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HLA-DQ-RECOGNIZING CD4 CELL REQUIREMENT FOR GENERATING HEPATITIS B SURFACE ANTIGEN-SPECIFIC SUPPRESSOR T CELLS

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For a more precise analysis of the immunological tolerance to hepatitis B virus surface antigen (HBsAg), we studied the induction mechanisms of HBsAg-specific suppressor T cells (Ts). The stimulation of peripheral blood mononuclear cells (PBMC) obtained from HB vaccinees with a high dose of HBsAg resulted in the generation of HBsAg-specific Ts. While these activated Ts were CD8 cells, their induction was abrogated by the deletion of CD4 cells prior to the in vitro culture, indicating that CD4 T cells were needed to induce CD8 Ts. An in vitro inhibition study of Ts induction with monoclonal antibodies to major histocompatibility complex class 2 antigens revealed that the induction of Ts was inhibited by anti-DQ but not by anti-DR. However, anti-DQ inhibition dose not directly affect the suppressive effect of Ts, indicating that HLA-DQ antigen might be required only for the induction of Ts. From these results, we can assume that immune suppression is initiated by the stimulation of CD4 cells by a molecular complex composed of a fragment of HBsAg and HLA-DQ antigen that is expressed on the surface of antigen presenting cells.

Introduction

The infection by hepatitis B virus (HBV) results in varied outcomes in infected hosts; there may be transient infection with a complete recovery or an immunologic tolerance to HBV surface antigen (HBsAg) which continuously produces HBsAg (carrier)[1]. Because chronic carriage of HBV is associated with chronic liver diseases, and understanding of the precise mechanisms involved in the specific immune response and immunologic tolerance to HBsAg is important. It has been reported that HBsAg-specific suppressor T cells (Ts) and their soluble factor(s) play important roles in the chronic HBV carrier state[2)3)] and immunological unresponsiveness to hepatitis B (HB) vaccine[4)5)]. The precise mechanism of the immune suppression of HBsAg, however, is presently still an enigma. Some investigators have reported a close linkage between a certain type of major histocompatibility complex (MHC) and immunological unresponsiveness to HBsAg in both human[6)7)] and mice[8)9)]. These results suggest that MHC gene products may have some function in the immunosuppressive response to HBsAg.

In the present study, we investigated the precise mechanisms whereby HBsAg-specific Ts are induced using in vitro systems for suppressor T-cell induction[10)].

Materials and Methods

Immunization and cell preparation

We studied two healthy consenting volunteers, one with artificially acquired anti-HBs by

vaccination and the other with naturally acquired anti-HBs. We gave each a booster dose of HB vaccine 20 μg/ml (B-mugen, Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan), and each volunteer served as a source of peripheral blood mononuclear cells (PBMC) for the following in vitro studies. PBMC were obtained from venous blood derived from these vaccinees by Ficoll-Hypaque density gradient. T cells were fractionated from PBMC by repeated sheep erythrocyte rosette formation, as previously described[11]. This method constantly yields a T-cell fraction consisting of >95% $CD3^+$ cells.

To obtain both $CD8^+$ and $CD4^+$ cells, an indirect OXRBC rosette-formation method[12] was employed. In brief, 2×10^7 PBMC were incubated at room temperature with 50 μl of either anti-CD4 (Cosmo Bio,Tokyo, Japan) or anti-CD8 (Cosmo Bio, Tokyo, Japan) for 30 min. Subsequently, we incubated the cells with anti-mouse immunoglobulin-coating OXRBC at for 60 min and layered them over a Ficoll-Hypaque density gradient. After they were centrifuged, we harvested both OXRBC-binding and -non-binding cells and then lysed the OXRBCs with 0.15% NH_4Cl. The purity of these cells was > 95% in positively selected (rosette-forming) cells and >85% in non-rosette-forming cells.

Monoclonal antibodies used in this study

Two distinct monoclonal antibodies to MHC class 2 antigens, anti-DR and anti-DQ (Cosmo Bio, Tokyo, Japan), were used in this study. Various doses of these antibodies and control myeloma proteins were added to in vitro cultures, either for the induction phase or the effector phase of HBsAg-specific Ts as described below.

In vitro culture for anti-HBs synthesis

We performd in vitro culture to make anti-HBs according to a previously reported method[13]. In brief, we cultured PBMC in RPMI 1640 supplemented with 2 mM of glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco Laboratory, Grand Island, NY, USA), and 10% fetal calf serum (complete medium) with 0.5 μg/ml pokeweed mitogen (Sigma Co, St. Louis, MO, USA) plus 0.2 μg/ml yeast-derived recombinant HBsAg (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan)[14]. We adjusted the cells to a concentration of 2.5×10^6/ml and cultured them for three days in 12 × 75-mm plastic tubes (Falcon 2054, Falcon, Lincoln Park, NJ, USA) in a 5% CO_2 atmosphere (the first culture). The cells were then washed thoroughly to remove antigen. After resuspension in complete medium without stimulants, they were cultured for an additional seven days at a density of 5×10^5/0.2 ml/well in 96-well, flat-bottomed culture plates (Falcon 3072, Falcon Co, USA) (the second culture). The titer of anti-HBs in the supernatant of these cultures was measured by commercially available EIA kit (Dinabot Ltd. IL, USA). Polyclonal IgG and IgM in the same culture supernatants were examined by ELISA method as described previously[3].

In vitro induction of HBsAg-specific Ts

HBsAg-specific Ts were generated according to the method described by Barnaba et al[10]. In brief, PBMC from the immunized volunteers wre separated into T cells and non-T cells as described above. 4×10^6 T cells were then cultured with 1×10^6 irradiated non-T cells (3000 rads) in 1 ml of complete medium in the presence of 20 μg/ml HBsAg in a 24-well, flat-bottomed culture plate (Falcon 3047, Falcon Co, USA). After five days of culture, the cells were collected, washed three times, resuspended in complete medium, and these activated cells were added to autologous PBMC (2×10^6/ml) of the first culture to make anti-HBs. The suppressive effect was expressed by % suppression calculated by the following formula: % suppression=[anti-HBs in the cultures of fresh PBMC alone] minus [anti-HBs in the culture with fresh PBMC] plus [high dose antigen stimulated PBMC (or T cells)]/anti-HBs in the cultures of fresh PBMC alone.

To examine the effect of anti-MHC class II antibodies, various doeses of either anti-DR or anti-DQ antibody was added into the mixture of

Table 1 Specificity of in vitro generated Ts

Added in vitro stimulated cells	Titer of antibody in culture supernatants		
	Anti-HBs(EIA U)	IgM(μg/ml)	IgG(μg/ml)
none	19.0	0.71	1.02
2.5 $\times 10^5$/ml	0.0	0.49	0.82
1.25$\times 10^5$/ml	0.0	0.79	1.00

All cultures contained 2.5$\times 10^6$/ml fresh PBMC to make anti-HBs.
Data were expressed as mean of triplicate cultures.

Table 2 Induction of Ts was inhibited by anti-DQ

	Ts	Ts precultivated with	Anti-HBs (mean±SD)		%Suppression
Exp. 1	none	none	39.6± 3.9	a	standard
	+(5$\times 10^5$/ml)	myeloma(50μg/ml)	15.2± 9.3	a	62
	+(5$\times 10^5$/ml)	anti-DQ(50μg/ml)	32.8± 3.5		17
Exp. 2	none	none	140.0±16.0	a	standard
	+(5$\times 10^5$/ml)	anti-DR	5.5± 2.1	a	96
	+(5$\times 10^5$/ml)	anti-DQ	107.0±42.3		24
Exp. 3	none	none	23.3±10.7	a	standard
	+(5$\times 10^5$/ml)	anti-DR	6.2± 3.2	a	73
	+(5$\times 10^5$/ml)	anti-DQ	25.0± 5.0		0

All cultures contained 2.5$\times 10^6$/ml fresh PBMC to make anti-HBs.
Data were expressed as mean of triplicate cultures.
a : $p<0.05$.

T cells and non-T cells with HBsAg. Five days after culture the T cells were harvested and their suppressive effect was examined as described above (induction phase; shown in Table 1). Simultaneously, the direct effect of these antibodies on Ts function were also examined by adding them into the mixture of in vitro generated Ts and fresh PBMC (effector phase; shown in Table 3).

Results

In vitro stimulated cells can suppress the synthesis of anti-HBs

As shown in Table 2, the synthesis of anti-HBs from PBMC was completely inhibited by adding the in vitro stimulated T cells, indicating that the stimulation of T cells with a high dose of HBsAg resulted in the generation of Ts. The specificity of such in vitro generated Ts was studied by their effect on the synthesis of polyclonal IgG and IgM. In vitro activated T cells were added to fresh PBMC from the same vaccinee, and the titers of anti-HBs, IgG and IgM in the supernatant of this culture were subsequently examined by ELISA. The results shown in Table 1 revealed that HBsAg-activated T cells suppressed only anti-HBs synthesis but not polyclonal immunoglobulin synthesis.

Characterization of in vitro induced Ts

To characterize these in vitro generated Ts, we attempted to delete either CD4 or CD8 T cells from in vitro stimulated T cells prior to their addition to the culture of fresh PBMC. As shown in Fig. 1, anti-HBs synthesis was suppressed by whole T cells (23.9 EIA U to 8.5 EIA U; 64.4% reduction) and by CD4 deleted T cells (CD8 cells; 52.3% reduction). However, such suppression was not observed by the deletion of CD8 cells from this T cells fraction. Thus, these results indicated that Ts generated by this in vitro stimulation were CD8 positive T cells.

$CD4^+$ T cells are needed for generation of

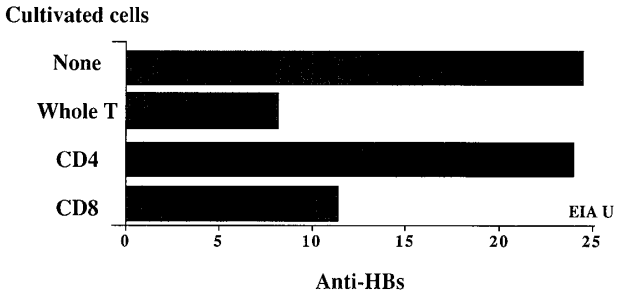
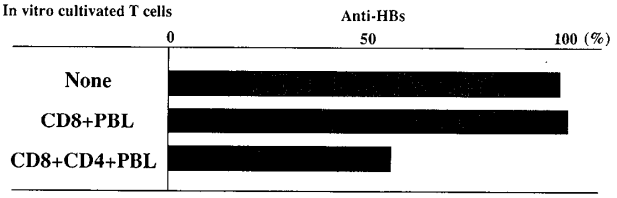


Fig. 1 Characterization of in vitro generated Ts
In vitro activated T cells were fractionated as described in Material and Methods, and 5 × 10^5/ml cells in each fraction were added to cultures containing 2.5 × 10^6/ml fresh PBMC with HBsAg plus pokeweed mitogen. Note that significant suppression was obtained by adding either whole T cells or CD8 cells but not CD4 cells.



Fig, 2 Requirement of CD4 cells to generate Ts
Either whole T cells (CD8 + CD4) or CD4 depleted T cells (CD8) were cultured with non-T cells plus 20 μg/ml HBsAg. By the depletion of CD4 cells prior to the culture, the induction of Ts was completely abrogated.

suppressor T cells

To study the induction mechanisms of HBsAg-specific Ts, we cultured either whole T cells or $CD4^+$ depleted cells ($CD8^+$ cells) with irradiated non-T cells as antigen presenting cells in the presence of HBsAg. Subsequently, we examined their suppressive effect on anti-HBs synthesis. As shown in Fig. 2, anti-HBs synthesis was reduced to 55.7% by adding T cells obtained from the cultures with irradiated non-T cells plus a high dose of HBsAg. However, the suppression of anti-HBs was not observed when using CD4 deleted cells with the some condition.

These results indicate that $CD4^+$ T cells were necessary for the generation of HBsAg-specific $CD8^+$ Ts.

Inhibition of Ts induction with anti-DQ antibody

To study the induction mechanism of Ts more precisely, we then examined the effect of antibodies to MHC class 2 antigens, anti-DR and anti-DQ, on suppressor T-cell induction. We cultured PBMC in a complete medium containing 20 μg/ml HBsAg with or without anti-DR, anti-DQ, or myeloma protein (control) for five days. We then harvested and assayed their suppressive effects on the anti-HBs synthesis in vitro culture. As shown in Table 2, the induction of Ts was completely inhibited by anti-DQ but not by either anti-DR or myeloma protein. Similar results were obtained in two other experiments using PBMC from different subjects. Our findings indicate that DQ antigen is necessary for Ts induction.

Table 3 Anti-DQ does not alter the effect of Ts activity

	Cultivared with	Anti-HBs (EIA U)		%Suppression
Effectors (PBL) 1.5×10^6	none	9.1	a	standard
	medium	1.2	a	87
	anti-DQ (50μg/ml)	2.4		74

All cultures contained 2.5×10^6/ml fresh PBMC to make anti-HBs.
Data were expressed as mean of triplicare cultures.
a : $p<0.05$.

Direct effect of anti-DQ on the function of in vitro-induced Ts

To further analyze the effect of anti-DQ on Ts, we examined whether anti-DQ had a direct inhibitory effect on the function of in vitro generated Ts. To address this question, anti-DQ was added to the in vitro cultures containing PBMC as the source material for anti-HBs and the in vitro-generated Ts. As shown in Table 3, we observed no remarkable difference in the functionality of Ts with or without the addition of anti-DQ, suggesting that anti-DQ did not directly inhibit the effect of suppressor T cells generated in vitro.

Discussion

It is well documented[13)14)] that a repeated

HBV vaccination results in the production of anti-HBs which have a potent inhibitory effect on HBV infection[13][14]. However, approximately 10% of healthy vaccinees did not develop anti-HBs after vaccination. This immunologic nonresponsiveness to HBV vaccine is at least partly due to the presence of HBsAg-specific Ts[4][5]. A similar Ts effect has also been reported in the immunologic tolerance to HBsAg among chronic carriers of HBsAg[2][3]. The precise mechanism for this effect of Ts, however, is still uncertain.

In this study, we first investigated whether in vitro stimulation of T cells with a relatively high dose of HBsAg can generate HBsAg-specific Ts. As shown in Table 1, T cells stimulated by a high dose of HBsAg had a potent suppressive effect on the synthesis of anti-HBs but not on those of polyclonal IgG and IgM, and these Ts belonged to CD8 positive T cells (Fig. 1).

The next question addressed in the present study and raised in previous reports concerning tetanus toxoid response[15] and KLH-specific immune response[16] was whether CD4 suppressor-inducer T cells (Tsi) were also required for the generation of $CD8^+$ Ts. Our results indicate that $CD4^-$ depleted cultures containing only CD8 cells plus irradiated non-T cells did not effectively generate Ts, and so $CD4^+$ Tsi might be needed to generate HBsAg-specific $CD8^+$ Ts. It is widely accepted that $CD4^+$ T cells recognize the class 2 molecules to be proliferated, and so we tried to determine which class 2 molecules these $CD4^+$ T cells recognized. Celis et al reported that HBsAg-specific helper T clones recognize the DR molecule on the surface of the antigen-presenting cells to be proliferated[17][18].

We also found that anti-DR antibody inhibited the synthesis of anti-HBs in vitro (data not shown). As shown in Table 2, Ts generation was inhibited by anti-DQ but not by anti-DR. In as much as anti-DQ might bind nonspecifically to the cells in the Ts-induction culture, this inhibitory effect of anti-DQ may result from the effect on the effector phase of the generated Ts, rather than on the induction of Ts itself. To exclude this possibility, we performed a similar inhibition study on a co-culture of Ts and fresh PBMC (referred to in Materials and Methods as "the first culture" for synthesizing anti-HBs). Because anti-DQ did not alter the effect of Ts in this culture (Table 3), we can conclude that the HLA-DQ molecule is important for the Ts induction phase but not for the effector phase.

Hirayama et al[19] have reported that the HLA-DR molecule stimulates CD4 cells to be proliferated against schistosomal antigens, but that, this response is suppressed by Ts controlled by HLA-DQw1 molecules in the nonresponder haplotype. Similarly, the importance of HLA-DQ molecule in the immunological non-responsiveness to HBsAg was also shown by Watanabe et al[7]. Therefore, taking these results and ours, we hypothesize that the immunological non-responsiveness in human is mediated by the sequential interaction among different cells; such as the first step is the stimulation of CD4 cells via the DQ molecule expressed on APC and subsequent activation of CD8 cells by these activated CD4 cells.

In mice (where the I-A molecule is comparable to the DQ molecule and the I-E molecule is comparable to the DR molecule) the MHC class 2 molecules act as restriction elements in the activation of helper T cells and Ts, respectively, in the immune response to LDH-B[20], IgG_2 myeloma protein[21], and F antigen. In those systems the antigen is presented to the helper T by the I-A molecule, whereas Ts are stimulated by the antigen in the context for the I-E molecule. Therefore, all results seem to indicate that two distinct MHC class 2 molecules, I-A and I-E in mouse and HLA-DR and HLA-DQ in human, control immune response and suppression, respectively. It has been predicted that the host's distinct immune response to HBV may result in the infected host's particular outcome[1].

Our findings that HLA-DQ molecules have an important function in the generation of HBsAg-

specific Ts that may negatively regulated immune response to HBsAg and these results will provide a due to the immune response mechanisms which result in distinct outcomes of HBV infection.

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HBs抗原特異的抑制性T細胞の誘導におけるHLA-DQ拘束性CD4陽性細胞の役割

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HBs抗原に対する免疫学的寛容状態の解明のために我々はHBs抗原特異的抑制性T細胞の誘導のメカニズムについて検討を行った．抑制活性をみるための方法としては，我々がこれまでに報告してきたin vitroにおけるHBs抗体産生系を用いた．まず，HBワクチンで免疫されたhigh responder (HBワクチンに対する高応答者)より得られた末梢血単核球をhigh doseのHBs抗原で刺激し，CD8陽性の抑制性T細胞を誘導した．この抑制性T細胞はHBs抗原に特異的であり，HBs抗体の産生のみを抑制していた．さらにこのCD8陽性抑制性T細胞の誘導にはCD4陽性細胞が必要であり，in vitroにおいてCD4陽性細胞を除くことにより抑制性T細胞の誘導は阻害された．この抑制性T細胞を誘導するCD4陽性細胞（suppressor-inducer T cell）はHLA class 2抗原のDQ抗原に拘束されており，抑制性T細胞を誘導する培養液中に，anti-DQを加えることにより抑制性活性の低下が認められた．この活性の低下は，anti-DQにのみ引き起されるものでありanti-DRや，myeloma proteinでは抑制活性の低下は得られなかった．また，このanti-DQにより阻害されるのはCD4 suppreessor-inducer T cellを介したCD8 suppressor T cell誘導の系であり，抗体産生系の培養中にanti-DQを加えても同様の抑制活性の低下は得られなかった．