

Cytokine profile of viral and autoimmune chronic active hepatitis

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Background: Patients with hepatitis have multiple immunologic abnormalities, which may be related to cytokine production.

Methods: We examined the *in vitro* production of interleukins (IL-2, IL-4, IL-6), tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) in purified peripheral blood mononuclear cells (PBMCs) of patients with hepatitis B virus positive (HBV), acute viral hepatitis (A-HBV), HBV+ chronic active hepatitis (HBV-CAH), and autoimmune-type chronic active hepatitis (AI-CAH).

Results: IFN- γ and TNF- α production were characteristically higher in patients with A-HBV than in healthy control subjects ($p < 0.001$). However, patients with AI-CAH produced highly elevated levels of IL-4 and IL-6 compared with patients with A-HBV and HBV-CAH and healthy control subjects. The cytokine profile (PBMC-induced IL-2, IL-4, IL-6, IFN- γ , and TNF- α production) is different in A-HBV, HBV-CAH, and AI-CAH disease. The increased cytokine secretion (IFN- γ and TNF- α in A-HBV and IL-4 and IL-6 in AI-CAH) could reflect altered relative frequencies of different cell phenotypes in these diseases.

Conclusion: Specific cytokine production may be important in the pathophysiology associated with diverse inflammatory states in patients with hepatitis. (J ALLERGY CLIN IMMUNOL 1993;92:902-8.)

Key words: Hepatitis B virus, autoimmune-type chronic active hepatitis, cytokines, interleukins, interferon- γ , tumor necrosis factor- α

Cytokines produced by cells of the immune system are known to regulate cell proliferation and differentiation and are implicated in the pathogenesis of various immune disorders.^{1, 2} The cytokine-mediated effects can modulate cellular interactions in an autocrine or paracrine manner. However, many of these substances are also pleiotropic; that is, they have effects on cells outside the immune system. Therefore the challenge for the future is to determine how the synthesis of this complex array of molecules (cytokines) is coordinated and regulated in human disease states. Interleukin (IL)-2, IL-4, IL-6, interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α) are some of the important cytokines and mediators of the inflammatory response.¹

A number of immunologic abnormalities have been reported in patients with various liver disor-

Abbreviations used

A-HBV:	Acute hepatitis B virus
AI-CAH:	Autoimmune-type chronic active hepatitis
CAH:	Chronic active hepatitis
Con A:	Concanavalin A
HBsAg:	Hepatitis B surface antigen
HBV:	Hepatitis B virus
IFN- γ :	Interferon- γ
IL:	Interleukin
PBMCs:	Peripheral blood mononuclear cells
PMA:	Phorbol myristate acetate
TNF- α :	Tumor necrosis factor- α

ders.³ We and others have reported the presence of circulating TNF- α and IL-6 cytokines in patients with chronic active hepatitis.^{4, 5} However, there is no detailed information available regarding the secretion and regulation of the production of various cytokines by the immune system in this hepatic disease. Because of the potential role of cytokines in the pathogenesis of various immune disorders, we explored the possibility that a profile of cytokine production by cells of the immune system might characterize acute and chronic active hepatitis. Therefore in the present study we examined the ability of peripheral blood mononu-

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clear cells (PBMCs) from patients with hepatitis B virus antigen positive (HBV), acute viral hepatitis (A-HBV), HBV+ chronic active hepatitis (HBV-CAH), and autoimmune-type chronic active hepatitis (AI-CAH) to produce IL-2, IL-4, IL-6, IFN- γ and TNF- α cytokines. Our observations show that patients with A-HBV produce highly elevated levels of IFN- γ and TNF α but low IL-4 levels compared with patients with HBV-CAH and AI-CAH. On the contrary, patients with AI-CAH produce more IL-4 and IL-6 than other patient groups and healthy control subjects.

METHODS

Patients

We studied three groups of patients with hepatitis. A-HBV, HBV-CAH, and AI-CAH groups each comprised 10 patients, and there were 10 normal control subjects. The diagnosis of disease in patients with hepatitis was established by clinical presentations and biochemical and serologic tests. Liver biopsy was performed on all patients with AI-CAH and HBV-CAH. The patient group with A-HBV, seven men and three women with a mean age of 24 ± 4 years, had antibodies to hepatitis B surface antigen (HBsAg+), demonstrated serum aspartate aminotransferase enzyme levels greater than 500 IU (normal, up to 40 IU), and had recent onset of hepatitis. The patients with HBV-CAH, eight men and two women with a mean age of 31 ± 5 years, had antibodies to HBsAg and aspartate aminotransferase enzyme levels five times greater than normal for more than 6 months.

The patients with AI-CAH, four men and six women with a mean age of 32 ± 6 years, had absolutely no serologic evidence of infection by hepatitis A, B, or C virus and no metabolic evidence of hepatitis. However, all patients with AI-CAH had highly elevated titers ($>1:180$) of serum anti-nuclear or anti-smooth muscle antibodies (as determined by indirect immunofluorescence; Electronucleonics Inc., Bethesda, Md.). The serologic studies for hepatitis virus antibodies were performed with commercially available kits (Abbott Laboratories, Chicago, Ill.). Patients were studied before commencement of immunosuppressive or antiviral therapy. Ten healthy control subjects, seven men and three women with a mean age of 30 ± 7 years, had no evidence of any disease.

Isolation of PBMCs

PBMCs were isolated from heparinized venous blood by density gradient centrifugation on histopaque 1077 (Sigma Chemical Co., St. Louis, Mo.), washed three times in RPMI-1640 medium (Gibco, Grand Island, N.Y.) and suspended at 1×10^6 cells/ml in RPMI-1640-10% AB+ serum (Flow Laboratories, Irvine, Scotland) supplemented with 2 ml/L L-glutamine, 50 μ g/ml streptomycin, and 100 μ g/ml penicillin.

PBMCs were analyzed with the following monoclonal antibodies: OKT3 (CD3+ T cells), OKT4

(CD4+ T cells), OKT8 (CD8+ T cells), OKB2 (CD24+ B cells), OKMI (CD11+ monocytes), anti-IL-2R (CD25+), and OKIa (HLA-DR+) (Ortho Diagnostics, Raritan, N.J.) by the indirect immunofluorescence technique described recently.⁶

PBMCs (1×10^6 cells/ml) were cultured at 37° C in media alone, with concanavalin A (Con A) (5 μ g/ml) or phorbol myristate acetate (PMA) (50 ng/ml) + ionophore A 23187 (1.0 μ mol/L) (Sigma) or lipopolysaccharide (5 μ g/ml) (*Escherichia coli* 0111 LPS, Difco Laboratories, Detroit, Mich.). After 48 hours, supernatants were harvested and stored at -70° C.

Cytokine assays

IL-2 production was measured as previously described in detail⁶ with the IL-2-dependent CTLL cell line. IFN- γ was measured in an ELISA in which two monoclonal anti-IFN- γ antibodies (Genzyme Corp., Boston, Mass.) were used as catcher and tracer antibodies, respectively. IL-4, IL-6, and TNF- α levels were each measured with commercially available specific ELISA kits (Genzyme Corp.), according to the manufacturer's protocol. In each assay the appropriate recombinant human cytokines were used to generate the standard curve. Sensitivities of the various assays were as follows: IL-2 (5 U/ml), IL-4 (18 pg/ml), IL-6 (10 pg/ml), IFN- γ (6 U/ml), and TNF- α (10 pg/ml).

RESULTS

The percentage distribution of B cells, monocytes, CD3+ total T cells, CD4+ helper/inducer and CD8+ suppressor/cytotoxic T-cell subsets, CD25+ (IL-2 receptor) and HLA-DR+ cells in PBMCs of six patients with A-HBV, HBV-CAH, and AI-CAH and in six healthy control subjects are given in Table I. As shown, CD4+ T cells increased and CD8+ T cells decreased in circulation of all patients with hepatitis compared with healthy control subjects. However, no major difference was evident in regard to B cells, monocytes, and T cells among various groups of patients with hepatitis. Patients with A-HBV, HBV-CAH, and AI-CAH had greatly increased percentages of CD25+ and HLA-DR+ cells in circulation compared with healthy control subjects ($p < 0.001$). However, CD25+ cells were more elevated in patients with AI-CAH than in those with A-HBV and HBV-CAH.

We measured the production of IFN- γ , IL-2, and IL-4 in PBMCs of patients with A-HBV, HBV-CAH, and AI-CAH after stimulation with Con A or PMA + ionophore. As mitogens we chose Con A, which delivers an activating signal via surface receptors,⁷ and PMA + ionophore, which bypasses surface receptors and mimics the effect of second messengers.⁸ The data for a total of 10 patients in each hepatitis group and 10

TABLE I. Phenotypic characterization of PBMCs

Subjects	B cells CD24+	Monocytes CD11+	T cells			Activation antigens	
			CD3+	CD4+	CD8+	CD25+	HLA-DR+
A-HBV	13.4 ± 3.2	10.6 ± 1.8	71.5 ± 5.2	53.2 ± 4.8	18.2 ± 2.3	17.3 ± 2.3	14.8 ± 3.0
HBV-CAH	12.8 ± 2.5	9.3 ± 1.8	70.6 ± 4.3	54.9 ± 4.6	16.6 ± 2.8	18.5 ± 5.3	19.5 ± 4.8
AI-CAH	10.8 ± 2.8	8.8 ± 1.4	73.1 ± 5.6	50.6 ± 5.8	17.5 ± 1.9	26.4 ± 5.8	17.2 ± 3.6
Normal	10.6 ± 2.4	8.2 ± 1.7	68.5 ± 4.8	43.5 ± 4.3	22.4 ± 3.2	1.2 ± 0.2	8.4 ± 1.3

Data shown are percentage distribution of cells in PBMCs. Results are expressed as mean(s) ± SD of six subjects.

healthy control subjects are given in Figs. 1 through 3.

Fig. 1 shows that IFN- γ production after Con A or PMA + ionophore stimulation was significantly elevated in patients with A-HBV as compared with healthy control subjects ($p < 0.001$). IFN- γ production was, however, low in patients with HBV-CAH and AI-CAH compared with those with A-HBV. IFN- γ was significantly elevated in patients with HBV-CAH and AI-CAH compared with healthy control subjects ($p < 0.01$). The IFN- γ production by unstimulated PBMCs was 16 ± 3 U/ml in patients with A-HBV, 10 ± 3 U/ml in patients with HBV-CAH, 8 ± 3 U/ml in patients with AI-CAH, and undetectable in healthy control subjects.

Fig. 2 shows that Con A and PMA + ionophore-induced IL-2 production was significantly decreased in all three groups of patients with hepatitis as compared with healthy control subjects ($p < 0.01$, $p < 0.02$, $p < 0.001$). The IL-2 production by unstimulated cells was below the sensitivity of the assay for both patients with hepatitis and healthy control subjects.

Fig. 3 shows that IL-4 production after both Con A and PMA + ionophore stimulation was significantly decreased in patients with A-HBV compared with those with AI-CAH and healthy control subjects ($p < 0.01$). In contrast, the IL-4 production from patients with HBV-CAH was within the normal range. However, it is interesting that PBMCs of patients with AI-CAH produced highly elevated levels of IL-4 ($p < 0.001$) compared with those from healthy control subjects. No IL-4 was detectable in unstimulated cells from the hepatitis groups or the control group.

There appear to be several mechanisms by which PBMCs releases IL-6 and TNF- α . Lipopolysaccharide is the most potent stimulator of IL-6 and TNF- α production by PBMCs. Therefore we have assessed the IL-6 and TNF- α production by PBMCs after stimulation with Con A and lipopolysaccharide (Figs. 4 and 5).

Fig. 4 shows that IL-6 production by PBMCs after stimulation with either Con A or lipopolysaccharide was significantly higher in patients with AI-CAH than in healthy control subjects ($p < 0.001$) and patients with A-HBV or HBV-CAH ($p < 0.01$). However, there was no major difference in IL-6 production between patients with A-HBV and those with HBV-CAH. IL-6 production by unstimulated cells was below the sensitivity of the assay.

Fig. 5 shows that production of TNF- α after stimulation with Con A or lipopolysaccharide was significantly higher in patients with A-HBV than in healthy control subjects ($p < 0.0001$) and patients with HBV-CAH or AI-CAH ($p < 0.001$). However, both HBV-CAH and AI-CAH groups demonstrated similar levels of TNF- α production. Unstimulated PBMCs from patients with A-HBV produced 76 ± 21 pg/ml TNF- α , those from patients with HBV-CAH produced 42 ± 18 pg/ml TNF- α , and those from patients with AI-CAH produced 21 ± 5 pg/ml TNF- α , but levels from healthy control subjects were undetectable.

DISCUSSION

Several cytokines are important mediators of the inflammatory response in infectious, autoimmune, and malignant disorders. They are produced by the immune cells and circulate in abnormal concentrations, inflicting tissue injury, inflammation, and pathophysiologic changes in human disease. Their secretion by the immune system is directly related to the factors that cause abnormalities in cell-cell interactions, proliferation, and function, which may be characteristic features of a particular disease. Earlier, cytokine levels were measured from serum samples of patients with various types of hepatitis.^{4, 5, 9} Many cytokines are not stable in serum because of the action of proteases and inhibitors, and if the serum is not protected, the cytokines are degraded and might produce aberrant values. Therefore measurement of in vitro production of

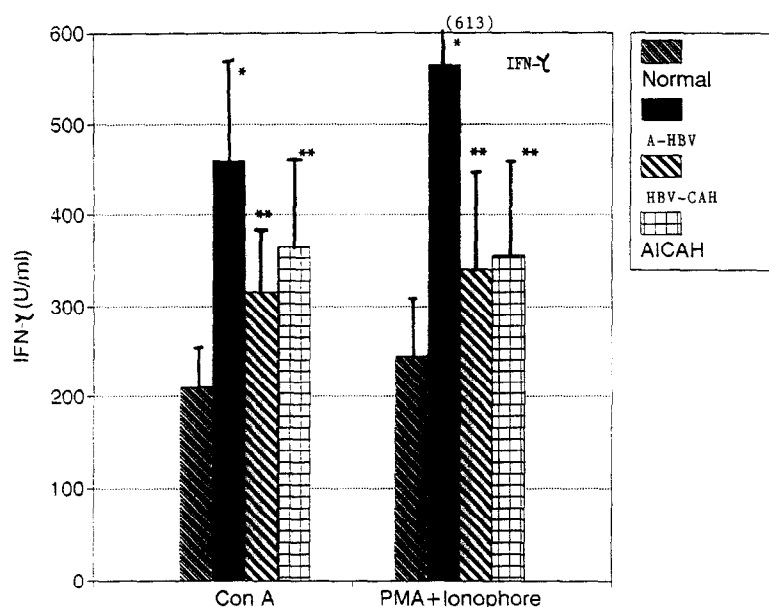


FIG. 1. IFN- γ production by PBMCs from patients with A-HBV, HBV-CAH, and AI-CAH after stimulation with Con A or PMA + ionophore; 1×10^6 cells/ml were cultured. * $p < 0.001$ versus normal control subjects (as determined by Student's t test); ** $p < 0.01$ versus normal control subjects. Data represent means $\pm$ SEM.

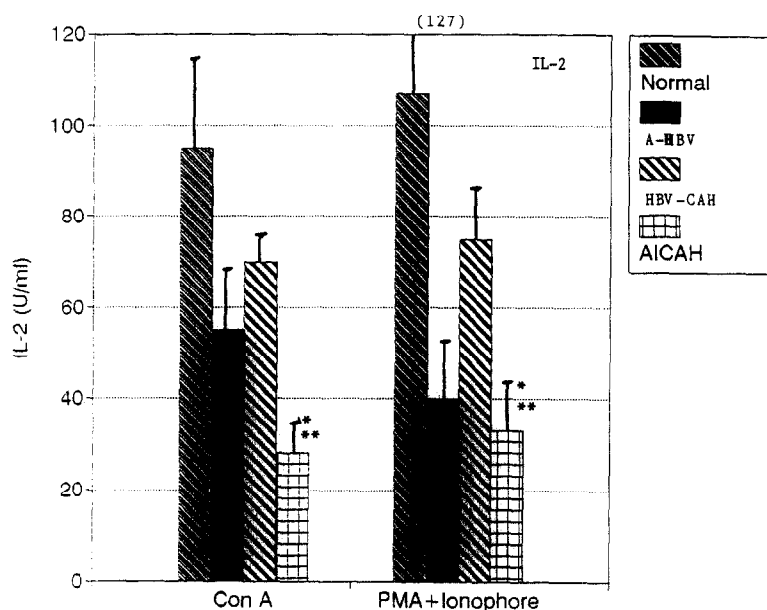


FIG. 2. IL-2 production by PBMCs from patients with A-HBV, HBV-CAH, and AI-CAH after stimulation with Con A or PMA + ionophore; 1×10^6 cells/ml were cultured. * $p < 0.001$ versus normal control subjects; ** $p < 0.001$ versus patients with HBV-CAH.

various cytokines by immune cells is a true indicator of cytokine involvement in the pathophysiology of a human disease.

We have obtained a characteristic cytokine profile from PBMCs stimulated with various agents for A-HBV, HBV-CAH, and AI-CAH. These studies demonstrate that PBMCs from all patients with hepatitis produced deficient levels of IL-2

but that production of IFN- γ , IL-4, IL-6, and TNF- α were different in each disease group. PBMCs of A-HBV specifically produced highly elevated levels of IFN- γ and TNF- α , but IL-4 and IL-6 production was highest in AI-CAH. The patients with HBV-CAH demonstrated a higher than normal production of IL-6 and TNF- α . Moreover, similar levels of IFN- γ and TNF- α

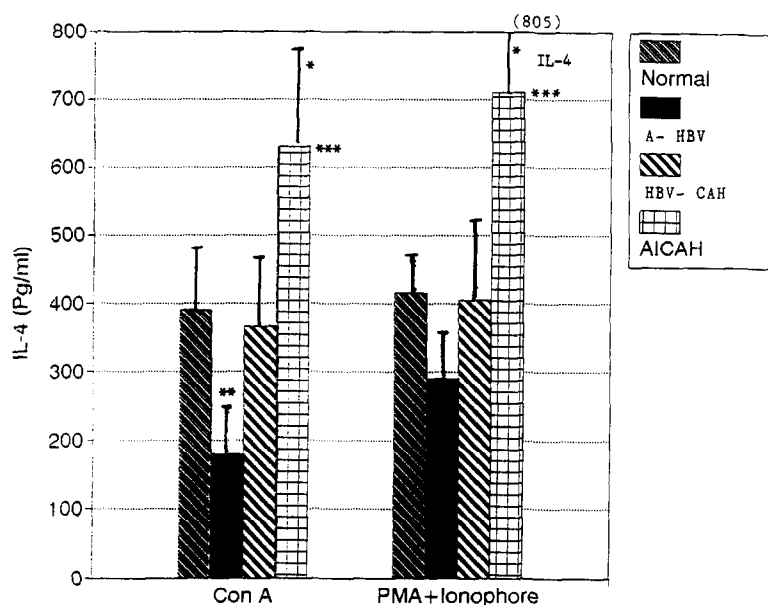


FIG. 3. IL-4 production by PBMCs from patients with A-HBV, HBV-CAH, and AI-CAH after stimulation with Con A or PMA + ionophore; 1×10^6 cells/ml were cultured. * $p < 0.001$ versus normal control subjects; ** $p < 0.01$ versus patients with AI-CAH and normal control subjects; *** $p < 0.01$ versus patients with A-HBV and HBV-CAH.

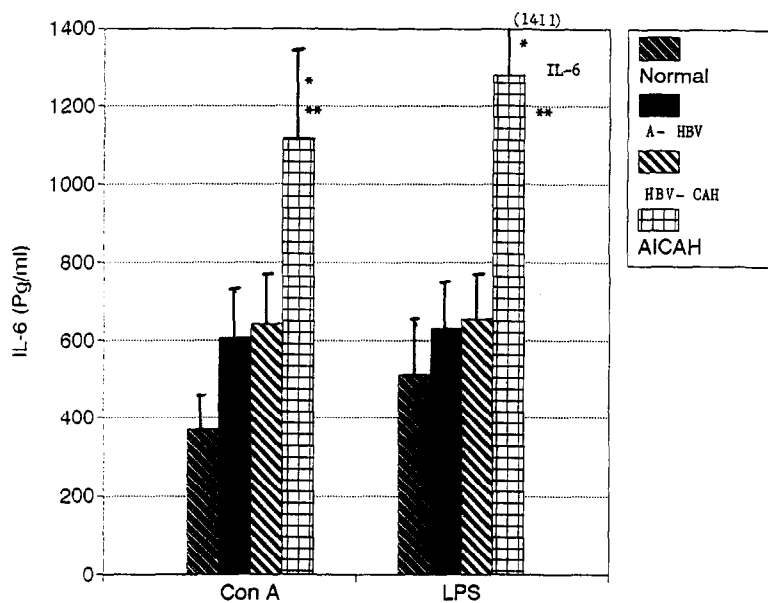


FIG. 4. IL-6 production by PBMCs from patients with A-HBV, HBV-CAH, and AI-CAH after stimulation with Con A lipopolysaccharide; 1×10^6 cells/ml were cultured. * $p < 0.001$ versus healthy control subjects; ** $p < 0.01$ versus patients with A-HBV and HBsAg + CAH.

production were evident when patients with HBV-CAH and those with AI-CAH were compared. It is interesting that HBV-CAH could be distinguished from AI-CAH on the basis of PBMC-induced IL-2, IL-4, and IL-6 production. Highly deficient IL-2 but enhanced IL-4 and IL-6 production were observed in AI-CAH as compared with HBV-CAH.

Because of its unique biologic activities, TNF- α

has long been believed to be a most crucial cytokine involved in the pathogenesis of several infectious diseases. However, IL-6 was shown to have a diverse array of activities in the immune system, including autocrine and pleiotropic growth factor activity, and is implicated in the B-cell hyperactivity and autoantibody production in autoimmune disease.¹⁰ Although IFN- γ itself has little or no direct inflammatory action, it

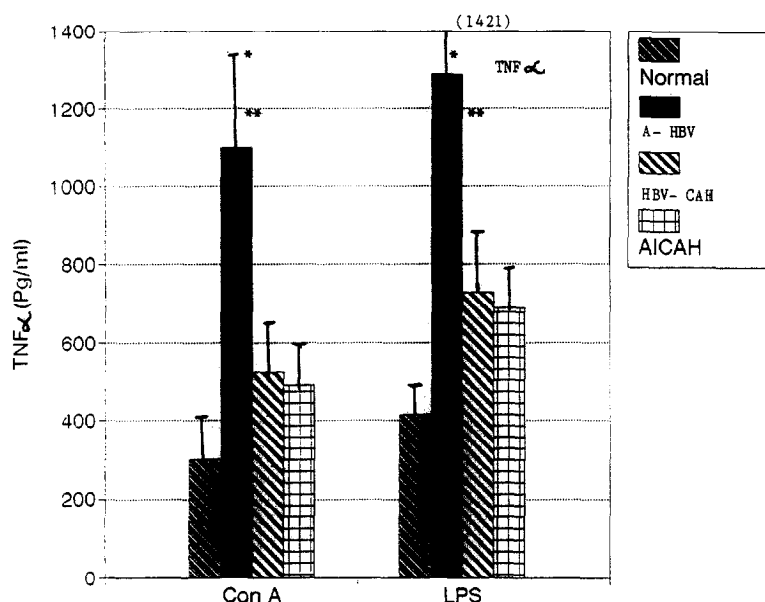


FIG. 5. TNF- α production by PBMCs from patients with A-HBV, HBV-CAH, and AI-CAH after stimulation with Con A or lipopolysaccharide; 1×10^6 cells/ml were cultured. * $p < 0.0001$ versus healthy control subjects; ** $p < 0.001$ versus patients with HBsAg + CAH and AI-CAH.

potentiates various activities of other cytokines that have direct inflammation-promoting activities.^{1, 2} Therefore TNF- α , IL-6, and IFN- γ constitute an important link in the cytokine network, and their abnormal production leads to various manifestations of disease.^{1, 2, 10}

The abnormalities in PBMCs with respect to highly enhanced IFN- γ and TNF- α production are the most characteristic and distinguishing features of A-HBV disease. It is tempting to speculate that production of these two proinflammatory cytokines may be very important in amplifying the inflammatory response and thus may be a relevant factor in the degree of clinical manifestation of this disease. Alternatively, the enhanced IFN- γ and TNF- α production might also act as a trigger for a heightened immune response in elimination of viral infection in A-HBV. In this regard, recent studies by Daniels et al.¹¹ have demonstrated that IFN- α therapy in long-term carriers of hepatitis B virus infection (HBV) resulted in clearance of HBsAg and the permanent inhibition of HBV with a resultant increase in the TNF- α production by PBMCs above the previously undetectable levels. Therefore it was suggested that elevated TNF- α levels may contribute to elimination of HBV in such cases.¹¹ The emerging roles of IFN- γ and TNF- α in the pathogenesis of A-HBV or clearance of viral infection in this disease clearly need further investigation.

Deregulated expression of the IL-6 gene and enhanced synthesis by the PBMCs of IL-6 have been implicated in the pathogenesis of various

autoimmune diseases¹⁰ and other immune disorders including infectious diseases.¹² IL-6 by itself induces B-cell hyperactivity and autoantibody production and therefore may be involved in tissue destruction.^{10, 12} A possible pleiotropic role for IL-6 may be a pathogenic factor in the causes of AI-CAH. Our demonstration of highly enhanced IL-6 production in AI-CAH suggests that the presence of hypergammaglobulinemia with various kinds of autoantibodies is likely a function of IL-6 in this disease. Because IL-6 is not only a B-cell differentiation factor but also a major regulator of acute-phase protein synthesis,¹⁰ the abnormal IL-6 production may explain the several symptoms observed in patients with AI-CAH.

Elevation of IL-4 production similar to IL-6 was also noted in PBMCs of patients with AI-CAH. The liver damage associated with AI-CAH is clinically and histologically similar to that of HBV-CAH, and there is also some evidence of viral causes (measles or rubella virus infection) in AI-CAH.¹³ All of our patients with AI-CAH had negative serologic test results for hepatitis A, B, or C virus infection. The trigger in PBMC abnormality or aberrant IL-4 and IL-6 production in AI-CAH is unknown. The identification of the factors for enhanced IL-4 and IL-6 production may provide information about the causes of this disease. Previously, enhanced IL-6 production was demonstrated in several diseases,^{10, 12} but elevated IL-4 and IL-6 levels together were noted only in scleroderma, an autoimmune rheumatic disease.¹⁴ IL-4 has been demonstrated to be a key cytokine

that induces CD23 expression on B cells, and it is also related to IgE synthesis in some allergic individuals.^{15, 16} There was no significant increase in IgE serum levels in AI-CAH. Therefore enhanced IL-4 synthesis caused by AI-CAH is a very puzzling immune system abnormality and phenomenon in this disease. This issue is to be elucidated further. However, it is important to note that cells of patients with other hepatic disease, such as A-HBV and HBV-CAH, produced either very low or almost normal levels of IL-4.

In conclusion, our data show that unregulated production of IFN- γ and TNF- α in A-HBV and IL-4 and IL-6 in AI-CAH may be one of the important events in the pathophysiology of these hepatic conditions. Interestingly, the autoimmunity in AI-CAH is characterized by immune alterations specific to enhanced IL-4 and IL-6 production. The differences in cytokine secretion among patients with various types of hepatitis may be a result of differences in the presence of activated CD25 $^{+}$ and HLA-DR $^{+}$ cells in PBMC (Table I). Alternatively, an intrinsic abnormality in cellular components (monocytes, T cells) could account for the characteristic cytokine production in each disease group. A major question concerns the significance of these cytokines in the inflammatory response. Hopefully, future studies will reveal the mechanism leading to abnormalities in the immune system to understand pathogenesis and also to design new therapeutic means for these very common and worldwide human diseases.

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