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HBV-specific lymphoproliferative and cytokine responses in patients with chronic hepatitis B

Johan Vingerhoets[1], Peter Michielsen[2], Guido Vanham[1], Eugène Bosmans[3], Wilma Paulij[4], Albert Ramon[5], Paul Pelckmans[2], Luc Kestens[1] and Geert Leroux-Roels[6]

[1]*Institute of Tropical Medicine, Laboratory of Immunology, Antwerp,* [2]*University Hospital, Department of Gastro-enterology, Antwerp, and* [3]*Eurogenetics, Tessenderlo, Belgium,* [4]*Organon Teknika, Boxtel, The Netherlands,* [5]*International Institute of Immunopathology, Cologne-Maastricht-Houston-Mexico, and* [6]*University Hospital, Department of Clinical Chemistry, Ghent, Belgium*

Background/Aims: Hepatitis B virus specific T cell reponses are crucial for viral elimination but their nature is not fully understood.

Methods: We studied the regulation of proliferation and cytokine production after antigenic stimulation in peripheral blood mononuclear cells from chronically HBV-infected patients and subjects with natural immunity after recovery from an acute infection. Proliferation and production of interferon-γ, IL-10 and tumor necrosis factor-α were determined after stimulation with HBcAg, HBeAg or HBsAg in the absence or presence of IL-12 or neutralizing antibodies to IL-12, interferon-γ, IL-4, IL-10 or tumor necrosis factor-α.

Results: Upon stimulation with HBcAg or HBeAg, peripheral blood mononuclear cells from chronic hepatitis B virus patients displayed a clear class-II restricted proliferative response (SI greater than 2.5). Both interferon-γ (less than 50 IU/ml) and IL-10 levels up to 600 pg/ml were detected. Proliferative or cytokine responses to HBsAg were very weak or absent. Addition of IL-12 to HBeAg-stimulated cultures increased the production of interferon-γ to more than 200 IU/ml in all patients and slightly increased the production of IL-10. Neutralization of IL-10 increased the HBeAg-induced interferon-γ production but had no effect on tumor necrosis factor-α production. Addition of anti-IL-4 or anti-tumor necrosis factor-α had no significant influence on proliferation or cytokine release. Importantly, in both chronic hepatitis B virus patients and naturally immune subjects, IL-12 induced proliferative and interferon-γ responses in peripheral blood mononuclear cells stimulated with HBsAg.

Conclusions: Our data indicate that peripheral blood mononuclear cells from chronic hepatitis B virus patients proliferate and produce interferon-γ and IL-10 upon HBeAg but not upon HBsAg stimulation. IL-12 augments the HBeAg-induced responses and, additionally, provokes proliferation and interferon-γ production in HBsAg-stimulated cultures.

Key words: Hepatitis B virus; HBeAg; HBsAg; Interferon-γ; Interleukin-10; Interleukin-12; T cell responses.

THE COURSE OF an infection with the non-cytopathic hepatitis B virus (HBV) is determined by the antiviral immune response of the host (1). The humoral immune response, in particular the generation of neutralizing anti-hepatitis-B-surface antigen antibodies (anti-HBs), is required for successful clearance of the virus. The cellular immune response contributes significantly to the elimination of the virus, but is also responsible for the liver damage caused by the lytic activity of hepatitis B virus-specific cytotoxic T lymphocytes (CTL) on HBV-infected hepatocytes (1). Increased hepatitis B core antigen (HBcAg) and HBeAg-specific T helper cell responses are observed in patients with acute self-limited HBV infection and are associated with viral elimination (2). In contrast, a chronic HBV infection is characterized by an inefficient immune response to HBsAg and weak but demonstrable responses to the nu-

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Correspondence: Johan Vingerhoets, Institute of Tropical Medicine, Unit of Immunology, Department of Microbiology, Nationalestraat 155, B-2000 Antwerpen 1, Belgium. Tel.: 32-3-247.62.26. Fax: 32-3-247.62.31.
e-mail: jvingerhoets@itg.be

cleocapsid antigens, HBcAg and HBeAg (3–6). Both proliferation and interferon-γ (IFNγ) production in response to HBeAg were shown to increase during acute exacerbation of the chronic disease (7,8). Recently, it has been shown that both increased proliferation and IFNγ production in response to HBcAg stimulation *in vitro* are closely connected to increased alanine aminotransferase (ALT) levels and eventual seroconversion to anti-HBe in IFNα-treated patients (9). These findings indicate that also in chronic hepatitis B infection, T cell responses to nucleocapsid antigens are crucial for the elimination of HBV. Depending on the pattern of cytokines, both human $CD4^+$ and $CD8^+$ T cells can be classified as Th1, Th0 or Th2-type cells, similarly to the murine system. Typical Th1 cytokines are IFNγ and TNFβ, which inhibit Th2- activity, while Th2-type cells secrete IL-4, IL-5 and IL-13. Both Th1 and Th2-type cells can produce IL-2 and IL-10 (inhibitor of Th1-activity). T cells producing a mixed Th1/Th2 pattern of cytokines have been designated Th0. Differentiation into one of these T helper subtypes depends on the presence of IL-12 (for Th1) or IL-4 (for Th2), on peptide-T cell receptor affinity and, possibly, on the presence of certain co-stimulatory molecules on the antigen-presenting cell. Few studies have been performed to define the nature of these T cell responses in chronic HBV patients. Both Th0 and Th1, but not Th2-type, clones could be generated from peripheral blood mononuclear cells (PBMC) of chronic hepatitis B patients and subjects with an acute, self-limited infection (3,6). In all previous studies, T cell reactivity to HBV antigens was determined by proliferation or IFNγ production. Recent studies have demonstrated Th0 and Th1 clones in blood and liver tissue upon mitogen or antigen-specific stimulation (10,11). Few data are available on the effect of regulatory cytokines on T cell proliferation and IFNγ production in response to HBV antigens in PBMC.

Our aim was to study the regulation of IFNγ in hepatitis B infection, and to compare this in PBMC from subjects with a non-protective versus a protective immune response. *In vitro* responses to HB-core and -surface antigens in chronically infected patients ($HBeAg^+$ or anti-HBe^+) and in naturally immune subjects (anti-HBs^+) were determined, and the role of IL-12 and other regulatory cytokines such as IL-10, IL-4 and TNFα in IFNγ production was investigated.

Patients and Methods

Patients

Blood samples were taken from 34 chronically HBV-infected patients and eight naturally immune subjects, who cleared HBV within a period of 1 to 5 years prior to blood collection. The following patient groups were included: nine asymptomatic HBsAg carriers with normal ALT levels and no clinical, histological, or biochemical signs of liver damage (HBeAg and HBV-DNA negative) (group I); ten chronic HBV patients with histological signs of cirrhosis but who were HBeAg and HBV-DNA negative (group II); and 15 $HBeAg^+$ patients who had elevated ALT levels and who were HBV-DNA seropositive (group III) (Table 1). HBV-DNA in sera was measured with the molecular hybridization assay (Genostics, Abbott Laboratories, North Chicago, IL, USA). None of the patients were receiving IFNα-therapy at the time of blood sampling. All patients and controls were repeatedly negative for serologic markers of HCV and HIV. All patients gave informed consent to the experiments according to the guidelines of the Ethical Committees of the University Hospital and the Institute of Tropical Medicine, Antwerp.

TABLE 1

Serological and clinical data from patients with chronic hepatitis B and control subjects that recovered from an acute HBV infection

	n	HBsAg	anti-HBs	HBeAg	anti-HBe	HBV-DNA	ALT[a]
Patients							
I	9	+	−	−	+	−	18±6 (1,9)
II	10	+	−	−	+	−	99±27 (6,10)
III	15	+	−	+	−	+	44±7 (15,15)
Naturally immune subjects							
IV	8	−	+	−	+	ND	ND

[a]ALT alanine aminotransferase (IU/ml), normal range=5–22 IU/l. The numbers of subjects with elevated ALT (>22 IU/l) are indicated within parentheses. ND=not determined.

Antigens, cytokines and monoclonal antibodies

Recombinant yeast-derived HBsAg (HBsAg, subtype ad) was kindly provided by Smith Kline Beecham Biologicals (Rixensart, Belgium). Recombinant HBcAg and HBeAg were generously provided by Organon Teknika (Boxtel, The Netherlands). HBcAg and HBeAg were derived from *E. coli* and purified using gel filtration (Sephacryl S300 HR, Pharmacia), as follows. Supernatant obtained after lysis of HBcAg or HBeAg producing *E. coli* cells was filtered (0.2 μm) and injected onto the column using a 50 mM TRIS buffer (pH 8.0) containing 10 mM EDTA and 5% sucrose as eluent. HBcAg or HBeAg reactive fractions were selected and pooled after dot-spot-blotting with an anti-HBc/HBe reactive murine mAb. *Candida albicans* recall antigen (CA, Pasteur Mérieux, France) was used as control antigen. All reagents were endotoxin free as determined by the Limulus amoebocyte lysate assay. In-

terleukin 12 (IL-12) was provided by Dr. M. Gately (Hoffmann-La Roche, Nutley, NJ, USA). The following neutralizing cytokine antibodies were used in the *in vitro* stimulation assays: anti-MHC class II (clone 9.3F10), anti-IL-10 (clone JES3-9D7, Biosource International, CA, USA), anti-IL-4 (Genzyme Corporation, Cambridge, MA, USA), anti-IL-12 (R&D Systems Europe). Anti-TNFα (clone MAL-1) and anti-IFNγ (clone MDL-1) were provided by Dr. E. Bosmans. All neutralizing antibodies were used at 10 μg/ml.

Lymphocyte purification and cryopreservation

Peripheral blood mononuclear cells (PBMC) were purified from EDTA-anticoagulated blood by Ficoll density gradient centrifugation. Subsequently, the cells were frozen as described previously (12). Briefly, 5–10×10^6 PBMC were suspended in 1 ml of cold heat-inactivated autologous serum and diluted in 1 ml of cold DMSO (20% in RPMI). Cell aliquots were placed at −80°C overnight and transferred to liquid nitrogen. Rapid thawing was performed at 37°C and cells were washed extensively. Then, cells were resuspended in complete medium consisting of RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) (all reagents from Gibco, Paisley, Scotland) and 10% pooled normal human serum (obtained from healthy donors seronegative for HIV, HBV and HCV). Viability, as determined by exclusion of eosin, always exceeded 90%.

Stimulation assays

PBMC were suspended at 3×10^6/ml in complete medium and transferred into 96-well round-bottomed plates at 75 μl/well. The different stimuli were added in quadruplicate wells also at 75 μl/well (total volume 150 μl/well). Dose titration experiments were performed and the following optimal antigen concentrations were used: HBsAg, 3 μg/ml; HBcAg, 1 μg/ml; HBeAg, 1 μg/ml and CA, 1/200 (final dilution). Proliferation of stimulated cells was determined after 6 days by addition of 0.4 μCi/well [^{3}H]-Thymidine (specific activity: 5.0 Ci/mmol) during the final 16 h of culture. Determination of thymidine uptake was done by liquid scintillation counting. Results were expressed as Δcpm of the mean of 4 to 6-fold cultures [Δcpm=cpm (stimulation with antigen) − cpm (stimulation without antigen)], or as stimulation index [SI=cpm (stimulation with antigen)/cpm (stimulation without antigen)]. A SI>2.5 was considered positive.

Measurement of cytokine production

Culture supernatants for determination of cytokine levels were collected at 2 and 6 days. The supernatants were stored at −30°C and thawed only once for analysis. IFNγ and TNFα were determined by commercial ELISA (Eurogenetics, Tessenderlo, Belgium). IL-10 and IL-4 were determined with an ELISA assay using capture and biotinylated detection monoclonal antibodies (clones JES3-9D7 and JES3-12G8 for IL-10 and clones 8D4-8 and MP4-25D2 for IL-4, respectively) from Pharmingen (San Diego, CA, USA). HRP-conjugated streptavidin was purchased from Jackson Immunoresearch Laboratories (West Grove, PE, USA). Recombinant cytokines diluted in culture medium were used as standards. The sensitivity of the different assays was 1 IU/ml, 80 pg/ml, 20 pg/ml and 5 pg/ml for IFNγ, TNFα, IL-10 and IL-4, respectively (data also shown in the figures).

Statistical analysis

Non-parametric tests were used throughout the study. The Mann-Whitney *U*-test or Kruskall-Wallis test was employed to compare two or more different subject groups. Paired datasets were analyzed using the Wilcoxon matched-pairs signed-ranks test. Correlation analysis was performed using Spearman's rank correlation test.

Results

Proliferative responses and cytokine production in PBMC from chronic hepatitis B patients upon stimulation with HBV antigens

The serological and clinical data of the three patient groups are shown in Table 1. Almost all patients, irrespective of their clinical classification, displayed a proliferative response to stimulation with HBeAg (median Δcpm: 10008±4804, 7166±8077, 7084±5701 cpm for group I, II and III, respectively, all SI>2.5) (Fig. 1A). No proliferation was seen in cultures without antigen (cpm<1000). The PBMC responses to HBeAg or HBcAg were completely inhibited by an anti-MHC class II mAb. Proliferative responses induced by HBcAg were comparable to those induced by HBeAg, and the responses to both antigens were correlated (r= 0.86, p<0.0005) (data not shown). Proliferation to HBsAg was not detected in any of the chronically infected patients (SI<2.5), whereas a clear response was present in vaccine recipients using identical culture conditions and antigen preparations (data not shown) (13).

To further characterize the immune response to HBeAg, we determined the production of IFNγ, IL-10 and IL-4 in PBMC cultures (Fig. 1B and 1C). Stimulation with HBcAg (data not shown) or HBeAg induced low levels of IFNγ in 31/34 patients while IL-10 was

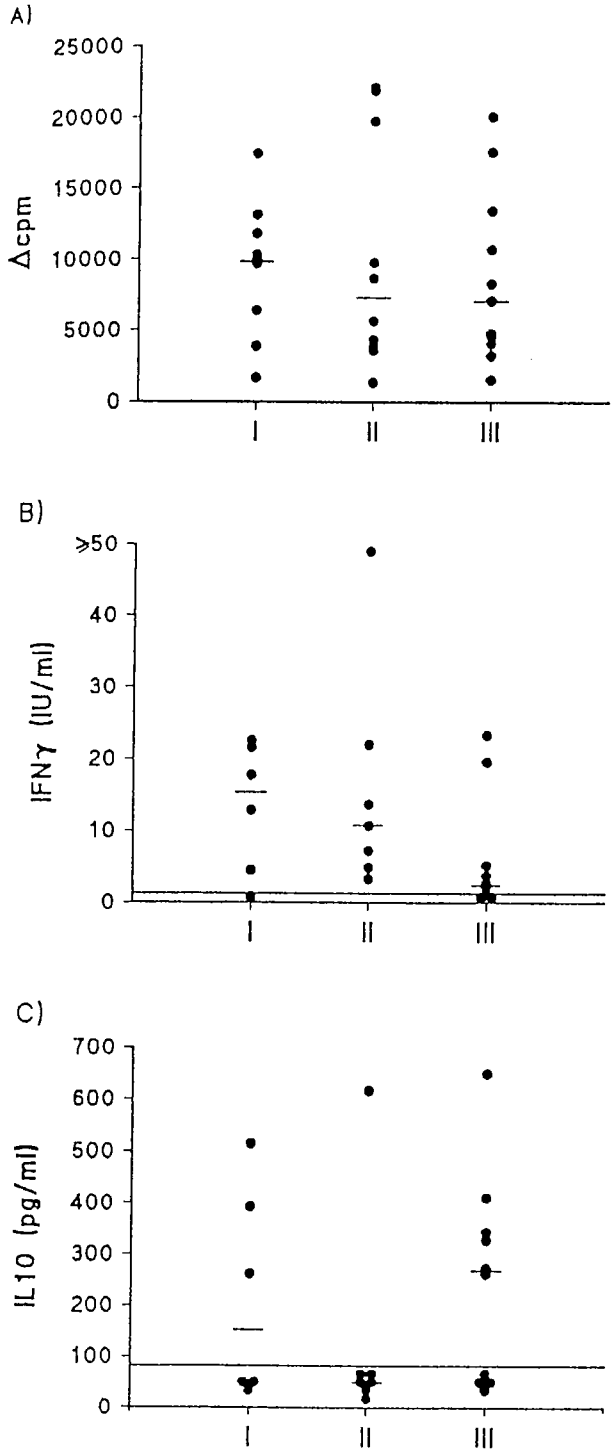

Fig. 1. (A) Proliferative responses and production of IFNγ (B) and IL-10 (C) on stimulation with HBeAg in patients chronically infected with HBV. Patients were classified as indicated in Table 1. For each patient, the results were expressed as Δcpm (A) or amount of cytokine in the supernatant (B, C). Each dot represents one patient and the median for each group is indicated by a horizontal dash. The cut-off levels for the cytokine assays are indicated by horizontal lines in panels B and C.

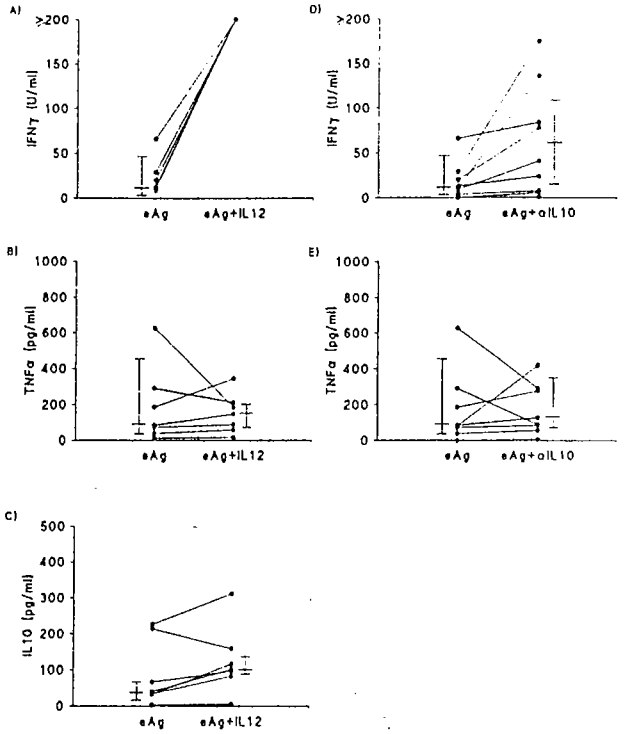

Fig. 2. Modulation of IFNγ (A, D), IL-10 (B) and TNFα (C, E) production by IL-12 (A, B, C) and anti-IL-10 (D, E) in PBMC from chronically infected patients. Each line represents one patient and the median±confidence interval are indicated for each group. Statistically significant differences (paired t-test) were obtained for IFNγ production ($p<0.0005$ for HBeAg plus IL-12 or plus anti-IL-10 versus HBeAg alone). IL-12 alone induced less than 20 IU/ml IFNγ, 200 pg/ml TNFα and no IL-10.

induced in 10/34 subjects. The production of IL-10 and IFNγ was similar at both time points examined (2 and 6 days), and no significant differences were observed between the patient groups. IL-4 output, measured at 2 or 6 days, was below the detection limit in all subjects. Stimulation with HBsAg did not induce IFNγ, IL-10 or IL-4 (data not shown). Since no differences were found between the patient groups, no further distinction was made between the patients in the subsequent experiments.

Effect of IL-12 and of neutralizing cytokine antibodies on cytokine production to HBeAg

Since IFNγ is an important factor in cellular immune responses to HBV infection, we added IL-12 or neutralizing antibodies to IL-4, IL-10 and TNFα to HBeAg-stimulated PBMC. The production of IFNγ, IL-10 and TNFα was measured in PBMC from 12 patients (Fig. 2). IL-12 alone induced low levels of IFNγ (<20 IU/ml), while anti-IL-10 did not induce any

IFNγ production in PBMC cultures. Addition of IL-12 to HBeAg enhanced IFNγ secretion in PBMC from all patients to levels ≥200 IU/ml ($p<0.0005$), while it only marginally increased IL-10 production (Fig. 2). Neutralization of IL-10 also significantly enhanced the production of IFNγ ($p<0.0005$) but to a lesser extent than IL-12. Neither IL-12 nor anti-IL-10 had a profound effect on the production of TNFα following stimulation with HBeAg. Addition of anti-IL-4 or anti-TNFα did not alter the cytokine production in PBMC from the patients (data not shown).

Comparison between chronically infected patients and recovered subjects

In order to determine a potential association of cytokine profile with immune protection, the responses in chronic HBV patients were compared to those in subjects who had spontaneously recovered from an acute infection between 1 and 5 years before blood sampling. In these experiments, PBMC were stimulated with HBeAg, HBsAg and the control antigen (CA). Figure 3 allows an easy comparison of proliferation and cytokine production of PBMC from eight patients and eight recovered controls. Both CA antigen and HBeAg induced proliferation and low levels of IFNγ production in patients and controls, whereas HBsAg stimulation did not result in a significant proliferative or cytokine response. IL-10 was only induced by HBeAg but not by HBsAg or CA in both groups. TNFα was induced by all three antigens, but the highest levels were observed with HBeAg. No differences were observed between patients and recovered controls for either proliferation or production of IFNγ, IL-10 or TNFα (Fig. 3).

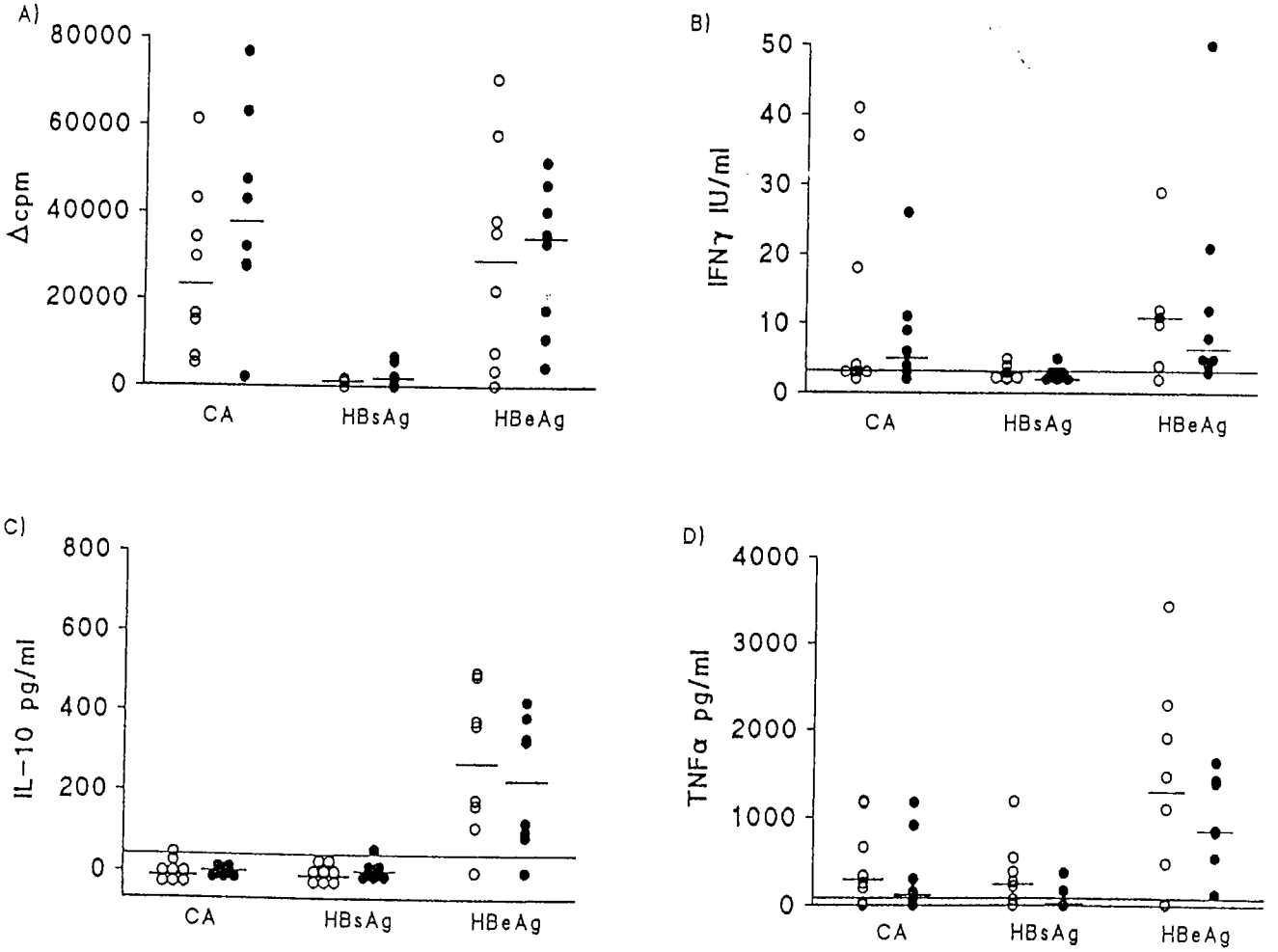

Fig. 3. Proliferative responses and cytokine production in eight chronically infected patients (closed symbols) and eight recently recovered control subjects (open symbols). Proliferation (A) and production of IFNγ (B), IL-10 (C) and TNFα (D) were compared after stimulation with control antigen (CA), HBsAg or HBeAg. Each dot represents one subject (Δcpm for proliferative responses and amount of cytokine produced). The median for each group is indicated by a horizontal line. The cut-off levels for the cytokine assays are indicated by horizontal lines.

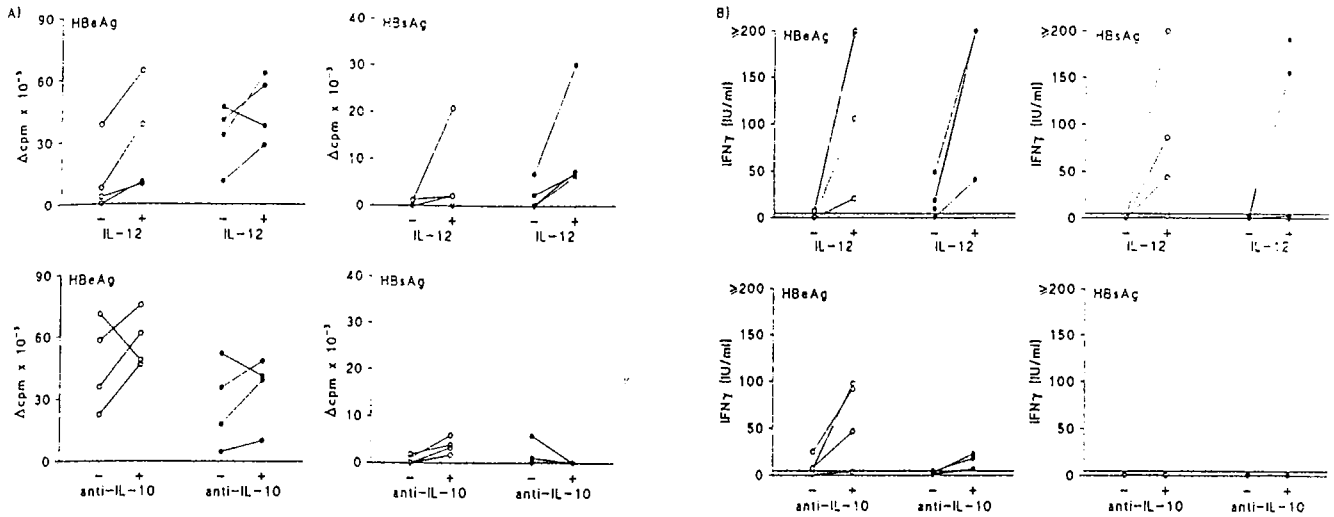

Fig. 4. Influence of IL-12 and anti-IL-10 on proliferation (A) and production of IFNγ (B) induced by HBeAg or HBsAg stimulation. Recently recovered subjects (controls, open symbols) were compared with chronically infected patients (closed symbols). Each line represents one subject. Statistically significant differences (Wilcoxon) were obtained for IFNγ production in PBMC from both patients and controls ($p<0.05$ for HBeAg plus IL-12 versus HBeAg alone). IL-12 stimulation alone induced no proliferative response and less than 20 IU/ml IFNγ.

IL-12 induces lymphoproliferation and IFNγ production in HBeAg- but also in HBsAg-stimulated cultures

Since HBsAg-specific cellular immune responses are important in protection and elimination of HBV, we examined the effects of IL-12 or anti-IL-10 on the responses to HBsAg in patients or recovered controls. PBMC from eight patients and eight controls were stimulated with HBeAg or HBsAg in the presence or absence of IL-12, in subjects 1 to 4, or anti-IL-10, in subject 5 to 8 (Fig. 4). Addition of IL-12 increased HBeAg-induced proliferation in 4/4 recovered controls and 3/4 patients. Whereas HBsAg alone did not induce proliferation, IL-12 plus HBsAg caused proliferative responses in 3/4 recovered controls and 4/4 patients. Anti-IL-10 enhanced the proliferation to HBeAg in 3/4 recovered controls and 3/4 patients, but it had only a marginal effect on the HBsAg- induced proliferation (Fig. 4A). Addition of IL-12 significantly augmented IFNγ levels induced by HBeAg ($p<0.05$ for controls and patients). While HBsAg alone did not induce IFNγ, the addition of IL-12 clearly increased the IFNγ levels in 3/4 controls and 2/4 patients. Neutralization of IL-10 enhanced the IFNγ production induced by HBeAg in 3/4 controls and in 2/4 chronic HBV patients. The neutralization of IL-10 did not induce detectable levels of IFNγ in HBsAg-stimulated cultures of controls or patients (Fig. 4B).

Enhancing effects of IL-12 on HBeAg-induced proliferation are mediated via IFNγ

To further delineate the effect of IL-12 on the lymphoproliferative response to HBeAg, we performed experiments in which PBMC from four chronic HBV pa-

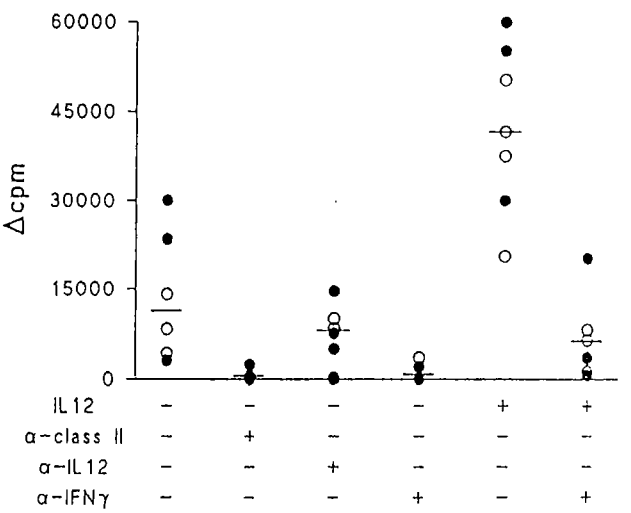

Fig. 5. Effect of IL-12, anti-MHC class II, anti-IL-12 or anti-IFNγ on the HBeAg-specific proliferation of PBMC from four recovered controls (open symbols) and four chronically infected patients (closed symbols). Each dot represents one subject and the median (for all subjects) is indicated by a horizontal line.

tients and four recovered controls were stimulated with HBeAg in the absence or presence of IL-12 or IFNγ neutralizing mAb. As a control, an mAb specific for MHC class II was added. The results show that neutralization of IL-12 slightly decreased the proliferative response to HBeAg. Neutralization of IFNγ almost completely blocked the proliferation, to the same level as obtained with the anti-MHC class II mAb (Fig. 5). Addition of IL-12 enhanced the response to HBeAg in all subjects, but this effect was abrogated by the neutralization of endogenous IFNγ (Fig. 5).

Discussion

In the present study, we showed that PBMC from chronically HBV-infected subjects and individuals who had recovered from an acute infection, proliferated and produced IFNγ as well as IL-10 upon stimulation with HBcAg or HBeAg, but not upon stimulation with HBsAg alone. The production of IFNγ in response to HBeAg stimulation could be enhanced by exogenous IL-12 or neutralization of the endogenously produced IL-10. Apparently, IFNγ production was responsible for the IL-12-induced increase in proliferation in response to HBeAg. Importantly, both proliferative responses and IFNγ production, which were absent in cultures stimulated with HBsAg alone, were strongly induced by simultaneous addition of IL-12 and HBsAg.

Our data did not show a clear relation between core-specific responses in PBMC and disease activity, as described by others (2,3,6,9,14). In these reports, fresh PBMC or T cell lines were used, whereas we used cryopreserved PBMC. The advantage of the present approach is that PBMC from a large group of subjects could be assayed in a limited number of experiments, thus reducing the day-to-day variability in these biological assays (12). Differences in the patient populations and in the definitions of disease severity between this study and previous reports could also have contributed to the dissimilar results.

Production of IFNγ and TNFα has been extensively studied in both serum and PBMC supernatants from chronic HBV patients (8,14,15). IFNγ is considered to be a major determinant for protection against viral infections, and both IFNγ and TNFα are known to down-regulate HBV gene expression in hepatocyte-like cells (16–18). The exacerbation of chronic disease and the seroconversion to anti-HBe in both chronic and acute infection are associated with an elevated production of IFNγ by PBMC upon stimulation with HBcAg or HBeAg (8,9,19). The production of IL-10 in HBeAg-stimulated PBMC from chronic HBV patients has not been documented yet. Since IL-10 is known to inhibit Th1-like activity, such as IFNγ production (20), its production could contribute to an impaired Th1-like response. Indeed, it has been suggested that Th2-like cells are responsible for maintenance of the chronic carrier state (21–24), whereas Th1-like cells could be important for viral elimination (25,26). IL-12 could direct the HBV-specific T cell response to a favourable Th1-type response (23,27). Our study showed that HBeAg induced not only IFNγ production but also considerable levels of IL-10. Furthermore, addition of IL-12 or neutralization of IL-10 augmented the production of IFNγ in HBeAg-stimulated PBMC from chronic HBV patients. Recent communications have also demonstrated a possible role of IL-12 in HBV clearance (28,29). Taken together, these data suggest that HBV induces an unbalanced cytokine profile, which is responsible for viral persistence.

The present study indicates that during HBeAg presentation to T cells, the production of both IFNγ and IL-12 is needed for an optimal response. The effect of IL-12 could also be counteracted by neutralization of endogenous IFNγ. It remains, however, difficult to determine the relative importance of Th1- or Th2-like cells in PBMC; other cell subsets such as NK cells, although these would not act in an HLA class-II restricted fashion, and monocytes can secrete IFNγ, IL-10 and other regulatory cytokines. Furthermore, neither the production of IL-4, which is the most typical Th2-cytokine, nor an effect of neutralizing anti-IL-4 was observed in this study.

An important observation in our study was the induction of IFNγ in HBsAg-stimulated PBMC from chronic HBV patients by IL-12. These data suggest that HBsAg-specific T cells exist in the circulation of the patients but that they are in a state of anergy. *In vivo*, this anergy could be secondary to an overproduction of immunosuppressive cytokines such as IL-10, which can be induced by HBeAg, as demonstrated in this study. Since our data do not show any difference in proliferation or cytokine production between chronic patients and recently recovered controls, a similar susceptibility to anergy induction might be present in the latter group. Anergy induction can be an active immunoregulatory mechanism to prevent overactivation of the antigen-specific T cell response and limit the hepatocellular injury during acute infection (30). Furthermore, the inability of chronic carriers to produce an efficient HBsAg-specific antibody response is possibly related to T cell unresponsiveness, since both B and T cell anergy to HBsAg stimulation are associated (1,31). In summary, PBMC from patients and recovered controls proliferated and produced IFNγ and IL-10 in response to stimulation with HBe

cAg but not HBsAg. IL-12 can augment the HBeAg-induced responses but can also induce proliferation and IFNγ production together with HBsAg in patients and controls. Very recent data show that IL-12 can inhibit the replication of HBV in transgenic mice through the induction of IFNγ (32). Further work will provide more information on the possible therapeutic applications of IL-12 in chronic hepatitis B (28,33).

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