gene products specifically in the liver[13,14], in order to resemble natural HBV infection in man. Although the time of developmental expression of HBc transgene products in these two lines of transgenic mice differ a little from that of BS10 mice, analysis of the T-cell response to recombinant HBcAg in these three lines of transgenic mice may suggest that circulating HBcAg induces tolerance of HBcAg reactive T cells while non-circulating HBcAg induces immunity rather than tolerance to HBcAg.

MATERIALS AND METHODS

DNA and transgenic mice

Three lines of HBV transgenic mice, BS10 (official designation, 1.2HB-BS10), SC33 and SPC were used. C57BL/6 (B6) mice (Clea Japan, Inc., Tokyo, Japan) were used as transgenic hosts. The production and characterization of 1.2HB-BS10 transgenic mice which contain the complete HBV genome DNA has been described.[7] SC33 mice were produced by microinjecting HBV core (HBc) gene ligated with serum amyloid P component (SAP) gene. SPC mice were made by microinjecting HBV core and precore gene conjugated with SAP gene. The constructs of transgenes used for microinjection are described in Fig. 1.

Quantitative analysis of HBe/HBcAg in the serum and the liver extract of transgenic mice

We used a commercially available ELISA kit (Abbot HBe EIA diagnostic kit; Dainabot, Tokyo) to measure HBe/HBcAg in diluted transgenic mouse serum and liver extract. This kit detects both HBeAg and HBcAg. HBcAg with known titre and purified recombinant HBcAg were used as a standard. To examine the developmental expression, livers from transgenic mice of various developmental stages were frozen and homogenized in extraction buffer (150 mM NaCl, 10 mM Na-K phosphate buffer pH 7·2, 0·2% Triton X-100, 1 mM phenylmethylsulphonyl fluoride) at the ratio of 0·1 g tissue/300 μl extraction buffer. The homogenates were centrifuged at 12,000 ***g*** for 10 min at 4 , and the supernatants were assayed by ELISA.

Antigens and immunization

Recombinant hepatitis B core antigen (HBcAg) was kindly provided by Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). This HBcAg produced in yeast was 100% pure by HPLC analysis.[17] Keyhole limpet haemocyanin (KLH) was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Purified protein derivatives (PPD) of BCG were purchased from Japan BCG Inc. (Tokyo, Japan). For primary immunizations, HBcAg (10 μg) was emulsified in complete Freund's adjuvant (CFA) and injected i.p. into a group of five mice. The booster immunization was performed with 10 μg HBcAg in incomplete Freud's adjuvant (IFA) 21 days after the primary immunization. Mice were bled from tail 4, 7 and 14 days after primary and booster immunizations. Anti-HBc antibody titre in the serum was assayed by ELISA.

Assay for antigen-specific T-cell proliferation

Antigen-specific T-cell proliferation was performed as described previously[18] with slight modifications. Briefly, 50 μl of HBcAg (300 μg/ml) or KLH (2 mg/ml) emulsified in CFA was injected into mice at the base of tail. Seven days later, draining inguinal and para-aortic lymph nodes were removed and single-cell suspensions made in Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY). After washing with HBSS once, lymph node (LN) cells were suspended in RPMI-1640 (Gibco) supplemented with 10% horse serum (Gibco), 2-mercaptoethanol (5×10^{-5} M), HEPES (10 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) (HOS medium). 6×10^5 cells were cultured in 0·2 ml horse serum (HOS) medium with varying concentrations of antigen in a 96-well microtitre plate (Falcon

no. 3075, Becton-Dickinson, Lincoln, NJ) for 5 days. Triplicate cultures were pulsed for the final 16 hr with 1 μCi of [^{3}H]TdR (ICN Biomedicals, Costa Mesa, CA). Proliferative responses were measured by the uptake of [^{3}H]TdR and expressed as a mean c.p.m. $\pm$ SD.

Cell separation

Purified B cells were obtained from spleen cells by treatment with monoclonal anti-Thy-1.2 antibody (F7D5, obtained from Serotec Ltd, Bicester, Oxon, U.K.) and rabbit complement (Cedarlane Laboratories, Ltd, Ontario, Canada). Purified T cells were obtained from inguinal and para-aortic LN cells of mice which were immunized with HBcAg at the base of the tail 7 days previously. Cells were incubated with appropriate amounts of rat anti-mouse IgM monoclonal antibody (Bet-2, obtained from ATCC) on ice. After 30 min, cells were washed three times with HBSS and incubated with sheep anti-rat IgG coated immunomagnetic beads (Dynal Inc., NY) at a ratio of 1 cell to 40 beads with constant rotation at 4 for 1 hr. Cells nonadherent to beads were separated by magnet, washed three times and used for experiments. Purity was checked by flow cytometric analysis using FACScan (Becton-Dickinson Inc.) and each lymphocyte population was more than 97% pure by staining with specific monoclonal antibody.

In vitro *culture for HBc antibody production*

HBcAg specific T-cell lines were produced and assayed according to the method as described previously[19] with slight modifications. Purified LN T cells were prepared as described above from mice immunized with HBcAg in CFA at the base of the tail 7 days previously. For *in vitro* antibody production, 1×10^7 spleen cells or splenic B cells were cultured with 3×10^3 HBcAg specific T-cell lines (for assay of B-cell tolerance) or 1×10^6 purified LN T cells from immunized mice (for assay of helper T cell activity) in the presence of varying amounts of HBcAg in 2 ml foetal calf serum (FCS) culture medium in a 24-well culture plate (Falcon no. 3047). After 2 days, cells were harvested, washed three times with HBSS to remove antigen, resuspended in 1 ml culture medium and incubated in a 24-well culture plate for 5 days. Supernatants from triplicate cultures were harvested and assayed for anti-HBc antibody by ELISA.

ELISA assay for anti-HBc antibody

A 96-well plate (Falcon no. 3915) was coated with HBcAg (10 μg/ml) for 1 hr at room temperature. The wells were blocked with 0·1% bovine serum albumin (BSA) in phosphate-buffered serum (PBS), pH 7·2. Fifty microlitres of culture supernatants or appropriately diluted sera were added to each well and incubated for 1 hr at room temperature. Unbound materials were washed with PBS containing 0·1% Tween 20. Plates were reacted with alkaline-phosphatase conjugated goat anti-mouse IgG + IgM (H + L) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD), followed by the reaction with *p*-nitrophenyl phosphate solution. The amount of anti-HBc antibody bound was measured by colorimetric analysis using Titertek Multiscan spectrometer (EFLAB, Helsinki, Finland) at OD_{405}. The results were converted to arbitrary units by comparison with standard anti-HBc serum.

RESULTS

Expression of HBe/HBc antigen in the serum and liver in transgenic mice

The expression of HBe/HBc antigen in three different lines of adult transgenic mice was examined. As shown in Fig. 2, BS10 transgenic mice contain relatively high amounts of HBeAg in the serum and HBcAg in the liver extracts. SPC transgenic mice contain small amounts (about 45 ng/ml, data not shown) of HBeAg in the serum but no detectable HBcAg in the liver extracts whereas SC33 transgenic mice contain HBcAg in the liver extracts but no HBeAg in the serum. As shown in Fig. 2, analysis of the developmental expression of HBcAg revealed that HBeAg was already detected in sera from BS10 mice from 1 day after birth and reached the adult level at 2–3 weeks of age. The HBcAg in BS10 liver extract was detected 2 days before birth and reached adult level at about 10 days after birth. The HBcAg in SC33 liver extracts was detected 2 days after birth and reached the adult level at 1 week. The amount of adult SC33 mouse liver HBcAg was about 10 times less than that of BS10 mouse.

In vivo antibody production of transgenic mice

To examine the ability of transgenic mice to produce anti-HBc antibody upon immunization with HBcAg, a group of five mice was immunized i.p. with 10 μg of HBcAg in CFA. Three weeks later, mice were boosted with 10 μg of HBcAg in IFA. They were bled 4, 7 and 14 days after the primary and the booster immunization. Sera were pooled and anti-HBc antibody titre assayed by ELISA. As shown in Fig. 3, B6, SC33 and SPC mice showed good antibody production in response to primary and secondary immunizations. BS10 mice, however, showed poor antibody responses even after the booster immunization. This indicates that BS10 mice are tolerant to HBcAg. SC33 mice contain anti-HBc antibody in the serum even before immunization. This indicates that HBc transgene products, although not secreted in the circulation, sensitized SC33 mice *in vivo* (see Discussion).

HBc-specific antibody production by B cells from transgenic mice

In order to examine whether the low responsiveness of BS10 mice to HBcAg is due to B-cell tolerance, the ability of B cells from transgenic mice to produce anti-HBc antibody was examined. HBcAg-specific T-cell lines derived from B6 mice were used as a source of helper T cells. These T-cell lines are HBcAg specific and I-A^b restricted since they respond to HBcAg but not to unrelated antigen, KLH or HTLV-I (a kind gift from Eizai Pharmaceutical Co., Tokyo, Japan) and are blocked by anti-$A\beta^b$ and anti-$A\alpha^b$ monoclonal antibodies (data not shown). Anti-Thy-1.2 antibody plus complement-treated spleen cells from B6 and transgenic mice were cultured with HBcAg-specific T-cell lines in the presence of HBcAg for 2 days. Cells were harvested and washed to remove HBcAg and recultured for 5 days. Anti-HBc-specific antibody in the culture supernatants was measured by ELISA. As shown in Fig. 4, there was no substantial difference of antibody titre between B6 and transgenic spleen B-cell cultures. This result indicates that B cells in BS10 mice are not tolerant to HBcAg. SC33 B cells produced high amounts of anti-HBc antibody by stimulation with T cells

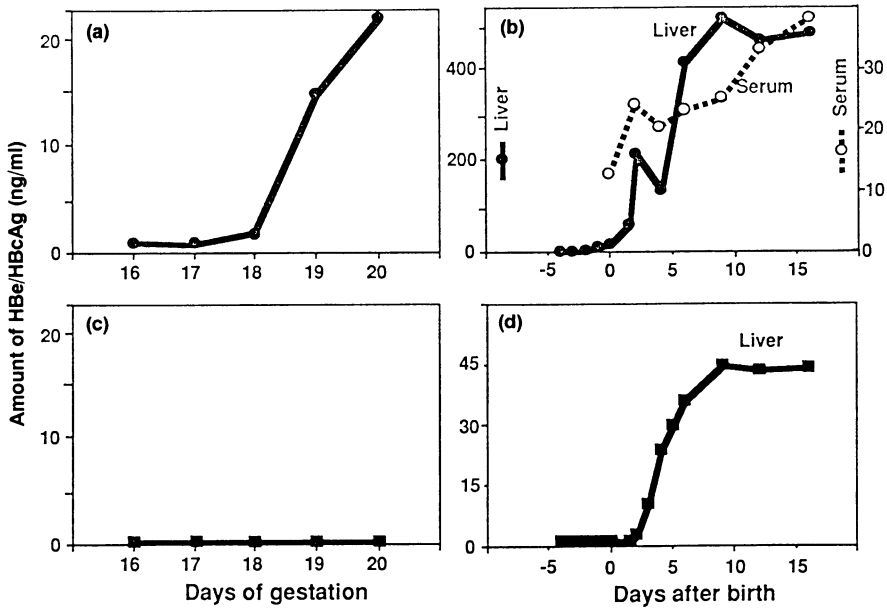

Figure 2. Developmental expression of HBc/HBeAg in BS10 and SC33 mice. (a) Developmental expression of HBcAg in prenatal mice of BS10. Fertilized eggs of BS10 mice obtained by *in vitro* fertilization were transferred into pseudopregnant foster mothers, which were killed at different days of gestation. The foetuses were examined whether they carried transgene or not by polymerase chain reaction, and the livers from transgene positive foetuses were collected. The liver extracts were prepared and assayed as described in Materials and Methods. (b) Change of HBe/HBcAg titre in the serum (○) and the liver extract (●) of BS10 mice. Each point represents a measurement of a pooled sample of three mice. (c) Developmental expression of HBcAg in prenatal mice of SC33. Measurement of HBcAg as described in (a). (d) Change of HBe/HBcAg titre in the liver extract of SC33 mice. Each value was an average of three to five mice. In both BS10 and SC33, the HBe/HBcAg levels reached that of adult mice by 16 days of age.

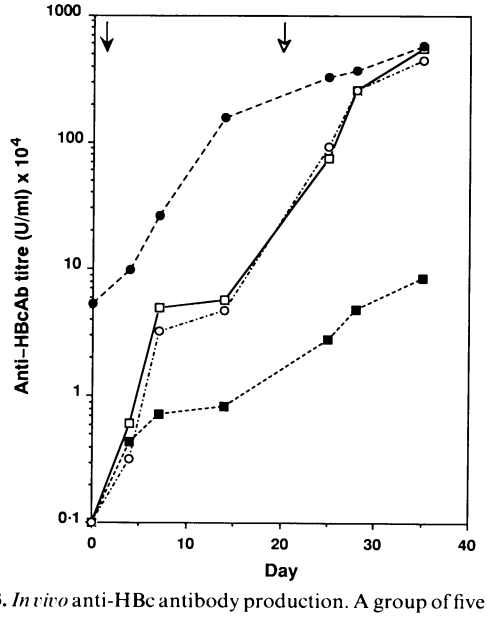

Figure 3. *In vivo* anti-HBc antibody production. A group of five mice was immunized with 10 μg HBcAg in CFA on Day 0(↓) and boosted with 10 μg HBcAg in IFA on Day 21(↓). B6 (—□—), BS10 (- - -■- - -), SCP (· - · ○ · - ·) and SC33 (- - -●- - -) mice were bled on Days 4, 7 and 14 after the primary and the secondary immunizations and pooled sera were assayed for anti-HBc antibody titre using ELISA. The antibody titre in the serum is expressed in arbitrary units by comparison with standard anti-HBc antiserum.

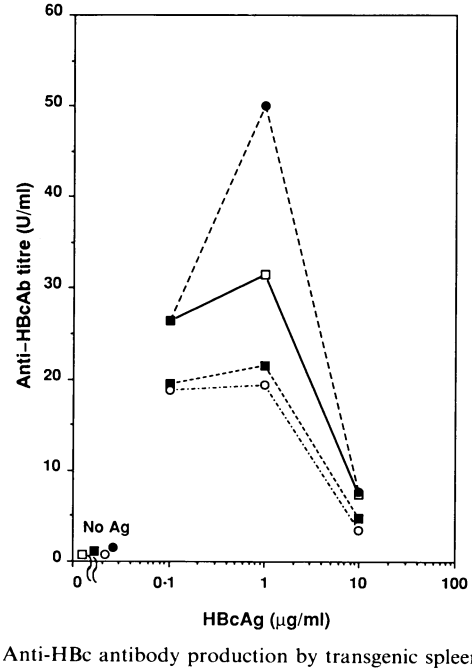

Figure 4. Anti-HBc antibody production by transgenic spleen B cells. 1×10^7 purified B cells from B6 (—□—), BS10 (- - -■- - -), SPC (· - · ○ · - ·) and SC33 (- - -●- - -) mice were cultured with 3×10^3 HBcAg specific T-cell lines and stimulated with varying concentrations of HBcAg. After culture for 2 days, cells were washed and recultured for 5 days. Anti-HBc antibody titre in the culture supernatants was assayed by ELISA.

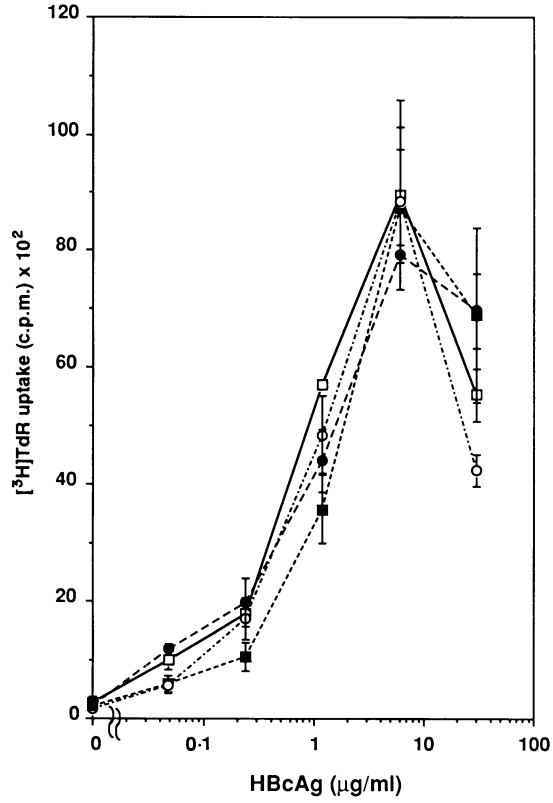

Figure 5. Antigen-presenting activity of transgenic spleen cells. 1×10^4 HBcAg specific T-cell lines were stimulated with HBcAg in the presence of 1×10^6 irradiated (33 Gy) spleen cells from B6 (—□—), BS10 (- - -■- - -), SPC (·—·○·—·) and SC33 (- -●- -) mice as antigen-presenting cells for 2 days. Proliferative responses were assayed by the uptake of [^{3}H]TdR for the final 16 hr.

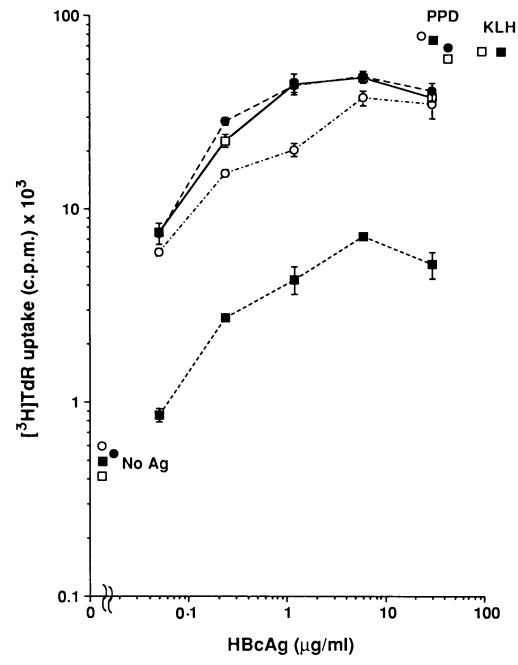

Figure 6. Antigen-specific T-cell proliferation assay of transgenic mice. A group of five mice was immunized with 10 μg of HBcAg in CFA at the base of tail. After 7 days, 6×10^5 inguinal and para-aortic LN cells from B6 (—□—), BS10 (- - -■- - -), SPC (·—·○·—·) and SC33 (- -●- -) mice were stimulated with varying concentrations of HBcAg or 50 μg/ml PPD for 5 days. Another group of five B6 (□) and BS10 (■) mice were immunized with 10 μg of KLH in CFA at the base of tail and LN cells were stimulated with 50 μg/ml KLH for 5 days. Proliferative responses were assayed by the uptake of [^{3}H]TdR for the final 16 hr.

and 1 μg/ml of HBcAg. This might reflect the existence of *in vivo* activated HBc-specific B cells in SC33 mice.

Antigen presentation by HBc transgenic mouse spleen cells

To evaluate the activity of antigen presentation of transgenic mice, 1×10^4 HBcAg-specific T-cell lines were stimulated with HBcAg in the presence of irradiated (33 Gy) spleen cells from B6, BS10, SC33 and SPC mice as antigen-presenting cells (APC). As shown in Fig. 5, T-cell lines showed almost equal proliferative responses against HBcAg plus APC from these strains of mice over a wide range of antigen concentrations. These results suggest that there is no defect of antigen-presenting activity in these transgenic mice. None of the transgenic APC stimulated T-cell lines in the absence of exogenous HBcAg. This indicates that circulating HBeAg in transgenic mice does not make enough Ag/MHC class II complex to stimulate specific T cells.

HBcAg-specific T-cell proliferation of HBc transgenic mice

In order to know whether T cells of transgenic mice are tolerant to HBcAg, a group of five mice were immunized with HBcAg in CFA at the base of tail. Seven days later, cells from draining lymph nodes were stimulated with HBcAg *in vitro* for 5 days. As shown in Fig. 6, LN cells from BS10 mice showed poor proliferative responses over a wide range of HBcAg concentrations compared to control B6 LN cells. SPC mouse LN cells consistently showed slightly decreased proliferative responses compared to B6 mouse LN cells in every experiment repeated more than six times. LN cells from SC33 mice showed similar proliferative responses compared to B6 LN cells. All the LN cell preparations showed similar proliferative responses against PPD, indicating that low responsiveness of BS10 LN cells was not due to the lack of appropriate immunizations. When immunized with KLH, LN cells from B6 and BS10 mice showed a similar degree of proliferation against KLH (Fig. 6). The proliferating cells were shown to be T cells by immunofluoresence analysis (data not shown). These results suggest that T cells of BS10 transgenic mice are specifically tolerant to HBcAg. SPC and SC33 T cells are not tolerant to HBcAg in spite of the fact that they contain HBeAg in the serum or HBcAg in the liver extracts. Non-immunized LN cells from these transgenic mice did not show any proliferative responses against HBcAg *in vitro* (data not shown).

T-cell tolerance in BS10 mice is not due to active suppression

To examine whether the T-cell tolerance observed in BS10 mice is due to active suppression, cell-mixing experiments were performed. HBcAg primed LN cells from BS10 and B6 mice were mixed at varying ratios and stimulated with HBcAg *in vitro*. As shown in Fig. 7, the proliferative response of B6 LN cells to HBcAg stimulation was not suppressed by adding BS10

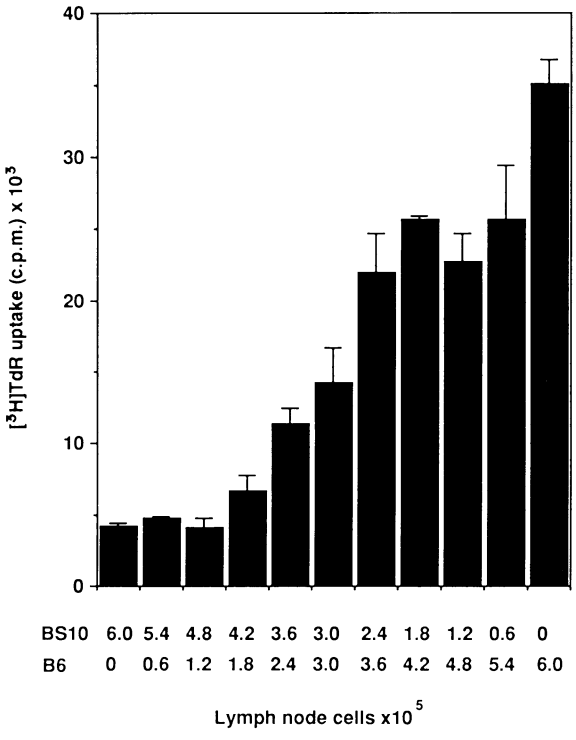

Figure 7. Absence of suppressive activity in BS10 mice. A group of five B6 and BS10 mice were immunized with HBcAg (10 μg) in CFA at the base of tail. Seven days later, draining LN cells from B6 and BS10 mice were mixed at varying ratios as indicated. Assays of proliferative responses were performed as described in Fig. 6.

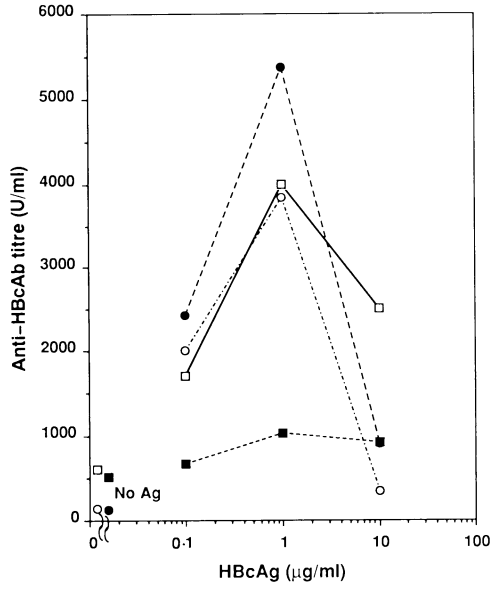

Figure 8. Helper activity of transgenic LN T cells. A group of five mice were immunized with 10 μg HBcAg in CFA at the base of tail. After 7 days, purified T cells from inguinal and para-aortic LN cells were obtained by the treatment with anti-IgM mAb (Bet-2) and magnetic beads to remove B cells. 1×10^6 purified T cells from B6 (—□—), BS10 (■), SPC (· ·○· ·) and SC33 (●) mice were cultured with 1×10^7 anti-Thy-1.2 plus C treated spleen cells from B6 mice which were immunized with HBcAg 2 weeks before. Cultures were stimulated with varying concentrations of HBcAg for 2 days, washed to remove antigen and recultured for 5 days. Anti-HBc antibody titres in the culture supernatants were assayed by ELISA.

LN cells, suggesting that no suppressive activity exists in BS10 mice.

Assay of helper T-cell activity

To examine the helper T-cell activity of transgenic mice, B6 or transgenic mice were immunized with HBcAg at the base of tail and draining LN cells were obtained 7 days later. Cells were cultured with purified splenic B cells from B6 mice which were immunized with HBcAg 2 weeks previously. Cultures were stimulated with varying amounts of HBcAg for 2 days, washed, and recultured for 5 days. Anti-HBc antibody titre in the culture supernatants was assayed by ELISA. As shown in Fig. 8, no helper activity was obtained from BS10 mouse LN cells. SPC and SC33 LN cells showed good helper activity as control B6 LN cells. This result suggests that BS10 helper T cells are tolerant to HBcAg.

DISCUSSION

We have analysed the HBcAg-specific immune responses of three different lines of HBV transgenic mice. These transgenic mice express different concentrations of HBeAg in the serum and HBcAg in the liver. BS10 mice, which contain the whole HBV genome, express relatively high concentrations of HBeAg in the serum and HBcAg in the liver. SPC mice, which contain HBV core and precore genes, express relatively low concentrations of HBeAg in the serum. SC33 mice, which contain only HBV core genes, do not express HBeAg at all in the serum but express HBcAg in the liver. HBcAg and HBeAg are different antigen specificities of the protein $p22^c$ encoded by the core gene. HBeAg, a 16,000 MW polypeptide, is a truncated form of $p22^c$ which contains some HBcAg determinants but in serum these are masked by certain serum proteins bound avidly to the protein. The precore sequence within the core gene open reading frame may affect the core gene-encoded polypeptides. Expression of the core gene alone can result in the formation of multimeric particles composed of $p22^c$ which may accumulate intracellularly. However, the expression of both core and precore gene sequences leads to the secretion of the 16,000 MW polypeptide with HBeAg specificity. Therefore, the SC33 mice could not produce HBeAg but there would be intracellular accumulation of HBcAg and the SPC mice may be predicted to produce HBeAg but little HBcAg. Analysis of transgene transcription in these mice revealed liver and kidney-specific expression of transgene mRNA in BS10 mice, which use the HBV gene promoter, as already reported.[7] SPC and SC33 mice employed the SAP component gene promoter and the transgene transcripts were detected only in the liver (K. Araki, J. Miyazaki and K. Yamamura, unpublished observations). The liver-specific transgene expression using SAP gene promoter has been reported in several different lines of transgenic mice.[13,14]

Although HBcAg and HBeAg are serologically distinct,[9,10] we were able to analyse HBcAg-specific T-cell responsiveness of these transgenic mice since HBcAg and HBeAg are highly cross-reactive at the T-cell level.[11,12] Our analysis revealed that BS10 mice, when immunized with HBcAg, showed an extremely low anti-HBc antibody response compared to the transgenic host

strain of B6 mice. Analysis of cellular site of low responsiveness of BS10 mice revealed that helper and proliferative T cells but not B cells are specifically tolerant to HBcAg. APC functions of BS10 mice were not defective. Also, there was no active suppression in the T-cell tolerance of BS10 mice. The T-cell tolerance in BS10 mice is specific and not due to generalized immune suppression observed in some lines of transgenic mice[20] since they respond effectively to unrelated antigens (PPD and KLH).

The mechanism of the T-cell tolerance in BS10 mice would provide important information as to the nature (circulating versus non-circulating) as well as the time of expression of self-antigens required for the tolerance induction of self-reactive T-cell clones. We speculate that the self-antigens responsible for T-cell tolerance in BS10 mice are circulating HBeAg from the following considerations. SC33 mice, which express HBcAg in the liver but do not express HBeAg in the serum, do not show T-cell tolerance but immunity to HBcAg. The lack of tolerance in SC33 mice might be due to the absence of circulating HBeAg in the serum since the transgene product ($p22^c$) is very likely to remain intracellular and in the absence of precore sequences it cannot become a secretory polypeptide with HBeAg specificity. Although the mechanism of the induction of immunity in SC33 mice is not clear (see below), this would suggest that expression of HBcAg in the BS10 liver may induce immunity to HBcAg in BS10 mice. However, BS10 mice, which express high amounts of HBcAg in the liver and HBeAg in the serum, are not immune but rather tolerant to HBcAg. One of the most likely explanations for this would be that although the HBcAg in BS10 liver might have the potential to induce immunity, it is not effective since T cells in BS10 mice are tolerant probably due to the absence of specific clones. This BS10 tolerance may be due to the presence of relatively high amounts of HBeAg in the serum from the very early developmental life. If this is the case, the apparent lack of or low T-cell tolerance (as observed in Fig. 6) in SPC mice, which express low amounts of HBeAg in the serum (but not HBcAg in the liver), suggests that the acquisition of tolerance depends on the amount of circulating antigens. The amount of HBeAg in SPC serum is about seven times less than that of BS10 serum. It is also possible, however, that the lack of or weak tolerance in SPC mice could be due to the presumed late appearance of circulating HBeAg. The time of developmental expression of HBeAg in SPC mice could not be determined due to the low amount. It is speculated to be on Days 2–3 after birth since SPC mice use the same promoter (SAP gene promoter) as SC33 mice.

It should be noted that small but significant amounts of anti-HBc antibody are produced in BS10 mice upon immunization with HBcAg. Also, BS10 mice show small but significant HBcAg specific T-cell proliferative responses. BS10 T cells which proliferate in response to high HBcAg concentration could bear T-cell receptors with high affinity. These results indicate that the tolerance in BS10 mice is not complete, further suggesting the quantitative nature of T-cell tolerance.

The second possibility would be that degraded products of the HBcAg in the BS10 liver circulate in the serum from a very early developmental stage of life (even before birth) and cause tolerance of HBcAg-reactive T-cell clones. Indeed, a small but significant amount of HBcAg was detected in the BS10 liver from 18 days of gestation. If this is the case, the induction of self-tolerance and immunity is critically dependent on the time of the appearance of self-antigens since SC33 mice, which also showed similar amounts ($\sim$ 10 ng/ml) of HBcAg in the liver 2 days after birth, are not tolerant but immune to HBcAg.

Other mechanisms would be possible (but seem to be unlikely in our opinion) for the T-cell tolerance in BS10 mice. Although not detectable on the surface of hepatocytes by immuno-histochemical examination (our unpublished data), the existence of large amounts of HBcAg in the BS10 liver might cause clonal inactivation of HBcAg reactive mature T-cell clones in the periphery. Since clonal inactivation in the periphery deals only with the mature T cells, the difference of developmental expression between BS10 and SC33 mice may not have any effect in this type of clonal inactivation. If this is the case, the clonal inactivation is dependent on the amount of HBcAg in the liver, since SC33 mice, which express less HBcAg, are not tolerant but immune to HBcAg. Creation of additional lines of transgenic mice with similar developmental but different quantitative expression of HBe/HBc transgene products would clarify these points.

None of the transgenic spleen cells are able to stimulate HBcAg specific T-cell lines in the absence of exogenous HBcAg (Fig. 6), suggesting insufficient circulating HBeAg in the serum to make Ag/class II complex for the activation of specific T cells. Also, we were not able to detect HBcAg-specific T-cell proliferation by transgenic mouse thymocytes in the absence of exogenous HBcAg (data not shown). Although these observations might contradict the ability of circulating HBeAg to induce tolerance of HBcAg-specific T cells, several reports suggest that the amount of antigen required for the positive and negative selection in the thymus is far less than that required for the activation of peripheral T cells.[21,23]

One of the interesting observations in this study is that SC33 mice, which do not secrete HBeAg in the circulation, contain, although small in amount, anti-HBc antibody in the serum even before immunization. Although cellular analysis suggests that B cells but not T cells in SC33 mice are sensitized *in vivo*, this would just reflect the sensitivity of assay. Indeed, immunization with HBcAg resulted in the accelerated and augmented antibody production of the secondary type immune response, which suggests the involvement of memory T cells. It could be that unsecreted HBcAg might be processed in such a way to sensitize T (and B) cells without causing any autoimmune response in SC33 mice (see below).

None of these HBV transgenic mice showed any evidence of hepatitis as diagnosed by the serum transaminases and by histological examination even after long-term observations for more than 1 year. Various attempts to induce hepatitis in these transgenic mice including transfer of HBcAg-hyperimmunized spleen cells or HBcAg-specific T-cell lines were not successful. Chisari *et al.* reported the development of hepatocellular inflammatory responses and eventual occurrence of hepatocellular carcinoma in HBs transgenic mice.[24] These HBs transgenic mice were also shown to be tolerant to HBsAg.[25] Moriyama *et al.*[26] reported that transient hepatitis was induced in HBV transgenic mice by the transfer of anti-HBs antibody or of anti-HBs $CD8^+$ T cells. There exist several differences between these transgenic systems including the target antigen (HBs versus HBc), amounts of transgene expression and transgenic host strain. It is possible that the amounts of expressed transgene products are too small in our transgenic mice for the induction of hepatitis. It is also possible that the host strain of transgenic mice may influence the immune response to hepatocellular

inflammatory responses. The responsiveness to HBcAg is reported to be under the immune response (Ir) gene control and B6 mice were reported to be intermediate responders to HBcAg.[12] It would be interesting to see the effects of introduction of high responder ($H\text{-}2^{k,s,d}$) or of low responder ($H\text{-}2^{p}$) haplotypes in these transgenic mice in hepatitis induction.

Finally, it is interesting to consider the relationship between our observations and chronic hepatitis B infection in man. HBV is not directly cytopathic and the immune response of the host against viral gene products appears to mediate hepatocellular tissue injury. Infants born to hepatitis B virus carrier mothers, who express a secreted HBeAg, invariably become persistently infected by HBV. BS10 mice, which express high amounts of HBeAg in the serum from the very early developmental life are tolerant to HBcAg at the T-cell level. Based on the observations in this paper, it may be speculated that transplacental transmission of HBeAg in man leads to depression of T-cell responsiveness to HBcAg in newborns subsequently exposed to HBV, thus leading to virus persistence. Similar observations were made by Milich *et al.*[4] using HBcAg transgenic mice.

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