

## Hepatitis B Virus Core Antigen Binds and Activates Naive Human B Cells In Vivo: Studies with a Human PBL-NOD/SCID Mouse Model

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**The hepatitis B virus (HBV) core (HBc) antigen (HBcAg) is a highly immunogenic subviral particle. Studies with mice have shown that HBcAg can bind and activate B cells in a T-cell-independent fashion. By using a human peripheral blood leukocyte (hu-PBL)-Nod/LtSz-Prkdc<sup>scid</sup>/Prkdc<sup>scid</sup> (NOD/SCID) mouse model, we show here that HBcAg also activates human B cells in vivo in a T-cell-independent way. HBcAg was capable of inducing the secretion of HBcAg-binding human immunoglobulin M (IgM) in naive human B cells derived from adult human and neonatal (cord blood) donors when these hu-PBL were transferred directly into the spleens of optimally conditioned NOD/SCID mice. No such responses were found in chimeric mice that were given hu-PBL plus HBV e antigen or hu-PBL plus phosphate-buffered saline. In addition, HBcAg activated purified human B cells to produce anti-HBc IgM in the chimeric mice, thus providing evidence that HBcAg behaves as a T-cell-independent antigen in humans. However, HBcAg-activated hu-PBL from naive donors were unable to switch from IgM to IgG production, even after a booster dose of HBcAg. Production of HBcAg-specific IgG could only be induced when hu-PBL from subjects who had recovered from or had an ongoing chronic HBV infection were transferred into NOD/SCID mice. Our data suggest that humans also have a population of naive B cells that can bind HBcAg and is subsequently activated to produce HBcAg-binding IgM.**

The hepatitis B virus (HBV) belongs to the family *Hepadnaviridae* and is the smallest DNA virus known. It is composed of an outer envelope consisting of three envelope proteins (large, middle, and small envelope proteins) and an inner protein capsid that contains the viral genome (22). The nucleocapsid of HBV is a 30- to 32-nm particle composed of multiple copies of a single polypeptide (P21). The intact structure exhibits HBV core (HBc) antigenicity. A nonparticulate form of HBc antigen (HBcAg), the HBV e antigen (HBeAg), is secreted in the serum during HBV infection. HBcAg and HBeAg share a 149-amino-acid homology and are therefore highly cross-reactive at the T-cell level. Despite this amino acid sequence similarity, the two HBV nucleocapsid proteins are recognized differently and induce different immune responses (9).

The particulate HBcAg is extremely immunogenic. It can function as both a T-cell-independent and a T-cell-dependent antigen (10). Immunization with HBcAg preferentially primes Th1-type cellular immune responses (11). HBcAg is an effective carrier of heterologous epitopes, and HBcAg-specific T cells support anti-envelope (anti-HBs), as well as anti-HBc, antibody production (4, 6, 12, 18). During chronic HBV infection, HBcAg is the only antigen that elicits a prominent immune response (9).

Secreted HBeAg is much less immunogenic. It serves an

immunomodulatory function in utero and induces T-cell tolerance to both HBeAg and HBcAg, which may predispose neonates born to HBeAg-positive mothers to persistent infection (13). In adults, circulating HBeAg modifies the immune response by deleting the inflammatory Th1 subset by Fas-mediated apoptosis while preferentially eliciting the noninflammatory Th2 subset of T cells (14).

Direct evidence for these unique qualities of HBcAg and HBeAg has been generated in the mouse, which is not a natural host of HBV. We have found evidence that the binding of HBcAg to naive murine B cells is dependent on a superantigen-like binding of a linear motif present in various variable-domain germ line genes (7). Because experiments with humans are limited by ethical constraints and in order to examine the immunogenicity of HBcAg for human lymphoid cells, we have developed a human peripheral blood leukocyte (hu-PBL)-NOD/severe combined immunodeficient (SCID) mouse model. Since the first report by Mosier et al. (16) that transfer of hu-PBL into SCID mice successfully resulted in stable long-term reconstitution of a functional human immune system, the ability of SCID mice to accept xenografts has been utilized in different research fields as a laboratory model that mimics the human immune system (3, 17, 21). In most models, hu-PBL were injected into the peritoneal cavities of recipient mice. This route leads to a predominant expansion of T cells, whereas the survival of B cells and their functional expression are minimal. Recently, we discovered that the transfer of hu-PBL directly into the spleens of optimally conditioned NOD/SCID mice led to a vigorous expansion of B cells and their differentiation into plasmacytoid cells (2). By using this hu-PBL-SCID mouse model, we show here that HBc particles

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(HBcAg) induce the production of HBcAg-binding immunoglobulin M (IgM) in the B cells of unprimed individuals that were transferred into NOD/SCID recipients.

#### MATERIALS AND METHODS

**Protein antigens.** HBcAg is a 21-kDa protein that assembles to form a nucleocapsid particle. It encompasses 183 amino acids. HBeAg shares amino acids 1 to 149 of HBcAg and contains an amino terminal extension of 10 amino acids encoded by the precore sequence of the precore-core gene. The HBcAg and HBeAg used in this study were of the ayw subtype. These recombinant proteins were produced in *Escherichia coli* and purchased from DiaSorin, Saluggia, Italy. The endotoxin contents of HBcAg (10 µg) and HBeAg (20 µg) were 1,362 and 3,702 EU, respectively. These were measured by using a quantitative chromogenic *Limulus* amoebocyte lysate test (Chromogenix AB, Mölndal, Sweden).

**Pretreatment of immunodeficient mice.** Homozygous Nod/LtSz-Prkdc<sup>scid</sup>/Prkdc<sup>scid</sup> (NOD/SCID) mice were bred and maintained under specific-pathogen-free conditions in the animal room of the Department of Clinical Chemistry, Microbiology and Immunology, Ghent University. All of the mice used in the present study were aged 9 to 12 weeks. One day before reconstitution with hu-PBL, NOD/SCID mice were irradiated (300 rads of gamma radiation) and injected intraperitoneally with 1 mg of TMβ1 in 0.5 ml of phosphate-buffered saline (PBS) to reduce endogenous mouse natural killer (NK) cell activity. TMβ1 is a rat monoclonal antibody directed against the mouse interleukin-2 receptor beta chain (20, 23). The study protocol used was approved by the local animal ethics committee.

**Blood donors and preparation of an hu-PBL suspension.** Blood was drawn from the following categories of donors: (i) five healthy adult donors who had never been exposed to HBV or hepatitis C virus (HCV), as demonstrated by the absence of all serological markers of past or ongoing HBV or HCV infection, as well as by the absence of HBV DNA and HCV RNA; (ii) two cord blood donors equally free of serological or molecular markers of HBV or HCV infection; and (iii) three adult donors with histories of HBV infection (one had fully recovered from an HBV infection, and two were chronically infected with HBV).

Mononuclear cells, referred to as hu-PBL, were isolated from fresh buffy coats or whole blood (drawn from the cubital or umbilical vein) by Ficoll-Hypaque (Nycomed Pharma, Oslo, Norway) centrifugation. The hu-PBL were suspended in PBS ( $2 \times 10^8$  cells/ml), always kept on ice, and transferred into the spleens of NOD/SCID mice as soon as possible. The purified human B cells were directly isolated from fresh buffy coats with Dynabeads M-450 pan-B (CD19) and Detachabeads (DynaL AS, Oslo, Norway). The procedure was performed in accordance with the manufacturer's guidelines. The purity of the B-cell suspension was analyzed by flow cytometry. The B-cell content always exceeded 95%, while the T-cell contamination was less than 2%.

**Intrasplenic injection of hu-PBL and in vivo immunization.** Immediately before intrasplenic engraftment of hu-PBL, recombinant HBcAg (rHBcAg; 10 µg per mouse), recombinant HBeAg (rHBeAg; 20 µg per mouse), or PBS (as an unstimulated control) was added to the suspension of hu-PBL. The NOD/SCID mice were anesthetized, and a subcostal cutaneous incision was made. The abdominal wall was then incised, the peritoneal cavity was opened, and the spleen was carefully exposed. Hu-PBL ( $10^7$  cells per mouse in 50 µl of PBS) were directly injected into the spleen. Subsequently, the spleen was repositioned in the abdominal cavity and the abdominal wall and the skin were sutured separately.

**Determination of total human immunoglobulins (IgG and IgM) and antigen-specific antibody in the plasma of hu-PBL-NOD/SCID mice.** Plasma of hu-PBL-NOD/SCID mice was collected at days 7 and 14 after hu-PBL transfer. The total human IgM and IgG content of NOD/SCID plasma was determined by in-house enzyme-linked immunosorbent assays (ELISA) as described previously (23).

The in vivo production of specific anti-HBc IgM, total anti-HBc antibody, and total anti-HBe antibody in the plasma of hu-PBL-NOD/SCID mice was measured with the commercial ELISA kits ETI-CORE-IGMK-2, ETI-AB-COREK-2, and ETI-AB-EBK (all from DiaSorin), respectively. The methods used for qualitative total anti-HBc and anti-HBe determination were competitive assays based on the ELISA technique. The method used for qualitative anti-HBc IgM determination was an immunometric assay based on the antibody capture ELISA technique. The cutoff values were calculated in accordance with the manufacturer's instructions. The mean cutoff absorbance of anti-HBc IgM at different experimental time points was  $0.140 \pm 0.007$ .

The anti-HBc IgG titer in the plasma of hu-PBL-NOD/SCID chimeric mice was determined by the method described by Maruyama et al. (8), with some modifications. rHBcAg (50 ng/well; DiaSorin) was used to coat microtiter plates (Nunc Immunoplates) overnight at 4°C. The plates were incubated for 1 h at 37°C with 100 µl of PBS containing 1% bovine serum albumin, 0.05% Tween-20,

and 5% heat-inactivated goat serum (blocking buffer). After removal of the blocking buffer, the murine plasma was diluted 1/50 to 1/12,800 with blocking buffer and added to the microtiter plates. The plates were kept for 2 h at 37°C and subsequently incubated with 100 µl of horseradish peroxidase-conjugated rabbit anti-human IgG (1/100,000; Dako AS) for 1 h at 37°C. After another three washes, the plates were developed by a final incubation for 30 min with tetramethylbenzidine (Sigma Chemical Co., St. Louis, Mo.) at room temperature. The enzymatic reaction was stopped with H<sub>2</sub>SO<sub>4</sub>, and the plates were read at 450 nm. A result was considered positive when the absorbance of the sample was three times as high as that of negative control plasma from a healthy donor.

**Flow cytometric determination of the frequency of naive B cells able to bind to HBcAg.** Human B cells were purified from spleens that had been surgically removed because of traumatic rupture. The use of human tissue for these studies was approved by the local ethics committee. The B cells were purified by using the B-cell-negative isolation kit from Dynal AS and following the manufacturer's guidelines. Purified human B cells ( $10^6$ /ml) were incubated for 30 min at +4°C without antigen or with HBcAg (5 µg/ml) or HBeAg (5 µg/ml). The cells were then washed three times and incubated with PBS-2% bovine serum albumin-5% normal human serum for 30 min at +4°C. The cells were washed again and incubated with a biotin-labeled human anti-HBc monoclonal antibody produced in our laboratory (Leroux-Roels et al.). Phycoerythrin-conjugated streptavidin (Becton Dickinson, Aalst, Belgium) was used as a second-step reagent together with fluorescein isothiocyanate-conjugated anti-CD19 antibody (Becton Dickinson). The stained cells were analyzed by flow cytometry.

**Statistical analysis.** Different groups were compared by using the Kruskal-Wallis H test. When the Kruskal-Wallis significance level was  $P < 0.05$ , Mann-Whitney U tests were applied for post hoc analysis.

#### RESULTS

**HBc particles induce the production of HBcAg-binding human IgM in hu-PBL-NOD/SCID mice transplanted with hu-PBL from healthy adult donors who have never been exposed to HBV.** Hu-PBL were isolated from fresh buffy coats derived from five different healthy adult donors (naive donors) who were free of serological and virological markers of past or ongoing HBV infection. The hu-PBL ( $10^7$  per mouse) were injected directly into the spleens of TMβ1-pretreated and irradiated NOD/SCID mice as described in Materials and Methods. Four experimental groups of mice were included. (i) Group 1 consisted of 19 animals that were injected with hu-PBL (derived from five donors) mixed with rHBcAg (10 µg per mouse) at the time of intrasplenic engraftment. (ii) Group 2 consisted of seven animals receiving hu-PBL (derived from two donors) mixed with rHBeAg (20 µg per mouse) at the time of cell transfer. (iii) Group 3 consisted of 16 animals receiving hu-PBL (derived from five donors) that were suspended only in PBS (as an unstimulated control). (iv) Group 4 consisted of nine animals that received only rHBcAg (10 µg per mouse in PBS) directly injected into the spleen without added hu-PBL.

Seven and 14 days after intrasplenic engraftment of hu-PBL, plasma was collected from all chimeric NOD/SCID mice ( $n = 51$ ). For anti-HBc IgM detection, this plasma was diluted 1/10, 1/100, 1/1,000, and 1/4,000, and for anti-HBc IgG detection, serial dilutions of 1/50 to 1/12,800 were used.

On day 7, HBcAg-binding human IgM was detected in 17 (89.5%) of the 19 mice that had received hu-PBL together with HBcAg (group 1) when sera were diluted 10-fold (Fig. 1A). On day 14, even higher levels of anti-HBc IgM were found and all (100%) of 15 murine plasma samples that were derived from HBcAg-immunized hu-PBL-NOD/SCID mice (group 1,  $n = 15$ , because 4 mice died before day 14) (Fig. 1B) scored strongly positive for HBcAg-binding human IgM. As shown in Table 1, the mean absorbance values for anti-HBc IgM in the HBcAg-immunized chimeric mice (group 1) at days 7 and 14

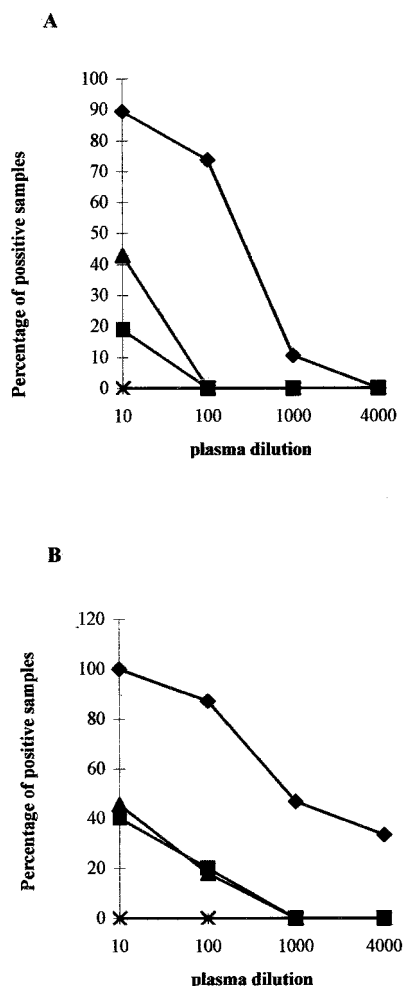


FIG. 1. Intrasplenic injection into conditioned NOD/SCID mice of HBcAg together with hu-PBL from healthy adult volunteers who had never been exposed to HBV induced the production of human HBcAg-binding IgM. On day 0,  $10^7$  hu-PBL were injected into the spleens of conditioned (300 rads of gamma radiation and 1 mg of TM $\beta$ 1 administered into the peritoneal cavity, both given on day -1) NOD/SCID mice. The four experimental groups examined were (i) group 1 ( $n = 19$ ), in which hu-PBL were given together with rHBcAg (10  $\mu$ g per mouse) (◆); (ii) group 2 ( $n = 7$ ), in which hu-PBL were given with HBeAg (20  $\mu$ g per mouse) (■); (iii) group 3 ( $n = 16$ ), in which hu-PBL were given in PBS (▲); and (iv) group 4 ( $n = 9$ ), in which HBcAg (10  $\mu$ g per mouse) was given without hu-PBL (×). The percentage of animals in each group which displayed a positive signal in the anti-HBc IgM ELISA at dilutions of 1/10 to 1/4,000 is shown. Results obtained at days 7 (A) and 14 (B) after cell transfer are presented.

were  $0.870 \pm 0.547$  (range, 0.086 to 2.3) and  $1.089 \pm 0.605$  (range, 0.295 to 2.176), respectively. These values are significantly higher ( $P < 0.005$ ) than those observed in the HBeAg-immunized chimeric mice (group 2) or in control animals that received hu-PBL in PBS (group 3) or HBcAg in the absence of human cells (group 4). Without added hu-PBL, HBcAg is unable to induce the production of anti-HBc IgM in pretreated NOD/SCID mice at day 7, as well as at day 14 (group 4).

Although a few mice from groups 2 and 3 had detectable anti-HBc IgM at a 1/10 dilution at both days 7 and 14, these

turned out to be low in absorbance compared to those in group 1 (Table 1). Whereas in 14 (74%) of the 19 animals in group 1 anti-HBc IgM was still detectable at a dilution of 1/100 at week 1, none of the animals from the other experimental groups had anti-HBc IgM titers of that magnitude (Fig. 1A). Two weeks following cell transfer (Fig. 1B), 13 (87%) of 15 HBcAg-immunized hu-PBL-NOD/SCID mice (group 1) had anti-HBc IgM detectable at a dilution of 1/100, 7 (47%) were still positive at 1/1,000, and 5 (33%) even scored positive at 1/4,000. In the other experimental groups, lower titers were measured at day 14. In group 2, only 1 of the 5 survivors had anti-HBc IgM detectable at a dilution of 1/100, and in group 3, only 2 (18%) of 11 were positive at this plasma dilution. The optical densities of these positive plasma samples (0.242, 0.159, and 0.295, respectively) were distinctly lower than those of the plasma samples from group 1 (Table 1).

All plasma samples were examined for the presence of total anti-HBe. In none of the four experimental groups could anti-HBe be observed (data not shown). Furthermore, anti-HBe-IgM was also measured in some experiments with an in-house ELISA but it could not be detected in any of four experimental groups of mice (data not shown).

Anti-HBc IgG could not be detected in the plasma of NOD/SCID mice receiving hu-PBL derived from HBV-naive cell donors, irrespective of the antigen that was given concomitantly. Even 3 weeks after the cell transfer and after an intravenous HBcAg boost at day 8 or 10, no class switch could be observed (three separate experiments; data not shown).

The plasma samples were also analyzed for the presence of total human IgM and IgG. No difference in the IgM and IgG concentrations was found in the animals in groups 1, 2, and 3. The mice in group 4 did not produce human or mouse IgM or IgG (data not shown).

To examine whether HBcAg from another source had the same antibody-inducing capacities, 2  $\mu$ g of yeast-derived recombinant HBcAg (from the laboratory of D.R.M.) was injected with  $10^7$  hu-PBL into three NOD/SCID mice. The levels of anti-HBc-binding IgM induced with this preparation were comparable to those raised with HBcAg produced in *E. coli*, suggesting that endotoxin is not required for anti-HBc IgM production.

**Specificity of HBcAg-binding human IgM.** The specificity of HBcAg-binding human IgM was studied in competition assays by using the anti-HBc and anti-HBe antibody detection kits from DiaSorin. The capacity of mouse plasma to compete for the binding of labeled human anti-HBc or anti-HBe antibodies to HBcAg and HBeAg, respectively, was examined. All of the plasma samples obtained 14 days after hu-PBL transfer from animals in group 1 (HBcAg added to hu-PBL) and 12 plasma samples from healthy blood donors (unexposed to HBV), including the five hu-PBL donors, were analyzed.

Figure 2 shows that plasma samples from all of the animals in group 1 ( $n = 19$ ) contain HBcAg-binding activity that inhibited the binding of labeled human anti-HBc antibodies from the kits by 21 to 89%. Plasma derived from the animals in groups 2 and 3 and the plasma of the 12 healthy controls did not display this inhibitory capacity. The mean inhibition values and standard deviations were  $1.45\% \pm 2.9\%$ ,  $3.49\% \pm 6.6\%$ , and  $6.07\% \pm 8.48\%$  for groups 2 and 3 and the healthy volunteers, respectively. This inhibitory capacity was not yet



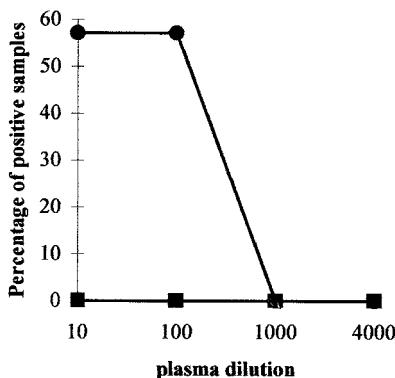


FIG. 4. Transfer of purified human B cells from healthy blood donors together with HBcAg into the spleens of conditioned NOD/SCID mice induces the production of HBcAg-binding human IgM. On day 0,  $2 \times 10^6$  purified B cells were injected together with  $10 \mu\text{g}$  of rHBcAg into the spleens of seven conditioned (300 rads of gamma radiation and 1 mg of TM $\beta$ 1 administered intraperitoneally on day -1) NOD/SCID mice (●). Five mice received B cells ( $2 \times 10^6$  cells per mouse) without added HBcAg (■). The percentage of animals in each group which displayed a positive signal in the anti-HBc IgM ELISA at dilutions of 1/10 to 1/4,000 is shown. The results shown were obtained 7 day after cell transfer.

stimulated to produce anti-HBc IgM, we isolated leukocytes from two cord blood specimens and transferred these into NOD/SCID mice. The sera of the two mothers who gave birth to the cord blood donors were negative for all serological and virological markers of HBV and HCV infection. Cord blood leukocytes (CBL;  $10^7$  cells per mouse) were injected into the spleens of 17 conditioned NOD/SCID recipients. Nine mice were given CBL and rHBcAg ( $10 \mu\text{g}$  per mouse) on day 0, and eight mice received CBL alone. Fourteen days after CBL transfer, all (nine [100%] of nine) plasma samples from the HBcAg-immunized group had anti-HBc IgM detectable at dilutions of 1/10 and 1/100. Five (56%) and two (22%) NOD/SCID mice had detectable antibodies at dilutions of 1/1,000 and 1/4,000, respectively (Fig. 5). Of the eight NOD/SCID mice that received CBL only, two died prematurely. Of the six plasma samples examined at day 14, only one (17%) had anti-HBc IgM detectable at a dilution of 1/100. No signal was present at higher dilutions. The mean absorbance of the plasma diluted 1/100 was  $1.4 \pm 0.8$  (range, 0.47 to 2.78) in the HBcAg-immunized mice, whereas this was only  $0.074 \pm 0.040$  (range, 0.032 to 0.151) in the HBcAg-deprived control group ( $P < 0.005$ ).

**PBL from donors with a past or ongoing HBV infection are able to produce anti-HBc IgM and IgG upon transfer into NOD/SCID recipients.** HBcAg induced the production of

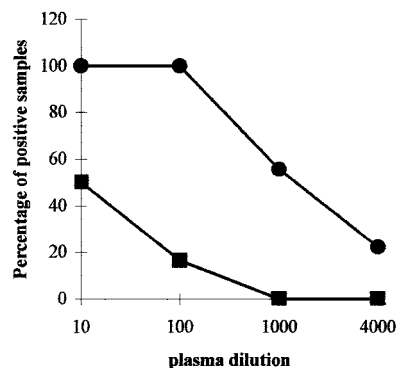


FIG. 5. Intrasplenic injection into conditioned NOD/SCID mice of HBcAg together with CBL from donors who have not been exposed to HBV induces the production of human HBcAg-binding IgM. On day 0,  $10^7$  human CBL were injected into the spleens of conditioned (300 rads of gamma radiation and 1 mg of TM $\beta$ 1 administered intraperitoneally on day -1) NOD/SCID mice. Nine animals were given CBL and HBcAg ( $10 \mu\text{g}$  per mouse) (●), and eight received CBL without HBcAg (■). On day 14, plasma was harvested and examined for the presence of HBcAg-binding IgM at dilutions of 1/10 to 1/4,000. The percentage of mice displaying a positive signal at each dilution is shown.

HBcAg-binding human IgM by leukocytes from HBV-naive adult donors, CBL, and purified B cells from HBV-naive adult volunteers upon transfer of the cells together with HBcAg into conditioned NOD/SCID recipients. However, anti-HBc IgG was never produced in this setting, even when the human cells were re-exposed to HBcAg in vivo in these mice. To examine whether hu-PBL derived from HBV-immune cell donors were able to produce anti-HBc IgG, hu-PBL were isolated from a person who had fully recovered from an HBV infection (HBsAg<sup>-</sup>, HBsAb<sup>+</sup>, HBcAb<sup>+</sup>, HBeAg<sup>-</sup>, HBeAb<sup>+</sup>, and HBV DNA<sup>-</sup>) and two chronically infected patients (HBsAg<sup>+</sup>, HBsAb<sup>-</sup>, HBcAb<sup>+</sup>, HBeAg<sup>+</sup>, HBeAb<sup>-</sup>, and HBV DNA<sup>+</sup>).

Hu-PBL ( $10^7$  cells per mouse) were injected into the spleens of conditioned NOD/SCID mice with ( $n = 8$ ) or without ( $n = 8$ ) rHBcAg ( $10 \mu\text{g}$  per mouse). Plasma was collected from these animals on days 7 and 14 after cell transfer. On day 7, anti-HBc IgM and IgG could be detected in the plasma from all of the animals and the titers of these antibodies increased with time (Table 2). In contrast to the results obtained with hu-PBL from HBV-naive donors, anti-HBc IgM and IgG production also occurred in animals receiving hu-PBL without HBcAg. However, the addition of HBcAg to the cells significantly enhanced HBc-specific antibody production ( $P < 0.05$ ). At both days 7 and 14, the mean absorbance of anti-HBc IgM in the mice receiving HBcAg boosters were higher than in the

TABLE 2. HBcAg-specific secondary immune responses in hu-PBL-NOD/SCID mice

Treatment	Day 7			Day 14		
	No. of mice	Anti-HBc-IgM mean absorbance $\pm$ SD (range)	Anti-HBc-IgG mean titer (range)	No. of mice	Anti-HBc-IgM mean absorbance $\pm$ SD (range)	Anti-HBc-IgG mean titer (range)
Hu-PBL + HBcAg	8	$2.58 \pm 0.41^a$ (1.75–3.00)	1/688 (<1/50–1/1,600)	7	$2.47 \pm 0.63^a$ (1.31–3.04)	1/8,243 <sup>a</sup> (1/100–1/12,800)
Hu-PBL + PBS	8	$0.30 \pm 0.41$ (0.03–1.15)	1/312 (<1/50–1/800)	6	$0.54 \pm 0.76$ (0.03–2.08)	1/1,492 (<1/50–1/6,400)

<sup>a</sup>  $P < 0.05$  compared to the control animal group (hu-PBL + PBS).

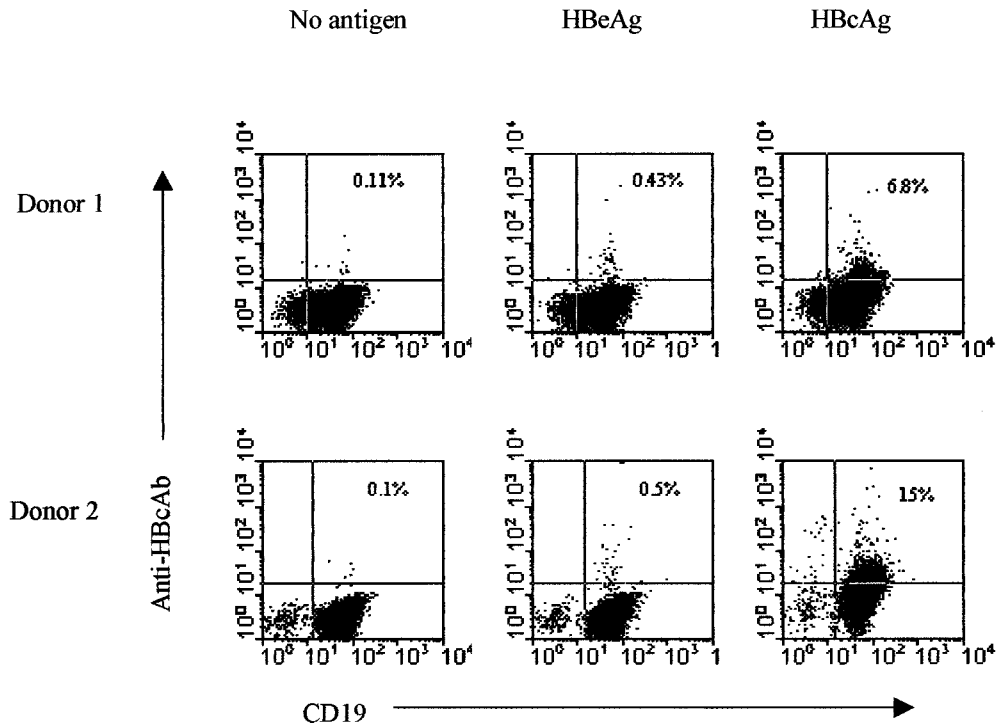


FIG. 6. Frequency determination of HBcAg-binding naive human spleen B cells by flow cytometry using direct binding of HBcAg. Human B cells were purified from two spleens. The cells were preincubated with HBcAg, with HBeAg, or without antigen (control) at 4°C for 30 min and subsequently stained with biotin-labeled human anti-HBc specific antibody (Ab) and anti-human CD19 antibody as described in Materials and Methods.

control animals not given boosters ( $P < 0.05$ ). On day 14, all mice receiving HBcAg boosters showed a positive signal for anti-HBc IgM in the plasma at a dilution of 1/4,000. This did not occur in NOD/SCID mice receiving hu-PBL without HBcAg. The endpoint dilution titers of anti-HBc IgG in the animals given HBcAg boosters were significantly higher than in the counterparts not given boosters ( $P < 0.05$ ).

**HBcAg binds to naive human spleen B cells in vitro.** Having demonstrated the ability of naive B cells to produce HBcAg-binding IgM, we wished to examine the direct interaction of HBcAg particles and B cells. Therefore, we purified human B cells from two spleens. The purity of these B-cell populations exceeded 90%. The binding of HBcAg to the naive B cells was demonstrated by using a biotin-labeled human monoclonal anti-HBc antibody, the specificity of which had been examined in a direct binding assay (HBcAg immobilized on a solid phase) and competition assays (using commercially available anti-HBc assays from DiaSorin and Abbott). The frequencies of HBcAg-binding B cells in these two donors were 6.8 and 15% (Fig. 6). B cells that were stained with biotin-labeled human anti-HBc antibody after incubation with HBeAg or without antigen showed a background staining of  $\leq 0.5\%$ .

## DISCUSSION

It has been clearly shown that HBcAg binds and activates naive murine B cells (15). In a companion paper, we report that the interaction between HBcAg and naive murine B cells seems to be dependent on a motif present in particular variable-domain regions, which is different from traditional anti-

gen-antibody interactions (7). Much less is known about the interaction between HBcAg and naive human B cells. In this study, we have investigated the in vivo response of human B cells to HBcAg by using the hu-PBL-NOD/SCID mouse model. Under these conditions, naive human B cells are able to produce HBcAg-binding human IgM, and the results of our studies strongly suggest that HBcAg behaves as a T-cell-independent antigen in humans as it does in mice. Hu-PBL from persons with a past or ongoing HBV infection are able to produce both anti-HBc IgM and IgG upon transfer into conditioned NOD/SCID mice.

We recently designed a conditioning regimen that strongly improved the survival of human cells transferred into SCID and NOD/SCID mice (23, 24). This method is based on the combined use of sublethal total-body irradiation (300 rads) and a rat antibody against the mouse interleukin-2 receptor  $\beta$  chain (TM $\beta$ 1) (20). When human cells are injected directly into the mouse spleen, human B cells become activated, expand vigorously, and transiently become the most prominent subset among the hu-PBL residing in the spleen (2). We have used this model to examine whether HBcAg displays the same unique immunological characteristics for human lymphoid cells that have been observed in the mouse (9, 10). The experiments presented here suggest that this is, indeed, the case. When hu-PBL from adult or neonate donors who have never been infected with HBV were exposed to HBcAg within the context of optimally conditioned NOD/SCID mice, the production of HBcAg-binding IgM ensued. The titer of this anti-HBc IgM rose with time. This production of anti-HBc IgM was

not observed in animals that were given hu-PBL (derived from the same donors) with HBeAg or without any antigen. In these experiments, HBeAg is an appropriate control antigen since (i) it is closely related to HBcAg, (ii) it is devoid of the conformationally dependent dominant B-cell epitope that conveys core antigenicity, and (iii) it is largely overlapping at the T-cell level. Because the HBcAg used here contained more endotoxin than the HBeAg used, we examined the effect of endotoxin on antibody production with great care. We noted that a mixture of lipopolysaccharide (4 to 10  $\mu\text{g}$  per mouse) and hu-PBL, upon intrasplenic injection into NOD/SCID mice, did not induce the production of anti-HBc IgM (data not shown). The inability of HBeAg to induce the production of anti-HBc IgM or anti-HBe antibodies in this model suggests that the capacity of HBcAg to induce the production of anti-HBc IgM is an intrinsic capacity of HBcAg rather than a consequence of endotoxin contamination.

In separate experiments not described here, we have examined whether another structural protein of HBV, namely, HBsAg, is able to induce the production of anti-HBs antibodies in cells from naive donors. Repeated experiments clearly showed that this is not the case. However, hu-PBL from immune cell donors (those who had recovered from an HBV infection or HBV vaccine recipients) can easily be induced to produce anti-HBs antibodies (2, 23).

The HBcAg specificity of the antibodies induced in HBcAg-stimulated hu-PBL-NOD/SCID mice was examined in a series of competition assays. The plasma derived at day 14 from all of the mice inoculated with hu-PBL plus HBcAg inhibited the binding of labeled anti-HBc to solid-phase-bound HBcAg, while no inhibition of the binding of anti-HBe to HBeAg was observed. Plasma from mice given hu-PBL with HBeAg or hu-PBL alone was devoid of such inhibitory activity.

In the mouse, Milich et al. clearly demonstrated that HBcAg behaves as a T-cell-independent B-cell antigen (10) by the binding and activation of naive B cells through HBcAg (15). To examine whether HBcAg is also able to activate B cells and induce anti-HBc antibody production in a T-cell-independent fashion in humans, we purified human B cells and transferred them into the spleens of conditioned recipient NOD/SCID mice without and with added HBcAg. Only under the latter condition could we detect anti-HBc IgM in the plasma of the recipient mice at days 7 and 14. This experiment suggests that HBcAg is also able to induce anti-HBc IgM production by direct activation of naive B cells in humans. This may explain why during an acute HBV infection, anti-HBc antibodies can be detected so early and in all patients. This unique quality of the core may also explain why, during a chronic HBV infection, anti-HBc IgM levels fluctuate throughout the course of the disease and follow the rise and fall of transaminase activities (inflammation and hepatocellular lysis) (5, 19).

Anti-HBc IgM titers were higher at day 14 than at day 7 when NOD/SCID mice were given hu-PBL, whereas anti-HBc IgM titers were lower at day 14 than at day 7 when purified human B cells were transferred. The latter is most likely due to the limited survival (less than 2 weeks) of purified human B cells in NOD/SCID mice (unpublished observation).

In contrast to the results obtained with nude mice, in which HBcAg administration induced the production of anti-HBc IgG (10), no anti-HBc IgG was found in our hu-PBL chimeras

generated with hu-PBL from naive donors. Despite extended follow-up (up to 3 weeks) and an additional HBcAg booster dose given intravenously on day 8 or 10, no class switch was observed. Observations beyond week 3 have been impossible because at around week 3, the hu-PBL chimeras succumb to severe graft-versus-host disease. Our data suggest that HBcAg is unable to stimulate human B cells further than the production of anti-HBc IgM. Even the abundant bystander help that is observed in our system (23, 24) is not able to promote isotype switching. Whether this process requires HBcAg-specific T-cell help or other cells and signals that are lacking in our model remains unresolved.

Anti-HBc IgG is easily induced and can be detected in the plasma of hu-PBL chimeras inoculated with HBcAg-primed cells. Transfer of hu-PBL from donors who had recovered from an HBV infection or had an ongoing HBV infection into the spleens of conditioned NOD/SCID mice leads to the production of anti-HBc IgG in these animals, even without the concomitant administration of HBcAg. The addition of HBcAg to the hu-PBL inoculum significantly enhanced anti-HBc IgM and IgG production. These results confirm and extend our previous observations (23, 24; unpublished data) that transfer of hu-PBL into optimally conditioned NOD/SCID mice in the absence of nominal antigen induces the production of IgG antibodies to the vast series of antigens to which the cell donor has been exposed. These may be vaccine antigens, such as tetanus toxoid, rubella virus, measles virus, or HBsAg (in HBV vaccinees), or microbial antigens to which the donor has been naturally exposed.

Finally, we demonstrated that naive B cells isolated from two human spleens contained HBcAg-binding fractions of 6.8 and 15%. This result is in line with the data obtained with mice by Lazdina et al. (7) and others (1, 15).

HBcAg may be recognized by the B-cell receptor, like any other classical antigen, or it may be endowed with B-cell superantigen qualities and thus interact with all B cells expressing a certain variable gene family, as suggested for the interaction between HBcAg and naive murine B cells (7). Analysis at the clonal level of the B-cell receptor of a series of HBcAg-binding IgM-producing human B cells may shed light on this question.

In summary, we have shown here that HBcAg is a unique antigen that is capable of inducing the secretion of HBcAg-binding human IgM in unprimed B cells derived from naive human blood donors and cord blood when these are transferred into NOD/SCID mice. In humans as well as in mice, HBcAg behaves as both a T-cell-independent and a T-cell-dependent antigen and clearly has the ability to bind a high number of naive B cells. Anti-HBc IgM production is T cell independent, while isotype switching of IgM to IgG seems to require additional signals. Production of HBcAg-specific IgG only occurs in mice receiving hu-PBL from persons who have recovered from an HBV infection or have an ongoing-chronic HBV infection.

The fact that HBcAg behaves so similarly in both humans (its natural hosts) and mice (not its natural hosts) further supports the notion that a very basic and conserved mechanism of immune recognition and/or signaling is involved (7). How these unique qualities of this structural antigen of HBV influence the course of the disease and if they are involved in the

induction and/or maintenance of chronicity are but a few of the questions that still need to be resolved.

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