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ORIGINAL ARTICLE

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Comparative analysis of HLA restriction and cytokine production in hepatitis B surface antigen-specific T cells from low- and high-antibody responders in vaccinated humans

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Abstract It is well known that individuals with low, or lack of, antibody production in response to hepatitis B surface antigen (HBsAg) exist in the human population. We have previously reported that HLA class I and class II genes are both involved in antibody production to HBsAg, and that specific alleles of HLA are associated with low and high antibody production. To elucidate further the mechanisms by which the diversity of antibody production to HBsAg is generated in humans, a total of 146 T-cell clones specific for HBsAg were produced from six healthy vaccinees (three low- and three high-antibody responders) and were examined for cytokine production and HLA restriction. It was found that the majority of the T-cell clones from the low-antibody responders were Th1- or Th0-like T cells (62% or 19%, respectively), whereas the majority of T-cell clones from the high-antibody responders were Th2-like T cells (77%), suggesting predominant expansion of Th1/Th0- and Th2-like T cells specific for HBsAg in the low- and high-antibody responders, respectively. This is the first evidence that the diversity of the response to HBsAg in humans is controlled by the activation of functionally distinct $CD4^+$ T-cell subsets, i.e., Th0, Th1, or Th2 T cells.

Key words HLA · Polymorphism · Th0, Th1 and Th2 helper T cells · HBsAg · Immune regulation

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Introduction

Hepatitis B virus infection is prevalent worldwide, and causes various types of clinical symptoms, including fulminant, acute, chronic active, and chronic persistent hepatitis; liver cirrhosis; and liver cancer; as well as producing an asymptomatic chronic carrier state. Vaccination with hepatitis B surface antigen (HBsAg) is performed worldwide, especially for hospital employees, because the neutralization of HBsAg by specific antibodies (Abs) is effective in preventing infection after accidental needle-stick exposure to HBsAg-positive blood (Hoofnagle et al. 1979; Itoh et al. 1986; Szmuness et al. 1980; Wainwright et al. 1989; Bocher et al. 1996). However, individual serum levels of HBsAg-specific Ab are diverse, and it has been reported that approximately 5% of vaccinees fail to produce the protective level of Abs to HBsAg after a standard vaccination schedule (Wismans et al. 1988).

We have previously reported that both HLA class I and class II genes are involved in the regulation of HBsAg-specific Ab production in the Japanese population (Mineta et al. 1996). HLA-DRB1*08032, DPA1*0103, DPB1*0402, DPB1*0202, and DPB1*1301 contribute positively to the anti-HBsAg antibody response, and HLA-A*2602, B70, DRB1*0405, DRB1*1101, and DQB1*0302 contribute negatively to the response. However, the combined correlation coefficient of the entire HLA gene family with HBsAg-specific Ab production is 0.50 by multiple regression analysis, indicating that not only the HLA multigene family but also other factor(s) significantly influence the immune response to HBsAg. In addition, we also found that even low-Ab responders showed HBsAg-specific T-cell proliferation in vitro in many instances (Mineta et al. 1996; Min et al. 1996). These observations tempted us to investigate cellular mechanisms to determine the diversity of the Ab response to this Ag in humans.

In the present study, we obtained peripheral blood mononuclear cells (PBMCs) from 92 vaccinees and analyzed the phenotypes after incubation of these cells with HBsAg in vitro, in terms of CD4, CD8, and CD56, and we investigated the association of Ab titer with these cell populations by flow cytometry. Because no specific change in cellular population was observed for low; or high-Ab responders by stimulation with HBsAg, we established a large number of HBsAg-specific T-cell clones from six healthy vaccinees (both low- and high-Ab responders), and we analyzed their cytokine production and determined HLA restriction. We report that HBsAg-specific T-cell clones obtained from low-Ab responders showed $CD4^+$ and Th1- or Th0-like cytokine production, whereas those from high-Ab responders showed $CD4^+$ and Th2-like cytokine production, providing evidence that the diversity of the Ab response to HBsAg in humans is regulated by the activation of functionally distinct $CD4^+$ T-cell subsets, i.e., Th0, Th1, or Th2 T cells.

Subjects and methods

Vaccination and radioimmunoassay (RIA)

After informed consent was obtained from each subject, i.e., 92 healthy Japanese medical students, they were immunized subcutaneously with 20 μg of recombinant (r)HBsAg vaccine (Derived Yeast, Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) three times, at 0, 4, and 24 weeks, respectively. Peripheral blood was collected from each subject 2 months after the last vaccination for the measurement of serum antibody titer, T-cell proliferation assay in vitro, and flow cytometry. Anti-HBsAg Ab titer was measured by RIA, using a commercial test system (Ausab; Abbott Laboratories, North Chicago, IL, USA).

For generating T-cell clones specific for HBsAg, six volunteers (three low- and three high-Ab responders, based on Ab titer), who had received their last vaccination 3 to 9 years ago, were revaccinated subcutaneously with 20 μg of HBsAg vaccine. Peripheral blood was collected from each of these subjects before and 4 weeks after the revaccination for the measurement of serum antibody titer, T-cell proliferation assay in vitro, and flow cytometry.

HLA typing

DNA was extracted from peripheral granulocytes of each of the six volunteers (donors) by the standard sodium-dodecylsulfate (SDS)-proteinase K digestion/phenol-chloroform extraction method. All subjects were genotyped for HLA class I (HLA-A) and class II (HLA-DRB1, DQA1, DQB1, DPA1, and DPB1) by the polymerase chain reaction (PCR)-single strand oligonucleotide probe (SSOP) method (Kimura et al. 1992; Date et al. 1996; Kimura and Sasazuki 1992). Briefly, 1 μg of genomic DNA was amplified for HLA genes with primers designed to specifically amplify each HLA gene, by 30 cycles of PCR in a thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA) with Taq DNA polymerase (Takara Taq; Takara, Kyoto, Japan). PCR products were hybridized with ^{32}P-labeled SSOP by dotblot hybridization. The HLA-B locus of HLA class I was serotyped using the microcytotoxicity test (Terasaki and McClelland 1964). The HLA DNA types of the six donors are shown in Table 1.

Generation of T-cell clones

The PBMCs from the fresh heparinized blood of each donor were separated by density gradient centrifugation, over Ficoll-Hypaque (Pharmacia, Uppsalia, Sweden). The PBMCs (1×10^5) were cultured in a 96-well round-bottomed plate (Corning, New York, NY, USA) with 10 μg/ml of recombinant HBsAg (rHBsAg) (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) in 200 μl of RPMI-1640 medium (Life Technologies, Grand Island, NY, USA), supplemented with 10% pooled human male serum, 2% l-glutamine (Life Technologies), and 1% penicillin-streptomycin (Life Technologies)(termed complete medium), at 37°C in a humidified 5% CO2/air atmosphere. Viable cells were collected by density gradient centrifugation, and then 0.5×10^6 responding cells were restimulated with irradiated (30 Gy) 2.5×10^6 autologous PBMCs prepulsed with 40 μg/ml of rHBsAg (HBsAg-pulsed irradiated auto PBMCs) in the presence of 100 U/ml of recombinant interleukin-2 (rIL-2) (Ajinomoto, Kobe, Japan). After two additional stimulations, a single cell was picked up, under microscopy, from among the responding cells, for further cultivation with 5×10^4 HBsAg-pulsed irradiated auto PBMCs. T-cell clones were restimulated every week

Table 1. HLA types of six donors

Donor	HLA type						
	HLA-A	HLA-B	HLA-DRB1	HLA-DQA1	HLA-DQB1	HLA-DPA1	HLA-DPB1
Donor A	0206/2401	52/39	0401/0901	0302/0302	0301/03032	0103/0201	0201/0501
Donor B	0201/0206	52/15	1502/0901	0103/0302	03032/06011	0201/02022	0901/0501
Donor C	1101/0201	39/35	0901/0901	0302/0302	03032/03032	0103/02022	0402/0501
Donor D	0206/3101	51/7	0101/0901	0302/0101	03032/0501	0103/02022	0201/0501
Donor E	2402/2407	62/35	0405/0901	0302/0302	03032/0401	0103/0201	0201/0901
Donor F	0206/2402	52/40	1502/0901	0103/0302	06011/03032	0103/0201	0201/0901

HLA-A, DRB1, DQA1, DQB1, DPA1, and DPB1 were genotyped by the polymerase chain reaction (PCR)-single strand oligonucleotide probe (SSOP) method. HLA-B was serotyped by the microcytotoxicity test

with HBsAg-pulsed irradiated auto PBMCs, four or five times, and then frozen until the assays were carried out.

T-cell proliferation assay

PBMCs (1×10^5 cells/well) were incubated in a round-bottomed 96-well plate in 200 μl of complete medium in the presence or absence of 5 μg/ml rHBsAg, or with 5 μg/ml purified protein derivatives (PPD) (Nihon BCG Seizo, Tokyo, Japan) for 1 week at 37°C in a humidified 5% CO2/air atmosphere. Twelve h before being harvested, cells were pulsed with 1 μCi (6.7 Ci/mmol) of [^{3}H]thymidine (ICN Biomedicals, Costa Mesa, CA, USA). Subsequently, cells were collected onto glass fiber filters (Wallac, Turku, Finland), using an automatic cell collecter (Wallac), and the incorporated radioactivity was quantified with a liquid scintillation counter (Wallac). Results were expressed as mean counts per min (Δ cpm) in triplicate cultures. The value for the background proliferation in the absence of antigen was subtracted. The stimulation index (SI) was obtained by dividing the mean cpm with HBsAg stimulation by that without stimulation. To assess the proliferative response of T-cell clones to HBsAg, 1×10^4 cells of each clone were incubated with 1×10^5 HBsAg-pulsed or non-pulsed irradiated auto PBMCs in 200 μl of complete medium, and proliferation was assayed by [^{3}H]thymidine uptake, as described above. The SI was obtained by dividing the mean cpm to HBsAg-pulsed irradiated auto PBMCs by that to non-pulsed irradiated auto PBMCs.

Flow cytometry and antibodies

Cells (1×10^4–1×10^5) were stained with fluorescein isothiocyanate (FITC) and phycoerythrin (PE)-conjugated or biotinylated monoclonal antibodies (MAbs) for 30 min at 4°C in phosphate-buffered saline (PBS) supplemented with 2% fetal calf serum (FCS) and 0.1% azide (FACS buffer). Then, the samples were washed three times with FACS buffer, resuspended, and analyzed on FACScan (Beckton Dickinson, San San Jose, CA, USA), using CellQuest software (Beckton Dickinson). The following MAbs were used for analysis: anti-CD4-PE or anti-CD4-biotin (Beckton Dickinson); anti-CD8-FITC or anti-CD8-biotin (Beckton Dickinson); anti-CD25-FITC (Pharmingen, San Diego, CA, USA) or anti-CD25-PE (Beckton Dickinson); anti-CD30-FITC (Pharmingen), anti-CD95 (Fas)-biotin (Pharmingen), and anti-Fas Ligand-biotin (Pharmingen); and anti-CD56-PE (Beckton Dickinson) and anti-TCR-αβ-FITC (Beckton Dickinson). Streptoavidin-R-PE (GIBCO BRL, Gaithersburg, MD, USA) was used to detect biotinylated reagents.

HLA restriction of antigen recognition

The HLA restriction of the HBsAg-specific T-cell clones was evaluated by adding MAbs against human HLA class II molecules or by using allogenic PBMCs (HLA matched or mismatched donors) as antigen-presenting cells (APCs) in the T-cell proliferation assay. The MAbs used were L243 (anti-HLA-DR), Leu 10 (anti-HLA-DP), and B7/21 (anti-HLA-DQ). All were purchased from Beckton Dickinson. HBsAg-pulsed irradiated auto PBMCs (1×10^5 cells) were incubated with MAbs for 1 h at 37°C, and then T-cell clones (1×10^4 cells) were added to the culture. In the experiment using allogenic PBMCs as APCs, 1×10^4 T cell clones were cultured with irradiated (30 Gy) allogenic PBMCs prepulsed with 40 μg/ml of rHBsAg. All culture was carried out in 200 μl of complete medium. Proliferation was assayed by [^{3}H]thymidine incorporation, as described above.

Enzyme-linked immunosorbent assay (ELISA)

T-cell clones (4×10^4 cells) were cultured with 1×10^5 HBsAg-pulsed or non-pulsed irradiated auto PBMCs, or with 1×10^5 irradiated auto PBMCs in the presence of 10 μg/ml p-phytohemayglutinin (PHA). After 24-h incubation, culture supernatants were collected and stored at −80°C. The concentrations of IL-4, interferon (IFN)-γ, IL-2, and IL-10 in the supernatants were quantified by ELISA, using commercially available human cytokine kits (Endogen, Woburn, MA, USA).

Results

Antibody titers and phenotypes of PBMCs in 92 vaccinees

Four weeks after the final immunization with HBsAg, serum and PBMCs of each subject were separated to examine the anti-HBsAg Ab titer, proliferative T-cell response to HBsAg, and the cellular population in PBMCs. Even low-Ab responders showed a proliferative T-cell response to HBsAg (Fig. 1). The PBMCs were incubated with or without HBsAg in vitro and the phenotypes of the proliferated cells were analyzed by flow cytometry, using anti-CD4, anti-CD8, and anti-CD56 MAbs. No clear correlation between Ab titer to HBsAg and the phenotype of the major population in PBMCs was observed, suggesting that the frequency of T-cells involved in regulating Ab production was not large even in the vaccinated subjects.

Generation of T-cell clones specific for HBsAg from low- and high-Ab responders

For further analyses of T cells in low-Ab responders, three low- and three high-Ab responders were revaccinated with HBsAg, and T-cell clones specific for HBsAg were obtained from their PBMCs. Three donors described as low-Ab responders showed less than 36 mIU/ml of anti-HBsAg Ab both before and after revaccination (Fig. 2A; donors A, B, and C). However, T-cell responses to HBsAg in vitro were observed in these donors (Fig. 2B; donors A, B, and C), indicating that the *Ir* gene defect hypothesis does not fit the mechanism of low-Ab response in these subjects. On the other hand, the three donors described as high-Ab responders showed between 52 and 602 mIU/ml of anti-HBsAg Ab before revaccination, and more than 7823 mIU/ml after

Fig. 1. Anti-hepatitis B surface antigen (*HBsAg*) antibody titer, proliferative T-cell response, and phenotype change in peripheral blood mononuclear cells (PBMCs) by stimulation with HBsAg in vitro. Ninety-two healthy volunteers were vaccinated with recominant (r)HBsAg, and antibody and T-cell proliferative response to HBsAg were analyzed as described in the text. PBMCs of each subject were cultured in the presence or absence of rHBsAg and doubly stained with anti-CD25 and the indicated antibody (Ab) (anti-CD56, anti-CD4, or anti-CD8). Only CD25-positive cells were analyzed for the expression of the indicated cell surface marker by flow cytometry. Data values presented (for Δ CD56, Δ CD4, and Δ CD8) were calculated by subtracting the proportion of the indicated marker in the absence of Ag from that in the presence of Ag

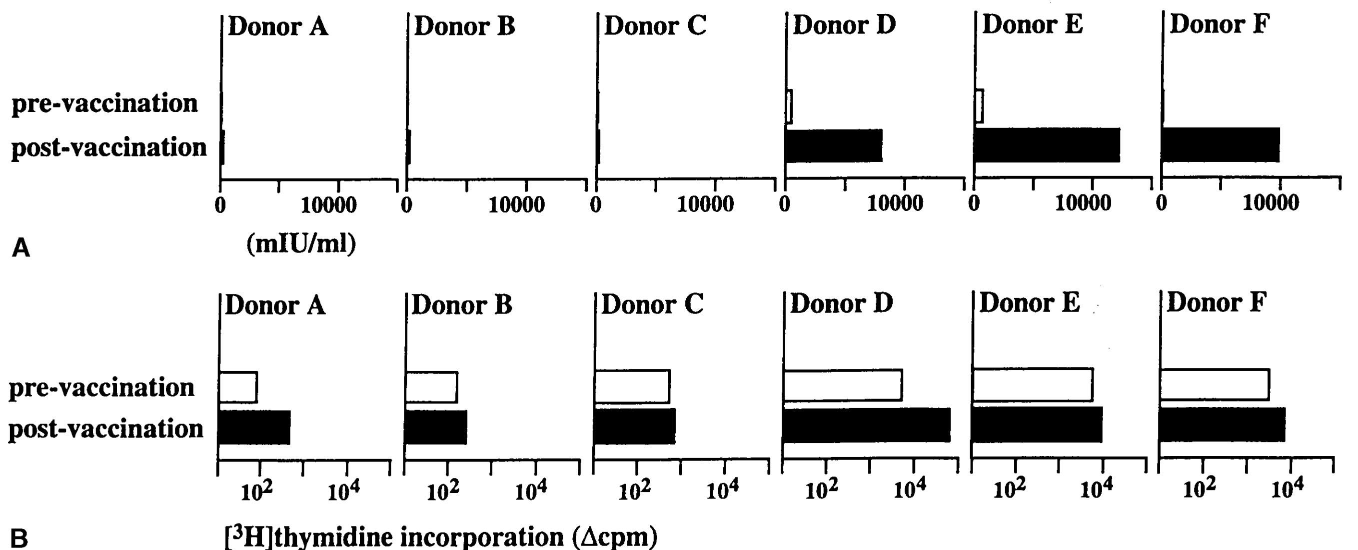

Fig. 2. **A** Anti-HBsAg antibody titer and **B** proliferative T-cell response in six subjects (three low-Ab responders [donors A, B, and C] and three high-Ab responders [donors D, E, and F]). *Open bars*, Antibody titer or T-cell proliferation pre-vaccination; *solid bars*, antibody titer or T-cell proliferation post-vaccination

revaccination (Fig. 2A; donors D, E, and F). The T-cell phenotypes in PBMCs were analyzed by flow cytometry. However, no significant difference in the proportions of $CD4^+$, $CD8^+$, $CD25^+$, $FasL^+$, or $CD30^+$ T cells was observed between low- and high-Ab responders, even though cells were cultured with rHBsAg (data not shown).

A total of 146 HBsAg specific T-cell clones were established. All clones showed proliferative responses to HBsAg (SI, ≥2.0) and also to PHA (data not shown). To determine the phenotypes of these clones, they were subjected to flow cytometry, using anti-CD4, anti-CD8, anti-CD56, and anti-TCRαβ Abs. All T-cell clones were $CD56^-$, $TCR\alpha\beta^+$, and $CD4^+$, except for 16 $CD8^+$ T-cell clones obtained from donor B (data not shown), indicating that the major fraction of HBsAg-specific T cells is the conventional $CD4^+$ TCRαβ T cell. The $CD8^+$ T-cell clones did not show cytotoxic activity to HBsAg-pulsed Epstein-Barr virus-transformed B cells (data not shown). Therefore, we focused on $CD4^+$ T-cell clones for further analyses.

Cytokine production of T-cell clones specific for HBsAg

All established $CD4^+$ T-cell clones were tested for cytokine production in terms of IL-2, IFN-γ, IL-4, and IL-10, by stimulation with HBsAg or PHA. The profiles of IL-4 and IFN-γ production of all T-cell clones are presented in Fig. 3. Th1-like cells were defined as cells secreting less than 6 U/ml of IL-4 and more than 90 U/ml of IFN-γ with HBsAg stimulation, and less than 26 U/ml of IL-4 and more than

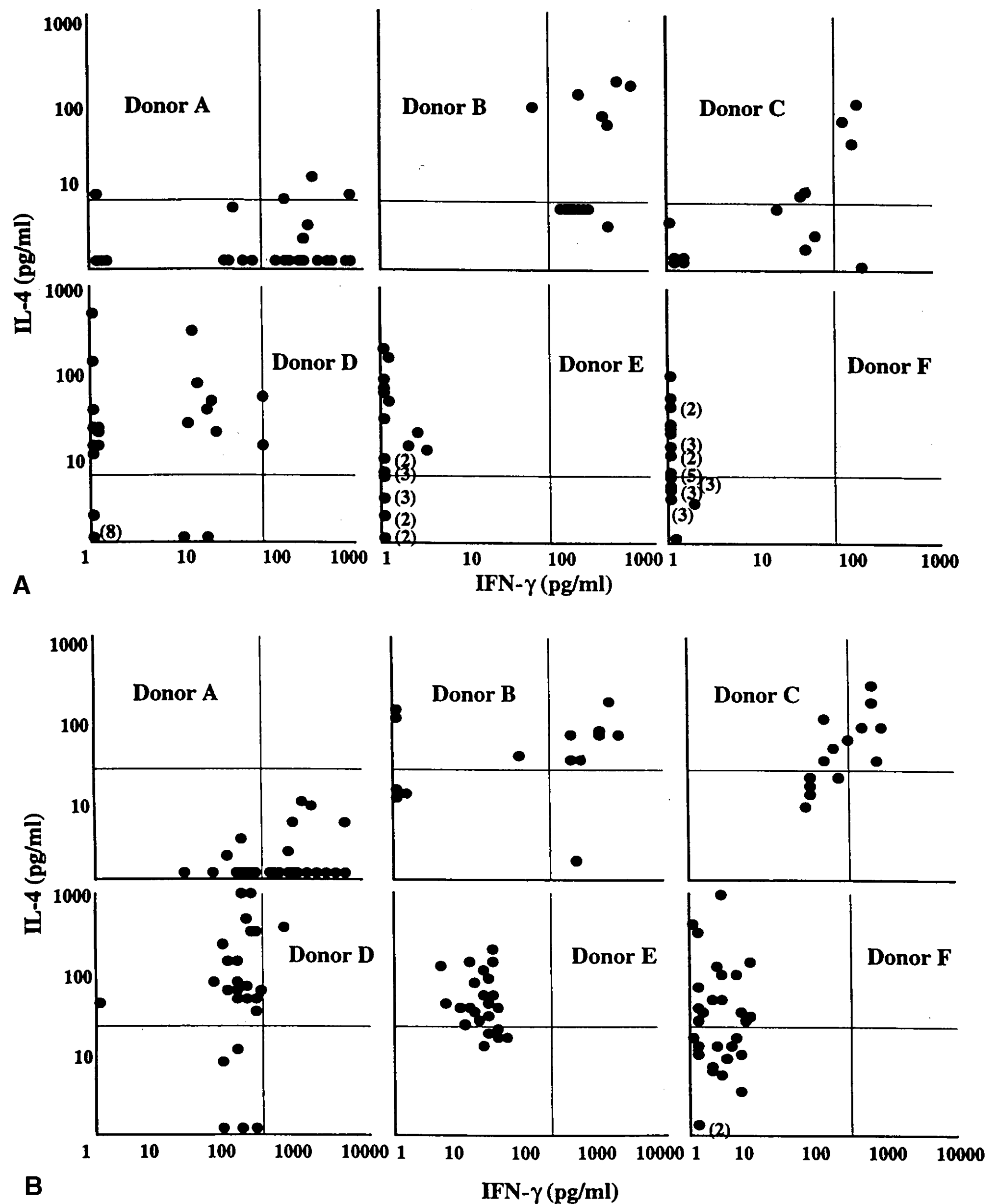

Fig. 3A,B. Cytokine production profiles of T-cell clones obtained from six subjects. A total of 130 T-cell clones were analyzed. T-cell clones were stimulated with **A** HBsAg and **B** phytohemaglutinin (*PHA*) in vitro, and the concentrations of interleukin (*IL*)-4 and γ-interferon (*IFN*) in the supernatant were measured by enzyme-linked immunosorbent assays (ELISAs) as described in the text. *Borderlines* for defining Th cell type are indicated. *Numbers in parentheses* indicate the numbers of clones that showed the same levels of cytokine production

344 U/ml of IFN-γ with PHA stimulation. Th2-like cells were defined as cells secreting less than 90 U/ml of IFN-γ and more than 6 U/ml of IL-4 with HBsAg stimulation, and less than 344 U/ml of IFN-γ and more than 26 U/ml of IL-4 with PHA stimulation. Th0-like cells were defined as cells secreting more than 6 U/ml of IL-4 and 90 U/ml of IFN-γ with HBsAg stimulation, and more than 26 U/ml of IL-4 and 344 U/ml of IFN-γ with PHA stimulation. With this definition, it appeared that the majority of T-cell clones from low-Ab responders secreted large amounts of IFN-γ or both IFN-γ and IL-4, resembling Th1- or Th0-like T cells, respectively. Sixty-two percent (32/52) of the T-cell clones were Th1-like T cells, and 19% (10/52) of the clones were Th0-like T cells, whereas the proportion of Th2-like T cells was only 8% (4/52). On the other hand, the majority of T-cell clones from high-Ab responders showed predominant secretion of IL-4. Seventy-seven percent (60/78) of the T-cell clones were Th2-like T cells, whereas only 1 clone showed Th1- or Th0-like cytokine production. These results suggest predominant expansion of HBsAg-specific Th1- or Th0-like T cells in the PBMCs of low-Ab responders. On the other hand, the largest fraction of HBsAg-specific T cells in the PBMCs of high-Ab responders was Th2-like T cells.

HLA restriction of the T-cell clones specific for HBsAg

The HLA restriction of the $CD4^+$ T-cell clones was examined either by blocking experiments with MAbs (anti-DR Ab, anti-DP Ab, and anti-DQ Ab) or by using HLA matched or mismatched allogenic PBMCs as APCs in proliferative assays. As shown in Table 2, in donor A, 19 clones were restricted to the DR molecule (DR4, DR9 or DR53) and 4 clones were restricted to the DP molecule (DP2). In donor F, 27 clones were restricted to the DR molecule (DR9 and DR15) and 2 clones were restricted to the DP molecule. This result indicated that HLA-DR was the major restriction molecule for T-cell clones from donors A and F. The restriction molecules of the T-cell clones from the other donors were determined by the same method, and the results are summarized, together with findings on T-helper subtypes, in Table 3. Based on our previous findings (Mineta et al. 1996), the HLA restriction of these T-cell clones was classified into four types; low-, high-, and neutral-HLA type restricted T-cells, and unknown type. Among the 52 clones from the low-Ab responders, 7 clones were low-HLA type restricted, 40 clones were neutral-HLA type restricted, and the restriction molecule of 5 clones was not known. On the other hand, among the 78 clones from the high-Ab responders, 17 clones were low-HLA type restricted, 59 clones were neutral-HLA type restricted, and the restriction molecule of 2 clones was not known.

Discussion

It is well known that there are individuals with low, or lack of, antibody production in the response to HBsAg in humans (Chiou et al. 1988; Varla-Leftherioti et al. 1990; Desombere et al. 1995; Egea et al. 1991; Kruskall et al. 1992; Watanabe et al. 1988; Hatae et al. 1992). The mechanisms of low responsiveness remain unclear, although several studies have revealed evidence relevant to the mechanisms. The absence of dominant immune response genes (Alper et al. 1989) or the presence of dominant immune suppression genes located in the MHC region (Watanabe et al. 1988; Hatae et al. 1992); defect of T-cell-APC interaction (Milich et al. 1984); selective killing of HBsAg-specific B cells by MHC-restricted cytotoxic T-lymphocytes (CTL) (Barnaba et al. 1990); imbalanced Th1/Th2 response to HBsAg (Vingerhoests et al. 1994; Hsu et al. 1996; Honorati et al. 1997; Bocher et al. 1999); and the elimination of HBsAg-specific T cells during thymic or postthymic repertoire maturation (Wismans et al. 1988) are mechanisms that have been proposed. Because the human population is largely outbred and carries varied HLA antigens, reflecting the marked polymorphism of the immune system, and because the population is exposed to diverse antigens, it is not easy to simply uncover the mechanisms of non/low responsiveness.

In our present study, the level of T-cell response in low-Ab responders was lower than that in the high-Ab responders. This may be explained by the absence of *Ir* genes, by a defect of T-cell-APC interaction, and/or by the elimination of HBsAg-specific T cells during repertoire selection in the low-Ab responders. However, T-cell responses specific for HBsAg observed in the low-Ab responders were significant, indicating that other mechanisms than those mentioned above exist and generate low-Ab responsiveness.

It has been demonstrated that CTL selectively killed HBsAg-specific B cells (Barnaba et al. 1990, and, accordingly, we examined a possible correlation between the level of Ab specific for HBsAg and the proportion of $CD8^+$ T cells in PBMCs incubated with HBsAg in vitro. Because no specific increase in the population of $CD8^+$ cells was observed in low-Ab responders and because the majority of HBsAg-specific T-cell clones from low-Ab responders were $CD4^+$, we dare to conclude that $CD8^+$ T cells do not have critical roles in low-Ab responsiveness to HBsAg, at least in the subjects analyzed.

The immune systems have been characterized as having two distinct types of helper T cells, Th1 and Th2 (Mosmann et al. 1986; Wierenga et al. 1991; reviewed in Romagnani 1991). The hallmark cytokine of Th1 cells is IFN-γ, and Th1 cells also produce IL-2, tumor necrosis factor (TNF), and leukotriene (LT), cytokines that mediate delayed-type hypersensitivity responses and macrophage activation. The signature cytokine of Th2 cells is IL-4, and Th2 cells also secrete IL-5, IL-9, IL-10, and IL-13, cytokines that provide help to B cells and are critical in the allergic response (Arthur and Mason 1986; Paliard et al. 1988; reviewed in Mosmann and Coffman 1989; reviewed in Paul and Seder 1994). Because of small sample sizes and/or differences in subjects (i.e., healthy volunteers or hepatitis patients) , it has been unclear whether the skewing of the immune response toward Th1 or Th2 is involved in the regulation of Ab production to HBsAg in humans. In the present study,

Table 2. Restriction molecules of T-cell clones obtained from donor A and donor F

Donor A	Proliferative response (Δ cpm)[a] [%][b]					
	Allo[d] PBMCs used as APCs		Antibodies used for blocking			
Clone	Allo 1[c]	Allo 2[c]	Anti-DR	Anti-DP	Anti-DQ	Restriction molecule
1	9,559 [22.2]	27,651 [64.2]	345 [0.8]	6,060 [14.1]	38,615 [89.7]	DR53
2	2,580 [41.8]	397 [6.4]	2,365 [38.3]	1,381 [22.4]	2,537 [41.1]	DP2
3	15,142 [93.5]	195 [1.2]	8,980 [55.4]	1,853 [11.4]	1,920 [11.9]	DQ3/DP2
4	578 [4.1]	1,458 [10.2]	10,012 [70.4]	1,552 [10.9]	13,319 [93.6]	DP5
5	9,570 [62.2]	1,130 [7.3]	174 [1.1]	2,790 [18.1]	7,222 [47.0]	DR9
6	58,407 [74.2]	168 [0.2]	25,155 [31.9]	8,665 [11.0]	65,780 [83.5]	DP2
7	5,262 [6.9]	41,606 [54.8]	467 [0.6]	16,588 [21.8]	57,389 [75.6]	DR4
8	12,753 [48.2]	12,049 [45.6]	3,789 [14.3]	7,848 [29.7]	23,380 [88.4]	DR53
9	4,921 [7.0]	39,491 [56.1]	10,784 [15.3]	6,571 [9.3]	47,537 [67.6]	DP
10	30,581 [455]	62,949 [93.6]	33,675 [50.1]	21,964 [32.7]	65,366 [97.2]	DR53
11	51,757 [83.6]	358 [0.6]	38,171 [61.6]	26,141 [42.2]	60,753 [98.1]	DR9
12	31,054 [101.1]	139 [0.5]	13,054 [42.5]	23,508 [76.6]	25,579 [83.3]	DR9
13	39,610 [83.6]	134 [0.3]	128 [0.3]	33,163 [70.0]	31,788 [67.1]	DR9
14	32,126 [55.1]	327 [0.6]	307 [0.5]	40,337 [69.2]	62,380 [107.1]	DR9
15	28,537 [39.3]	46,513 [64.1]	287 [0.4]	35,290 [48.6]	68,151 [93.9]	DR53
16	12,019 [52.6]	223 [1.0]	131 [0.6]	4,851 [21.2]	20,234 [88.6]	DR9
17	142 [0.3]	24,155 [47.9]	118 [0.2]	17,100 [33.9]	43,190 [85.6]	DR4
18	30,902 [44.2]	57,460 [82.2]	1,065 [1.5]	28,395 [40.6]	56,240 [80.5]	DR53
19	1,355 [29.2]	3,490 [75.3]	115 [2.5]	2,022 [43.6]	2,425 [52.3]	DR4
20	11,322 [38.4]	26,901 [91.2]	332 [1.1]	27,539 [93.4]	38,568 [130.9]	DR53
21	35,738 [50.2]	54,722 [76.8]	1,906 [2.7]	63,356 [88.9]	82,302 [115.6]	DR53
22	8,053 [17.6]	35,429 [77.4]	370 [0.8]	27,425 [59.9]	46,322 [101.2]	DR4
23	290 [0.5]	39,205 [68.7]	152 [0.3]	14,771 [25.9]	32,919 [57.7]	DR4
24	431 [5.6]	1,186 [15.6]	135 [1.8]	1,534 [20.2]	9,482 [124.4]	DR4
Donor F Clone	Allo 3[c]	Allo 4[c]	Anti-DR	Anti-DP	Anti-DQ	Restriction molecule
1	28,280 [74.3]	141 [0.4]	9,025 [23.7]	21,104 [55.4]	40,343 [105.9]	DR9
2	24,743 [44.7]	42 [0.1]	16,807 [30.4]	26,303 [47.5]	24,342 [44.0]	DR9
3	26,117 [185.1]	175 [1.2]	2,905 [20.6]	6,392 [45.3]	10,370 [73.5]	DR9
4	16,276 [82.8]	217 [1.1]	3,659 [18.6]	10,500 [53.4]	15,732 [80.0]	DR9
5	4,464 [107.1]	69 [1.7]	180 [4.3]	968 [23.2]	3,141 [75.3]	DR9
6	24,671 [91.6]	237 [0.9]	7,941 [29.5]	15,679 [58.2]	18,231 [67.7]	DR9
7	538 [6.6]	315 [3.9]	6,981 [86.0]	363 [4.5]	7,220 [88.9]	DP
8	383 [10.5]	13,167 [361.3]	115 [3.2]	1,030 [28.3]	2,561 [70.3]	DR15
9	123 [0.2]	18,340 [33.4]	2,849 [5.2]	26,404 [48.1]	34,687 [68.2]	DR15
10	171 [1.6]	653 [6.2]	2,674 [25.4]	142 [1.4]	3,988 [37.9]	DP
11	3,237 [139.9]	4 [0.2]	2,213 [95.6]	553 [23.9]	1,771 [76.5]	DR9
12	14,633 [127.0]	210 [1.8]	2,213 [19.2]	7,102 [61.7]	13,151 [114.2]	DR9
13	5,836 [144.6]	21 [0.5]	342 [8.5]	1,700 [42.1]	4,739 [117.4]	DR9
14	22,347 [247.4]	173 [1.9]	956 [10.6]	5,591 [61.9]	9,041 [100.1]	DR9
15	32,825 [163.3]	92 [0.5]	2,545 [12.7]	10,845 [54.0]	17,000 [84.6]	DR9
16	42,511 [280.8]	114 [0.8]	3,337 [22.0]	6,647 [43.9]	13,300 [87.9]	DR9
17	14,104 [268.1]	260 [4.9]	825 [15.7]	2,999 [57.0]	5,674 [107.9]	DR9
18	14,762 [378.3]	179 [4.6]	548 [14.0]	1,139 [29.2]	3,581 [91.8]	DR9
19	18,224 [204.9]	233 [2.6]	788 [8.9]	4,484 [50.4]	8,393 [94.3]	DR9
20	49,122 [202.3]	245 [1.0]	3,632 [15.0]	19,320 [79.6]	27,019 [111.3]	DR9
21	19,363 [234.0]	320 [3.9]	865 [10.5]	4,033 [48.7]	6,560 [79.3]	DR9
22	12,351 [564.5]	194 [8.9]	190 [8.7]	1,148 [52.5]	1,848 [84.5]	DR9
23	6,482 [252.4]	185 [7.2]	575 [22.4]	1,034 [40.3]	2,288 [89.1]	DR9
24	53,396 [343.2]	230 [1.5]	3,401 [21.9]	8,097 [52.1]	14,714 [94.6]	DR9
25	16,111 [174.1]	219 [2.4]	2,514 [27.2]	5,660 [61.2]	15,774 [170.4]	DR9
26	35,809 [180.2]	173 [0.9]	2,147 [10.8]	9,630 [48.4]	19,298 [97.1]	DR9
27	5,881 [124.6]	1,276 [27.0]	1,467 [31.1]	2,874 [60.9]	5,477 [116.1]	DR9
28	3,680 [239.1]	163 [10.6]	350 [22.7]	776 [50.4]	1,000 [65.0]	DR9
29	44,893 [372.3]	142 [1.2]	4,973 [41.2]	10,784 [89.4]	16,857 [140.0]	DR9

[a] Δ cpm was calculated by subtracting the count in the absence of hepatitis B surface antigen (HBsAg) from that in the presence of Ag in the experiment using allo peripheral blood mononuclear cells (PBMCs) as antigen-presenting cells (APCs). In the blocking experiment, Δ cpm was calculated by subtracting the count in the presence of blocking antibody (Ab) from that in the absence of Ab in the condition with HBsAg
[b] Percent response in the experiment using allo PBMCs as APCs was calculated by dividing the Δ cpm, shown in this table, by that using auto PBMCs as APCs. Percent response in the blocking experiment was calculated by dividing Δ cpm, shown in this table, by Δ cpm calculated by subtracting the count in the absence of HBsAg from that in the presence of Ag in the condition without blocking Ab
[c] The DNA types of HLA class II of allo PBMCs are: allo 1, HLA-DRB1*0405/0901, -DQA1*0302/0302, -DQB1*03032/0401, -DPA1*0103/0103, -DPB1*0201/0402; allo 2, HLA-DRB1*0401/1101, -DQB1*0301/0301, -DPB1*1401/1401 (DPA1 and DQA1 are not genotyped); allo 3, HLA-DRB1*0405/0901, -DQA1*0302/0302, -DQB1*0401/03032, -DPA1*0103/0103, -DPB1*0201/0402; allo 4, HLA-DRB1*1101/1502, -DQA1*0103/05013, -DQB1*0301/06011, -DPA1*02022/0201, -DPB1*0901/1401
[d] For explanation of the terms "allo" and "auto" PBMCs, see text

Table 3. Summary of restriction molecule and T-helper (Th) type of T-cell clones obtained from six donors

Donor A			Donor B			Donor D			Donor E			Donor F		
Clone	Restriction molecule	Th	Clone	Restriction molecule	Th	Clone	Restriction molecule	Th	Clone	Restriction molecule	Th	Clone	Restriction molecule	Th
1	DR53	Th1	1	DR	Th1	1	DP2	Th2[a]	1	DR4	Th2	1	DR9	ND
2	DP2	Th1	2	DP	Th1	2	DP2	Th1[a]	2	DQ3	Th2	2	DR9	Th2
3	DQ3/DP2	Th1	3	DR	Th2	3	DP2	Th2	3	DR4	ND	3	DR9	Th2
4	DP5	Th1	4	DP	Th1	4	DP2	Th2	4	DR4	Th2	4	DR9	Th2
5	DR9	Th1	5	DP	Th1	5	DP2	Th2[a]	5	DR4	ND	5	DR9	ND
6	DP2	Th1	6	DR	Th0	6	DP2	Th2[a]	6	DR9	Th2	6	DR9	Th2
7	DR4	Th1	7	DR	Th0	7	DR1	Th2[a]	7	DR9	Th2[a]	7	DP	Th2
8	DR53	Th1	8	DR	Th0	8	DP2	Th2[a]	8	DR4	Th2[a]	8	DR15	ND
9	DP	Th1	9	DP	Th1	9	DP2	Th2	9	DR4	Th2[a]	9	DR15	Th2[a]
10	DR53	Th0	10	DQ	Th1	10	DP2	Th2[a]	10	DR4	Th2[a]	10	DP	Th2
11	DR9	Th1	11	DQ	Th1	11	DP2	Th2[a]	11	DR4	Th2[a]	11	DR9	Th2
12	DR9	Th1	12	DP	Th1	12	DP2	Th2	12	DR4	Th2[a]	12	DR9	Th2
13	DR9	Th1[a]	13	DR	Th0	13	DR9	Th0	13	DR9	Th2[a]	13	DR9	Th2
14	DR9	Th1	14	DR	Th0	14	DP2	Th2	14	DR4	ND	14	DR9	ND
15	DR53	Th1				15	DP2	Th2	15	DR4	Th2	15	DR9	Th2
16	DR9	Th1				16	DP2	Th2	16	DQ3	Th2	16	DR9	Th2[a]
17	DR4	Th1	Donor C			17	DP2	Th2[a]	17	DR4	ND	17	DR9	Th2
18	DR53	Th1	Clone	Restriction molecule	Th	18	DP2	Th2	18	DP2	Th2	18	DR9	ND
19	DR4	Th1				19	DP2	Th2	19	DP	Th2[a]	19	DR9	ND
20	DR53	Th1				20	DP2	Th2	20	DR4	Th2	20	DR9	ND
21	DR53	Th1	1	DR53	Th2	21	DP2	Th2	21	DP2	Th2	21	DR9	ND
22	DR4	Th1[a]	2	DR53	Th0	22	DP2	Th2	22	DR4	Th2[a]	22	DR9	Th2[a]
23	DR4	Th1[a]	3	DP	Th2[a]	23	DR1	Th2[a]	23	DR4	Th2	23	DR9	ND
24	DR4	Th1[a]	4	DR53	Th0	24	DP2	Th2				24	DR9	ND
			5	DR9	Th0	25	DP2	Th2				25	DR9	ND
			6	DR53	Th1	26	DP2	Th2				26	DR9	Th2[a]
			7	DR9	Th2[a]							27	DR9	Th2[a]
			8	DR9	Th0[a]							28	DR9	ND
			9	DR9	ND							29	DR9	Th2[a]
			10	DQ3	ND									
			11	DP	ND									
			12	DR9	ND									
			13	DQ3	ND									
			14	DQ	ND									

T-helper cell types of $CD4^+$ T-cell clones were defined by interleukin (IL)-4 and γ-interferon (IFN)-secreting profile against HBsAg or phytohemaglutinin (PHA), [a]shown in Fig. 3
ND, Not defined

large numbers of T-cell clones specific for HBsAg were analyzed, and it was found that, in vaccinated humans, Th1- or Th0-like T cells were predominantly expanded in low-Ab responders, whereas Th2-like T cells were expanded in high-Ab responders. Therefore, our finding indicates that one of the underlying causes of low responsiveness is the lack of a Th2-like response.

One explanation for the selective activation of a distinct $CD4^+$ T-cell subset by HBsAg vaccination in humans may be provided by the notion that the HLA class II associated with low- or high-Ab responsiveness presents particular peptide antigens to Th0-type T cells and induces them to differentiate into Th1- or Th2-like T cells, respectively. Because the sample size in the present study was too small for statistical analysis, this notion should be tested in future experiments in which much larger numbers of low- and high-Ab responders are analyzed.

We previously reported that both HLA class I and HLA class II genes were dynamically involved in Ab production to HBsAg in vaccinated humans (Kruskall et al. 1992; Hatae et al. 1992; Watanabe et al. 1990; Mineta et al. 1996). However, the correlation coefficient for the presence of the entire HLA gene family was assumed to be 0.5, suggesting that other genetic or environmental factors are involved in the Ab production (Mineta et al. 1996). It has been reported that, in vivo, the priming of Th1 or Th2 cells may be influenced by a number of other factors, including APCs or the cytokine milieu in the lymphoid tissue at the site of inoculation (reviewed in O'Garra and Murphy 1994; Gajewski et al. 1991). In addition, several transcription factors, including T-bet, NFAT, c-Maf, and GATA-3, have been shown to regulate the differentiation of Th0 into Th1/Th2 (reviewed in Glimcher and Murphy 2000). Therefore, polymorphism in these factors may provide an alternative explanation for the selective induction of Th1- and Th2-like cells in low- and high-Ab responders, respectively.

In summary, our data presented here suggest that, in vaccinated humans, low-Ab immune responsiveness to HBsAg is caused, in part, by the predominant induction of a Th1- or Th0-like T-cell response specific for this antigen, resulting in a lack of Th2-like T cells, while high-Ab responsiveness is caused by the predominant induction of Th2-like T cells. Our finding will provide clues for future investigations to uncover the mechanisms governing immune regulation.

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